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Development, Polymorphism and Cross-Species Transferability of Genomic SSR Markers in *Duabanga Moluccana*, an Indigenous Tree Species from Sarawak

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Abstract: In this study, we used ISSR-suppression methods to develop a set of SSR markers for *Duabanga moluccana*. It is an indigenous fast growing tropical tree species. A total of 44 SSR regions were identified and specific primer pairs were designed. The SSR motifs contained perfect compound with 24 (54.5%) occurrences, followed by the imperfect compounds with 8 (18.2%), simple perfect with 8 (18.2%) and the simple imperfect repeats with 4 (9.1%). The newly identified SSR markers were characterized by screening 20 individuals of *D. moluccana* seedlings. Among 43 primer pairs tested, 25 (58.1%) SSR markers amplified the desired PCR products and 115 alleles were detected. The number of alleles per locus ranged from 2 to 8, with a mean value of 4.60. Polymorphism Information Content (PIC) values ranged from 0.225 to 0.792, with an average of 0.604. A success rate of transferability of *D. moluccana* SSR markers varied, ranging from 84% in *Duabanga grandiflora*, 36% in *Neolamarckia cadamba*, 24% in *Canarium odontophyllum* and 28% in *Shorea parvifolia*. These SSR markers herein could be used to generate useful baseline genetic information for effective selection of plus trees, provenance trials and establishment of forest Seed Production Areas (SPAs) of *D. moluccana* in the selected forest reserves for tree plantation and improvement activities. Besides, the transferability of the newly developed SSR markers across a range of species and genera suggests their potential usefulness for a variety of population genetic studies.

Keywords: *Duabanga moluccana*, Simple Sequences Repeats (SSRs), Microsatellites, ISSR-Suppression Method, Forest Plantation and Population Genetics

Introduction

The demand for quality wood is projected to increase dramatically in line with global consumption requirements. This increasing demand is mainly forced by global population growth and rise in socio-economic levels (FAO, 2010). The global consumption of industrial round wood is estimated to increase from 1,707 million m^3 in 1990 to 2,436 million m^3 in 2030 (FAO, 2009). However, the slow-growing of natural forests are unable to meet current global demand for wood, resulting in the loss and degradation of natural forests (Fenning and Gershenzon, 2002). The development of high-yielding with short rotation plantation forests is vital to supply the bulk of humanity's wood needs on a long-term basis. It is also important to ensure a sustainable supply of high genetic quality seedlings for planted forest development worldwide to maximize adaptability and yield potentials under stress-site condition (Goel and Behl, 2001).

With advances in genomics research, there has been a remarkable progress in the development of an array of potential molecular markers, including RAPD, RFLP, AFLP, SSRs and other markers for monitoring forest tree improvement activities, such as measuring genetic variation in breeding populations, germplasm

identification, verifying controlled crosses and estimating seed orchard efficiencies (Neale *et al*., 1992). As explained by Westman and Kresovich (1997), DNA-based markers play a vital role to detect variation for both coding and non-coding DNA sequences from nuclear and organelle genomes. Nowadays, these molecular markers have proven their utility in fields like taxonomy, physiology, embryology, genetic engineering, etc (Joshi *et al*., 1999; Mondini *et al*., 2009).

Simple Sequence Repeats (SSRs) or microsatellites are becoming a popular DNA marker for genetic analysis in plants. According to Saha *et al*. (2003), SSRs are a class of repetitive DNA that is a ubiquitous component of eukaryotic genomes. Such loci are found scattered throughout the genome and inherited in a Mendelian fashion (Moon *et al*., 1999). SSRs are consisting of a short motifs, typically mono-, di-, tri-, or tetranucleotide repeats, which are repeated several times (Mahalakshmi *et al*., 2002). They almost invariably show extensive polymorphism, due to the variability in SSR repeat length as a consequence of slippage during DNA replication or unequal crossing over. The hyper-variability (with mutation rates ranging from 10^{-2}-10^{-6} per locus per generation) in species and populations is the key feature of SSRs as molecular markers (Chistiakov *et al*., 2006). To exploit SSRs as DNA based-markers, they are assayed by PCR with specifically designed primers to match unique sequences flanking the SSR region.

SSRs display high information content, as they are co-dominant and highly multiallelic. Furthermore, they are usually transferable across closely related species and it has been reported some classes of SSRs constitute an important source of quantitative genetic variation, coding for functional elements of protein molecules and serve as regulatory elements of transcription (Kashi *et al*., 1997; Collevatti *et al*., 1999). Therefore, these markers have contributed greatly to the understanding of mating systems and pollen dispersal patterns (Garcia *et al*., 2005), construction of genetic maps (Brondani *et al*., 2006) and forensics (Craft *et al*., 2007). Additionally, the attractive attribute of this marker is especially in the case of species which show a low level of genetic variation, inbred populations and geographically close populations (Rakoczy-Trojanowska and Bolibo, 2004). Butcher *et al*. (1999) and Ho *et al*. (2006) also reported the use of SSR markers in monitoring the genetic effects of forest management practices and fragmentation on genetic diversity and gene flow in several forest tree species.

Conventionally, isolation of SSR loci involves construction of a genomic library, screening with repeat oligonucleotide probes for the identification of positive clones, designing and synthesis of primers (Roy *et al*., 2004). However, these tasks are usually labour-intensive, time-consuming and expensive because the proportional of SSRs to the entire genome is generally low (Lian *et al*., 2001). In addition, the recovery rate of useful SSRs is low due to non-specific amplification and monomorphic loci (Hayden and Sharp, 2001). An alternative method is by searching the SSR-containing sequences from the available databases, e.g. EMBL and GenBank. This method is cost-effective, simple and relatively quick but only applicable to species that are well represented in the databases (Rakoczy-Trojanowska and Bolibok, 2004).

Duabanga moluccana Blume or locally known as sawih is a timber species belonging to the family Sonneratiaceae. The wood of *D. moluccana* confers various advantages for the timber industry including production of wood works and products, such as plywood, veneer, blockboard and interior joinery. Additionally, it is suitable for interior paneling, matches, moulding and pulping (CIRAD, 2003). Owing to its fast-growing ability, *D. moluccana* has been now identified as a species of great potential for planted forests development in Sarawak. To date, the genetic information and molecular markers of this species are still scanty. Thus, the objectives of this study were to develop a set of SSR markers specific for genotyping *D. moluccana* using ISSR-suppression methods and to evaluate the possibility of cross-species transferability of SSRs among tree species. Two ISSR-suppression methods were applied in developing SSR markers for *D. moluccana* as described by Lian *et al*. (2001) and a modified protocol of Lian *et al*. (2006). As explained by Lian *et al*. (2001), such methods are relatively simple without enrichment and screening procedures. The methods have been widely used by many researchers in developing SSR markers for species with little genomic information (Tamura *et al*., 2005; Sui *et al*., 2009; Qosim *et al*., 2011; Xie *et al*., 2011; Liu *et al*., 2013).

Materials and Methods

Plant Materials and DNA Isolation

The fresh young leaf samples of *D. moluccana* seedlings were collected from the nursery of the Forest Tree Seed Bank, Sarawak Forestry Corporation (SFC), Sarawak. Total genomic DNA was isolated using a modified CTAB method (Doyle and Doyle, 1990).

Cloning and Sequencing of ISSR Amplified Fragments and Primer Design

Five SSR primers, namely $(AC)_{10}$, $(AG)_{10}$, $(GTG)_6$, $(AC)_6(AG)_5$ and $(TC)_6(AC)_5$ were used to amplify the fragments flanked by two SSR sequences arranged in opposite orientations from the DNA of *D. moluccana*. PCR was performed using a Palm Cycler (Corbett, USA). Reaction in a volume of 25 µL consists of 20 ng of genomic DNA, 10 pmol of the primer, 0.2 mM of each dNTP, 1× PCR buffer, 2.5 mM $MgCl_2$ and 0.5 U of *Taq* DNA polymerase (Invitrogen, USA). The PCR was

performed at 94°C for 2 min, 40 cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, followed by final extension at 72°C for 10 min. The amplified PCR products were purified from agarose gel by using QIAquick® Gel Extraction Kit (QIAGEN, Germany) and then ligated into pGEM®-T Easy Vector System (Promega, USA). Colony PCR with M13 forward and reverse sequence primers were performed to identify the presence of positive clones. Then, the recombinant plasmid was isolated and purified according to the Wizard®*Plus* SV Minipreps DNA Purification system protocol (Promega, USA). The recombinant plasmids DNA were sequenced using the ABI Prism™ Bigdye ™ terminator cycle sequencing Ready reaction kit V.3.1 (Applied Biosystems, USA) and analysed on a ABI 3730XL capillary DNA sequencer (Applied Biosystems, USA). Subsequently, an Initiating Primer (IP1) designed from the sequenced region at one end of the SSR and for nested PCR another IP2 based on the sequence between IP1 and the SSR sequence. IP1 and IP2 primers were designed using PRIMER 3.0 v.0.4.0 (Rozen and Skaletsky, 2000). These two primers were used in order to determine other sequence flanking the SSRs by a genome walking method. All the primers were designed based on the following criteria: Primer length of 20-25 bp, GC content 40-60% and T_m between 57-63°C. The primers were synthesized by Bio Basic Inc. (Canada).

Construction of DNA Libraries

Adaptor-ligated, restricted DNA libraries were constructed according to Siebert *et al*. (1995). Approximately 7 µg of DNA was separately digested in a 100 µL reaction volume with 100 U of a restriction enzyme (*Alu*I, *Eco*RV, *Hae*III, *Rsa*I and *Ssp*I) at 37°C for overnight. Then, DNA was extracted with CIA (24:1) and precipitated by adding $^1/_{10}$ volume of 3 M NaOAc (pH 5.2) and 3 volumes of 99.5% ethanol. The mixture was precipitated at -20°C for at least 30 min and following by centrifugation at 13,000 rpm for 5 min. Subsequently, the pellet was washed with 70% ethanol, dried before dissolved in 40 µL of ddH_2O. The digested DNA was then ligated to 1.4 µg of a blunt adaptor overnight at 16°C using a T4 DNA Ligation Kit (Invitrogen, USA). After treatment, the ligated fragments were precipitated by ethanol as described above, dissolved in 180 µL of ddH_2O and stored at -30°C until future analysis. As Adaptor Primers (AP) for nested PCR, AP1 (5'-CCATCCTAATACGACTCACTATAGGGC-3') and AP2 (5'-CTATAGGGCACGCGTGGT-3') were also prepared.

Identification of the Sequence beyond the Determined ISSR Sequences

Two steps PCR amplification from adaptor-ligated restricted DNA libraries were performed using the primers prepared based on sequences of each ISSR fragment (IP1 and IP2) and adaptor primer (APl and AP2). A primary reaction was conducted in a 25 µL reaction mixture containing 1 µL of the adaptor-ligated DNA, 0.2 mM of each dNTP, 5 pmol adaptor primer AP1 and ISSR-specific primer IP1, 1× PCR buffer, 1.5 mM $MgCl_2$ and 0.5 U of *Taq* DNA polymerase (Invitrogen, USA). The PCR was performed using Mastercycler Gradient Thermal Cycler (Eppendorf, Germany) with temperature cycling conducted as follow: Initial denaturation step at 94°C for 2 min, followed by 35 cycles of 1 min at 94°C, 30 sec at an annealing temperature of 62°C and 2 min at 72°C, followed by l cycle of 1 min at 94°C, 30 sec at an annealing temperature of 62°C and 5 min at 72°C and concluded with a 10 min extension at 72°C. The secondary reaction was conducted with 1 µL of a 100-fold dilution of the primary PCR product using the adaptor primer AP2 and ISSR-specific primer IP2. The same reaction mixture and PCR conditions as in the primary PCR was used, except that the annealing temperature and cycle number were reduced to 60°C and 29 cycles. The PCR product was examined using a 1.5% agarose gel. A single major band was excised, cloned and then sequenced as described above. Subsequently, the other primer (IP3) from the newly-defined flanking sequence was designed for amplifying of the region containing a SSR.

SSR Repeats Identification and Primer Design

The SSR regions were initially identified from the sequences using SSR Finder (http://www.geboc.org/ssr/ssr.html). Motifs were searched included di-, tri-, tetra-, penta as well as compound repeats composed of di- and tri- repeats and imperfect repeats. In the perfect SSRs, the minimum number of repeat units for dinucleotides is five and four repeat units for trinucleotides. In the compound and imperfect repeats, the minimum length of di-, tri- and tetranucleotide repeats is five units. Imperfects repeats are defined as having no more than one disruptive element of length ≥ 1 and ≤ 20 bp (Berube *et al*., 2007). The flanking sequences of the repeat motifs were used to design specific primers. These SSR-specific primer pairs were designed according to the following criteria: 20-25 bp with annealing temperature between 50-60°C and to give an expected product size of 100-400 bp, using the PRIMER 3.0 v.0.4.0 (Rozen and Skaletsky, 2000).

SSR Marker Validation and Polymorphism

To investigate the desirable properties of each isolated SSR loci, 20 *D. moluccana* seedlings were selected and then genotyped. Reaction in a volume of 25 µL consists of 10-20 ng of template DNA, 0.2 mM of each dNTP, 5 pmol of each designed primer pair, 1× PCR buffer, 1.25 mM of $MgCl_2$ and 0.5 U of *Taq* DNA polymerase (Invitrogen, USA). Amplifications were

conducted in Mastercycler Gradient PCR (eppendorf, Germany) for 5 min at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at optimal annealing temperature and 1 min at 72°C, followed by final extension of 5 min at 72°C. The PCR products were electrophoresed using a 3.5% (w/v) metaphor agarose gel (Cambrex, USA) in 1× TBE and stained with Gel Star® nucleic acid gel stain (Lonza, Rockland, ME USA). The allele sizes were determined by referring to 25 bp DNA ladder (Invitrogen, USA) and 100 bp DNA ladder (Promega, USA). The transferability of these SSR markers was further investigated on four different tree species, *Duabanga grandiflora*, *Neolamarckia cadamba*, *Canarium odontophyllum* and *Shorea parvifolia*. The variability of SSR markers at each locus: the number of alleles (A), Polymorphism Information Content (PIC), expected heterozygosity (H_e) and deviations from Hardy-Weinberg Equilibrium (HWE) were tested using Power Marker 3.25 (Liu and Muse, 2005) and POPGENE 1.32 (Yeh *et al*., 1997).

Results

SSR Identification and Primer Design

A total of 44 microsatellite repeats were identified in *D. moluccana* genome. Twenty SSR repeats (48%) were identified based on Lian *et al*. (2001) and the remaining microsatellite repeats (52%) were based on a modified protocol of Lian *et al*. (2006). The SSR database contained simple perfect and simple imperfect microsatellites constituted by di- and trinucleotides and, perfect/imperfect compounds. The most numerous class was the perfect compound with 24 (54.5%) occurrences, followed by the imperfect compounds (8 or 18.2%), simple perfect (8 or 18.2%) and the simple imperfect repeats (4 or 9.1%) as shown in Fig. 1. Out of the 44 identified SSR repeats, specific primer pairs were successfully designed for 43 SSR repeats. However, primer pair could not be designed for $(ATT)_4$ due to a high concentration of A and T nucleotides that is unsuitable for primers designing.

SSR Marker Validation and Polymorphism

Of the 43 SSR primer pairs designed, 26 (60.5%) [18 SSR primers derived from Lian *et al*. (2001) and 8 derived from Lian *et al*. (2006)] amplified a product of the expected size. The remaining 17 (39.5%) SSR primers produced unexpected PCR product size or multiple banding patterns. These primers were then eliminated from further analysis. A total of 20 *D. moluccana* seedlings collected from the nursery of the Forest Tree Seed Bank were genotyped to determine the polymorphism level of each newly developed SSR primer pairs. Of the 26 SSR primers analysed, 7 (28%) were derived from simple perfect repeats, 4 (16%) from simple imperfect, 10 (40%) from compound perfect and 4 (16%) from compound imperfect. All these SSR primers were able to produce the expected products with one or two bands in all or some of the *D. moluccana* individuals (Fig. 2). This is consistent with the *D. moluccana* as a diploid species (2n = 48). Besides that, the co-dominant nature of SSR markers are enabled to distinguish homozygous individuals (one band or two copies of the same allele) from heterozygous individuals (two bands or two different alleles) in diploid species as shown in Fig. 2.

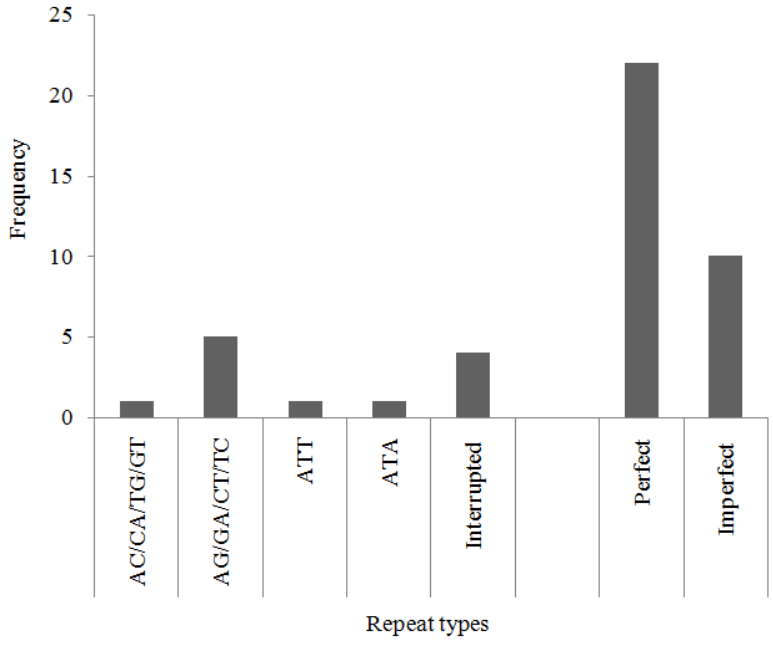

Fig. 1. Frequency of different types of SSR repeat motifs identified in *D. moluccana* genome

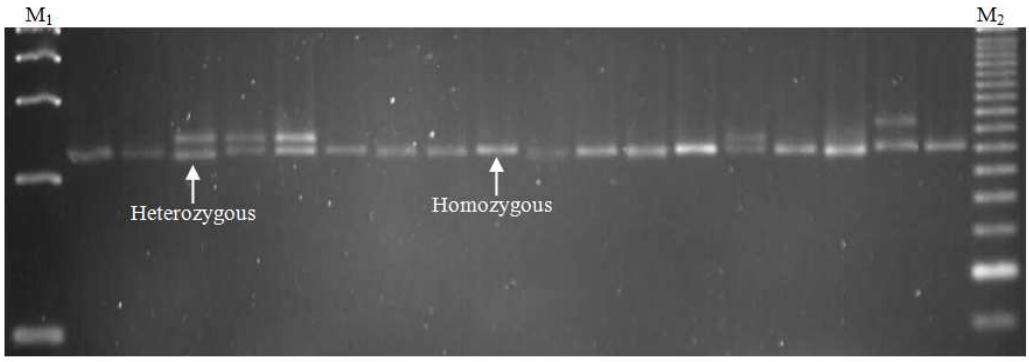

Fig. 2. Polymorphism of a SSR marker, DMTCAC11 in *D. moluccana*. Electrophoresis of amplified products on 3.5% metaphor agarose gel. M_1: 100 bp DNA ladder (Promega, USA). M_2: 25 bp DNA ladder (Invitrogen, USA)

Table 1. Characteristics of the 25 SSR markers developed for *D. moluccana*

Locus	Repeat motif	Primer sequence (5'-3')	A	T_a (°C)	Product size range (bp)	PIC	H_e	Deviation from HWE	Method
DMAC01*	$(GA)_3CACC(GA)_7$	F: GTACAGCACGCACATGACAC R: GTAGGCCGCTATGAAACCAG	5	55	193-210	0.668	0.715	0.019	Lian *et al*. (2001)
DMAC02*	$(GT)_5GC(GT)_2$	F: TCAGTCTCAGCAAGTGTGTGC R: ATCTTCAGCCTCTCCTTCG	4	52	152-165	0.473	0.544	0.001	Lian *et al*. (2001)
DMAC03*	$(CA)_3(AG)_4$	F: AAGGGATGTGTTTCGGAGTG R: TGGCTAGGCCTTTCGTTAAG	2	55	200-225	0.225	0.255	0.000	Lian *et al*. (2001)
DMAC04*	$(AT)_3(GT)_8$	F: CACGTGTCAACACTTTCAGCTA R: GTGGTTACTTTTGTTAGTCCAATCC	4	53	180-210	0.562	0.606	0.000	Lian *et al*. (2001)
DMAC05*	$(GT)_5$	F: TCTCCTTAAATCTCGTCTTGTGC R: GATGGCATGCAACAACCTC	4	52	150-175	0.592	0.586	0.000	Lian *et al*. (2001)
DMAC06*	$(TAG)_3(TAA)_3$	F: GGCTGAGGTCATGTCAGAGTC R: AAATTGGCACACGCATTTAG	6	52	230-250	0.650	0.654	0.000	Lian *et al*. (2001)
DMAC07*	$(AC)_7(GT)_3$	F: GATTGGCAACTCCATCCAAG R: CACTCATGTCACCGAAATGG	3	60	175-220	0.528	0.430	0.000	Lian *et al*. (2001)
DMAC08	$(TG)_2ACAAA(TG)_5$	F: AATGCGTGTGCCAATTTTAC R: GCGACACTGATACCTAAGTCTGTT	3	52	130-175	0.488	0.574	0.253	Lian *et al*. (2001)
DMAG02*	$(CT)_8$	F: AGCAGGGAGCTTACATTTC R: ACAGTGTCCACAACCTCTCG	5	60	175-240	0.690	0.685	0.000	Lian *et al*. (2001)
DMAG09*	$(AG)_{10}$	F: GGATTCTGTTCACGATTTTCG R:TCACCGCCAACCTCTCTAAG	6	59	200-275	0.739	0.752	0.000	Lian *et al*. (2001)
DMAG04*	$(GAT)_3GTT(GAT)_3$	F: AGAAGCTGGCAGAAAAATGC R: GCGAGAGAAAGCAAAAGGTC	7	54	150-250	0.785	0.811	0.092	Lian *et al*. (2001)
DMAG05	$(CT)_{19}$	F: TGTGTGCTCATTGCTTTCTTG R: GGCTCTCTCTTCGTCTATTTTGG	8	52	237-350	0.739	0.766	0.000	Lian *et al*. (2001)
DMAG06*	$(CT)_4TTTT(CT)_3$ $G(TTTTC)_2$	F: TCATCACGAGACGACTGACC R: GGGGTACAATCTAAATTGATCGAG	4	55	225-275	0.675	0.725	0.000	Lian *et al*. (2001)
DMAG07*	$(TCC)_3CC(ATT)_2$	F: CTGCTGCCGCTACAGAACTC R: GAGAGAGGAGCGAAGGGAAG	2	53	200-300	0.365	0.480	0.000	Lian *et al*. (2001)
DMAG08*	$(GACA)_2C(AG)_4$	F: CCATTATCTGCTTCCCTTCG R: ATATCATCCCGCCGCTTC	5	53	148-200	0.534	0.579	0.000	Lian *et al*. (2001)
DMAG10	$(CT)_8$	F: TTCCTCTCCTCGGTTTCTTG R: TGCGTCTCTTCTTCCTTGAAC	7	51	180-250	0.745	0.758	0.306	Lian *et al*. (2001)
DMGTG02*	$(CCA)_2A(CAC)_3$	F: ACTCCCTCCTCTCCATCTCC R: GGACAAGCGACTCCACTGAC	3	60	130-190	0.460	0.515	0.000	Lian *et al*. (2001)
DMACAG01	$(AC)_6(AG)_9$	F: $(AC)_6(AG)_5$ R:TGAGCTCATTGTAGTAGAGAACAAG	7	55	230-300	0.792	0.801	0.064	Lian *et al*. (2006)
DMACAG07*	$(AC)_6(AG)_5$	F: $(AC)_6(AG)_5$ R: CGAATGAGGCCGAAACTTAG	4	56	295-330	0.554	0.568	0.000	Lian *et al*. (2006)
DMTCAC04	$(ATA)_7$	F: GGGGTACGGTTGAGTACAGC R: AGTGAAGCGCAGGGTATTTG	5	54	200-280	0.650	0.611	0.807	Lian *et al*. (2006)
DMTCAC05*	$(GT)_5(GA)_6$	F: AAATACCCTGCGCTTCACTC R: $(TC)_6(AC)_5$	3	56	225-275	0.539	0.614	0.000	Lian *et al*. (2006)
DMTCAC07*	$(GT)_5(GA)_6$	F: ATTGCCTCAGGTATGCAATC R: $(TC)_6(AC)_5$	4	59	200-250	0.608	0.636	0.019	Lian *et al*. (2006)
DMTCAC08*	$(CT)_6(CA)_6$	F: $(TC)_6(AC)_5$ R: AGCTGCCTAGCACCTCTGTC	5	56	225-375	0.678	0.649	0.008	Lian *et al*. (2006)
DMTCAC11*	$(GA)_{19}$	F: CTGGCTATAACAGGGCCAAA R: CACACACACACTATACTCCACCTC	5	59	200-275	0.729	0.767	0.001	Lian *et al*. (2006)
DMTCAC13*	$(TC)_6(AC)_6(AT)_3$	F: $(TC)_6(AC)_5$ R: GCAAGCTTGAGGATTTCGAG	4	52	205-270	0.628	0.660	0.000	Lian *et al*. (2006)
Mean			4.60			0.604	0.630		

A = Number of alleles; T_a = Annealing temperature; PIC = polymorphism information content; H_e = Expected heterozygosity (Nei's, 1973); *significance departure from HWE ($p<0.05$)

Table 2. Cross-species amplification of *D. moluccana* SSR markers

SSR Primer	GD1	GD2	NC1	NC2	CO1	CO2	SP1	SP2
DMAC01	+	+	MP	-	MP	MP	MP	MP
DMAC02	+	+	-	-	-	-	-	-
DMAC03	+	+	+	+	+	+	+	+
DMAC04	+	+	+	-	+	+	+	+
DMAC05	+	+	-	-	-	-	-	-
DMAC06	+	+	+	-	-	-	-	-
DMAC07	-	-	-	-	-	-	-	-
DMAC08	+	+	-	-	-	-	+	+
DMAG02	-	-	-	-	-	-	-	-
DMAG04	+	+	-	-	-	-	MP	-
DMAG05	+	+	+	-	+	+	+	-
DMAG06	+	+	-	-	-	-	-	-
DMAG07	+	+	+	+	-	-	MP	-
DMAG08	+	+	+	-	+	MP	+	+
DMAG09	+	+	+	-	+	+	+	+
DMAG10	+	+	-	-	-	-	-	-
DMGTG02	+	+	-	-	-	-	MP	MP
DMACAG01	+	+	MP	-	MP	MP	MP	-
DMACAG07	-	-	MP	-	MP	MP	MP	MP
DMTCAC04	-	+	-	-	-	-	-	-
DMTCAC05	+	+	MP	-	MP	MP	MP	MP
DMTCAC07	-	-	MP	-	-	-	MP	-
DMTCAC08	+	+	+	-	+	+	+	+
DMTCAC11	+	+	-	-	-	-	-	-
DMTCAC13	-	+	MP	+	MP	MP	-	-

Duabanga grandiflora (DG), *Neolamarckia cadamba* (NC), *Canarium odontophyllum* (CO) and *Shorea parvifolia* (SP).
"+" indicates expected amplifiable products; "MP" indicates multiple PCR products without expected sizes and "-" indicates unexpected PCR product sizes or without PCR products.

From the 26 primers tested, only 13 were able to amplify all the *D. moluccana* samples. Meanwhile, null alleles were detected at the remaining 13 SSR primers; with DMAG03 primer pair carried the highest number of null alleles (15 null alleles). Therefore, this DMAG03 primer pair was excluded from further characterization.

A total of 115 alleles were detected across the 25 loci. The number of alleles detected per locus ranged from two (DMAC03 and DMAG08) to eight (DMAG06) with a mean value of 4.6 (Table 1). This mean value was higher than other studies of *Brassica rapa* (1.9 alleles per locus) (Tamura *et al*., 2005), *Citrus sinensis* (2 alleles per locus) (Novelli *et al*., 2006) and *Arachis hypogaea* (2.44 alleles per locus) (Cuc *et al*., 2008). The PIC values ranged from 0.225 (DMAC03) to 0.792 (DMACAG01). Twenty-three out of 25 SSR loci were considered highly informative with PIC more than 0.5, while two primers (DMAC03 and DMAG08) were categorized in reasonably informative loci (0.25 < PIC < 0.5) (Botstein *et al*., 1980). In general, PIC value is positively correlated with the number of alleles and the frequency of the alleles (Buchanan and Thue, 1998). This is consistent with the present study as most of the SSR loci with a large number of alleles displayed higher PIC (Table 1).

Cross Species Amplification of SSR Markers

The transferability of *D. moluccana* SSR markers was further investigated on four different tree species, *Duabanga grandiflora*, *Neolamarckia cadamba*, *Canarium odontophyllum* and *Shorea parvifolia*. The success of SSR markers amplifications was evaluated by the positive amplification of a PCR band of the expected size. Results for cross-species amplification are summarized in Table 2. The success rate was varied among species, ranging from 84% in *Duabanga grandiflora*, 36% in *Neolamarckia cadamba*, 24% in *Canarium odontophyllum* and 28% in *Shorea parvifolia*. Of the 25 SSR primer pairs tested, six (24%) primers (DMAC03, DMAC04, DMAG05, DMAG08, DMAG09 and DMTCAC08) amplified at least one individual in all species analysed. Among these transferable *D. moluccana* SSR primers, two were simple perfect, three were compound perfect and one was compound imperfect repeats. As reported by Ekue *et al*. (2009), SSR markers based on perfect repeats were likely to be more conserved than those harbouring imperfect repeats. On the other hand, four loci (DMAC07, DMAG02, DMACAG07 and DMTCAC07) failed to amplify expected PCR products in all species tested.

Discussion

Two methods were used for SSR markers development in *D. moluccana,* namely Lian *et al*. (2001) and a modified protocol of Lian *et al*. (2006). Based on Lian *et al*. (2001), it involves four major steps: (a)

amplifying SSRs using a SSR-primer [$(AC)_{10}$, $(AG)_{10}$, $(CT)_{10}$ and $(GTG)_6$] to produce fragments flanked by SSR sequences; (b) designing a primer based on the region between the two SSR sequences; (c) sequencing the region from the primer to the side flanking the SSRs by a walking method and (d) designing another primer from the newly defined flanking sequence for amplification of the region containing a SSR. The second method was based on Lian *et al*. (2006) with slightly modifications for isolating compound SSR markers. First, the fragments flanked by compound SSR sequence at both end were amplified from DNA using compound SSR primers, i.e., $(AC)_6(AG)_5$ and $(TC)_6(AC)_5$. These amplified sequences were cloned and sequenced. A locus-specific primer was designed from the sequence flanking the compound SSR. This is relatively simple method compared to Lian *et al*. (2001) because without adaptor-ligated DNA libraries construction. Then, a common compound SSR primer and locus-specific primer were used as a SSR marker to amplify each compound SSR region. The frequency distribution of repeat motif was varied in *D. moluccana*. The dinucleotide repeats were the most abundant in *D. moluccana*. This finding was consistent with the other studies such as in *Trifolium repens* (93%) (Kolliker *et al*., 2001), *P. trichocarpa* (72.4%) (Tuskan *et al*., 2004) and *Hevea brasiliensis* (96.1%) (Yu *et al*., 2010) that dinucleotide repeats were the most abundant in plant systems. Toth *et al*. (2000) also reported that dinucleotides are about 1.5-fold more common than other repeat motifs (tri-, tetra- and pentanucleotides) in genomic DNA.

In simple perfect dinucleotides, AG/GA/CT/TC (83.3%) was observed more frequently than AC/CA/TG/GT (16.7%). Among AG/GA/CT/TC repeat motif, two SSRs had more than 10 repeats and three contained 8 and 10 repeats. AG/GA/CT/TC was found to be the longest simple repeat motif with 19 repeat units (38 bp). The greater number of AG/GA/CT/TC repeats relative to AC/CA/TG/GT repeats in *D. moluccana* genome is consistent with findings from other tree species, such as *Shorea curtisii* (Ujino *et al*., 1998), *Eucalyptus* spp. (Brondani *et al*., 1998), *Populus tremuloides* (Dayanandan *et al*., 1998), *Caryocar brasiliense* (Collevatti *et al*., 1999), *Hevea brasiliensis* (Roy *et al*., 2004) and *Pinus resinosa* (Boys *et al*., 2005). Pfeiffer *et al*. (1997) also reported that there is one $(AG)_n$ microsatellite per 194 kb and one $(AC)_n$ per 406 kb in the *Picea abies* nuclear genome. In contrast, higher frequency of AC/TG repeats was reported in other species such as tobacco (Lagercrantz *et al*., 1993), *Pinus strobus* (Echt and May-Marquardt, 1997), *H. brasiliensis* (Yu *et al*., 2010) and *Acacia* sp. (Butcher *et al*., 2000).

Two perfect trinucleotide repeat motifs were detected meanwhile another two trinucleotide were in perfect and imperfect compounds, respectively. The longest repeat array found in prefect trinucleotide was ATA, repeated for seven times. Simple perfect dinucleotide repeats (75%) was frequently observed compared to trinucleotide repeats (25%). In agreement with earlier studies, dinucleotides are frequently found in non-coding regions, while trinucleotides are expected to be most abundant SSR class found in coding regions (Berube *et al*., 2007). Lagercrantz *et al*. (1993) also stated that trinucleotide SSRs are relatively infrequent in plants compared with vertebrates and other organisms. Compound repeat motifs were also detected. Out of 32 compound repeats identified, 24 were perfect repeats (75%) and 8 were imperfect repeats (25%). Most of the compound perfect repeats were characterized by the presence of two to three dinucleotide repeat motifs with varying repeat length. A relatively long stretch of compound perfect repeat containing AC/TG and AG/TC motifs with a repeat length of three and nine, respectively. Among the compound imperfect repeats, GT seemed to occur at a higher frequency, which interrupted by one to six non-repeat nucleotides. Besides the GT motifs, different motifs comprising di-, tri-, tetra- and penta-nucleotide also existed as part of compound imperfect repeats.

The differences in the abundance of SSR motifs among plant genera are not well understood. Moreover, the number and repeat type of SSR are influenced by the restriction enzyme used to size fractionate the genome during DNA libraries construction (Hamilton and Fleischer, 1999). Therefore, although various frequencies of SSR repeat motifs were detected, these frequencies do not represent the actual distributions of SSRs throughout the *D. moluccana* genome. This is because as only five ISSR primers were used in amplifying fragments flanked by SSRs. In this study, it was also found that more than one SSR repeats in a single clone. This indicates that the SSRs are organized in clusters. According to Oliveira *et al*. (2006), DNA repair system also plays an important role in determining the SSR distribution in different species.

Multiple bands were produced due to multiple primer binding sites along the genome. This phenomenon is quite common since SSR sequences may be associated with the repetitive genomic DNA (Smith and Devey, 1994). Moreover, multiple products seem to be associated with compound SSRs developed based on a modified protocol of Lian *et al*. (2006). This is because 12 out of the 16 primers were from this category. One of the reasons that lead to multiple products might due to the use of universal primers [$(AC)_6(AG)_5$ or $(TC)_6(AC)_5$] as one of the primers in the compound SSR marker developments. Thus, this subsequently will increase the possibility of amplifying the undesired products.

Null alleles are commonly detected in SSR analysis. It refers to any individuals repeatedly fail to amplify any allele at just one SSR primer while all other primers amplify normally. These null alleles are usually attributed to a mutation within primer-binding sites flanking the microsatellite or amplification conditions problems (Yu *et al*., 1999). As consequence, this may completely inhibit primer binding and leading to the loss of products or giving a faint band (Gupta and Varshney, 2000) and therefore, some of the heterozygotes might be incorrectly genotyped as homozygotes (Selkoe and Toonen, 2006). In this study, re-amplifications on such samples were carried out to exclude the possibility of failure during PCR amplification. According to Rallo *et al*. (2000), the detection of null alleles in SSR loci is quite common especially in highly outbreeds heterozygous species. Moreover, the presence of null alleles has been reported in a wide range of taxa, including humans (Callen *et al*., 1993), fish (Jones *et al*., 1998), insects (Liewlaksaneeyanawin *et al*., 2002; Li *et al*., 2009) and plants (Sefc *et al*., 1999; Sousa *et al*., 2005; Pastorelli *et al*., 2003; Yazdani *et al*., 2003).

PCR amplification of SSR loci typically produce stutter or shadow bands. These bands are usually differs in size from the actual SSR allele by multiple of the repeat unit length. In this study, stutter bands were detected in DMAG04, DMAG05 and DMAG10. Example of stutter bands produced by DMAG05 primer is shown in Fig. 3. The stutter bands were mainly occurred in the perfect dinucleotides (DMAG05 and DMAG10) and simple imperfect trinucleotide repeats (DMAG04). A similar result was also reported by Valk *et al*. (2005) as dinucleotides are usually display higher stutter than tri- and tetranucleotide repeats. For example, three bands per individual were detected at DMAG05 locus (L1, Fig. 3). To study the stutter bands, sequence analysis of these three bands was carried out. The results showed that the stutter band (DMAG05-allele 2) lacks two CT repeat units at position 125-128 bp (4 bp) relative to the actual SSR band (DMAG05-allele 3) (Fig. 4). Besides that, a substitution of T (DMAG05-allele 3) to C (DMAG05-allele 2) was also observed at SSR region (at position 100 bp). In agreement with Walsh *et al*. (1996), the longer alleles (DMAG05-allele3) in heterozygous individuals exhibit a greater degree of stutter band formation than the shorter alleles (DMAG05-allele 1) at the same locus. Walsh *et al*. (1996) also reported that stutter bands are primarily caused by the slippage of *Taq* DNA polymerase during amplification. As explained by Shinde *et al*. (2003), *Taq* DNA polymerase slippage rate increases with the number of repeat units and inversely correlated with repeat unit length.

Another well-known reason is the addition of an extra A residue to the 3'-end of the amplification product by the *Taq* DNA polymerase used in PCR. The formation of stutter band is also affected by other factors such as length of the repeat flanking sequence, denaturation temperature and number of PCR cycles (Olejniczak and Krzyzosiak, 2006). Stutter bands are usually complicated with the interpretation of DNA profiles due to difficulties in allele determination and heterozygotes may be confused with homozygotes (Weising *et al*., 2005). According to Butler (2005), stuttering can be reduced by using SSR primers with longer repeat units, SSR alleles with imperfect repeat units and DNA polymerases with faster processivity.

To verify the newly developed SSR markers amplified the targeted SSR repeats, the selected PCR products were cloned and sequenced. For instance, the DMAG05 primer pair amplified the desired SSR region in *D. moluccana* genome and the SSR flanking region was highly conserved (Fig. 5). It was also observed that SSR alleles exhibited complex patterns of mutation including changes in the number of SSR repeat units, base substitution and insertions/deletions (indels) within and outside the microsatellite motif. A substitution of mononucleotide T to C and G to C were observed at positions 84 bp and 128 bp, respectively. A substitution at position 84 bp was interrupted the $(CT)_{19}$ long perfect repeat motif into $(CT)_2CC(CT)_{12}$ imperfect repeat motif. Such interrupted motifs tend to reduce the slippage rate (Ellegren, 2004) and leads to the birth of new SSRs (Sethy *et al*., 2006).

As explained by Kruglyak *et al*. (1998), such mechanism is important in the life cycle of SSRs which is essentially a balance between expansion by slippage and degradation by point mutations. Harr *et al*. (2000) also stated that the interruptions in a repeat track may be only a transitional state and could be removed by DNA replication slippage or reverse mutations. Addition of CT repeat units in repetitive tracts of DMA05-allele 1 (Fig. 5) was caused by polymerase slippage during DNA replication or slipped strand mispairing. Besides, it was also found that another 3 bp additions (at position ranged from 117 to 119 bp) flanking the SSR region (DMA05-allele 1) (Fig. 5). Such addition is a major contributor to a length polymorphism and subsequently increased the product size from 164 bp (DMA05-allele 2) to 174 bp (DMAG05-allele 1).

The mutation patterns of SSRs are far more complex which may not only be based on repeat number but also the region flanking the SSRs (Rallo *et al*., 2000). Indel of a single base or even long DNA fragments in the flanking regions has been reported as a source of variation in SSRs (Lia *et al*., 2007). According to Folkertsma *et al*. (2005), the indels in the flanking regions are more frequent and thus responsible for most of the variation in allele size rather than changes in SSR repeat. This phenomenon is also observed in maize (Matsuoka *et al*., 2002) and almond (Xie *et al*., 2006) that the allelic divergence at SSR loci is caused by the indels in their flanking sequences.

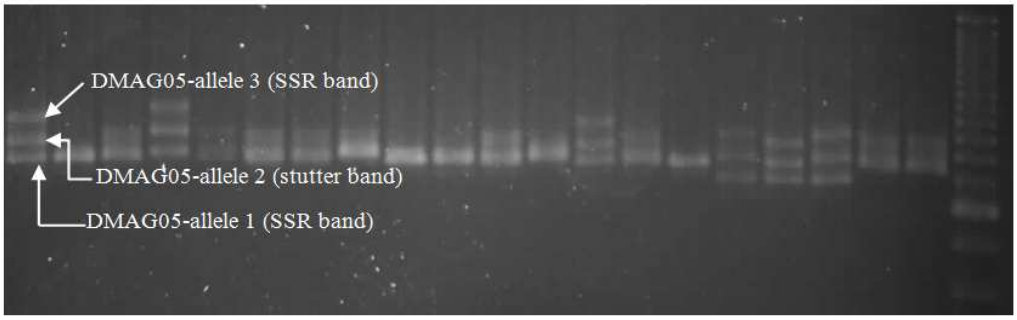

Fig. 3. Polymorphism of a SSR marker, DMAG05 in *D. moluccana*. Electrophoresis of amplified products on 3.5% metaphor agarose gel. M: 25 bp DNA ladder (Invitrogen, USA)

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                 Forward primer binding site
                 >>>>>>>>>>>>>>>>>>>>
DMAG05-allele1   TGTGTGCTCATTGCTTTCTTGATCACGTGAAAAAGGCTTTGTTTTTGTGGGCTGGGCGGC  60
DMAG05-allele2   TGTGTGCTCATTGCTTTCTTGATCACGTGAAAAAGGCTTTGTTTTTGTGGGCTGGGCGGC  60
DMAG05-allele3   TGTGTGCTCATTGCTTTCTTGATCACGTGAAAAAGGCTTTGTTTTTGTGGGCTGGGCGGC  60
                 ************************************************************

DMAG05-allele1   CCCTCTGCCTTGCTTTGCCTCTCTCTCTCTCTCTCTCTGTCTGTCT-------------- 106
DMAG05-allele2   CCCTCTGCCTTGCTTTGCCTCTCTCTCTCTCTCTCTCCCTCTCTCTCTCTCTCTCTCT  120
DMAG05-allele3   CCCTCTGCCTTGCTTTGCCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT  120
                 ************************************   ** ***

DMAG05-allele1   --------GCGTTGTGTCTCTCTGGTTGTGGTGGAGACCAAAATAGACGAAGAGAGAGCC  158  (CT)10GTCTGTCT
DMAG05-allele2   CTCT----GCGTTGTGTCTCTCTGGTTGTGGTGGAGACCAAAATAGACGAAGAGAGAGCC  176  (CT)10CC(CT)12
DMAG05-allele3   CTCTCTCTGCGTTGTGTCTCTCTGGTTGTGGTGGAGACCAAAATAGACGAAGAGAGAGCC  180  (CT)25
                         ************************************************

                                                  <<<<<<<<<<<<<<<<<<<<<<<
                                                  Reverse primer binding site
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Fig. 4. Sequence alignments of three SSR alleles in *D. moluccana* amplified by DMAG05. Asterisks (*) indicate nucleotide conservation. The repeat regions are marked in bold and point mutation is highlighted in grey. Gaps (-) indicate the absence of nucleotides in given alleles

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                 Forward primer binding site
                 >>>>>>>>>>>>>>>>>>>>
DMAG05-allele1   TGTGTGCTCATTGCTTTCTTGATCACGTGAAAAAGGCTTTGTTTTTGTGGGCTGGGCGGC  60
DMAG05-allele2   TGTGTGCTCATTGCTTTCTTGATCACGTGAAAAAGGCTTTGTTTTTGTGGGCTGGGCGGC  60
                 ************************************************************

DMAG05-allele1   CCCTCTGCCTTGCTTTGCCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTGTGT  120
DMAG05-allele2   CCCTCTGCCTTGCTTTGCCTCTCCCTCTCTCTCTCTCTCTCTCTCTC----------T  110
                 ***********************  *************************          *

DMAG05-allele1   CTGCGTTGTGTCTCTCTGGTTGTGGTGGAGACCAAAATAGACGAAGAGAGAGCC  174 (CT)19
DMAG05-allele2   CTGCGTTCTGTCTCTCTGGTTGTGGTGGAGACCAAAATAGACGAAGAGAGAGCC  164 (CT)2CC(CT)12
                 *******  ************************************************

                                                  <<<<<<<<<<<<<<<<<<<<<<<
                                                  Reverse primer binding site
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Fig. 5. Sequence alignment of SSR alleles from two different individuals of *D. moluccana*. Asterisks (*) indicate nucleotide conservation. The repeat regions are marked in bold and point mutations are highlighted in grey. Gaps (-) indicate the absence of nucleotides in given alleles

Such allele size variation was considered deviations from the expectations of Stepwise Mutation Model (SSM) (Lia *et al*., 2007). As explained by Ellegren (2004) many theoretical models fail to accurately describe allele frequency distributions in natural populations. Bhargava and Fuentes (2010) also stated that SMM and Two Phase Model (TPM) may only provide adequate measures in populations that are closely related than unrelated taxa.

The level of polymorphism of an SSR usually increases with repeat number (Ellegren, 2004). Xu *et al*. (2008) demonstrated that SSR markers with length more than 30 bp were significantly more polymorphic than other markers with length less than or equal to 30 bp. In this study, a 15 repeats DMACAG01 marker $[(AC)_6(AG)_9]$ was found to be more polymorphic (PIC = 0.792) than the longest repeat microsatellites, DMAG05 (19 repeats) $[(CT)_{19}]$ (PIC = 0.785) and DMTCAC11

$[(GA)_{19}]$ (19 repeats) (PIC = 0.729). Therefore, our results showed that the number of repeat units is not always associated with the degree of polymorphisms. Similar results were also observed in other studies, e.g., Dayanandan *et al*. (1998) in *Populus tremuloides*, Brondani *et al*. (1998) in *Eucalyptus* spp., Yu *et al*. (1999) in *Phaseolus vulgaris,* Chiba *et al*. (2003) in *Cucumis melo*, Rallo *et al*. (2000) in *Olea europaea* and Ma *et al*. (2009) in genus *Fagopyrum*.

Mutation rates vary considerably among different repeat motifs. In this study, dinucleotide loci showed a higher polymorphism level than trinucleotide loci in term of number of alleles as well as PIC values. For example, the number of alleles and PIC value of DMACAG01 with compound perfect dinucleotide repeats $(AC)_6(AG)_9$ (7 alleles) (PIC = 0.792) were higher compared to DMTCAC04 with simple perfect trinucleotide repeats $(ATA)_7$ (5 alleles) (PIC = 0.650). Schug *et al*. (1998) had estimated that dinucleotide repeats mutate at rates 6.4 and 8.4 times higher than tri- and tetranucleotide repeats, respectively in *Drosophila melanogaster*. Meanwhile, the average PIC value among dinucleotide repeats (0.48) was reported to be higher than trinucleotide repeats (0.33) in Pigeonpea (Odeny *et al*., 2007). Similar differences in levels of polymorphisms between these two classes were also reported in *Melaleuca alternifolia* (Rossetto *et al*., 1999), *P. abies* (Scotti *et al*., 2002), *Lycopersicon esculentum* (He *et al*., 2003) and *Persea Americana* (Ashworth *et al*., 2004). Li *et al*. (2004) also stated that dinucleotide motifs are usually associated with more repeat numbers and making them the best source of highly polymorphic SSR markers.

Deviation from Hardy-Weinberg Equilibrium (HWE) was analyzed using Chi-square test ($p<0.05$). In this study, 20 loci deviated significantly from HWE ($p<0.05$) (Table 1). This might be due to the excess of homozygotes and the presence of null alleles (Selkoe and Toonen, 2006). They further explained that homozygotes excess (also known as heterozygote deficit) can be due to biological realities of violating the criteria of an ideal population, such as strong inbreeding, selection or against for a certain allele. In addition, the *D. moluccana* seedlings used for SSR polymorphism analysis were collected from the nursery of Forest Tree Seed Bank, Sarawak Forestry Corporation. These seedlings are originated from the same source from the Batu Niah, Sarawak and thus, this might cause the increase in the number of homozygotes or genetic similarity among the seedlings.

Cross-species amplification of SSRs loci is considered as a cost-effective approach in developing locus specific markers for new species (Yasodha *et al*., 2005). According to Kutil and Williams (2001), a high sequence similarity among primer binding sites and repeat motifs is treated as orthology for trans-species SSRs. In plants, successful transferability of SSR markers between species has been demonstrated in numerous taxa, including *Vitis* (Arnold *et al*., 2002), *Pinus* spp. (Gonzalez-Martinez *et al*., 2004), *Ficus* spp. (Nazareno *et al*., 2009) and *Salix/Populus* spp. (Hoshikawa *et al*., 2009).

The relatively high transferability of *D. moluccana* SSR markers was observed in *D. grandiflora*, as 21 out of 25 markers resulted into amplicons of expected size. This indicates that a very high level of sequence conservation exists in DNA sequence flanking SSRs between *D. moluccana* and *D. grandiflora.* On the other hand, a relatively large evolutionary distance may explain the low success rate of transferability of *D. moluccana* (Sonneratiaceae) SSR markers to an unrelated taxa belonging to the *N. cadamba* (Rubiaceae), *C. odontophyllum* (Burseraceae) and *S. parvifolia* (Dipterocarpaceae). As explained by Rao *et al*. (2007), there is significant inverse relationship between SSRs performance and evolutionary distance of the species. Rossetto (2001) also stated that the cross-species amplification of SSR loci was 76.4% at genus level and 35.2% at the family level. Moreover, other factors such as transposition, chromosomal rearrangements and insertions/deletions also could affect the transferability of the SSR markers across species. In the present study, the level of polymorphism of each transferable SSR marker was not determined since only two samples per species were tested. Overall, the results demonstrated that the *D. moluccana* SSR markers were transferable on other tree species and would become useful tools for population genetic studies.

Conclusion

This study has demonstrated that ISSR-suppression PCR methods are suitable for developing SSR markers for species with little genomic information. It is a relatively simple and rapid without enrichment and screening and the time for procedures has also been shortened. The development of an array of SSR markers in this study would be very useful for genetic diversity study among natural populations of *D. moluccana*. In fact, this is the first available information of genomic SSR markers in *D. moluccana*. Knowledge of the levels and patterns of genetic diversity is valuable for tree breeders to accurately identify genetically diverse populations, selection of plus trees, provenance trials and establishment of Seed Production Areas (SPAs) of *D. moluccana* in the selected natural stands for tree improvement and conservation activities.

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Author's Contributions

Kit-Siong Liew: Participated in all the experiments, data analysis and contributed to the writing of the manuscript.

Wei-Seng Ho: Participated in some of the experiments, coordinated the data analysis and contributed to the writing of the manuscript.

Shek-Ling Pang: Designed the research plan and organized the study and participated in the data-analysis and contributed to the revision of the manuscript.

Julaihi Abdullah: Designed the research plan and organized the study, including plant sampling.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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