Development and characterization of microsatellite markers in sawih tree (*Duabanga moluccana* Blume) using ISSR-suppression PCR techniques

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Abstract *Duabanga moluccana* (or locally known as sawih) is an indigenous fast growing tropical tree species that confers various advantages for the timber industry and for planted forests development. In this paper, we isolated and characterized 8 polymorphic microsatellite markers from the *D. moluccana* genome using ISSR-suppression PCR techniques. The number of alleles and PIC values ranged from 3 to 8 alleles per locus and from 0.488 to 0.792, respectively. Three microsatellite loci were deviated from Hardy-Weinberg equilibrium ($P < 0.05$). The transferability rate ranged from 24 to 100 % among the three indigenous tree species tested. This indicates that the newly developed microsatellite markers would be useful tools for population genetic studies on *D. moluccana* and other indigenous tree species.

Keywords Microsatellite markers · ISSR-PCR · *Duabanga moluccana* · Planted forest · Population genetics

Rapid socio-economic changes in the world are having profound impacts on all sectors, including forestry. While demand for wood products is increasing, so is the demand for environmental services of forests. However, these slow growing trees with long generation intervals are unable to meet current global demand for wood, resulting in the loss and degradation of natural forests by logging (Fenning and Gershenzon 2002). The alternative now is to farm trees in plantations composed

of fast-growing species with short rotation cycle of 6 to 8 years. The rationale is that natural forests at the most produce about 3 m³/ha/year of timber, whereas plantations can produce annually from 10 to 30 m³/ha of timber (Krishnapillay and Razak 2001). Hence, plantations development will serve as a strategy for maintaining a sustainable supply of timber and at the same time, reducing the logging pressure on natural forests for wood production to an acceptable level. In the present study, we have developed and characterized microsatellite markers from the genome of *Duabanga moluccana* (sawih) that would be useful for generating baseline genetic information for tree improvement and conservation programmes of this species. *D. moluccana* has been targeted as one of the fast growing indigenous tree species for planted forest development in Sarawak, Malaysia (Ho et al. 2010). It is widely distributed at 15–750 m altitude on damp clay-rich fertile soils, especially in high light intensity areas such as rivers-banks, forest-edges, logged-over forests, road-sides, abandoned cultivation sites and limestone hills (Bojo 1995). The trees are of great economic importance for production of various wood works and products such as plywood, veneer and pulping.

Microsatellite markers were isolated using two ISSR-suppression PCR techniques as proposed by Lian et al. (2001, 2003, 2006). This method has been used by researchers in developing microsatellite 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). Total genomic DNA was extracted from young leaves of *D. moluccana* according to the protocol modified from Doyle and Doyle (1990). Five ISSR primers, (AC)₁₀, (AG)₁₀, (GTG)₆, (AC)₆(AG)₅ and (TC)₆(AC)₅ were employed. The ISSR-PCR amplification was carried out in a Mastercycler Gradient PCR (Eppendorf, Germany). The amplification products were resolved on 1.5 % agarose gels and fragments of 400 bp - 3 kb were recovered by using the QIAquick gel

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Table 1 Primer sequences and characteristics of eight polymorphic microsatellite markers in *Duabanga moluccana*

Locus	Repeat motif	Primer sequence (5'-3')	T _a (°C)	Product size range (bp)	A	PIC	GenBank accession no.
DMAC01*	(GA) ₃ CACC(GA) ₇	F: GTACAGCACGCACATGACAC R: GTAGGCCGCTATGAAACCAG	55	193-210	5	0.668	HQ679905
DMAC08	(TG) ₂ ACAAA(TG) ₅	F: AATGCGTGTGCCAATTTTAC R: GCGACACTGATACCTAAGTCTGTT	52	130-175	3	0.488	HQ679906
DMAG05	(CT) ₁₉	F: TGTGTGCTCATTGCTTTCTTG R: GGCTCTCTCTTCGTCATTTTGG	54	150-250	7	0.785	HQ679908
DMAG06*	(CT) ₄ TTTT(CT) ₃ G (TTTTC) ₂	F: TCATCACGAGACGACTGACC R: GGGGTACAATCTAAATTGATCGAG	52	237-350	8	0.739	HQ679909
DMAG10	(CT) ₈	F: TTCCTCTCCTCGGTTTCTTG R: TGCCTCTCTTCTTCTTGAAC	51	180-250	7	0.745	HQ679910
DMACAG01	(AC) ₆ (AG) ₉	F: ACACACACACACAGAGAGAGAG R: TGAGCTCATTGTAGTAGAGACAAG	55	230-300	7	0.792	HQ679907
DMTCAC04	(ATA) ₇	F: GGGGTACGGTTGAGTACAGC R: AGTGAAGCGCAGGGTATTTG	54	200-280	5	0.650	HQ679911
DMTCAC11*	(GA) ₁₉	F: CTGGCTATAACAGGGCCAAA R: CACACACACACTATACTCCACCTC	59	200-275	5	0.729	HQ679912

T_a = Annealing temperature of the primer pairs; A = Number of alleles

* Indicates significant deviation from Hardy-Weinberg equilibrium ($P < 0.05$)

extraction kit (Qiagen, Germany). The purified fragments were ligated with pGEM-T-Easy Vector (Promega, USA) overnight and then transformed into JM109 *E. coli* competent cells (Promega, USA). The recombinant plasmids were isolated and purified according to the Wizard® Plus SV Minipreps DNA Purification system protocol (Promega, USA). The purified plasmids DNA were sequenced using Applied Biosystems 3730xl DNA Analyzer. Then, the initiating primers (IP1 and IP2) were designed from the ISSR region of sequences with Primer3 v.0.4.0 (Rozen and Skaletsky 2000). Adaptor-primers for nested PCR, AP1 (5'CCATCC TAATACGACTCACTATAGGGC3') and AP2 (5'CTATAG GGCACGCGTGGT3') were also prepared.

Genomic DNA was separately digested with *AluI*, *EcoRV*, *HaeIII*, *RsaI* and *SspI* restriction enzymes. The restricted fragments were ligated to a specific adaptor (consisting 48-mer blunt adaptor: 5'GTAATACGACTCACTATAGGGCAC GCGTGGTTCGACGGCCCGGGCTGGT3' and an 8-mer with the 3-end capped by an amino residue: 5'ACCAGCCC-NH₂3') using a T4 DNA ligase (Invitrogen, USA). Next, the sequence of the other flanking region of each microsatellite was determined by the genome walking method (Siebert et al. 1995). The primary PCR reaction was performed with each constructed DNA library using IP1 and AP1 primers. The secondary PCR reaction was then performed with a 100-fold dilution of primary PCR products using IP2 and AP2 primers. The resultant single band in some libraries were cloned and sequenced as described above. A total of 44 microsatellite repeats were identified by using SSRfinder (<http://www.csufresno.edu/ssrfinder/>). Twenty microsatellite repeats were identified based on Lian et al. (2001) and the remaining microsatellite repeats were based on Lian et al. (2006)

protocols, and 43 microsatellite repeats were selected for primer design using the Primer3 v.0.4.0 (Rozen and Skaletsky 2000).

A total of 20 *D. moluccana* individuals were genotyped to assess the polymorphism of each microsatellite locus. PCR was performed in 25 µl reaction mixtures containing 1 × PCR buffer, 0.2 mM dNTPs, 1.25 mM Mg²⁺, 5 pmol of each primer, 10–30 ng of template DNA and 0.5 U of *Taq* DNA polymerase (Invitrogen, USA). The PCR conditions were: 3 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, T_a °C annealing temperature for 30 s, 72 °C for 1 min, followed by 5 min at 72 °C. Alleles at individual locus were separated using 3.5 % metaphor agarose gel and stained with Gelstar® nucleic acid gel stain (Lonza, USA). Validation of all microsatellite loci was also conducted by DNA sequencing

Table 2 Cross-species amplification of *Duabanga moluccana* microsatellite markers in three indigenous tropical tree species

SSR locus	<i>D.grandiflora</i>	<i>N.cadamba</i>	<i>S.parvifolia</i>
DMAC01	+	MP	MP
DMAC08	+	–	+
DMAG05	+	+	+
DMAG06	+	–	–
DMAG10	+	–	–
DMACAG01	+	MP	MP
DMTCAC04	+	–	–
DMTCAC11	+	–	–

“+” indicates expected amplifiable products; “MP” indicates multiple PCR products without expected sizes and “–” indicates unexpected PCR product sizes or without PCR products

using Applied Biosystems 3730xl DNA Analyzer. The variability at each locus: the number of alleles (A), polymorphism information content (PIC) 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). The presence of null allele was also tested using Micro-Checker Version 2.2.3 (Oosterhout et al. 2004).

Of the 43 primer pairs tested, 25 amplified the expected PCR products meanwhile 17 produced unexpected PCR products or multiple bands. One primer pair (DMAG03) did not generate amplification products in most of the *D. moluccana* samples. Eight out of 25 primer pairs yielded polymorphic PCR products and selected for further characterization (Table 1). Allelic diversity ranged from 3 to 8 alleles per locus, with an average of 5.88 and the PIC values ranged from 0.488 to 0.792, with an average of 0.70. This result was also comparable to other published findings using the same method of developing microsatellite markers (Sui et al. 2009; Liu et al. 2013). Three loci (DMAC01, DMAG06 and DMTAC11) deviated significantly from HWE ($P < 0.05$) due to the excess of homozygotes and the presence of null alleles.

The transferability of these markers was further investigated to other indigenous tree species. Some of the markers either had produced no clear PCR products or amplified multiple PCR products without expected sizes. The success rate of transferability was varied, ranging from 100 % in *Duabanga grandiflora*, 12.5 % in *Neolamarckia cadamba* and 25 % in *Shorea parvifolia* (Table 2). This indicates that the newly developed markers would be useful tools for population genetic studies on *D. moluccana* and other indigenous tropical tree species.

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