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PRIMER NOTE

Development of microsatellite markers in *Gonystylus bancanus* (Ramin) useful for tracing and tracking of wood of this protected species

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Abstract

Ten polymorphic microsatellite markers have been developed for *Gonystylus bancanus* (Ramin), a protected tree species of peat swamp forests in Malaysia and Indonesia. Eight markers were also shown to be polymorphic in other *Gonystylus* species. The markers will enable assessing the amount of genetic variation within and among populations and the degree of population differentiation, such that donor populations can be selected for reforestation projects. They may be used for tracing and tracking of wood in the production chain, so that legal trade in this Convention on International Trade in Endangered Species of Wild Fauna and Flora-protected timber species, derived from specifically described origins, can be distinguished from illegally logged timber.

Keywords: CITES, logging, peat swamp, Sarawak

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Peat swamp forests are threatened tropical ecosystems because of extensive forest conversion into agricultural land and other uses. As a typical peat swamp tree, *Gonystylus bancanus* (Ramin) still has a wide distribution in Malaysia and Indonesia, but the number of adult individuals within populations is rapidly decreasing, even though the species is protected (IUCN 2006). For the Sarawak government's effort to restore them, ecological and population-genetic aspects of this species should be considered. This includes the amount of genetic variation present and the degree of population differentiation, which is important information for selection of the most suitable donor populations for reforestation. To be able to study the population genetics, we have developed microsatellite markers.

Genomic DNA of *Gonystylus bancanus* was isolated from leaf samples of 20 trees from two populations in Sarawak (Malaysia) with the QIAamp DNA Stool Mini Kit (QIAGEN). In addition, DNA was extracted from 47 samples of seven other *Gonystylus* species, also collected in Sarawak (Table 2). Di- and trinucleotide repeat microsatellite loci were isolated from a genomic DNA library using an enrichment procedure after Karagyozov *et al*. (1993) as modified by and described in detail by van de Wiel *et al*. 1999) and Esselink *et al*. (2003). In short, 500 ng genomic DNA was restricted–ligated with *Alu*I, *Rsa*I or *Hae*III, pooled and amplified. Microsatellite-containing fragments were selected by hybridization to Hybond N + membranes with separately spotted synthetic oligonucleotides: $(GT)_{12}$ $(GA)_{12}$ $(TCT)_{10}$ $(TGT)_{10}$ $(TGA)_9$ $(AGT)_9$ $(CGT)_8$ $(GCT)_8$ $(GCC)_7$. The fragments were eluted from the membranes with increasing stringencies, up to 0.1% sodium dodecyl sulphate for 10 min at 100 °C. Eluted fragments were precipitated, re-amplified and cloned. The clones were screened for the presence of repeat sequences by spotting and hybridization with ^{33}P-labelled oligonucleotides. We obtained 2446 colonies of which 193 (7.9%) were positive: 134 from the (GT) enrichment, 19 from the (GA) enrichment, and only 40 from all trinucleotide motifs together. Twenty-seven unique repeats were identified with sufficient flanking sequences to enable primer design using PRIMERSELECT (DNAStar). Reverse primers were

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Table 1 Characteristics of the microsatellite markers developed

Locus*	EMBL Accession no.	Repeat motif†	Primer sequences (5′–3′)	Dye§	Calculated T_m	Size of cloned fragment (bp)	Quality¶	Number of alleles**	Effective number of alleles††	H_O	H_E
WGb06	AM423091	$(CGG)_{11-4}$	F: CTTGGCCAGATCGACCCTCGACTT R: GTTTGTTCCCCCGTCGGTGATGATTTTC	NED	62	207	1	5	1.7	0.3	0.425
WGb17	AM423093	$(TCT)_{7-1}$	F: GTAGTCATTAACGGTGGGAAGGTT R: GTTTGTTATCAATGCACTACAAAGACC	NED	50	315	1	12	7.3	0.85	0.886
WGb22	AM423096	$(GT)_{18}$	F: GCACGCAAGGCCTATCTGAGTGTA R: GTTTGAGGGCATGTGGTAGAGGGAAACT	NED	57	290	2	9	4.4	0.65	0.79
WGb23	AM423097	$(GT)_{21}$	F: TTGAGAGGGCGACACATTGAGAGT R: GTTTGCATATGTGTATGGCATGTGATGTA	6-FAM	58	> 311	2	7	4	0.6	0.769
WGb24	AM423098	$(GT)_{18}$	F: AGGGCACAACTTCATGGGCATCA R: GTTTGAACCCGAACTTATAGGTGACAGC	HEX	53	341	2	9	5.2	0.75	0.827
WGb29	AM423099	$(GT)_{23-2}$	F: ATGATAGTCACGCAAATGATGGTG R: GTTTCCGCCCTAAAGTTGCAATAAAGT	NED	53	129	2	11	6.5	0.65	0.867
WGb32	AM423101	$(GA)_{23}$	F: CTCAAAACACAATCAACAGAAAAT R: GTTTCTTTGATGAAGACGAAGAAATGT	6-FAM	51	312	2	16	10.3	0.8	0.926
WGb37	AM423104	$(GT)_9$	F: ACGCGTGCAGTGGGCAGGTG R: GTTTCCAAAACCAGAAGGCTCAAAACAA	HEX	55	170	1	7	4.1	0.7	0.777
WGb38	AM423105	$(GT)_{11}$	F: TTTTCTTTGCCAAGGATTTATCTT R: GTTTAATTATTGGAGCAGCACACTACAA	HEX	52	280	1	6	3.4	0.65	0.723
WGb39	AM423106	$(GT)_{11}$	F: CATGGCTTGCCAAATATACATAGG R: GTTTAAGGCAAGGCTTCATCAAATCTTC	6-FAM	53	156	2	5	2.6	0.6	0.633

*In addition, nine other polymorphic microsatellite markers of poorer quality [rated according to Smulders *et al*. (1997) as quality 3 (many stutter bands that hamper accurate genotyping) and quality 4 (additional products amplified)] were submitted to the EMBL database as well: AM423089, AM423090, AM423092, AM423094, AM423095, AM423100, AM423102, AM423103 and AM423107; †minus sign denotes mismatch; ‡including pigtail (GTTT; Brownstein *et al*. 1996); §the forward primers were dye-labelled; FAM- and HEX-labelled primers as well as unlabelled primers were from Biolegio (Nijmegen, The Netherlands). NED-labelled primers were from Applied Biosystems; ¶quality rating according to Smulders *et al*. (1997); quality 1: clear products, easily scorable; quality 2: few stutter bands but well scorable; **in 20 plants from two populations on Sarawak; ††*Ne*, H_O and H_E calculated with POPGENE 1.32 (Yeh *et al*.; http://www.ualberta.ca/~fyeh/info.htm); Hardy–Weinberg and linkage disequilibrium among loci was calculated with FSTAT 2.9.3 (Goudet, J. 2001. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available from http://www2.unil.ch/popgen/softwares/fstat.htm).

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Table 2 Result of the cross-species amplification of the 10 microsatellite markers on samples from seven other *Gonystylus* species

Microsatellite marker	Species (number of plants tested)						
	G.affinis (6)	*G.borneensis* (4)	*G.micranthus* (9)	*G.lucidulus* (9)	*G.forbesii* (2)	*G.nobilis* (8)	*G.stenosepalus* (9)
WGb06	P*	Pn	P	P	P	P	Pu
WGb17	Pu	Pu	P	Pu	P	Pnu	Pn
WGb22	—	—	—	— †	—	— †	— †
WGb23	—	—	—	—	—	—	—
WGb24	Pn	Pn	Pn	P	P	P	P
WGb29	P	P	P	P	P	P	P
WGb32	Pn	Pn	P	Pn	P	Pn	P
WGb37	P	P	P	P	P	P	P
WGb38	P	Pu	P	P	P	Pn	P
WGb39	P	P	Pu	Pu	P	Pu	P

*P, amplification of polymorphic bands; *n,* no amplification in two or more samples; u, probably null alleles (inferred from an excess of homozygotes); –, no amplification; †amplification observed in one plant, which was heterozygous.

pigtailed by adding a GTTT sequence at the 5′–end. This was carried out as *Taq* DNA polymerases may add a nontemplate adenosine to the 3′-end of some products during the polymerase chain reaction (PCR). Pigtailing leads to nearly 100% adenylation of the 3′–end of the forward strand (Brownstein *et al*. 1996), which facilitates the interpretation of the peaks and therefore contributes to accurate genotyping (Bredemeijer *et al*. 1998). Amplification was performed in 20-µl reaction volume containing 10 ng of genomic DNA, 4 pmol of each primer, 100 µM of each dNTP, 10 mM Tris-HCL pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2–2.5 mM $MgCl_2$ and 0.2 U Goldstar*Taq* DNA polymerase (Eurogentec). PCR conditions were 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 45 s and a final extension at 72 °C for 10 min, using a slope of 1 °C/s, on an MJ PTC200. Amplification products were separated on a 6% denaturated polyacrylamide gel and visualized by silver staining (Promega Silver Sequence DNA Sequencing System). Of the 27 loci, 19 were polymorphic. In line with the relative frequency of repeats in the positive clones, most of these were (GT) repeats, and only two (WBg05 of poor quality and WBg06 of good quality) were trinucleotide repeats.

We selected the most easily scorable markers (see Table 1). For these 10 markers, we amplified samples from 20 plants with fluorescently labelled primers, separated them on an ABI PRISM 3700, and analysed the results using GENOTYPER 3.1 (Applied Biosystems). The markers amplified 5–16 different alleles. The effective number of alleles ranged from 1.7 (for WBg06, the only trinucleotide repeat marker) to 10.3. Consistent with the outbreeding behaviour of the tree species, most sampled trees were heterozygous at these polymorphic loci. Observed heterozygosity was between 0.6 and 0.85 for all markers except WBg06, for which it was only 0.3. No deviation from Hardy–Weinberg was detectable, and the loci were not in linkage disequilibrium with each other. The percentage positive clones (7.9%) and the fraction of polymorphic markers from these clones (9.8%) are in the range of about 10% efficiency for each of the steps, as we have often found in microsatellite enrichments in other species, including tree species (van de Wiel *et al*. 1999; Van der Schoot *et al*. 2000; Pastorelli *et al*. 2003).

We also tested the 10 markers on samples from seven other *Gonystylus* species on an ABI PRISM 3700 (Table 2). WBg23 did not amplify in any species, and WBg22 only rarely, but the eight other markers consistently amplified polymorphic bands in the other species, although with some drop-out of samples (depending both on marker and species). Null alleles were very likely present in some species for markers WBg17 and WBg39.

The microsatellite markers developed here will enable studying genetic diversity and population differentiation of Ramin populations in Malaysia and Indonesia, such that donor populations can be selected for reforestation projects. In addition, they may be useful for tracing and tracking of wood in the production chain (Koopman & Diemont 2004), so that legally logged wood (from specific sites or plantations) can be distinguished from illegally logged wood.

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