ONLINE RESOURCES

Novel polymorphic microsatellite markers for the helophytic plant species *Hanguana malayana* (Jack) Merr. (Commelinales: Hanguanaceae)

SITI NURFAZILAH ABDUL RAHMAN, ASYRAF MANSOR, PETER C. BOYCE and AHMAD SOFIMAN OTHMAN*

School of Biological Sciences, Universiti Sains Malaysia (USM), 11800 Minden, Penang, Malaysia

[Rahman S. N. A., Mansor A., Boyce P. C. and Othman A. S. 2012 Novel polymorphic microsatellite markers for the helophytic plant species *Hanguana malayana* (Jack) Merr. (Commelinales: Hanguanaceae). *J. Genet*. **91**, e15–e17. Online only: http://www.ias.ac.in/jgenet/OnlineResources/91/e15.pdf]

Introduction

Hanguana malayana (Jack) Merr. is a large to massive colonial dioecious helophyte producing extensive spongy stolons and reproduce both sexually and asexually. It is a plant of open lowlands along muddy banks of large rivers, and the margins of freshwater bodies, and freshwater swamp forest. It ranges from Sri Lanka to western Micronesia (Caroline Islands: Palau), south as far as northern Australia and north to the Philippines (Luzon), and peninsular Thailand below the Isthmus of Kra (Siti Nurfazilah *et al.* 2010). Throughout its range, *H. malayana* is morphologically stable, leading to the speculation that the majority of populations may be clonal, reproducing primarily by asexual processes.

Clonal plant populations are made up of a few genets and numerous genetically similar ramets (Li *et al.* 2006). A genet is composed of all tissues originating from single sexually produced zygote, whereas a ramet is an independent part of a genet arisen through clonal replication (Harper 1977). To understand the diversity of population structures and the dynamics of clonal plants, such as *H. malayana* which reproduce by both asexual and sexual reproduction, genetic studies are required.

The goal of this study was to identify and characterize microsatellite containing sequences for *H. malayana*. These data will be invaluable for a broad range of applications in genetic research of *H. malayana,* including population structure, and spatial distribution of genets and ramets.

Microsatellite markers are highly polymorphic, with high variability of repeating units (Pung *et al.* 2000; Mizuki *et al.* 2005) and further, are easily and economically assayed by polymerase chain reaction (PCR) (McCouch *et al.* 1997; Temnykh *et al.* 2000). Use of microsatellite markers enable detection of high levels of allelic diversity, and are therefore ideal for ecological and population studies.

Material and methods

Microsatellites were isolated from *H. malayana* following Jones *et al.* (2001) and Edwards *et al.* (1996), with modifications as per Nguyen *et al.* (2007). Approximately 500 ng of genomic DNA was digested with *Rsa*I, and ligated to *Mlu*I adaptor. The PCR was performed in a total volume of 50 μL containing 10× PCR buffer, 2 mM dNTPs, 5 U AmpliTaq Gold (Applied Biosystems, California, USA), 25 mM $MgCl_2$, 5 ng of ligated DNA, and 500 ng of one of the oligos of the *Mlu*I adaptor. PCR thermal conditions were: 10 min at 95°C, followed by 30 cycles of a three-step PCR at 94°C for 15 s, 60°C for 1 min, and 72°C for 3 min, and a final extension of 72°C for 15 min. PCR products were hybridized to a Hybond-N+ membrane consisting of 5–14 repeats of dinucleotide, trinucleotide, and tetranucleotide motifs, $(A)_{21}$, $(AT)_{20}$, $(AAT)_{15}$, $(GA)_{20}$, $(CA)_{20}$, $(AG)_{20}$, $(GT)_{20}$, $(AGA)_{15}$, $(ACA)_{15}$ and $(CAT)_{15}$, $(CTA)_{15}$, $(AAC)_{15}$, $(AAG)_{15}$, $(ATC)_{15}$ at 50°C for 18–48 h. Unhybridized DNA was removed, and the membrane boiled to elude the hybridized products. The eluted DNA was amplified using the same conditions as above, then ligated into pGEM®-T Easy Vector (Promega, Wisconsin, USA), transformed into JM109 *Escherichia coli* competent cells (Promega), and plated on agar containing ampicillin, IPTG and X-gal. Plasmids from recombinant colonies were amplified using TempliPhi (GE Healthcare, Buckinghamshire, UK). After blue–white screening, 61 positive clones were sequenced. A total of 24 primer sets were designed using the Primer3 Plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), and synthesized. Optimization of amplification conditions for each locus followed, and the variability of isolated markers was assessed in 88 individuals of *H. malayana* from Gopeng, Perak in peninsular Malaysia. Genomic DNA was extracted from leaf tissue of *H. malayana* using cetyltrimethyl ammonium bromide (CTAB) methodology (Doyle and Doyle

*For correspondence. E-mail: sofiman@usm.my.

Keywords. Hanguanaceae; microsatellite markers; SSR; *Hanguana malayana*.

Table 1. Characteristics of nine polymorphic microsatellite loci in *Hanguana malayana* (Jack) Merr.

Locus	Primer sequences (5′ →3′)	Repeat motif	Size range (bp)	N_A	T_a (°C)	H_o	H_e	HWE *P* value	Accession no.
HM01	F: 5′- TGTTGTGCAGAGAGACTGAGG-3′ R: 5′- GCAGTCATTTCCTTGGGACA-3′	$(GA)_{17}$	161–163	3	58	0.920*	0.510	>0.0001	HQ891861
HM02	F: 5′- CACGGCAGTCTGATGAATTT-3′ R: 5′-TGCGAACTCCAAACATCGTA-3′	$(CAA)_7$	143–148	3	58	0.966*	0.523	>0.0001	HQ891862
HM03	F: 5′- GACTAGGATCTCATCCTCCTCTG-3′ R: 5′- ACAGGACTCCGCTTAGACCA-3′	$(GA)_{24}$	153–156	5	61	0.443	0.456	>0.0001	HQ891863
HM04	F: 5′- CTGGGACTATTGCTCCCTCA-3′ R: 5′- CACAGCTCCCTCACTCACAA-3′	$(GAA)_{18}$	151–159	8	61	0.943*	0.535	>0.0001	HQ891864
HM05	F: 5′- TTGTTTTCTTGGGCAGAAGG-3′ R: 5′- TGGACTAGTGGATGAACTTGAGA-3′	$(GA)_{18}$	139–154	12	58	0.966*	0.824	>0.0001	HQ891865
HM06	F: 5′- CAACGGACTTGCCTCCAAC-3′ R: 5′- TCTGGACTATCTCGCATTTCA-3′	$(GA)_{25}$	138–144	5	58	0.954*	0.670	>0.0001	HQ891866
HM07	F: 5′- GATCTGGACTAACAGATTAAAGAAGC-3′ R: 5′- GATGGGCCAACAAGTATTGC-3′	$(TGCG)_7$	392–396	3	58	0.506	0.550	>0.0001	HQ891867
HM08	F: 5′- CTGGACTAGCTGATGCCACA-3′ R: 5′- TTCCTTCCTTTCACATTATCACAA-3′	$(CT)_{20}$	299–309	9	58	0.952*	0.847	>0.0001	HQ891868
HM09	F: 5′- TTAAGCTGTTGGAGTATTGTCATT-3′ R: 5′- TCTGGACTAAATGGCCTTGG-3′	$(CT)_{20}$	235–240	6	55	0.216	0.288	>0.0001	JF268670

N_A, number of alleles; T_a, annealing temperature; H_o, observed heterozigosity; H_e, expected heterozigosity; *Denotes significant departures from Hardy–Weinberg equilibrium.

1987). PCR amplifications were conducted in a total reaction volume of 25 μL, consisting of 1.5 μL of template DNA (approximately 100 ng), 1× Magnesium Free Green GoTaq® Flexi buffer, (1.5–3.0) mM $MgCl_2$, 2 mM dNTPs mixtures, 10 nmol of each forward and reverse primer, and 1 U *Taq* DNA polymerase (Promega). Amplification was performed using a Thermal Cycler PTC-200 DNA Engine (MJ Research, Massachusetts, USA). The PCR profile was initially denaturated at 94°C for 2 min, followed by annealing of 35 cycles of 94°C for 30 s (see table 1, annealing temperature), and 72°C for 1 min, and finally 1 cycle of 72°C for 10 min.

Fragment analysis was conducted using an ABI3730XL sequencer, GeneMapper v4.0 analysis software, and Peak Scanner v1.0 (Applied Biosystems). Number of alleles (N_A) was calculated using POPGENE version 1.31 (Francis and Yang 1999). Expected heterozygosity (H_e), observed heterozygosity (H_o), and significant linkage disequilibrium for all loci was analysed using Arlequin v 3.0 software (Excoffier *et al.* 2005).

Results and discussion

Of the 24 microsatellite markers tested, nine loci were polymorphic and remaining loci were not easily amplified, or produced stutter bands. The nine loci are the first polymorphic microsatellite markers reported for *H. malayana*. Information obtained from each microsatellite is summarized in table 1. The number of alleles ranged from 3 to 12 with an average of 5.67 alleles per locus. The observed and expected heterozygosities ranged from 0.216 to 0.989 and from 0.288 to 0.842, respectively. Significance of departures from Hardy–Weinberg equilibrium (HWE), and linkage disequilibrium (LD) between pairs of microsatellite loci were tested by using Arlequin v3.0 (Excoffier *et al.* 2005). Six loci (HM01, HM02, HM04, HM05, HM06, and HM08) showed significant excess of heterozygotes and deviation from HWE. The deviation from HWE at all six loci is probably owing to *H. malayana* being primarily a clonal plant reproducing asexually. Observed heterozygosities of the loci were higher than expected ($H_o > H_e$), perhaps due to asexual reproduction. There was significant pairwise LD for loci HM01, HM02, HM03, HM04, HM05, HM06, HM07 and HM08. It is possible that somatic mutations produce genetic variation in this particular *H. malayana* population. Herms and Mattson (1992) suggested that somatic mutations provide clonal organisms with a mechanism for enhancing their survival conditions. Clonal populations are often assumed to have low levels of recruitment through seed, and are expected to become dominated by only one or few large genets (Shimizu *et al.* 2002).

In conclusion, these novel microsatellite markers show sufficient levels of polymorphism to be used as molecular markers for accurate population genetics studies, and for assessing genetic variations.

Acknowledgements

We are grateful for the assistance of Amir Shahrudin, Rahmad Zakaria, Nurul Salmi Abdul Latip, and Khairul Nasirudin Abu Mangsor from the School of Biological Sciences, Universiti Sains Malaysia. This study was funded by Research University Grant from the Universiti Sains Malaysia, Penang, and funds from the Malaysian Remote Sensing Agency. The first author is gratefully acknowledges a USM fellowship for financial support.

References

Doyle J. J. and Doyle J. L. 1987 A rapid isolation procedure for small amounts of leaf tissue. *Phytochem. Bull.* **19**, 11–15.

Edwards K. J., Barker J. H. A., Daly A., Jones C. and Karp A. 1996 Microsatellite libraries enriched for several microsatellite sequences in plants. *BioTechniques* **20**, 758–760.

Excoffier L., Laval G. and Schneider S. 2005 Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evol. Bioinformatics Online* **1**, 47–50.

Francis C. Y. and Yang R. C. 1999 PopGene v. 1.31, Microsoft Window–based freeware for population genetic analysis. University of Alberta and Center for International Forestry Research.

Harper J. L. 1977 *Population biology of plants*. Academic Press, London, UK.

Herms D. A. and Mattson W. J. 1992 The dilemma of plants: to grow or defend. *Q. Rev. Biol.* **67**, 283.

Jones E. S., Dupal M. P., Kolliker R., Drayton M. C. and Forster J. W. 2001 Development and characterization of simple sequence repeat (SSR) markers for perennial ryegrass (*Lolium perenne* L.). *Theor. Appl. Genet.* **102**, 405–415.

Li W., Wang B. and Wang J. 2006 Lack of genetic variation of an invasive clonal plant *Eichhornia crassipes* in China revealed by RAPD and ISSR markers. *Aquat. Bot.* **84**, 176–180.

McCouch S. R., Chen X., Panaud O., Temnykh S., Xu Y., Cho Y. G. *et al.* 1997 Microsatellite marker development, mapping and applications in rice genetics and breeding. *Plant Mol. Biol.* **35**, 89–99.

Mizuki I., Tani N., Ishida K. and Tsumura Y. 2005 Development and characterization of microsatellite markers in a clonal plant, *Dioscorea japonica* Thunb. *Mol. Ecol. Notes* **5**, 721–723.

Nguyen T., Baranski M., Rourke M. and McPartlan H. 2007 Characterization of microsatellite DNA markers for a mahseer species, *Tor tambroides* (Cyprinidae) and cross-amplification in four congeners. *Mol. Ecol. Notes* **7**, 109–112.

Pung C. C., Tan S. G., Rusea G. and Yusoff K. M. 2000 Diversity and genetic differentiation of Malaysian Oaks (*Quercus* spp.) revealed by microsatellite markers. Proceedings of the 4th National Congress on Genetics, pp. 88–90, Genting Highlands, Malaysia.

Shimizu Y., Ando M. and Sakai F. 2002 Clonal structure of natural populations of *Cryptomeria japonica* growing at different positions on slopes, detected using RAPD markers. *Biochem. Systemat. Ecol.* **30**, 733–748.

Siti Nurfazilah A. R., Ahmad Sofiman O., Mohd Fahmi A. B. and Boyce P. C. 2010 Studies on *Hanguana* (Commelinales, Hanguanaceae) for Sunda II: Five new forest species from Peninsular Malaysia and recircumscription of *Hanguana malayana*. *Willdenowia* **40**, 205–219.

Temnykh S., Park W. D., Ayres N., Cartinhour S., Hauck N., Lipovich L. *et al.* 2000 Mapping and genome organization of microsatellite sequences in rice (*Oryza sativa* L.). *Theor. Appl. Genet.* **100**, 697–712.

Received 21 August 2011, in revised form 20 October 2011; accepted 21 October 2011
Published on the Web: 9 March 2012