Journal of Tropical Forest Science 30(2): 252–258 (2018)
https://doi.org/10.26525/jtfs2018.30.2.252258

DEVELOPMENT OF MICROSATELLITES USING NEXT-GENERATION SEQUENCING FOR *ACACIA CRASSICARPA*

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Submitted May 2017; accepted November 2017

Acacia crassicarpa is a tropical forestry species that is increasingly being planted in South-East Asia. Using next- generation sequencing, we developed 12 nuclear microsatellite markers for *A. crassicarpa* and optimised them for assay in three multiplex sets. We tested the 12 loci on 34 *A. crassicarpa* samples and found that polymorphism ranged from 4 to 8 alleles per locus (average = 5.8). The loci were easy to score in terms of binning and their reproducibility and polymerase chain reaction success rate were high. Given their usability and polymorphism we believe that these 12 loci will be useful for DNA profiling and mating system analysis with direct application in breeding programmes and the conservation of wild populations. Six of the markers also amplified products in related *A. mangium* and *A. auriculiformis*, with four being polymorphic in three samples of each species. Therefore, these markers added more broadly to the genomic resources available in *Acacia*.

Keywords: DNA profiling, genetic improvement, simple sequence repeats (SSR), multiplex microsatellites, *Acacia* silviculture, genetic resources

INTRODUCTION

Plantation forestry using *Acacia* species is increasingly providing an alternative to native forest harvesting in Southeast Asia (Nambiar & Harwood 2014). A key challenge for improving the sustainability of *Acacia* forestry in Asia, is increasing the efficiency of breeding programmes (Griffin et al. 2015). The long-lived nature of trees presents a barrier to the rapid development of improved varieties using traditional breeding approaches. As such, modern tree breeding programmes are increasingly applying molecular markers to improve the efficiency of production and planting systems (Butcher et al. 2000, Ng et al. 2005, Grattapaglia & Kirst 2008). Molecular markers are useful for DNA profiling (fingerprinting) of individuals and tracking clones (Kirst et al. 2005) and hybrids (Le et al. 2016); revealing the genetic structure of breeding populations (Freeman et al. 2007, Jones et al. 2006, Le et al. 2017); identifying paternity and outcrossing rates in open pollinated seed orchards (Griffin et al. 2010, Patterson et al. 2004), including in *Acacia* (Le et al. 2016, 2017, Muhammad et al. 2017); and are expected to form the basis of marker-assisted selection programmes in the near future (Grattapaglia & Kirst 2008, Muranty et al. 2014, Muhammad et al. 2017).

Microsatellite markers have been developed to assist breeding in two of the main acacia plantation species *A. mangium* and *A. auriculiformis* as well as their widely deployed hybrid (Butcher et al. 2000, Ng et al. 2005). However, no molecular markers have been specifically developed for the increasingly important species, *A. crassicarpa*. *Acacia crassicarpa* is native to northern Australia and southern Papua New Guinea (McDonald & Maslin 2000). It is one of the fastest growing tropical acacias, and has the best mechanical strength and fibre properties among eight species tested in Kalimantan by Laurila (1995). It is also an excellent fuel wood, and is used in restoration programmes, particularly in *Imperata* grasslands in Indonesia (McDonald & Maslin 2000). There are now over 700,000 ha of *A. crassicarpa* plantations in South-East Asia, mainly in Indonesia (Nambiar & Harwood 2014). An operational set of microsatellite markers would benefit both breeding and genepool conservation in *A. crassicarpa*.

Microsatellite primers are often transferable between closely related species (e.g. Butcher et al. 2000, Nevill et al. 2008). However, despite belonging to the same taxonomic section, Juliflorae (McDonald & Maslin 2000), this does not appear to be the case between *A. mangium* and *A. crassicarpa*. Of the seven microsatellite (simple sequence repeats) primers developed for *Acacia mangium* (Butcher et al. 2000), only four produced clear amplification products in *A. crassicarpa*, and those loci were monomorphic and likely to be uninformative (Tran Duc Vuong, personal communication). Therefore, given recent advances in microsatellite discovery from next-generation sequencing (Grover & Sharma 2016), we decided to take a *de novo* approach to marker development for *A. crassicarpa*. This study aimed firstly to identify microsatellite regions in *A. crassicarpa* and develop primers for their assay in cost-effective multiplex combinations; secondly to examine allelic variation in the loci in *A. crassicarpa*; and thirdly to assess the transferability of the loci to closely related species.

MATERIALS AND METHODS

Marker development

Sequence analysis

Acacia crassicarpa has a relatively large genome at 1350 Mbp (Mukherjee & Sharma 1995), which is approximately double that of *A. mangium* at 635 Mbp (Blakesly et al. 2002). In order to capture an ample number of reads to find microsatellites of an appropriate length and repeat sequence type, a 1/4 run was undertaken on the Genome Sequencer FLX Titanium platform at Flinders University Adelaide, Australia. Sequencing of *A. crassicarpa* sample from a cultivated plant which was part of a commercial breeding programme in Indonesia was successful. Using cultivated plant was convenient, and ensured that the markers developed would be directly applicable to the breeding stock—additional optimisation could be required if a sample was taken from a genetically divergent wild population. The sample used had the highest quality and concentration of DNA among the 34 samples extracted. All DNA sequences are available at the Sequence Read Archive (http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?study=SRP065046). The DNA sequences were analysed for appropriate microsatellite regions using the bioinformatics software QDD. A search was run to identify sequences containing eight or more repeats of any di- to hexanucleotide sequence, capable of being amplified to yield a polymerase chain reaction (PCR) product of 90–450 nucleotides (bp) in length. QDD identified 872 potentially useful microsatellite sequences, including 226 imperfect (compound or interrupted) repeats and 646 perfect repeats. The majority (91.6%) of perfect repeats were dinucleotide repeats and the remainder were trinucleotide (7.6%) and tetranucleotide (0.8%) repeats.

Initial screening of microsatellite loci

For initial screening, we selected primer pairs for 48 possible microsatellite loci based on the following criteria:

(1) Only loci with perfect repeats were included, because these were more likely to be polymorphic than imperfect repeats (Smulders et al. 1997)
(2) Microsatellites with flanking regions containing mononucleotide repeats, excessive noncoding DNA and/or poor primer-binding sites (in apparently rapidly evolving regions or within 10 bp of the microsatellite sequence) were rejected
(3) Where possible microsatellites having the longest repeat numbers were chosen, since higher repeat numbers were reported to give rise to higher polymorphism (Smulders et al. 1997)
(4) A range of PCR product sizes between 90 and 340 bp were selected to allow multiplexing of the final set of loci
(5) Tri- and tetranucleotide repeats were given priority, as these might be easier to score than dinucleotide repeats (Rongwen et al. 1995)
(6) All primers were chosen to have an annealing temperature of approximately 60 °C using the software Primer3 to facilitate multiplexing.

In order to test the primers, DNA was extracted from 34 *A. crassicarpa* samples, each representing a unique individual, using DNeasy plant minikit (QIAGEN) and suggested QIAGEN protocol, with the exception that the incubation temperature was reduced to 55 °C, following Griffin et al. (2010). Unlabelled

primer pairs were used to initially test for amplification products, and PCR was conducted for all 48 loci separately using a panel of four *A. crassicarpa* samples. The PCR reaction mix contained 1× Mango *Taq* reaction buffer. 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 2 U Mango *Taq*, 0.2 µM of each primer and 10 ng DNA in a 25 µL reaction. Thermocycling was conducted at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, followed by a final extension of 72 °C for 10 min. PCR products were visualised on agarose gels stained with GoldView™.

The initial screening showed that of the 48 primer pairs tested, 31 successfully amplified products for all samples on the first attempt, while 17 produced little or no amplification (data not shown). The 31 successful primer pairs were further tested using eight different *A. crassicarpa* samples, and the products were separated on 2% agarose gels to detect size polymorphisms. Loci that failed to amplify in more than one sample, or showed no detectable size polymorphism using the eight *A. crassicarpa* samples, were rejected. The 20 loci showing the most consistent amplification at 55 °C, combined with the highest apparent degree of polymorphism, were selected as candidates for multiplexing (Table 1).

Screening microsatellite loci for multiplexing

The software Multiplex Manager was used to determine the best multiplex combinations of the 20 chosen loci. The most efficient combination involved four sets of loci, two containing four loci each and two containing six loci each. Fluorescently labelled forward primers were purchased from (FAM and HEX) and (NED and PET), and the 20 loci were screened using a panel of seven *A. crassicarpa* samples. The PCR mix contained 1× multiplex PCR mastermix (QIAGEN), all multiplexed primers (labelled and unlabelled) at 0.2 µM each, except 6-FAM-labelled primers which were used at 0.1 µM each, and 4 ng template DNA per 10 µL reaction (see Table 1 for primer sequence, dye used for each primer pair and multiplex composition). Thermocycling conditions were as follows: denaturation at 95 °C for 15 min; followed by 30 cycles of 94 °C for 30 s, 55 °C for 90 s, and 72 °C for 60 s, followed by a final extension of 72 °C for 15 min. Following PCR, the presence of amplified DNA was verified by running 5 µL of product on a 2% agarose gel. An aliquot of each PCR reaction (1 µL) was dried and sent to the Australian Genome Research Facility (AGRF), Plant Genomics Centre, University of Adelaide, for capillary fragment separation on the DNA analyser. Fragment data from AGRF were analysed in our laboratory using the software GeneMapper to bin (score) alleles for each locus.

Based on results from this round of testing, five loci (cras11, 15, 16, 18 and 19; Table 1) were rejected on the grounds of excessive/artefactual peaks or failure to bin as predicted (e.g. binning inconsistently relative to the expected motif length), and the remaining loci were recombined into three multiplex sets. Locus cras34 was also dropped due to difficulty in multiplexing it with other loci.

Selection and evaluation of the final set of microsatellite loci

The 14 remaining loci were tested on all 34 *A. crassicarpa* individuals and two more loci were deemed unsuitable (cras39 and 46; Table 1), leaving 12 loci (listed as retained in Table 1) in three multiplex sets (A, B and C; Table 1). These loci were used to genotype all remaining *A. crassicarpa* samples including replicates of samples 2, 4, 6 and 8.

Marker evaluation

The genetic information content of the final 12 microsatellite loci was calculated using data from the 34 *A. crassicarpa* individuals. The software INEst (Chybicki & Burczyk 2009) was used to calculate the statistics *A* (number of alleles), H_o (observed heterozygosity), H_e (expected heterozygosity), *F* (Wright's inbreeding coefficient) and *f*n (frequency of null alleles) (Table 2). The program GenAlEx version 6.5 was used to calculate the chi-square probability of deviation from Hardy–Weinberg equilibrium for each locus, and the probability of identity for each locus as well as the cumulative probability of identity (Table 2). Reproducibility was tested by running replicates of samples 2, 4, 6 and 8 in separate PCRs on separate occasions and comparing allele scores across runs. To test cross-species transferability, the final 12 primer pairs were tested on three samples each of *A. mangium* and *A. auriculiformis*. DNA extraction and PCR procedures for these samples were as described above for *A. crassicarpa*.

Table 1 Details of the 20 microsatellite loci selected for multiplex testing in *Acacia crassicarpa*

Locus	Allele range (bp)	Repeat motif	Forward primer	Dye label	Reverse primer	Test result	Multiplex set
cras01	74-92	AAT	GCTCACCTTCATTTCCACAAA	6-FAM	CAGGATGTTATTGACCTCTGAGC	Retain	B
cras07	96-114	AAT	TAGCGAGGCGACCTTACAAC	PET	GAACGTGGAGGAAGCAGG	Retain	B
cras09	101-117	AAC	ATGGCGTCGTTCCATGTAGT	HEX	AAACAAAATCCATCCACTATTCA	Retain	C
cras11	101-113	AGT	CGCACCAGATAGGATGAAGA	PET	CTTGGATGCTATTGTGGCCT	Artefactual peaks	-
cras13	117-127	AT	CACGAATCAACCAAGAAGCA	HEX	TCCAGTGGAGCCTATTATTCTTTC	Retain	B
cras15	109-123	AAAT	TGAAAGCCACATTATGTCTTGG	NED	CATTTGGTTTGCATGGTCTG	Not multiples of 4	-
cras16	109-127	ACT	AAGGCGATATTTAGAGGCCC	HEX	CATGGTTTCTCACGATAAGGG	> 2 alleles	-
cras18	122-156	ACAT	TCCGTCATTATTTTCCCTCTGT	HEX	TCAGGTATTTGGTAAGAGTAAATTGTC	Extra peak	-
cras19	118-134	AAT	CAACTTTTAAGATGAGGCCGA	PET	AATTTCGACACGTTAGGATACG	> 2 alleles	-
cras20	136-178	AT	GCCAGATTGTGCATGTTGTT	6-FAM	TGGTAGTAGAGGGTAAAGTATTACAGC	Retain	A
cras21	127-161	AT	CAGGGATCCAATTTAACCGA	6-FAM	GAACGCGCTTCAAGGATTAC	Retain	C
cras24	130-145	AGG	GAAGGAGCTGTGAATGGAGC	NED	CACTCAACCTTCCCTCTTCG	Retain	B
cras28	157-167	AT	TAATCCCTGGCCACATTGTT	6-FAM	TGCGTCTTTGTTTGTATGGAG	Retain	B
cras33	174-183	AAT	TCGTCAGGCTGATGGATGTA	NED	AGACGCTACAGTGGTCTGGG	Retain	C
cras34	175-195	AT	ACTATAAGGATGTTGCGGCG	6-FAM	TGGAAGTTGTTTAAGTGGAGCA	Overlaps other loci	-
cras35	187-197	AC	TGAGGATGAAACCAAATAAGCC	HEX	CACAGCGAGTTTTCTCTTATACCA	Retain	A
cras39	168-238	AAT	TCCCAATTTTGCTTCTGATTC	PET	GAACTCGAGCTCAACAGCCT	> 2 alleles	-
cras43	246-260	AT	GGGTGGTTTGAGGTAACAAGG	6-FAM	GCCCTCCCTAGTAGACCCAC	Retain	A
cras46	283	AG	ATTCACCTTCCGCAATTTCA	HEX	CCCTAATCACATTGCAACCC	Monomorphic	-
cras47	303-313	AC	CTTGCTTTCAAATTCCCCTG	NED	TGACAATAGCATGGTTCAGCA	Retain	A

The 12 loci in bold were retained and optimised to be assayed in three multiplex sets (A, B and C)

RESULTS AND DISCUSSION

Polymorphism

The number of alleles per locus varied from four to eight with an average (*A*) = 5.8 (Table 2). This level is equivalent or slightly higher than that found in the related forestry species *A. mangium* (two to seven alleles/locus; Butcher et al. 2000) and *A. auriculiformis* (two to eight alleles/locus; Ng et al. 2005), where marker development was undertaken using traditional library enrichment methods prior to the advent of next-generation sequencing. Observed heterozygosity in the sampled genotypes was close to that expected for natural populations with most loci being in Hardy–Weinberg equilibrium. Wright's inbreeding coefficient (*F*) was very low across all loci and averaged only 0.05. However, despite this we did detect significant deviation from Hardy–Weinberg equilibrium at three loci (cras13, cras21 and cras24; Table 2). This was not surprising given that our samples came from a breeding programme consisting of individuals from different native provenances. Nor was it uncommon, for example Ng et al. (2005) found divergence from Hardy–Weinberg equilibrium in 7 of the 15 markers tested in *A. mangium*. One process that can drive deviation from Hardy–Weinberg equilibrium is the presence of null alleles, but there was little or no evidence for null alleles in the marker set (Table 2). The impact of deviation from Hardy–Weinberg equilibrium in the three loci here is likely to be minimal in individual-based genotyping applications such as DNA profiling for clone identification and ramet testing. However, if markers are used for population level analysis, care should be taken when including these three loci.

The cumulative probability of identity (PI +; Table 2) estimated the likelihood that two different individuals would have the same multi-locus genotype by chance. The 12 loci produced a cumulative probability of identity of 1.6×10^{-9}. It has been suggested in wildlife forensic cases that a probability of identity between 0.001–0.0001 (depending on populations size) is sufficent to distinguish individuals (Waits et al. 2001). Therefore the markers developed here will be very effective for individual genotyping applications and should be powerful enough to allow parentage analysis.

Reproducibility and usability

Analysis of repeat samples showed that allele scores were identical for replicates across all loci in all samples (0.0% error for a total of 96 allele scores). Error rates were not reported in the *A. mangium* and *A. auriculiformis* studies mentioned above, but in other forest trees allelic error rates can be as high as 8% in similar

Table 2 Genetic diversity parameters for the 12 final microsatellite loci developed for *Acacia crassicarpa*

Locus	*A*	*Ho*	*He*	*F*	*fn*	HW prob	PI	PI +
cras01	7	0.82	0.71	-0.16	0.00	0.70	0.159	1.6×10^{-1}
cras07	7	0.68	0.76	0.11	0.02	0.57	0.095	1.5×10^{-2}
cras09	6	0.44	0.50	0.11	0.03	0.98	0.290	4.4×10^{-3}
cras13	6	0.53	0.51	-0.04	0.00	0.00	0.259	1.1×10^{-3}
cras20	6	0.65	0.67	0.03	0.01	0.99	0.164	1.9×10^{-4}
cras21	8	0.74	0.76	0.03	0.00	0.00	0.102	1.9×10^{-5}
cras24	5	0.56	0.73	0.23	0.08	0.03	0.135	2.6×10^{-6}
cras28	5	0.56	0.59	0.06	0.00	0.99	0.240	6.1×10^{-7}
cras33	4	0.59	0.70	0.16	0.05	0.47	0.151	9.2×10^{-8}
cras35	4	0.38	0.36	-0.06	0.00	0.99	0.460	4.3×10^{-8}
cras43	7	0.68	0.72	0.06	0.00	0.99	0.131	5.6×10^{-9}
cras47	5	0.53	0.51	-0.04	0.00	0.89	0.285	1.6×10^{-9}
Average	5.8	0.60	0.63	0.05	0.02	0.64	0.206	

A = number of alleles, *Ho* = observed proportion of heterozygotes, *He* = expected (unbiased) proportion of heterozygotes, *F* = Wright's inbreeding coefficient, *fn* = frequency of null alleles, HW prob = probability of deviation from Hardy-Weinberg equilibrium, PI = probability of identity by locus and PI + = cumulative probability of identity

microsatellite studies (Bloomfield et al. 2011). The markers were also generally easy to score, with the exception of cras13 which required more careful checking due to stutter peaks. We noted that care should also be taken not to overload products from loci cras07 and cras09 during fragment separation as both loci had alleles close to the 100 bp standard peak. These alleles did not interfere with scoring of the standard peak unless grossly overloaded.

Transferability to other species

Transferability of these markers to *A. mangium* and *A. auriculiformis* found six primer pairs did not amplify (Table 3). Two primer pairs (cras09 and cras35) produced products that differed in size from those of *A. crassicarpa*, but these were not polymorphic in either *A. mangium* and *A. auriculiformis* across the small number of samples tested. Primer pairs cras13 and cras24 produced polymorphic products for both *A. mangium* and *A. auriculiformis*. Cras20 and cras43, (multiplex set A) were labelled with the same dye making them impossible to distinguish. One of these was polymorphic in both *A. mangium* and *A. auriculiformis*, and the other was polymorphic in *A. auriculiformis* (Table 3). We concluded that cras09, 13, 20, 24, 35 and 43 were potentially useful microsatellites for *A. mangium* and *A. auriculiformis*, and recommend further testing of these six loci using larger numbers of the target taxa.

Table 3 Transferability of the final 12 microsatellite loci developed in *Acacia crassicarpa* to *A. mangium* and *A. auriculiformis*

Locus	*A. mangium*	*A. auriculiformis*
cras01	-	-
cras07	-	-
cras09	mono	mono
cras13	poly	poly
cras20	poly	poly/mono*
cras21	-	-
cras24	poly	poly
cras28	-	-
cras33	-	-
cras35	mono	mono
cras43	poly	poly/mono*
cras47	-	-

The success of transferability is indicated for each loci: - = no product; mono = monomorphic, poly = polymorphic; *cras20 and cras43 used the same dye and in *A. auriculiformis* could not be distinguished, one is monomorphic and one is polymorphic

Acacia crassicarpa, A. mangium and *A. auriculiformis* all belong to the same taxonomic section, Juliflorae, subgenus *Phyllodineae* (McDonald & Maslin 2000). A large transferability study by Butcher et al. (2000) found that markers developed in *A. mangium* transferred most effectively to other species within section Juliflorae compared with other sections, subgenera and genera, as has been observed in *Eucalyptus* (Nevill et al. 2008). Section Juliflorae includes some of the most important acacia forestry species in South-East Asia (e.g. *A. mangium, A. auriculiformis, A. crassicarpa, A. cincinnata*) and it is possible that the markers identified as transferable here will also work in other members of this section. Thus, as well as their direct utility in *A. crassicarpa*, the transferable markers contribute to the growing genetic resources available for economically significant *Acacia* species in South-East Asia (Butcher et al. 2000, Nambiar & Harwood 2014, Ng et al. 2005).

CONCLUSIONS

We developed a robust set of 12 microsatellite loci, optimised in three multiplex sets, with reliable amplification, high polymorphism, and high scoring reproducibility in *A. crassicarpa*. These markers are suitable for genotyping to determine clonal identity, estimate outcrossing rates, undertake paternity analysis, and for general quality control—for example checking ortet–ramet matches in selection programmes. Six of the 12 markers are transferable to related species and contribute to the genetic resources available for acacia forestry species in South-East Asia.

ACKNOWLEDGEMENTS

We thank Gardner M for access to the sequencing platform at Flinders University, Adelaide, Australia. For assistance in the laboratory we thank Wise S. We also thank Griffin Tree Improvement Pty Ltd who provided funds and plant material for the project.

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