

Novel Microsatellite Markers for Conservation of Australian Native *Samadera bidwillii*

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Abstract

Microsatellite markers were developed for *Samadera bidwillii*, a nationally listed vulnerable shrub or small tree, to enable investigation of its genetic structure and diversity within and among populations from its known distribution throughout coastal areas mainly in fragmented and disturbed lands from Mackay to Gympie, Queensland, Australia. The loci were tested for cross-amplification in related *Samadera* species. Ten polymorphic microsatellite markers were isolated and characterised from an enriched library of *S. bidwillii*, which exhibited di- and trinucleotide repeats. The mean number of alleles per locus ranged from 1.3 to 2.5 and mean expected and observed heterozygosities ranged from 0.06 to 0.33 and from 0.03 to 0.26, respectively in five populations. All loci successfully amplified in six other closely associated *Samadera* species also reported from Australia. Developed loci can be used in genetic diversity, population structure and gene flow studies with an emphasis on the conservation of *S. bidwillii* and related species.

Keywords

454 Sequencing, Conservation, Microsatellites, *Samadera*, Native Shrub

1. Introduction

Samadera bidwillii (Simaroubaceae, a family known for medicinal compound quassinoids) is a native shrub listed as “vulnerable” (Australian EPBC Act 1999 and Queensland Nature Conservation (Wildlife) Regulation 2006) [1]. The species is restricted to Queensland and known in approximately 40 locations distributed from Mackay to Gympie [2]. The species occurrence has been extended by a coal mine within the Callide Ranges near Biloela in Central Queensland [3]. However, continued loss of its range is occurring due to insect damage, fire,

weed invasion, grazing and land clearing. These factors might have contributed to the reduction of genetic diversity level within populations leading towards potential risk of becoming endangered species. Information on partitions of genetic variation within and among populations is a pioneer for its conservation. To support the development of a conservation plan, understanding of the genetic variation in the species, its structure and gene flow patterns at the population level is vital. This genetic variation can be determined by differences in the number of small repeated DNA segment copies called microsatellites. Microsatellites are codominant, highly polymorphic and hyper-variable, robust and highly informative tools to assess inter-population comparisons. Earlier, microsatellite markers were developed in Australian threatened species [4] [5] and other Simaroubaceae genera [6] [7] [8] but not for the *Samadera* genus. The aim is to develop new microsatellite markers, which can be useful to find genetic variation at the population level in *S. bidwillii* but are also helpful in cross-amplification in other *Samadera* species that are also threatened.

2. Materials and Methods

Total genomic DNA was extracted using DNeasy Plant Mini-kit (Qiagen, Germany) from silica gel dried leaves of reference sample collected from an adult shrub sampled in a natural population located in the narrow strip of road reserve in Nikenbah, Queensland. To check the patterns of genetic variation, DNA was extracted from leaves of 30 adults each collected from five substantial populations of *S. bidwillii* (Figure 1). For cross amplification check, DNA was extracted from herbarium specimens except for *Samadera* sp. St Mary samples, which were collected from a natural stand found within St Mary State Forest (Figure 1).

The reference DNA elute sequenced at the Australian Genome Research Facility (AGRF, Brisbane), using GS-FLX Titanium KLR70 chemistry (454 Roche Applied Science, Germany) to build an enriched library. The sequences were trimmed to suitable length and filtered for redundancy and multiple copies in the sequence dataset using CLC Genomics Workbench 6.0 software (CLC Bio, Denmark). Microsatellites having a minimum of five repeats for di-nucleotides to hexanucleotides were identified using QDD v2.1 pipeline [9]. The possible primer combinations were viewed in an MS Excel (Microsoft Corp., Seattle) output file.

Unlabelled forward and reverse primer pairs for at least 60 microsatellite loci were targeted for design with suitable flanking regions using Primer 3 web 4.0.0 [10] [11]. Selection criteria such as product size within 100 to 400 bp, optimal primer size 20 bp, melting temperature 59°C, GC content 50%, and zero GC clamp were left default. Primer lengths less than 18 bp and more than 23 bp were eliminated from selections. Optimisation of PCR conditions on a Mastercycler® gradient thermocycler (Eppendorf, Germany) was performed for each locus using four randomly selected genomic DNA samples of *S. bidwillii*. The reaction

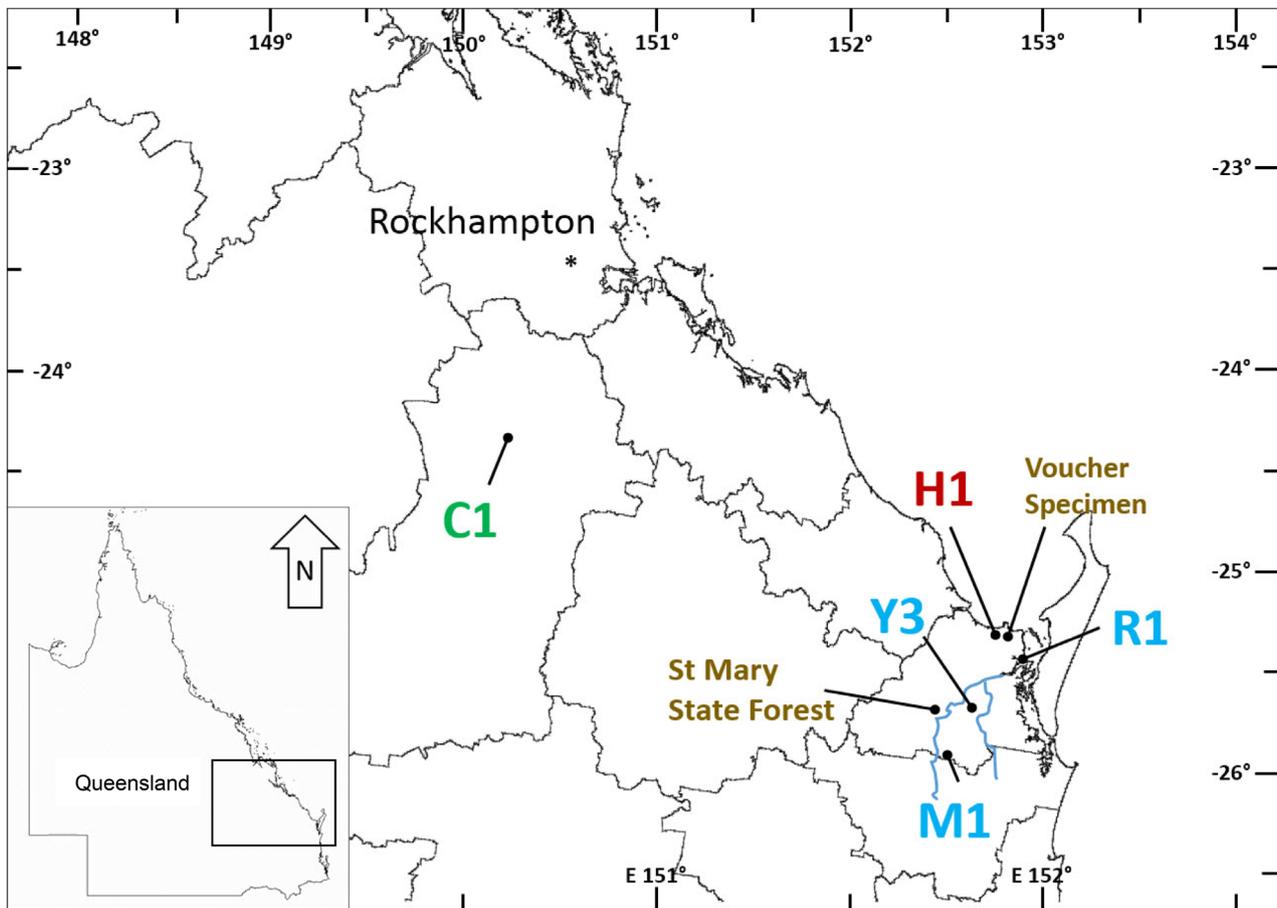


Figure 1. Location map of *Samadera* populations and voucher specimen used in this study. Geographic locations for the study populations: C1—Callide; H1—Hervey Bay; R1—River Heads, Queensland; Y3—Young State Forest, Tinana; M1—Mt Bauple. All populations are located in Queensland, Australia.

mix contained 1 μL genomic DNA, 1 x reaction buffer, 0.2 mM of each dNTPs, 1.5 mM MgCl_2 , 0.4 μM of each primer, 250 ng BSA and 0.25 U of *Taq* F1 DNA polymerase (Fisher Biotec, Australia) in a total volume of 12.5 μL . The reaction buffer was made up with 67 mM Tris-HCl with pH 8.8, 16.6 mM $(\text{NH}_4)_2\text{SO}_4$, 0.45% Triton X-100 and 0.2 mg/mL gelatin. A touchdown gradient program was set with an initial 3 min of denaturation at 95°C; 14 cycles at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s (example of 60°C - 53°C), and further 21 cycles at 94°C for 30 s, annealing at 53°C for 30 s and extension at 72°C for 30 s. The final extension was for 10 min at 72°C followed by incubation at 15°C. A temperature gradient decrease of 0.5°C in each PCR cycle was imposed for first 14 cycles.

The amplified PCR product was then visualised under UV on 1.5% agarose 0.6 \times TBE gel (140 volts; 40 min) containing 0.1 $\mu\text{L}/\text{mL}$ EtBr and sized with 100 bp DNA ladder (Fisher Biotec, Australia). Suitable primers with single and clear band patterns were identified at suitable annealing temperature and tested for potential polymorphism on 16 randomly selected DNA samples across the populations. The reaction was performed using the touchdown cycles with the same

PCR conditions by setting primers annealing at 55°C for 30 s in last 21 cycles. Subsets of potentially polymorphic primers were identified from images that showed the combination of repeatable clear bands within the expected size range by running PCR mix on 3% agarose 0.6 × TBE gel electrophoresis (140 volts; 110 min) (Figure 2).

The forward primer of each pair was labelled with one of the FAM, VIC, NED or PET fluorescent dyes to enable multiplexed reaction mixes for fragment analysis of all DNA samples [12]. For that, each mix was made up with 1 µL of 50 times diluted PCR product, 0.5 µL 600 LIZ Size Standard v2.0 (Applied Biosystems, USA) and 8.5 µL Hi-Di™ Formamide (Applied Biosystems, USA). These master mixed samples were denatured for three minutes at 95°C on heat plate followed by quick cooled in ice before analysed using POP 7™ polymer (Applied

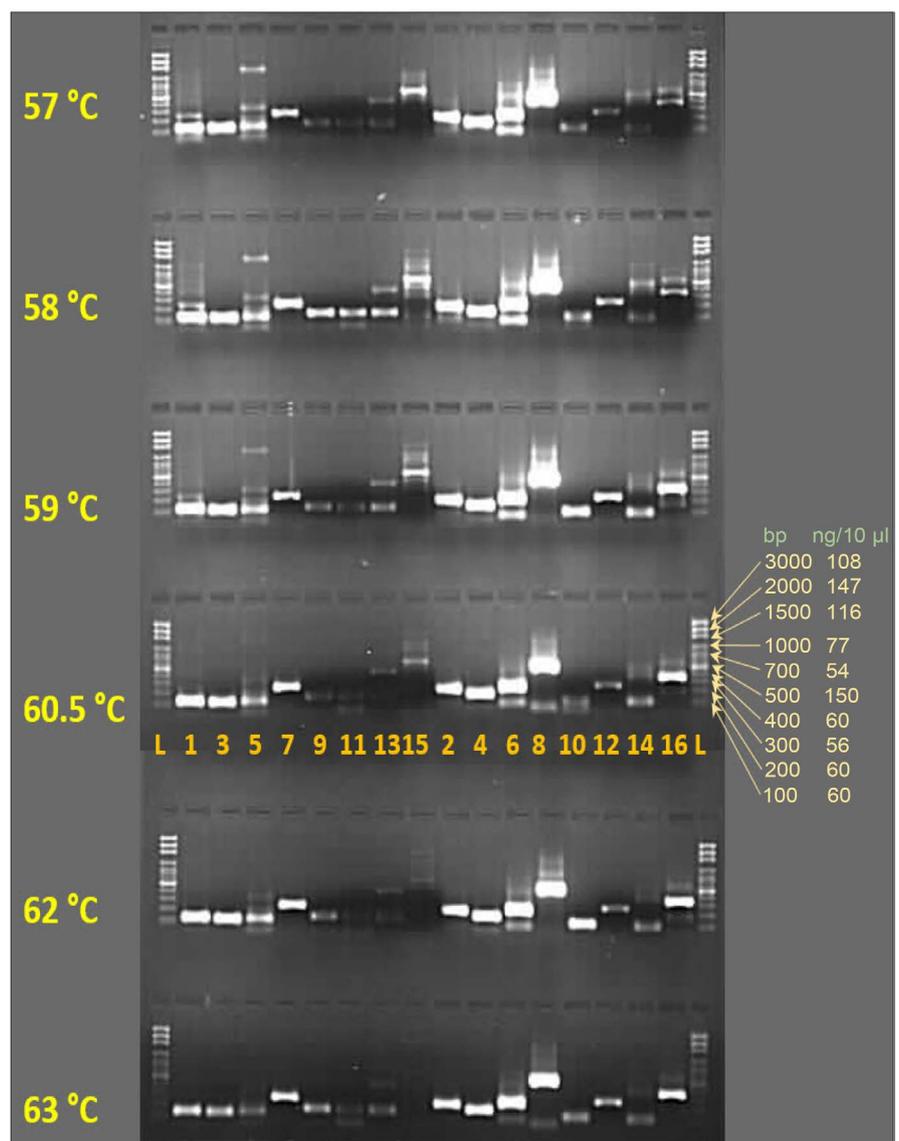


Figure 2. Primer bands patterns at different temperature gradients visualised under UV lights on 1.5% agarose gel. Primer SB01 to SB16, L = 100 bp ladder.

Biosystems, USA) under standard conditions in 8 capillaries AB 3500 Genetic Analyser (Applied Biosystems, USA).

Fragment sizes of the alleles were determined relative to the internal size standard using GeneMarker v.2.4.0 software (SoftGenetics, USA). Scoring was double-checked manually for consistency and accuracy. MICRO-CHECKER v2.2.3 [13] was used to check for evidence of extreme stuttering, large allele dropout and null alleles based on 1000 bootstraps and 95% confidence interval.

Parameters used to assess genetic diversity included a number of alleles per locus (A), observed (H_o) and expected heterozygosity (H_e) and multilocus matches analysis (unique MLGs) were calculated using the GenAlEx 6.5 software package [14]. A contingency Chi-squared test was performed for each locus to determine if each population site deviated significantly from expectations under the Hardy-Weinberg Equilibrium (HWE) using GenAlEx 6.5. In addition, six other *Samadera* species from Queensland and New South Wales were also tested for cross-amplification with the same-screened loci.

3. Results and Discussion

DNA extracted from leaf samples of *S. bidwillii* using the DNeasy[®] Plant Mini-kit resulted in a right quantity of DNA. Australian herbarium specimens assigned to *S. bidwillii* were reported to contain alkaloids and tannins, and these chemicals are known to interfere with DNA extraction in woody plants [15]. Thus, fast, reliable and high-quality DNA extraction methods are needed for *S. bidwillii* leaves. The DNeasy[®] Plant Mini-kit provided high-throughput isolation of high-quality DNA by removing impurities such as proteins, polysaccharides and enzyme inhibitors from *S. bidwillii* leaf samples [16].

The genomic DNA enriched library resulted in 508 sequences with a read length ranging from 150 to 529 (mean 372) nucleotides and 7488 possible primer combinations matching with quality criteria implemented in QDD pipeline. Next-generation sequencing was adopted as it is able to provide large volumes of data within a short time, making it superior to other technologies such as Sanger sequencing [17]. The result was an integration of microsatellite detection and a redundancy check performed to avoid amplification of multiple PCR products [18]. A total of 516 SSR regions were identified, having 364 di-, 133 tri-, 11 tetra- and 8 hexanucleotide repeats (with a minimum of five subunits for all repeats). According to Reference [19], there is no strict definition of the number of repeats required and even small repeats number could detect polymorphisms [20]. According to Reference [21], SSR-flanking regions also display microsatellite polymorphism, therefore right size DNA sequence with clear flanking regions from any repeated bases also equally crucial in selecting primers [20]. DNA sequences without microsatellite markers were discarded from this process.

Of the carefully designed 60 microsatellite loci, 57 were found simple repeats and three were compound repeats. All loci ranged from 5 - 12 subunits with a product size from 102 to 332 bp. Subsets of potentially polymorphic primers

were verified from images that showed a combination of repeated clear bands within the expected size range (obtained during optimisation of PCR conditions) for each locus obtained from four randomly selected genomic DNA samples.

Based on the variations recorded within 16 random DNA samples of *S. bidwillii*, 18 loci were selected for further testing (**Table 1**). The other 42 loci were discarded with no amplification or polymorphism was detected by gel electrophoresis after sequencing. Thus, the efficiency of simple sequence repeats development from the DNA sequencing data was 3.5% obtaining potential loci. Among the 18 loci, 10 carried a di-nucleotide motif and 8 carried a tri-nucleotide motif.

The 18 microsatellite loci were further tested using 150 *S. bidwillii* samples collected from five populations (each containing 30 samples). Of 18 microsatellite loci, a total of eight loci were detected with null alleles, or with inconsistent scorable bands. According to Reference [22], null alleles can cause deviation from *HWE*, causing substantial bias in the population genetic analysis. Hence, the eight microsatellite loci were discarded from further testing (**Table 1**).

Ten polymorphic microsatellite loci consisting of a total of 28 scorable alleles with firm and precise peaks were scored for the five population samples. In all loci, the number of alleles per locus was found to range from one to four, with the highest in locus SB37 (**Table 2**). The average mean number of alleles per locus (A) ranged from 1.3 to 2.5. H_O varied from 0 to 0.53 and H_E from 0 to 0.65, which was found to be comparatively higher than H_O across all loci. Based on the locus-wise observed and expected heterozygosity (H_E), SB37 has the maximum heterozygosity (0.53 and 0.65, respectively). Similar results have been recorded for *S. amara* (Simaroubaceae) [23] and *Davidsonia jerseyana* (an Australian endangered tree species) [24]. The deficiency of heterozygotes could be due to self-incompatibility or clonality in *S. bidwillii* observed during periodic field visits. There were a total of 69 unique genotypes recorded in multilocus matches analysis, ranged from one (C1) to 30 (H1). This variation may be due to phenotypic plasticity or mutation in *S. bidwillii* populations, where considerable differences in plant height, stem diameter leaf size and flower colour have been observed during field trips to collect leaves for this study.

A contingency Chi-squared test was performed for each locus to determine whether each population site deviated significantly from expectations under *HWE* in GenAEx 6.5. The results revealed significant departures for the loci SB34 and SB40 ($p < 0.05$) (**Table 2**). Furthermore, six other *Samadera* species from Australia were also tested with 10 loci (**Table 3**).

Satisfactory amplifications were achieved except for *Samadera* sp. from the Moonee Creek samples (which were found difficult to score for locus SB40). The number of alleles ranged from one to five, indicating that this set of loci could be used in population genetic studies involving species interrelation and delineation for associated *Samadera* species. Earlier studies proved successful as cross-species amplification of microsatellite markers among closely related taxa [25] [26] [27],

Table 1. Characteristics of eighteen primer pairs developed for *Samadera bidwillii*.

Locus		Primer sequences (5'-3')	Repeat motif	Size range (bp)	T _A (°C)	Label	GenBank accession no.
SB01*	F:	GAGGACATTCTGGACGCGAG	(AGG) ₁₀	120 - 126	56	FAM ^a	KP765244
	R:	ACACTTCAGTCTTCCAGCCC					
SB43	F:	AACTTTCATGCACATTCTTTCCA	(AT) ₅	186	56	VIC ^a	-
	R:	GGTATCATTTTCGCACTTTCATCA					
SB05	F:	CACATTCCAACAGGACCAACA	(AG) ₁₀	117	56	NED ^a	-
	R:	ACTTCTTTGTACGGGGATGT					
SB48*	F:	TGCAATTGTTAAGCAGAATGAGA	(AAG) ₇	211 - 217	56	NED ^a	KP765252
	R:	GGAACATTCTTGGGCTGTGC					
SB09*	F:	ACAGCTTAGTCTTTTAGTGCTCA	(AG) ₅	146 - 150	56	PET ^a	KP765245
	R:	TGGGCTGCTTCATAAATCTGA					
SB12*	F:	TCTTCGCTTCGTCGTTAAGGA	(AGC) ₃ (AGG) ₆	237 - 243	56	PET ^a	KP765246
	R:	GCCAAACAAGCAAAACAACAAGT					
SB03	F:	GCTAAGGTGAAAGAGCCCAT	(AG) ₆ (TG) ₃	113	56	FAM ^b	-
	R:	GGGCCATTGGAGACTCTGAC					
SB17*	F:	AGAGGCTCCATAATAATTGGACA	(AG) ₇	237 - 241	56	FAM ^b	KP765247
	R:	AACCCTACCACATTCCTGCG					
SB16	F:	AACAAGCTCATCCCCACTCG	(AT) ₁₀	332	56	FAM ^b	-
	R:	ACTTCTCACACCTGGATTTCACT					
SB27	F:	CTGGCTGGCTCGAGAAAAC	(AAT) ₅	166	56	VIC ^b	-
	R:	CAAGTTGGTTTGCAAGTGGGA					
SB37*	F:	ATCCTCAAGAAGCTACGAAGAG	(AC) ₇	172 - 178	56	NED ^b	KP765250
	R:	GGATTGGTAAGTTAGAAGTCTCT					
SB58	F:	GCCGTTATTCCTTCTCCCC	(AAT) ₉	157	56	PET ^b	-
	R:	TGTTCAACGTCTCCTGCTGG					
SB52*	F:	TTCCGTACGCTGACAGTCAC	(AGC) ₆	88 - 100	56	FAM ^c	KP765253
	R:	CTTCTTGGTGGCGGTGGTAT					
SB57	F:	TGCGGTGAAACTTCCTTGAA	(AT) ₁₁	170	56	FAM ^c	-
	R:	GCCAACCATGAGCACTACAA					
SB40*	F:	GCTGAAAAGTCTTTGTGTTTGC	(ACG) ₇ (ACA) ₃	271 - 283	56	FAM ^c	KP765251
	R:	GATCAGCGTGGACAGCAACA					
SB34*	F:	TGAGCAATCCATCCGAGGTG	(AG) ₅	143 - 147	56	VIC ^c	KP765249
	R:	CGTACTTGGCATGCGAGGTA					
SB28	F:	GCAGCAAAGCCGAAGAAATTC	(AT) ₁₂	183	56	NED ^c	-
	R:	GCGGAATGGTGAATACGGAAC					
SB32*	F:	ACAAACACTTCATTCCTTTGGCA	(AAT) ₅	143 - 155	56	PET ^c	KP765248
	R:	TGGAAACGGGGAAAAGTAAA					

T_A = annealing temperature. *Polymorphic loci that were used in the further study, while other loci presented in the table were not. ^{a,b,c}Indicates loci combination group that were mixed in the same capillary electrophoresis run.

Table 2. Patterns of variation observed across five populations of *Samadera bidwillii*.

Locus	C1 (n = 30)			H1 (n = 30)			R1 (n = 30)			Y3 (n = 30)			M1 (n = 30)		
	A	H _O	H _E												
SB01	1	0.00	0.00	2	0.07	0.06	3	0.27	0.36	3	0.10**	0.16	3	0.17***	0.36
SB09	1	0.00	0.00	2	0.20	0.18	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00
SB12	1	0.00	0.00	2	0.07***	0.18	2	0.17	0.15	2	0.07	0.06	2	0.03***	0.10
SB17	1	0.00	0.00	3	0.40	0.47	2	0.33	0.39	2	0.30	0.26	2	0.07	0.06
SB32	2	0.10	0.10	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00
SB34	1	0.00	0.00	3	0.50	0.50	3	0.20*	0.31	2	0.43	0.43	2	0.50	0.49
SB37	1	0.00***	0.00	4	0.53	0.65	1	0.00***	0.00	1	0.00***	0.00	1	0.00***	0.00
SB40	2	0.20	0.18	3	0.38	0.57	2	0.33	0.39	2	0.10	0.15	2	0.13*	0.23
SB48	2	0.00***	0.32	3	0.33	0.42	1	0.00	0.00	2	0.07	0.06	2	0.03	0.03
SB52	1	0.00	0.00	2	0.13**	0.28	3	0.43	0.49	2	0.03	0.03	2	0.27	0.28
Mean	1.3	0.03	0.06	2.5	0.26	0.33	1.9	0.17	0.21	1.8	0.11	0.12	1.8	0.12	0.16
SE	0.2	0.02	0.03	0.3	0.06	0.07	0.3	0.05	0.06	0.2	0.05	0.04	0.2	0.05	0.06

n = sample size; A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity. *P < 0.05, **P < 0.01 and ***P < 0.001 indicate a significant deviation from Hardy-Weinberg equilibrium expectation.

Table 3. Cross-amplification of microsatellite loci in other Australian congeneric species.

Locus	<i>Samadera</i> sp. Tozer Range (n = 5)		<i>Samadera</i> sp. Dam Creek (n = 6)		<i>Samadera</i> sp. St Marry (n = 30)		<i>Samadera</i> sp. Mt Goonaneman (n = 4)		<i>Samadera</i> sp. Mt Nardi (n = 7)		<i>Samadera</i> sp. Moonee Creek (n = 2)	
	Location: Queensland	A	Location: Queensland	A	Location: Queensland	A	Location: Queensland	A	Location: New South Wales	A	Location: New South Wales	A
SB01	114 - 120	2	114 - 126	3	120 - 123	2	120	1	120	1	120	1
SB09	146 - 166	3	146 - 166	1	146 - 152	3	148	1	148 - 150	2	148	1
SB12	237 - 243	2	237 - 243	2	228 - 243	4	237 - 243	2	237 - 243	2	237	1
SB17	239	1	227 - 237	3	239 - 241	2	237 - 241	3	231 - 241	3	231 - 239	2
SB32	143 - 155	2	143 - 155	2	155	1	155	1	155	1	155	1
SB34	143	1	143 - 147	2	143 - 147	3	143 - 145	2	143	1	143	1
SB37	172	1	172 - 182	4	172 - 182	5	172	1	172 - 182	3	172 - 174	2
SB40	271 - 280	2	271 - 280	2	280	1	280	1	280 - 283	2	NA	-
SB48	205 - 214	2	205 - 214	2	211 - 217	3	214	1	211	1	217	1
SB52	97	1	97	1	97 - 100	2	100	1	88 - 97	2	97	1

n = sample size; A = number of alleles; NA = Nonspecific Amplification. Species sample/voucher information: *Samadera* sp. St Mary, St Mary State Forest, *Samadera* sp. Tozer Range (AQ 727812, AQ 749148); *Samadera* sp. Dam Creek (AQ 490886, AQ 672751, AQ 565375, AQ 772859, AQ 558744, AQ 533148); *Samadera* sp. Mt Goonaneman (AQ 768, AQ 622748, AQ 650561, AQ 668777); *Samadera* sp. Mt Nardi (CANB 567803, CANB 597565, CANB 597560, CANB 542646, CBG 9003549, CBG 8900669-2); *Samadera* sp. Moonee Creek (CANB 509611, CANB 537288). Voucher specimen code with AQ: Brisbane herbarium; CANB & CBG: Canberra herbarium.

and these could save time and cost for laboratory cloning and microsatellite screening in characterising many species [28].

4. Conclusion

Ten polymorphic microsatellite markers for the vulnerable species *S. bidwillii* were developed and characterised for the first time on record. The efficiency of simple sequence repeats development was found to be 3.5% of the total DNA sequencing data from the developed genomic library to obtain potential loci. The 454 sequencing approach offered a fast and straightforward alternative to traditional methods for microsatellite isolation in genetically unknown and narrowly distributed threatened species. The method has proved appropriate for the ten reported markers suitable for *S. bidwillii* population genetics study. Primer sequences of ten loci are available with their estimates of expected heterozygosity. The average number of alleles per locus ranged from 1.3 to 2.5 and expected heterozygosity varied from 0 to 0.65. The microsatellite markers that were developed are useful tools for measuring genetic variation, estimation of gene flow and characterisation of the spatial population genetic structure of *S. bidwillii*. Furthermore, these markers are also useful for devising reliable collection and conservation procedures for *S. bidwillii* and related species of the genus *Samadera*.

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