Short title: Simmons et al. *Gossia gonoclada* microsatellites

Characterisation and transferability of microsatellite markers for the endangered rainforest tree, *Gossia gonoclada* (Myrtaceae).

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ABSTRACT

- Premise of the study: *Gossia gonoclada*, is nationally endangered due to habitat loss, weed invasion and the introduction of myrtle rust. Microsatellite DNA markers were developed for this plant species to study genetic diversity and trace parentage for conservation and restoration purposes.

- Methods and Results: Double digest restriction-site associated DNA sequencing (ddRADseq) libraries were generated for *Gossia bidwillii* and *Gossia punctata* and used to develop 24 nuclear microsatellite markers, 21 of which were found to be polymorphic in *G. gonoclada*. The number of alleles per locus ranged from 1 to 7, and the observed and expected heterozygosity ranged from 0.130 to 0.609 and from 0.159 to 0.731 respectively. Twenty-three of these markers amplified in *G. bidwillii* and 24 to *G. fragrantissima*.

- Conclusions: The set of 24 microsatellites markers will be useful for future conservation efforts for *G. gonoclada* and aid in genetic studies of other *Gossia* species.

Key words: Australia; conservation; microsatellite primers; rainforest; *Gossia gonoclada*; Myrteae; population genetics

INTRODUCTION

Gossia gonoclada (F.Muell. ex Benth.) N.Snow & Guymer (Myrtaceae, Tribe Myrteae) is an endangered dry rainforest tree that is restricted to highly urbanised south-east Queensland, Australia (AGRT, 2001; DSEWPaC, 2012). Once thought to be extinct, *G*. *gonoclada* (syn. *Austromyrtus gonoclada*) was rediscovered in the 1980's and actions were undertaken with the aim of arresting the decline of the species in the wild and maintaining viable *in situ* populations (AGRT, 2001). Recovery actions at the time included increasing the number of wild individuals (73 individuals from nine populations) by propagating and augmenting existing populations and new locations with clonal material from 21 genets in the early 2000's (AGRT, 2001). The invasive fungal plant pathogen *Austropuccinia psidii* (Beenken 2017) (myrtle rust) is host specific to Myrtaceae and has spread rapidly across the east coast of Australia since its introduction in 2010 (Carnegie & Lidbetter, 2012; Pegg et al. 2014). Myrtle rust is now recognised as a significant threat to the survival of *G*. *gonoclada* and it's 19 congeners of north-eastern Australia, New Caledonia and Papuasia (DSEWPaC, 2012; Taylor et al. 2016). Research aimed at understanding the myrtle rust infection process, risk and resistance is currently underway (T Taylor pers. comm.).

The development of genetic markers for *G. gonoclada* may be beneficial for identifying any genotypes and breeding groups that exhibit greater resistant to myrtle rust, as well as for assessment of genetic diversity and structure for conservation genetic purposes, in *Gossia* more generally. Double digest restriction-site associated DNA sequencing (ddRADseq) is a technique in which a set of DNA fragments representative of an organism's genome can be generated using next generation sequencing (Peterson et al. 2012). Sequence reads obtained from ddRAD libraries can be screened to identify and develop single nucleotide

polymorphic (SNP) and microsatellite (simple sequence repeat; SSR) DNA markers for use in the genotyping of plants and animals. The ddRAD protocol is useful for this purpose as it is based on the sequencing of size selected DNA fragments associated with specific restriction enzyme cut sites and therefore allows thousands of homologous loci to be compared among closely related individuals. The protocol has a relatively low per sample cost and allows rapid identification of a high number of polymorphic microsatellite loci for potential use in genetic analyses (Peterson et al. 2012; Yang et al. 2016). In this paper, we report on a ddRADseq approach to the development of the first set of microsatellite markers for *Gossia*.

METHODS

Leaf material was sampled from herbarium specimens of *Gossia bidwillii* (BRI AQ0816622) and *G. punctata* (BRI AQ0816621) (Appendix 1). The samples were ground using a TissueLyser II mill (QIAGEN, Germany) and gDNA extracted from 20 mg of the resultant material using a NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany) with the PL1 lysis buffer as per the manufacturer's protocol but with an 60 minute cell lysis step. The quality and purity of the DNA isolates were assessed using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Willington, USA) and by electrophoresis on a 1% agarose gel. DNA concentration was assessed for all samples using a Qubit 2.0 fluorometer (Thermo Fisher Scientific). Additional leaf samples were collected from 23 wild sourced *G. gonoclada* plants used in the recovery propagation program and from two populations of *G. bidwillii* and *G. fragrantissima* to assess both conspecific and congeneric cross-amplification of the microsatellite primers (Appendix 1). Representative voucher specimens were lodged at the Queensland Herbarium (BRI) (Appendix 1). Total genomic DNA was extracted from the leaf

material desiccated in silica gel tissue using QIAGEN DNeasy® Plant Mini Kits (QIAGEN Valencia, CA, USA) following manufacturer's instructions.

Sequencing libraries were prepared for single *G. bidwillii* and *G. punctata* samples following a ddRAD protocol based on Peterson et al. (2012) (see https://molecularbiodiversity.wordpress.com/home/protocols/). Briefly, 120 ng of genomic DNA was double-digested overnight using the restriction enzymes *Eco*RI-HF and *Mse*I in CutSmart buffer (New England Biolabs, Ipswich, USA). Unique double-stranded DNA adapters containing in-line barcodes were ligated to the cut-site overhangs followed by size-selection of 300-800 bp fragments using Agencourt AMPure XP beads (Beckman Coulter, Beverly, USA). Incorporation of index sequences (based on Illumina TruSeq LT indices) including Illumina flow cell attachment sequences was undertaken by qPCR using a KAPA Real-Time Library Amplification Kit (Kapa Biosystems, Wilmington, USA). After AMPure XP bead purification of the PCR products and quantification with a Qubit, the ddRAD libraries were pooled at equal concentrations. The library pool was subjected to agarose gel electrophoresis and fragments 400-500 bp selected by gel excision followed by purification using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA). The recovered library fragments were quantified by Qubit and prepared for sequencing on an Illumina MiSeq using a MiSeq Reagent Kit v3 600 cycles PE (Illumina, San Diego, USA). Sequencing metrics were assessed in Illumina BaseSpace (https://basespace.illumina.com) and reads exported in FASTQ format. The reads were de-multiplexed based on their adapter sequences using the script *process_radtags.pl*, which is a component of the STACKS pipeline (Catchen et al., 2013). De-multiplexed reads were paired and merged then *de novo* assembled in Geneious v7.2 (Biomatters, Auckland). Only high

quality paired-end reads that successfully merged were used in downstream development of markers.

From the *G. bidwillii* libraries 251,742 merged reads were generated with a mean length of 322 bp. The corresponding figures for *G. punctata* were 129,499 merged reads and a mean length of 319.5 bp. The reads were screened for microsatellite loci having a minimum of six repeats for di-nucleotides, and four repeats for tri- and tetra-nucleotides, using the QDDv2b pipeline (Meglécz et al., 2010) and primers designed using the program PRIMER 3 (Rozen & Skaletsky, 2000). The resulting 1519 loci were sorted based on PCR product size, repeat class, repeat length, GC content and multiplexing potential; 68 unlabelled microsatellite primer pairs (37 from *G. bidwillii* and 31 from *G. punctata*) were selected for evaluation of amplification and optimisation. Twenty-four primer pairs that consistently amplified single bands within the expected size range (13 from *G. bidwillii* and 11 from *G. punctata*) were labelled with fluorescent dyes for multiplexing in fragment analysis (Table 1).

Polymerase chain reaction (PCR) amplification was performed using reaction volumes of 11.5 µl containing approximately 25 ng of *Gossia* spp. genomic DNA, 1 x reaction buffer (67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg/mL gelatine), 0.2 mM of each dNTP, 2 mM MgCl2, 0.5 U F1 Taq polymerase (all reagents Fisher Biotech, Brisbane, Australia) and 0.2 µM forward primer, 0.2 µM reverse primer, with the forward primer of each pair being end-labelled directly with one of four fluorescent dyes (VIC, NED, PET, Applied Biosystems, Scoresby, Australia; FAM, Geneworks, Thebarton, Australia) to enable multiplexing in fragment analysis. Amplification was performed on an Mastercycler Nexus Gradient (Eppendorf, Hamburg, Germany) with the following cycling conditions: denaturation at 95 °C for 3 mins; 35 cycles of 94 °C for 30 secs, specific annealing

temperature (Table 2) for 30 secs, 72 °C for 45 secs; final elongation step at 72 °C for 10 mins.

PCR products were multiplexed according to dye sets and size ranges to avoid overlap, and then separated on an AB 3500 Genetic Analyser (Applied Biosystems, Scoresby, Australia). Fragment sizes were determined relative to internal lane standard (GS-600 LIZ; Applied Biosystems, Scoresby, Victoria, Australia) and manually checked in the SoftGenetics GeneMarker v2.7.0 software (Millennium Science, Mulgrave, Australia). All 24 primers were scored for *G. gonoclada*. The presence of null alleles, scoring errors, and large allele dropouts were checked for all loci using MICROCHECKER v2.2.3 (Van Oosterhout et al. 2004). The resultant multi-locus genotypes were used to calculate allelic frequencies, mean number of alleles per locus (*A*), mean expected heterozygosity (*He*), and mean observed heterozygosity (*Ho*) for each locus using GenAlEx 6.5 (Peakall & Smouse, 2012; Table 2).

Twenty-one of the 24 loci were polymorphic for *G. gonoclada* (Table 2) with an average of 2.917 alleles per locus (SE ± 0.324; Table 2). Mean observed heterozygosity was 0.244 (SE ± 0.039) while mean expected heterozygosity was 0.368 (SE ± 0.050; Table 2). All microsatellite loci have been deposited in GenBank (Table 1). Using the same PCR conditions described above, 23 and 24 loci were cross-compatible to congeners *G. bidwillii* and *G. fragrantissima* respectively (Table 2). Seventeen loci were polymorphic for both congeners. This is fewer polymorphic loci than were observed in *G. gonoclada* but is potentially a consequence of smaller sample size. There was an average of 2.375 alleles per locus for both *G. bidwillii* and *G. fragrantissima* (SE ± 0.268, 0.254 respectively), with a mean observed heterozygosity of 0.261 (SE ± 0.058) for *G. bidwillii* and 0.239 (SE ± 0.062) for *G.*

fragrantissima and mean expected heterozygosity of 0.343 (SE ± 0.052) for *G. bidwillii* and 0.399 (SE ± 0.054) for *G. fragrantissima* (Table 2).

CONCLUSIONS

In this study, we developed 24 microsatellite markers for *Gossia* identified from *G. bidwillii* and *G. punctata* ddRADseq libraries. Of the suite of markers, 21 were found to be polymorphic in *G. gonoclada*, 23 in *G. bidwillii* and 24 in *G. fragrantissima*. The successful transfer of the markers to *G. gonoclada* and *G. fragrantissima* which occur in different phylogenetic clades to *G. punctata* and *G. bidwillii* (Fernando unpubl. data), suggests that the markers are likely to be transferrable to a wide range of *Gossia* species and possibly closely related genera in tribe Myrteae, such as *Austromyrtus*. These microsatellites markers will be useful for identifying *G. gonoclada* genotypes that may be more resistant to myrtle rust and for genetic diversity studies that may inform future population enhancements and recovery actions for this species (Gustafsson, 2000; Kingsford & Watson, 2011; Vallee *et al*., 2004).

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TABLES

Table 1. Details of 24 microsatellite markers developed for *Gossia* with allele size ranges observed in *G. gonoclada*.

Locus	Primer sequence (5'-3')	Repeat Motif	Size (bp)	*Ta* (°C)	Genbank Accession	Fluorescent label	Multiplex Mix
Gb3	F: CGTACAATTGGTGGTGGTGC R: CGCCCACAATTAGAGTTCACG	AG	100-116	57	MF769341	VIC	1
Gb4	F: TAGGATTGGCAGCTGGAACG R: AAATCATAACACGCGCACCC	AG	112-130	57	MF769346	FAM	2
Gb5	F: CGTCAAGTTTCAATCTTTGTGC R: CCACTTTCCTAATCAGTGTGC	AG	114-124	57	MF769352	NED	2
Gb6	F: CCACAAGCGGTAGGTAAAGC R: GTTGAAGCAGAACGTCGGTG	AT	89-97	57	MF769343	NED	1
Gb7	F: TTCTTGTCGGTCACCACTCG R: CCCTCTCGAAAGTTCACACCT	AG	114-129	57	MF769359	FAM	1
Gb10	F: GGTACGTAGAAGTGGCAAAC R: ACAGATAACTTGCGCATGGC	AG	138-142	57	MF769356	PET	2
Gb14	F: CCAGAAGGCTCTTGGGATCG R: TCCTCTTGCATTCTCTGTACGC	AG	147-153	58.5	MF769342	VIC	1
Gb18	F: CGTCTTGGACCATTGAAGTGC R: CGGTATTGTATCCGCAACGC	AG	155	57	MF769357	NED	2
Gb21	F: AAAGCCTCGTTTCCCTCTCG R: CGGTCAACATTTCCAAGGCG	AG	221-243	57	MF769350	VIC	2
Gb22	F: AAGCCACAGACCTTCCACG R: TCTGTTTGGTTAGGGAGCTCA	AG	202-222	57	MF769338	FAM	1
Gb24	F: ACACCTTCGATTCCTCTCTGC R: GGGAAACGATGCATAAAGTCA	ACG	225-234	57	MF769354	NED	2
Gb25	F: ACCGGCAAATACAATGTGCG R: CGTTGTACAGTTACGCGTGC	AT	228-236	57	MF769347	FAM	2
Gb26	F: AGAATGGGTCGTATCTGCGC R: TGAAGCTTCCCACGAACCAA	AG	220-252	57	MF769355	PET	1
Gp1	F: CTGAATCGCGTGTAAAGGGC R: TTCCAGGAAAGTTGCGGTGA	AGG	86-110	57	MF769337	FAM	1
Gp2	F: ACCATATGCCACCAACCTCG	AG	95-101	57	MF769353	PET	2

Gp5	F: CGTCCATCAATCCCATTACGC R: ATTCCCACTTCGTGTCCCAT	AGG	110-131	57	MF769349	VIC	2
Gp11	F: GGCAATCAACATCCACTCGC R: TGATTGGCCCTGATTCGTCC	AG	193-216	57	MF769358	FAM	2
Gp12	F: ATAGCAAACCCGGGACATCG R: TCGGTTATGTGAGCTTGCTCT	AC	198-208	57	MF769360	VIC	1
Gp14	F: CCCACAAGCTCGATCCCTC R: CCAGCTGCGATTTCACCTTC	AAG	208-221	57	MF769345	PET	1
Gp16	F: TTGGAAGAGCTATTCATAGGCG R: GGAAGCTATGTTATCCTCTTGA	AT	215-227	57	MF769344	NED	1
Gp22	F: TTTACGCGAAACCCACAAGC R: GAGTGGGTGCTGACTCCAAG	AG	298-314	57	MF769339	FAM	1
Gp24	F: GGTAGGACTGGAAACTCGGG R: GCGATTCACAGTCACAGCAA	AG	291-295	57	MF769351	VIC	1
Gp25	F: CCACGTGCGAAGAACATGAC R: CGTTCATCGCGACCCTACAT	AG	310-334	57	MF769348	FAM	2
Gp30	F: GGATTGACGATTTCTCACTTGC R: AGTCGGACTATATTTGGCTCG	AG	318-334	57	MF769340	FAM	1

Note, *Ta* (°C) = annealing temperature.

Table 2. A summary of variation detected in three *Gossia* species at 24 microsatellite loci. Collection information for the sampled plants are available in Appendix 1.

	Gossia gonoclada (n=23)			*Gossia bidwillii* (n=5)			*Gossia fragrantissima* (n=5)		
Locus	*A*	*Ho*	*He*	*A*	*Ho*	*He*	*A*	*Ho*	*He*
Gb3	5.000	0.304	0.703	2.000	0.000	0.320	2.000	0.000	0.320
Gb4	4.000	0.348	0.413	4.000	0.600	0.580	4.000	0.400	0.480
Gb5	2.000	0.174	0.159	1.000	0.000	0.000	1.000	0.000	0.000
Gb6	2.000	0.087	0.083	2.000	0.000	0.320	1.000	0.000	0.000
Gb7	2.000	0.000	0.231	4.000	0.400	0.580	1.000	0.000	0.000
Gb10	2.000	0.130	0.258	2.000	0.200	0.180	2.000	0.000	0.320
Gb14	2.000	0.391	0.405	4.000	0.400	0.480	2.000	0.400	0.320
Gb18	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000
Gb21	3.000	0.261	0.507	3.000	0.667	0.667	3.000	0.333	0.611
Gb22	2.000	0.304	0.466	5.000	0.400	0.720	5.000	0.400	0.740
Gb24	4.000	0.478	0.542	1.000	0.000	0.000	2.000	1.000	0.500
Gb25	3.000	0.391	0.559	2.000	0.200	0.500	4.000	0.200	0.660
Gb26	3.000	0.000	0.204	-	-	-	1.000	0.000	0.000
Gp1	5.000	0.435	0.682	3.000	0.400	0.540	4.000	0.600	0.580
Gp2	2.000	0.609	0.499	2.000	1.000	0.500	2.000	1.000	0.500
Gp5	4.000	0.136	0.650	4.000	0.600	0.580	4.000	0.600	0.660
Gp11	1.000	0.000	0.000	2.000	0.400	0.480	2.000	0.000	0.444
Gp12	1.000	0.000	0.000	3.000	0.200	0.340	2.000	0.000	0.320
Gp14	2.000	0.261	0.227	1.000	0.000	0.000	4.000	0.200	0.700
Gp16	2.000	0.000	0.083	2.000	0.000	0.320	3.000	0.200	0.620
Gp22	6.000	0.522	0.731	4.000	0.600	0.660	3.000	0.200	0.340
Gp24	3.000	0.273	0.310	1.000	0.000	0.000	1.000	0.000	0.000
Gp25	2.000	0.500	0.375	3.000	0.200	0.460	2.000	0.200	0.420
Gp30	7.000	0.261	0.755	1.000	0.000	0.000	1.000	0.000	0.000

Note: *A* = number of alleles; *He* = expected heterozygosity; *Ho* = observed heterozygosity; *n* = number of individuals sampled; - = locus did not amplify.

APPENDIX

Appendix 1. Voucher information for *Gossia* accessions used in this study.

Species	**Voucher specimen accession no.** [a]	**Collection locality**	**Geographic Coordinates**	**No. of individuals sampled**
G. gonoclada	BRI AQ0431237	Nosworthy Park	-27.5402, 152.9924	1
G. gonoclada	BRI AQ0664196	Aminga St	-27.5235, 152.9591	1
G. gonoclada	BRI AQ0600581	Cliveden Ave	-27.5491, 152.9927	1
G. gonoclada	BRI AQ0546691	Murray's Property	-27.6629, 153.1611	16
G. gonoclada	BRI AQ0662936	Murray's Rd	-27.6630, 153.1611	1
G. gonoclada	BRI AQ0637291	Alexander Clark Park	-27.7069, 153.1925	2
G. gonoclada	BRI AQ0588356	Usher Park	-27.6402, 153.1591	1
G. punctata	BRI AQ0839799	Bahr's Scrub	-27.7553, 153.1661	1
G. fragrantissima	BRI AQ0816615	Currumbin Valley	-28.1884, 153.4279	4
G. fragrantissima	BRI AQ0732325	Mooloolah	-26.7565, 152.9260	1
G. bidwillii	BRI AQ0675362	Northbrook	-27.3069, 152.6882	1
G. bidwillii	BRI AQ891239	Mudlo	-26.0133, 152.2162	1
G. bidwillii	BRI AQ891240	Oakview	-26.1581, 152.3288	1
G. bidwillii	BRI AQ891241	Oakview	-26.1472, 152.3200	1
G. bidwillii	BRI AQ891242	Mudlo	-26.0189, 152.2171	2

a One voucher per population lodged at the Queensland Herbarium (BRI), Brisbane, Australia.