Development and cross-species amplification of microsatellite markers from the endangered Wee Jasper Grevillea (*Grevillea iaspicula*, Proteaceae)

Susan E. Hoebee[1,2,3]

1. CSIRO Plant Industry, GPO Box 1600, Canberra, ACT 2001, Australia
2. School of Resources, Environment and Society, The Australian National University, Canberra, ACT 0200, Australia
3. Current address: Department of Botany, La Trobe University, Bundoora, Victoria 3086, Australia; e-mail: s.hoebee@latrobe.edu.au

Introduction

Grevillea is the largest genus of the Proteaceae (c. 357 species; Makinson 2000) and all but two species are Australian endemics. Grevilleas have diverse life-history characteristics, *e.g.,* habits (prostrate shrubs – trees), mating systems (obligate outcrossers – mixed mating with varying degrees of self-fertility) and pollination syndromes (bird – insect). This diversity and the high proportion of species listed as rare or threatened (176 of 357 species, ROTAP; Briggs and Leigh 1996) has led to several studies investigating factors and processes determining the genetic structure of *Grevillea* species (*e.g.,* England *et al.* 2001, 2002; Hoebee and Young 2001; Llorens *et al.* 2004; Holmes *et al.* 2008).

Informative molecular markers are essential for assessing diversity, gene flow and population dynamics. Several methods are available (see Glaubitz and Moran 2000) but microsatellites (or simple sequence repeats, SSRs) are particularly ideal, especially when they can be used in a variety of species. Within *Grevillea*, SSR primers have only previously been developed from *G. macleayana* (McGill.) Olde & Marriott (England *et al.* 1999). Here, the development of seven polymorphic SSRs from a *G. iaspicula*-derived genomic library is described. In addition, cross-species amplification of these markers is assessed, along with the polymorphism of six *G. macleayana*-derived SSR primer pairs in *G. iaspicula* McGill.

Grevillea iaspicula is endangered and has an extremely restricted and highly fragmented distribution in the Wee Jasper region of New South Wales, Australia (Hoebee and Young 2001). Conservation recommendations for this species have been in place since 1976 and an official recovery plan was established in 1991 (Butler *et al.* 1991). The mating system and genetic structure of *G. iaspicula* was assessed using allozymes (Hoebee and Young 2001), but high resolution SSR markers are necessary to evaluate contemporary connectivity of the extant populations via paternity and parentage analyses.

Abstract

Grevillea iaspicula is a highly restricted species occupying limestone outcrops near Wee Jasper, New South Wales (Australia). To allow future assessment of the ecological processes shaping the structure of *G. iaspicula* populations, seven polymorphic microsatellite (SSR) loci were developed from a partial genomic library. Amplification and polymorphism of six previously derived *G. macleayana* SSR primer pairs in the target species, *G. iaspicula*, were also assessed and cross-species amplification of SSR primers among several grevillea species is discussed.

Keywords: *Grevillea*, microsatellites, Proteaceae, SSRs, transferability

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Materials and Methods

Total genomic DNA was extracted from leaf tissue of *G. iaspicula* following Doyle and Doyle (1990). DNA was digested with *Sau*3AI, ligated into pUC18 and used to create a partial genomic library of c. 5500 clones containing 400-900 bp DNA inserts. Hybridisation of all combinations of 5′ end-labelled [γ^{32}P]ATP di- and tri-nucleotide combinations, excluding AT/TA, identified over 180 positive clones. Plasmid DNA from positive clones was isolated using Wizard™ *Plus* Minipreps DNA purification systems (Promega). Of these, 67 were sequenced using ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

Primers were developed for four pure and three interrupted dinucleotide SSRs as well as a single interrupted trinucleotide SSR (Table 1), following Löffert *et al.* (1997), and tested on nine *G. iaspicula* accessions (three plants from each of three *G. iaspicula* populations). The loci were amplified in a 20 µL PCR reaction (final concentration: 1 x PCR Buffer, 2.5 mM $MgCl_2$, 200 µM dNTPs each, 10 pmol/µL of each primer, 1 Unit *Taq* DNA polymerase, and 50 ng genomic DNA) using a touchdown procedure (2 cycles of 94°C for 1 min, 55°C for 30 sec, 72°C for 1 min, with sequential decrease of annealing temperature by 1°C every second cycle until 30 cycles of 94°C for 1 min, 50°C for 30 sec, 72°C for 1 min) and visualised on 3% agarose

Table 1. Details of primers developed for eight microsatellite loci in *G. iaspicula* with the structure of the repeat motif in, and the size (base pairs) of, the cloned allele. Primer and product information is followed by amplification results from a subset of nine individuals visualised on 3% agarose gels (P = polymorphic, M = monomorphic), as well as the number of alleles (N_A), heterozygosity (H_e) and product size range determined from 99 reproductive plants used in further analyses and where forward primers were radioactively labelled and loci visualised on 5% denaturing polyacrylamide gels. Polymorphism of some loci has also been established in other species, including GI7 (see text).

Locus	Repeat motif	Primer sequences (5′-3′)	Product size (bp)	Product % GC	Optimal annealing temperature (°C)	Amplification Results	N_A / H_e / Product range (bp)	GenBank accessions
GI1F	$(GA)_4A(GA)_9$	CTCTCTTATCTCTCAAACTCAG	158	47.5	53.0	P	Not tested	DQ858446
GI1R		CTATTCGACGAAACCAAC						
G14F	$(AC)_{11}$	AACCATAAGGGCGACAAG	180	41.7	53.3	P	5 / 0.504 / 176-182	DQ858447
GI4R		GCCTACAGATATGGTGGAAC						
GI5F	$(TC)_8$	CAATAGCAGCAATAATGGAC	78	48.7	51.4	P	Not tested	DQ858448
GI5R		GCCCAGAGAAACAAAAAG						
GI6F	$(AG)_{13}$	AGCCACTTGTCTATCACTATC	93	44.1	50.0	P	Not tested	DQ858449
GI6R		TCTATCTATCCCCACTCTTC						
GI7F	$(TG)_2TA(TG)_7$	TCAACCTCTCTCCCTCTCAC	224	41.5	53.4	M	-	DQ858450
GI7R		CCTCCCAACCCATACATAC						
GI8F	$(GA)_2CT(GA)_9$	TTGTGGTGGGTAGAAGAG	442	41.2	53.4	P	Not tested	DQ858451
GI8R		TTGATGAAGAGTATGTGAGG						
GI9F	$(AG)_{13}$	GACAAAACCTTCCCAACC	206	43.7	53.8	P	9 / 0.795 / 190-212	DQ858452
GI9R		TCCATAATCGCATCTTCC						
GI10F	$(TTC)_8T(TTC)_2$	TGTAATATCGTACCACTGACC	126	42.1	51.4	P	Not tested	DQ858453
GI10R		TGTAAACTCAACCCCCTC						

gels. Additionally, six *G. macleayana* SSR primer pairs, which according to England *et al.* (1999) resulted in PCR product using *G. iaspicula* template, were re-tested for both amplification quality and polymorphism as described above.

A subset of six unlinked SSR loci (GI4, GI9, GM13, GM15, GM25 and GM37) was used to estimate heterozygosity in 99 reproductive *G. iaspicula* individuals (Tables 1 and 2). Loci were tested for Burrow's composite measure of linkage disequilibria between pairs of loci following Weir (1979) and were corrected for multiple tests using the Bonferroni procedure. Amplification followed the procedures described above with the exception that each forward primers was end-labelled with [γ^{33}P]ATP prior to PCR. Amplified products were diluted with 20 µL formamide loading dye and 2 µL of the mix was electrophoresed through a 5% denaturing polyacrylamide gel. The gels were fixed using standard procedures and the amplified products were visualised following 2 days exposure to BIOMAX™MR Scientific Imaging Film (Kodak).

Results and Discussion

Of the eight *Grevillea iaspicula*-derived loci, seven showed evidence of polymorphism (Table 1). The remaining locus (GI7) was monomorphic for all nine accessions (Table 1); it must be noted, however, that the resolution obtainable on agarose is insufficient to state this as absolute. With this in mind, GI7 has been shown to be polymorphic in other studies using fluorescently labelled primers and capillary electrophoresis (e.g. Llorens 2004; Whelan *et al.* 2006).

Four of the six *G. macleayana*-derived loci (GM13, GM15, GM25 and GM37) consistently amplified well in the *G. iaspicula* samples and all were polymorphic (Table 2). The observed size ranges in *G. iaspicula* typically bounded the original cloned allele (Table 2). In the one instance where observed allelic ranges exceeded the cloned allele size (GM15) it did so by only 10 base pairs. In *G. iaspicula*, GM12 and GM29 resulted in poor amplification and multiple priming sites, respectively. GM29 appeared to amplify two loci, only one of which was variable. This putatively polymorphic locus was much larger than the expected size of c. 141 base pairs (England *et al.* 1999) and it was not pursued further. Subsequent research by England *et al.* (2002) found variation at the GM29 locus to be within the left flanking sequence and not within the SSR motif.

Cross-species amplification of SSR loci is known to decrease with increasing evolutionary distance between source and non-source taxa (England 1999; Rossetto 2001). Nevertheless, England *et al.* (1999) showed that several *G. macleayana*-derived microsatellite markers were transferable to other grevilleas, including *G. iaspicula*. Treatment within the *Flora of Australia* suggests that these two species are distantly related: *G. macleayana*, with toothbrush-style inflorescences, is placed within the *Pteridifolia* group (*Aspleniifolia*/*Hookeriana* subgroup**)**, as opposed to the placement of *G. iaspicula* within the *Floribunda* group (*Rosmarinifolia* subgroup) with spider-like inflorescences (Makinson 2000). The work presented here showed that not only were these markers transferable but that some loci are also polymorphic.

Table 2. Transferability and polymorphism of six *Grevillea macleayana* primers developed by England *et al.* (1999) to the target species, *G. iaspicula*. Heterozygosity was determined from 99 reproductive plants. Code key: ✓ = single polymorphic locus amplified in complete population assays; × = poor priming or multiple priming sites.

Locus	Repeat motif*	Expected product in *G. macleayana* (bp)*	*Grevillea iaspicula*			
			Transferability	Product range (bp)	No. of alleles	Heterozygosity (*He*)
GM12	$(AC)_9$	295	×	-	-	-
GM13	$(CT)_{23}$	139	✓	129-141	7	0.707
GM15	$(CA)_{10}$	134	✓	144-163	10	0.865
GM25	$(CT)_{15}$	252	✓	250-264	4	0.623
GM29	$(CT)_{15}$	141	×	-	-	-
GM37	$(CT)_8$	136	✓	130-154	7	0.786
*From cloned allele						

Several of the *G. iaspicula*-derived SSR loci are also transferable and polymorphic in other species from this genus. For example, GI7 and GI9 are polymorphic in *G. macleayana* (Whelan *et al.* 2006), *G. caleyi* R.Br. and *G. longifolia* R.Br (Llorens 2004), and *G. repens* F.Muell. ex Meisn. (Holmes *et al.* 2008); GI9 is also polymorphic in *G. acanthifolia* A.Cunn. and *G. sphacelata* R.Br (Celebrezze 2002); and GI8 is polymorphic in *G. longifolia* (Llorens 2004). There is also evidence to suggest that the some loci amplify in other genera within the Proteaceae (*e.g., Macadamia*; J. Neal, pers. comm.). Transferability of SSR markers both within *Grevillea* and within the Proteaceae has both great economic potential and conservation value in that several proteaceaeous species are important forestry and crop species and, as noted above, many *Grevillea* species are rare or threatened taxa. The availability of genetic markers will lead to a better understanding of the population genetic structures of these species, which in turn will inform their utilisation or conservation.

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