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Primer Note

Development of 23 polymorphic microsatellite loci in invasive silver wattle, *Acacia dealbata* (Fabaceae)[1]

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- *Premise of the study:* Microsatellite markers were developed for silver wattle, *Acacia dealbata* (Fabaceae), which is both an ornamental and an invasive weed species. It is native to southeastern Australia and invasive in Europe, Africa, Asia, and the Americas.
- *Methods and Results:* The pyrosequencing of a microsatellite-enriched genomic DNA library of *A. dealbata* produced 33,290 sequences and allowed the isolation of 201 loci with a minimum of seven repeats of microsatellite motifs. Amplification tests led to the setup of two multiplex PCR mixes allowing the amplification of 21 loci. The polymorphism of these markers was evaluated on a sample of 32 individuals collected in southeastern Australia. The number of alleles and the expected heterozygosity varied between two and 11, and between 0.11 and 0.88, respectively.
- *Conclusions:* The level of polymorphism of this set of 23 microsatellites is large enough to provide valuable information on the genetic structure and the invasion history of *A. dealbata*.

Key words: *Acacia dealbata*; Fabaceae; invasion; microsatellites; pyrosequencing; silver wattle.

Silver wattle, *Acacia dealbata* Link (Fabaceae), is a tree species native to southeastern Australia, where it is widespread and common. It has been introduced since the 18th century in various parts of the world for ornamental or forestry purposes (Kull et al., 2011). It is now considered an invasive species in southern Europe, South Africa, India, Madagascar, New Zealand, Chile, and California (Kull et al., 2011). In those regions, *A. dealbata* invades woodland and disturbed environments where it outcompetes native species. *Acacia dealbata* has a long and complex history of worldwide introductions from Australia, at least since its introduction in the early 18th century in Europe (Kull et al., 2011). Silver wattle is the focus of research both because it is a model for the impact of landscape fragmentation in its native area (Broadhurst and Young, 2006) and because it is a famous invader (Lorenzo et al., 2010), classified on the Delivering Alien Invasive Species Inventories for Europe (DAISIE) list as one of the 100 European invaders with the most significant ecological impacts (DAISIE European Invasive Alien Species Gateway, 2006). In both cases, the use of highly polymorphic genetic markers could help answer questions related to the spatial genetic structure of *A. dealbata* populations.

Variable genetic markers such as microsatellite markers are a common tool used to infer invasion routes of invading species and specifically to identify the sources of invasive populations (Estoup and Guillemaud, 2010). They are also commonly used in conservation biology studies to decipher the impact of environmental disturbance on the genetic structure of tree species (e.g., Aldrich et al., 1998). Recently, next-generation sequencing technologies have been used to obtain very large numbers of shotgun sequences of genomic DNA from which microsatellites could be isolated (Guichoux et al., 2011). This method requires a large effort of sequencing to obtain a sufficient number of sequences containing microsatellites. Microsatellite enrichment of the genomic DNA prior to sequencing allows a drastic cost reduction (Malausa et al., 2011). Here we present the isolation and properties of 23 polymorphic microsatellite loci of *A. dealbata* using 454 GS FLX Titanium (Roche Applied Science, Penzberg, Germany) pyrosequencing of a microsatellite-enriched genomic DNA library.

METHODS AND RESULTS

Total genomic DNA was extracted from leaf material of a plant collected in southeastern Australia (34°30′51.498″S, 148°49′53.3892″E) using the DNeasy Plant Kit (QIAGEN, Hilden, Germany). We followed the procedure of Malausa et al. (2011) for enrichment and sequencing. Genomic DNA was submitted to sonication, ligation to standard adapters, and purification on a NucleoFast PCR

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plate (Macherey-Nagel, Düren, Germany). DNA was denatured and then hybridized for 20 min at 56°C to eight biotin-labeled oligonucleotides, the sequences of which are microsatellite motifs [$(AG)_{10}$, $(AC)_{10}$, $(AAC)_8$, $(AGG)_8$, $(ACG)_8$, $(AAG)_8$, $(ACAT)_6$, and $(ATCT)_6$].

We then used primers corresponding to the adapters (5′-GTTTAAGGCCTAGCTAGCAGAATC-3′ and 5′-GATTCTGCTAGCTAGGCCTT-3′) to amplify the microsatellite-enriched DNA. Preparation of the enriched library for emulsion PCR, sequencing, and analytical processing using the 454 GS FLX Titanium followed the manufacturer's protocols. We then sorted the obtained sequences, removed the sequence of the adapters, and selected sequences with desirable properties using QDD version 1 (Meglecz et al., 2010).

Among the 33,290 sequences obtained, a total of 201 sequences longer than 80 bp, containing microsatellite motifs with at least seven uninterrupted repeats and flanking regions free of tandem repetitions, were used to design PCR primer pairs. We tested the 201 primer pairs for amplification on seven individuals, and then tested the successful primer pairs for fluorescent PCR on 32 individuals collected in southeastern Australia, between latitudes 34°37′43.1″S and 34°29′08.9″S and between longitudes 148°50′28.3″E and 148°45′23.9″E (see Appendix 1 for individual coordinates). A representative voucher specimen (CANB77329, deposited in the Australian National Herbarium [CANB], Canberra, Australia) was previously collected in the same region as the samples studied here.

These 32 individuals were used to test the amplification of these loci according to the following procedure: PCR amplifications were performed in a 10-μL volume containing 1× QIAGEN Multiplex Master Mix, 0.2 μM of each primer, and ca. 20 ng of genomic DNA extracted from individual leaves using the DNeasy Plant Kit (QIAGEN). The amplification reactions were performed in an Eppendorf (Hamburg, Germany) Mastercycler thermocycler and included a 15-min denaturation step at 95°C; followed by 35 cycles of 30 s at 94°C, 1.5 min at 56°C, and 1 min at 72°C; followed by a final extension step at 60°C for 30 min. Fifty primer pairs gave positive PCR amplification of the predicted size for the seven individuals. They were then used in fluorescent PCR to amplify

Table 1. Characteristics and variability of 23 microsatellite loci of *Acacia dealbata* based on 32 individuals sampled in southeastern Australia.

Locus	Primer sequences (5′–3′)	Repeat motif[a]	Allele size range (bp)[b]	5′ end-labeled dye	Multiplex marker set[c]	GenBank accession no.
Ad-33	F: GAGAAGAGAAAGGGGATGGG	$(GA)_8$	159–169	FAM	2	KP702738
	R: CCATCATTATTGTTTGTCCCG					
Ad-41	F: TGAGATTATAAGCTTCTGGTAAAAAG	$(GA)_8$	102–108	PET	2	KP702739
	R: AAAAGCCACGCCCTTTAGTT					
Ad-45	F: TCTAATAAAAGAACAGCAACGCA	$(AC)_8$	74–87	NED	2	KP702740
	R: AATTGGTTTGCATGTGATGG					
Ad-47	F: CCCACATAAAGAAGAAACTAGTGAA	$(AG)_8$	89–95	PET	1	KP702741
	R: CGTGGGAAAGAAACATGGAT					
Ad-48	F: TAGCTTTCCTCGGATCCCTT	$(AC)_9$	196–214	VIC	2	KP702742
	R: ACACATCAGAACACTGGGCA					
Ad-49	F: CTTCACAAGCAAAAGGGCAC	$(AG)_8$	168–184	FAM	1	KP702743
	R: CAGCCATGTTCATGGTTAAAA					
Ad-54	F: TGCAGCCAGAAAGTGAAATG	$(TTC)_8$	131–143	NED	1	KP702744
	R: GAGAAACTAAAGATGAAGACGGAGA					
Ad-59	F: CCCAGGTCAAACGCAGTAAT	$(TC)_{10}$	167–175	NED	1	KP702745
	R: CTGTCAAACACCAGAAAGGTAGA					
Ad-63	F: CCTTTTCCCCACATTCTCCT	$(TC)_9$	120–133	VIC	NA	KP702746
	R: CGTCCCGCAATTTCTTATGT					
Ad-66	F: TGTGAACGAAGCACAAGACC	$(CA)_8$	161–165	VIC	1	KP702747
	R: TGTGAGCAACGACATTGACA					
Ad-70	F: CTCCCATCTCAGATCTTGGC	$(GT)_8$	131–132	PET	1	KP702748
	R: CAAGCAATGTTCCACTCCCT					
Ad-86	F: TTTGAAAGCATGCTCCATTTT	$(AC)_8$	106–122	NED	1	KP702749
	R: GCCTTCCCTCCGTTAGAATC					
Ad-89	F: TCATTCTGCACGTCATTTCC	$(CT)_{11}$	80–96	NED	1	KP702750
	R: CACAGGGCTTTGTATTTGTATCTTT					
Ad-97	F: GCAGTTAAAACCTCGAAGGC	$(GT)_8$	92–96	FAM	1	KP702751
	R: CTGAACTTGATCACTTCATGGT					
Ad-116	F: TTGGGTTGAATTCCTTTCTCA	$(AC)_9$	127–135	VIC	NA	KP702752
	R: GTATGCCTCGGAGATGGTGT					
Ad-126	F: TCCACCCAAGAACTGATGAA	$(AC)_{11}$	111–121	NED	2	KP702753
	R: CAGGCGTCCCATAAACTTGT					
Ad-127	F: GATGGTTATGGTGCCTCAAG	$(GT)_9$	106–136	VIC	1	KP702754
	R: GGCCTAAACCACAAAGGTGA					
Ad-137	F: ACCCTCAACCCTGACTTCCT	$(AC)_9$	114–128	VIC	2	KP702755
	R: AACCCTCAATCCTGCAAGTG					
Ad-145	F: CGTGAAAATGGCCTGTAGGT	$(AG)_{10}$	234–284	NED	1	KP702756
	R: CCTTCAACTCATTCACCATCTC					
Ad-173	F: TCTCAACCTCAAACTGCGAA	$(TG)_8$	150–160	NED	2	KP702757
	R: GAGGTGAAGGCCAAATGAAG					
Ad-176	F: TGACTGTGCGGAATTCATCT	$(AC)_9$	114–130	FAM	1	KP702758
	R: TCCTTATAAACAATGCGACATAGA					
Ad-177	F: GCCAGCAAAACAAACACAGA	$(CA)_8$	149–151	PET	1	KP702759
	R: CTGCAGTCCTAAATGTCAGTGC					
Ad-201	F: GACAAGTCTTGCCATGATGTTT	$(TG)_8$	197–210	VIC	1	KP702760
	R: CCATCCACTTTTTGCCTGAT					

[a] Repeat motifs are those of the pyrosequenced alleles.

[b] Size ranges are based on the allele scoring performed on capillary electrophoresis data.

[c] Two sets of possible multiplex reactions are listed. NA indicates that the primer pair produces unspecific products in multiplex.

TABLE 2. Variability of the 23 microsatellite loci developed for *Acacia dealbata* based on 32 individuals sampled in southeastern Australia.

Locus	*A*	H_o	H_e	HW[a]
Ad-33	6	0.677	0.651	
Ad-41	4	0.344	0.579	*, FDR
Ad-45	4	0.438	0.588	*, FDR
Ad-47	4	0.656	0.613	
Ad-48	6	0.5	0.569	
Ad-49	9	0.813	0.858	
Ad-54	5	0.438	0.449	
Ad-59	5	0.29	0.457	*, FDR
Ad-63	5	0.406	0.458	
Ad-66	3	0.531	0.478	
Ad-70	2	0.125	0.119	
Ad-86	7	0.531	0.472	
Ad-89	6	0.4	0.525	
Ad-97	3	0.29	0.263	
Ad-116	4	0.625	0.668	*, FDR
Ad-126	6	0.625	0.58	
Ad-127	8	0.633	0.791	
Ad-137	7	0.563	0.606	
Ad-145	11	0.296	0.876	*, FDR
Ad-173	6	0.594	0.555	
Ad-176	6	0.313	0.427	*
Ad-177	2	0.438	0.437	
Ad-201	5	0.531	0.58	

Note: *A* = number of alleles; H_e = expected heterozygosity of Nei (1987); H_o = observed heterozygosity; HW = first type error of the probability test of Hardy–Weinberg equilibrium.

[a]An asterisk (*) indicates a significant test at the 5% level, and "FDR" indicates that the test is significant after the false discovery rate adjustment for multiple testing.

the 32 individuals as described above but with forward primers labeled with a fluorescent dye on the 5′ end, and with 25 cycles of PCR instead of 35. Twenty-three polymorphic microsatellites could be unambiguously scored using an ABI 3700 sequencer (Applied Biosystems, Waltham, Massachusetts, USA) with the GeneScan 500 LIZ Size Standard (Applied Biosystems) and GeneMarker software (version 1.75; SoftGenetics LLC, State College, Pennsylvania, USA) (Table 1). The sequences containing the 23 microsatellites led to no significant similarity when blasted against National Center for Biotechnology Information (NCBI) nonredundant (NR) and expressed sequence tags (dbEST) databases.

The number of alleles measured on 32 individuals sampled from a single Australian population varied from two to 11, and Nei's expected heterozygosity (Nei, 1987) varied from 0.11 to 0.88 and was larger than 0.5 for 14 loci (Table 2). Deviations from Hardy–Weinberg equilibrium (HWE) and linkage equilibrium were tested using GENEPOP 4.0 (Rousset, 2008). We took into account multiple testing (in the case of HWE tests) and nonindependence between tests (in the case of linkage tests) by using the false discovery rate (Benjamini and Hochberg, 1995) and the sequential Bonferroni (Sokal and Rolf, 1995: p. 236) adjustments, respectively. Five loci (Ad-145, Ad-41, Ad-59, Ad-116, and Ad-45) significantly departed from HWE after false discovery rate adjustment and displayed significant heterozygote deficiency (Table 1). Linkage disequilibrium was detected for three pairs of loci (between Ad-116 and Ad-137, Ad-116 and Ad-97, and Ad-49 and Ad-86). Twenty-one microsatellite loci could be amplified in two sets of PCR multiplex mixes, one with 14 and the other with seven primer pairs (Table 1). The two other primer pairs produced unspecific amplifications when used in multiplex PCR and thus were not included in any multiplex mix.

CONCLUSIONS

We chose to present all 23 primer pairs although some of them may display Hardy–Weinberg or linkage disequilibrium because those disequilibria may be specific to the population sampled for the current study. The microsatellite markers developed here for *A. dealbata* display a moderate to large level of polymorphism in individuals sampled in natura in the native area of the species. This level of polymorphism should, however, be large enough for population genetic analyses to provide valuable information regarding the biology and worldwide invasion routes of *A. dealbata.*

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Appendix 1. Sampling information of the 32 *Acacia dealbata* individuals used in this study.

Sample name	GPS coordinates
AD100	34°31′45.4″S, 148°48′09.4″E
AD120	34°31′52.5″S, 148°48′53.2″E
AD14-1	34°32′44.5″S, 148°50′28.3″E
AD140	34°31′39.4″S, 148°49′15.3″E
AD156	34°29′46.9″S, 148°49′32.8″E
AD161	34°29′45.0″S, 148°49′33.4″E
AD181	34°30′32.2″S, 148°49′56.7″E
AD200	34°30′39.1″S, 148°49′39.7″E
AD220	34°30′38.8″S, 148°49′31.0″E
AD240	34°30′27.7″S, 148°50′05.9″E
AD250	34°29′46.0″S, 148°48′42.1″E
AD261	34°30′03.8″S, 148°48′39.2″E
AD270	34°30′07.0″S, 148°48′38.0″E
AD290	34°30′55.3″S, 148°48′24.6″E
AD310	34°32′18.2″S, 148°48′51.7″E
AD311	34°32′21.4″S, 148°48′53.2″E
AD330	34°37′42.5″S, 148°47′12.2″E
AD340	34°37′43.1″S, 148°47′12.2″E
AD360	34°33′22.8″S, 148°49′40.6″E
AD380	34°34′31.0″S, 148°48′20.0″E
AD400	34°34′30.6″S, 148°48′19.0″E
AD420	34°34′47.8″S, 148°48′44.8″E
AD440	34°32′44.9″S, 148°50′27.8″E
AD460	34°32′28.2″S, 148°48′59.8″E
AD480	34°32′56.8″S, 148°49′09.8″E
AD500	34°29′08.9″S, 148°45′23.9″E
AD520	34°30′40.5″S, 148°46′13.3″E
AD540	34°30′43.9″S, 148°46′13.2″E
AD560	34°30′44.6″S, 148°46′23.7″E
AD581	34°30′57.2″S, 148°45′35.1″E
AD600	34°30′57.2″S, 148°45′31.7″E
AD612	34°30′59.0″S, 148°45′31.6″E