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Isolation and characterisation of microsatellite markers for the dwarf sheoak *Allocasuarina humilis* (Casuarinaceae), an important understorey species in ecological restoration

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Introduction

The dwarf sheoak *Allocasuarina humilis* (Otto & A.Dietr.) L.A.S.Johnson (Casuarinaceae) is a dioecious wind-pollinated woody shrub endemic to the biodiverse Southwest Australian Floristic Region (SWAFR) of Western Australia. It occurs throughout most of the SWAFR, where it is found in vegetation communities ranging from heath to forest (Western Australian Herbarium 1998–). Plants grow to 2 m high, resprout from a lignotuber following fire and are thought to be very long-lived. Plants are diploid ($2n = 20$; Barlow 1959), flower from May to November (Western Australian Herbarium 1998–) and infructescences contain multiple glabrous samaras 5-6 mm long with a short truncate wing (Wilson and Johnson 1989) that may facilitate some wind dispersal. Seed dispersal via emus has also been noted (Calviño-Cancela et al. 2006).

Abstract

Eight polymorphic microsatellite markers were developed for *Allocasuarina humilis* (Otto & A.Dietr.) L.A.S.Johnson, a common woody shrub endemic to south-west Australia that is a common component of the understorey across a range of vegetation types. The markers were isolated using 454 shotgun sequencing and tested on 48 individuals from two natural populations. We found six to 19 alleles per locus (mean 12.1) and observed heterozygosity ranged from 0.208 to 0.956 (mean 0.710). The markers will be used to assess population structure and diversity in *A. humilis*, with the aim of developing appropriate seed sourcing strategies for ecological restoration.

Key words: *Allocasuarina*; microsatellites; SSRs

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Allocasuarina humilis is a common understorey component of ecological restoration programs for areas disturbed by logging, mining and roadworks and is also used in community-based restoration projects. Seed sourcing has been mostly conducted in areas local to restoration sites, following the principles of local provenance and the guidelines of the previous Western Australian Forest Management Plan (Conservation Commission of Western Australia 2004). However, with a fully outcrossing breeding system and the potentially widespread dispersal of pollen via wind, local sourcing of seed may not be the most appropriate strategy for *A. humilis*.

Molecular analysis can reveal patterns of gene flow, genetic diversity and population connectivity to better inform the seed sourcing decision-making process. Restoration outcomes are greatly improved when information on a species' genetic characteristics is incorporated into the planning phase of restorations (Godefroid et al. 2011). Here, we developed eight high resolution microsatellite markers for *A. humilis* with which to evaluate its genetic diversity and population structure, to assist in developing appropriate seed sourcing strategies for ecological restoration.

Materials and methods

Genomic DNA was extracted from freeze-dried leaf material from one *A. humilis* plant using the Doyle and Doyle (1987) CTAB method with 1% w/v polyvinylpyrrolidone (MW 40000) added to the extraction buffer. Shot-gun sequencing was conducted at the Australian Genomic Research Facility (Brisbane, Australia) using 4 µg DNA on a GS-FLX Titanium machine (Roche, 454 Life Sciences) following Gardner et al. (2011). We used QDD 1.3 (Meglécz et al. 2010) with default parameters to screen raw sequences for ≥ eight di- to hexa-base repeats. Primers were designed using Primer3 within QDD. Of the 539 unique loci, 10 were chosen for polymerase chain reaction (PCR) trials following recommendations in Meglécz et al. (2010) and Gardner et al. (2011). Loci were preferred that had pure microsatellite motifs ≥8 repeats in length that were >20 bp from primers.

Universal M13 tails (5'- CACGACGTTGTAAAACGAC) were appended to each forward primer at the 5' end, and the complement sequence was modified with a 5' fluorescent label (FAM, VIC, NED or PET). Singleplex polymerase chain reaction (PCR) amplifications were performed in an 8 µL reaction mix containing 20 ng template DNA (extracted from freeze-dried leaf material using the method detailed above), 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 0.2 mM of each dNTP, 2.0–2.75 mM $MgCl_2$, 0.15 µM fluorescent labelled M13 primer, 0.03 µM forward primer, 0.15 µM reverse primer, 0.05 µL *Taq* DNA polymerase. Most reactions benefited from addition of 0.5 M betaine to the PCR mix (Table 1). Loci were amplified using one of two programs in an Eppendorf gradient thermocycler: (1) 95°C for 4 min; 15 cycles of 30 s at 94°C, 30 s at 65 to 50°C with a stepdown of 1 °C per cycle, and 72°C for 45 s; 30 cycles of 15 s at 94°C, 15 s at 50°C, and 45 s at 72°C; 8 min at 72°C; (2) as for Program 1, but the initial amplification step involved 25 cycles with a stepdown of 0.6°C per cycle and an extension phase of 80 s at 72°C. Products were visualised on 8% polyacrylamide gels stained with ethidium bromide, and PCR reactions were further optimised for $MgCl_2$ and/or betaine (as per Table 1). PCR products were visualised on an Applied Biosystems 3730 capillary sequencer and scored manually using GeneMapper 4.0 (Applied Biosystems).

Of the 10 loci screened, one did not amplify a product and one was monomorphic. The remaining eight loci were used to genotype and assess genetic diversity in 48 individuals from two natural populations (Table 1). The Donnybrook population is situated in open Jarrah forest 170 km south of Perth, while Lesueur is 210 km north of Perth in Kwongan heath. Both populations contained several hundred plants. Linkage disequilibrium among pairs of loci and deviations from Hardy-Weinberg equilibrium (HWE) were assessed using exact tests in Genepop 4.2 (Rousset 2008), applying the Bonferroni correction. Null allele frequencies were estimated for each locus using INEst 1.1 (Chybicki and Burczyk 2009) and genetic diversity parameters were estimated with Genepop 4.2.

Results and discussion

Evidence for null alleles and deviations from HWE was found for Ahum8 in both populations and for Ahum15 at Mt Lesueur (Table 1). As no deviations from HWE were found at other loci and the species is dioecious, the observed deviations were most likely caused by null

Table 1. Primer sequences and characteristics of ten microsatellite loci isolated from *Allocasuarina humilis*. See main text for details of PCR programs. Allele size range and number of alleles (N_A) are based on 48 individuals from two natural populations. Observed and expected heterozygosity (H_o, H_e) and null allele frequencies are based on 24 individuals within each natural population. One locus was monomorphic and one did not amplify.

Locus	Primer sequence (5′-3′)a	GenBank accession	Repeat motif	$MgCl_2$ (mM)	PCR progr	Size range (bp)	N_A	Donnybrook (n = 24) (33° 34′ 38.3″ S, 115° 51′ 9.2″ E)			Mt. Lesueur (n = 24) (30° 04′ 03.3″ S, 115° 07′ 40.5″ E)		
								H_o	H_e	Null allele	H_o	H_e	Null allele
Ahum6	F: TAAGCACGGAAAGAAACGCT	KM374578	$(AT)_9$	2.5	2[b]	104-128	10	0.826	0.820	0.034	0.500	0.705	0.105
	R: GGTCTTCATATTCAATCTCTTGGG												
Ahum8	F: CCACTGTTATGGCCTACAGGA	KM374579	$(AG)_{11}$	2.5	1[b]	94-122	12	**0.522***	0.853	**0.141***	**0.542***	0.893	**0.144***
	R: ACACAGTCAAGACGGCTTTC												
Ahum9	F: TGATGAATGTAATGAAGGCTGC	KM374580	$(GA)_9$	2.5	1	97-131	14	0.500	0.553	0.044	0.792	0.650	0.024
	R: TGGATTTAAGCTACCTGATGGA												
Ahum15	F: CCAACCAATCTACAGAACAGCA	KM374581	$(AT)_{10}$	2.5	1[b]	186-236	17	0.591	0.827	0.096	**0.208***	0.920	**0.272***
	R: TCTAACGTTCTGTCAAGCAATCA												
Ahum16	F: GCTGTCAGGTCTGGGTAAATTC	KM374582	$(CT)_9$	2.5	1[b]	159-191	12	0.833	0.789	0.028	0.875	0.815	0.025
	R: TGTACACGTTGGAGTTGTGGA												
Ahum18	F: AAGGAAATCACCCATAATCGAA	KM374583	$(ATT)_{20}$	2.25	1[b]	136-199	19	0.956	0.896	0.019	0.956	0.947	0.019
	R: AGCAAATTTGTCCAACACCC												
Ahum22	F: CAAGGCTTGAACCAAAGATCA	KM374584	$(AT)_8$	2.5	1[b]	129-141	6	0.667	0.768	0.064	0.667	0.576	0.043
	R: GCTGTACATGTTGGCAAGTCA												
Ahum24	F: TTTTGAGCGTATGCAATGGG	KM374585	$(GT)_{10}$	2.5	1[b]	110-122	7	0.783	0.808	0.042	0.583	0.662	0.076
	R: TCACCATTTAAATTGAGGGTCT												
Ahum11	F: TTTCTTTGCTTTGCTTTCTATGTTG	KT224416	$(AC)_8$	2.5	1[b]	90	0						
	R: ACGTCAATTGATCCAAAGAAA												
Ahum14	F: TCCCGGGATTGTAAGTGTTG	KT224417	$(TTCTGA)_9$	2.5	1[b]	191	1						
	R: TATCTCCTGCGAGACGTGTG												

a Forward primers include an M13 universal sequence appended to the 5′ end (5′- CACGACGTTGTAAAACGAC)

b 0.5M betaine included in PCR

* Significant deviation from Hardy-Weinberg equilibrium or significant estimate of null allele frequency ($P < 0.05$)

alleles rather than inbreeding. There was no evidence for linkage disequilibrium between any loci in either population. Among the 48 screened individuals, the number of alleles per locus ranged from six (Ahum22) to 19 (Ahum18), with a mean of 12.1. We detected moderate to high genetic diversity, with expected heterozygosity ranging from 0.553 to 0.947 (mean 0.789) and observed heterozygosity from 0.208 to 0.956 (mean 0.710).

These microsatellite markers are the first to be developed for a Western Australian *Allocasuarina* and have doubled the number of markers available for the genus. Reliable polymorphic microsatellites have proved difficult to isolate in *Allocasuarina* (Lamont 2010; Lamont et al. 2012; L. Broadhust pers. comm; this study). Six microsatellite markers have previously been developed for *A. verticillata* (Lam.) L.A.S.Johnson (Broadhurst 2011) and two for *A. emuina* L.A.S.Johnson (Lamont et al. 2012). Consistent with the relatively recent radiation of the genus (Steane et al. 2003), the markers developed by Broadhurst (2011) and Lamont et al. (2012) had high rates of transferability among *Allocasuarina* species both within and across sections (Lamont 2010). The eight microsatellite markers developed here for *A. humilis* are therefore expected to be transferable to other *Allocasuarina* species for which assessment of genetic characteristics may be required for conservation or other purposes. These markers are being used to evaluate the patterns of genetic diversity, divergence and structure among *A. humilis* populations in the SWAFR in order to inform appropriate seed sourcing strategies for restoration of disturbed or degraded land.

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References

Barlow, B.A. (1959). Chromosome numbers in the Casuarinaceae. *Australian Journal of Botany* **7**, 230–237.

Broadhurst, L.M. (2011). Genetic diversity and population genetic structure in fragmented *Allocasuarina verticillata* (Allocasuarinaceae) – implications for restoration. *Australian Journal of Botany* **59**, 770–780.

Calviño-Cancela, M., Dunn, R.R., van Etten, E.J.B. and Lamont, B.B. (2006). Emus as non-standard seed dispersers and their potential for long-distance dispersal. *Ecography* **29**, 632–640.

Chybicki, I.J. and Burczyk, J. (2009). Simultaneous estimation of null alleles and inbreeding coefficients. *Journal of Heredity* **100**, 106–113.

Conservation Commission of Western Australia (2004). *Forest Management Plan 2004–2014*. Conservation Commission of Western Australia, Perth, Western Australia.

Doyle, J.J. and Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**, 11–15.

Gardner, M.G., Fitch, A.J., Bertozzi, T. and Lowe, A.J. (2011). Rise of the machines – recommendations for ecologists when using next generation sequencing for microsatellite development. *Molecular Ecology Resources* **11**, 1093–1101.

Godefroid, S., Piazza, C., Rossi, G., Buord, S., Stevens, A.-D., Aguraiuja, R., Cowell, C., Weekley, C.W., Vogg, G., Iriondo, J.M., Johnson, I., Dixon, B., Gordon, D., Magnanon, S., Valentin, B., Bjureke, K., Koopman, R., Vicens, M., Virevaire, M. and Vanderborght, T. (2011). How successful are plant species reintroductions? *Biological Conservation* **144**, 672–682.

Lamont, R. (2010). *Conservation genetics and ecology of the endangered heathland shrub, Allocasuarina emuina*. PhD Thesis, University of the Sunshine Coast, Sippy Downs, Queensland.

Lamont, R.W., Stokoe, R.L. and Shapcott, A. (2012). Ecological genetics of the wind-pollinated, tetraploid, *Allocasuarina emuina* L. Johnson (Casuarinaceae) from southeast Queensland reveals montane refugia for coastal heath during the last interglacial. *Australian Journal of Botany* **60**, 718–734.

Meglécz, E., Costedoat, C., Dubut, V., Gilles, A., Malausa, T., Pech, N. and Martin, J.-F. (2010). QDD: a user-friendly program to select microsatellite markers and design primers from large sequencing projects. *Bioinformatics* **26**, 403–404.

Rousset, F. (2008). GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* **8**, 103–106.

Steane, D.A., Wilson, K.L. and Hill, R.S. (2003). Using matK sequence data to unravel the phylogeny of Casuarinaceae. *Molecular Phylogenetics and Evolution* **28**, 47–59.

Western Australian Herbarium (1998–). *FloraBase – the Western Australian Flora*. Department of Parks and Wildlife. Accessed 3 March, 2015. http://florabase.dpaw.wa.gov.au

Wilson, K.L. and Johnson, L.A.S. (1989). Casuarinaceae. *Flora of Australia* **3**, 100–135.