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PERMANENT GENETIC RESOURCES

Microsatellite markers isolated from a polyploid saltbush, *Atriplex nummularia* Lindl. (Chenopodiaceae)

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Abstract

***Atriplex nummularia* is a polyploid Australian saltbush which has been identified as a suitable species for use in the rehabilitation of agricultural land affected by salinity. We isolated 12 polymorphic loci for a preliminary assessment of genetic variability and structure within the species as a basis for a breeding programme. Preliminary screening of loci in 40 individuals from two populations revealed multibanded genotypes consisting of up to seven alleles in a single individual, with up to 29 alleles observed at a single locus. The multibanded patterns are consistent with the polyploid status of this species.**

Keywords: *Atriplex*, genetic diversity, microsatellites, polyploidy, SSR

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Dryland salinity is a common consequence of European agricultural practices. The clearing of vegetation in Australia's agricultural regions has resulted in the salinization of over 2.2 million ha of land and this is expected to reach 17 million ha if current trends continue. Attempts to rehabilitate salinized land often involve the planting of salt-tolerant plant species which lower the water table and provide landholders with a fodder crop. In Australia, the dioecious, perennial saltbush shrub *Atriplex nummularia* Lindl. found throughout arid and semi-arid regions, has been identified as such a species. *A. nummularia* is octoploid with $n = 36$ and $2n = 36$II and the base number of the genus is considered to be $x = 9$ (Nobs 1980). The species is highly tolerant to salt and also contains high levels of crude protein, making it ideal fodder for livestock. Here, we present 12 polymorphic microsatellite markers for *A. nummularia* which will be used to describe levels of genetic variability across the range of the species and in a breeding programme.

Genomic DNA was extracted from the frozen (–70 °C) leaf material of one individual of *A. nummularia* as in Byrne *et al*. (1998). Microsatellite DNA loci were isolated following the protocol described by Glenn & Schable (2005). Linker ligated DNA was denatured and hybridized to one of two biotinylated microsatellite oligonucleotide mixes [mix 1: $(AC)_{13}$, $(AACC)_5$, $(AACG)_5$, $(AAGC)_5$, $(AAGG)_5$, and $(ATCC)_5$ (Savannah River Ecology Laboratory) or mix AG: $(AG)_{13}$], and captured on Streptavidin MagneSphere Paramagnetic Particles (Promega). Microsatellite-enriched DNA was eluted from the magnetic particles and amplified in polymerase chain reactions (PCR) using SuperSNX24 Forward (Savannah River Ecology Laboratory) as the single primer in the reactions. PCR products were cloned into TOP10 *Escherichia coli* cells (Invitrogen) using TOPO TA Cloning Kits (Invitrogen), allowing direct selection of recombinants via disruption of the lethal *E. coli* gene, *ccdB*. A total of 1344 positive clones were isolated and inserts for 768 clones were amplified by PCR with M13 forward and reverse primers. PCR products were screened by dot blot hybridization onto Hybond-N + nylon membranes (GE Healthcare) using equal concentrations (1 nM each) of biotin-labelled dinucleotide $(AC)_{13}$ and $(AG)_{13}$ probes. Positive clones were detected using a CDP-Star Universal Alkaline Phosphatase detection kit (Sigma) and the membranes exposed to radiography film. Dot blot screening showed good correlation between the intensity of the detection reaction and the length of the microsatellite sequence in the plasmid insert. Seventy-seven of the 143 ExoSAP-treated and sequenced PCR products contained microsatellites and one sequence was duplicated. Eighty PCR products were initially selected for sequencing by product size, 25 of these contained microsatellites. A further 63 PCR products were selected for sequencing based on dot blot hybridization, and of these, 52 contained microsatellites. Dot blot screening showed good correlation between the intensity of the detection

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Table 1 Characterization of 12 microsatellites in *Atriplex nummularia*

Locus/Accession no.	Primer sequences (5′–3′)	Repeat motif	N_b	N_i (± SE)	N_m	T_A (°C)	$MgCl_2$ (mM)	Allele size range (bp)
1AB9	F: CTCATATCCAGCCTAAATTCAACC	$(GT)_9GGT(GT)_{15}$	21	1.50 ± 0.21	7	56	1.00	150–204
EU305053	R: AAAATGGGCAGTCATCCAAC	$GGT(GT)_3GGT(GT)_6$						
1AF1	F: GAACACAGGCTATTCCCAAG	$(CA)_8$	6	2.57 ± 0.09	4	56	1.25	147–156
EU305054	R: GGAAAGAACATGTGAGAGTTCG							
1AG5	F: CTGCAAGCTAGCGTTCATGC	$(TG)_{10}$	4	1.79 ± 0.10	3	56	0.75	151–160
EU305055	R: ACTCCTCCTATGGCCCTATCTC							
1B12	F: TGTAGCTGGAAGCGGTAAAG	$(TC)_{27}$	21	3.66 ± 0.21	7	56	1.25	233–284
EU305056	R: GAAGCGGTGGTAAATTCCTG							
1C2	F: TGTAAGCCTTGGTAGCAGACC	$(GA)_{14}$	44	4.48 ± 0.18	7	56	1.50	274–352
EU305057	R: ACGCGATTCCCTGTAAAGAC							
2D4	F: TCCTTTCCGAACCTTCTTTG	$(TC)_{13}$	5	1.28 ± 0.09	3	56	1.75	169–182
EU305058	R: GACCTCGTGGAGGATGTATTG							
2F2	F: ATAGGCGACAAGCGAACAAC	$(AG)_{12}$	8	2.62 ± 0.13	4	56	1.00	195–207
EU305059	R: GCTTTTCCAACTCCACCACA							
2H8	F: GCCGTCGATCTTTCTTCTGT	$(AG)_{15}TAGA(AG)_4$	16	2.15 ± 0.16	4	56	2.75	366–398
EU305060	R: GCGCCTCAATCTGTTCATC							
3D6	F: TGGGTGATATGGCTTCCA	$(AG)_{14}TG(AG)_5$	29	3.48 ± 0.16	5	56	1.25	249–301
EU305061	R: ACGAAGCTCTTGGTGTCAGG							
10C1	F: TCTCCCTCTCCTATCACATGG	$(TC)_{19}$	24	4.60 ± 0.16	6	56	1.75	188–233
EU305062	R: GCCTTTCTTGTTGCATCGTC							
10G3	F: TCAGTCACAAGAGCTAAATTCGAC	$(TC)_{12}$ ... $(TC)_5(AC)_3$	12	2.41 ± 0.15	5	56	1.50	191–218
EU305063	R: CAGGAAACTCAAACAAATCTGG							
10H3	F: CGTGCAGAATGTATGGTGGA	$(GA)_{22}$	20	2.37 ± 0.15	4	56	1.25	191–226
EU305064	R: CAAGTCGCGTCAGGATCATA							

N_b, total number of observed bands; N_i, mean number of bands per individual; N_m, maximum number of bands per individual; T_A, annealing temperature.

reaction and the length of the microsatellite sequence in the plasmid insert.

PCR primer pairs were designed from the flanking sequences for 20 of the positive clones identified using Primer 3 (Rozen & Skaletsky 2000) and Amplify 1.2 (Engles 1993) software. Unlabelled primer pairs were tested for amplification and polymorphism using one individual from each of three populations of *A. nummularia*, four populations of *Atriplex amnicola* Paul G. Wilson and one population of *Atriplex bunburyana* F. Muell. Preliminary PCR mixtures contained 50 mM KCl, 20 mM Tris HCl (pH 8.4), 0.2 mM of each DNTP, 0.3 mM forward and reverse primer, 0.5 U of *Taq* polymerase, 20 ng of DNA and 1.5 mM $MgCl_2$. Amplification was carried out using a standard three-step PCR profile (30 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s with initial denaturation and final extension periods of 96 °C for 2 min and 72 °C for 5 min, respectively) in an Eppendorf Mastercycler ep gradient S thermocycler. Products were subject to electrophoresis on 8% nondenaturing polyacrylamide gels and stained with ethidium bromide. Subsequent PCRs were conducted with variable $MgCl_2$ concentrations to improve product quality. A total of 12 primer pairs successfully amplified reliable PCR products in all the individuals tested.

The forward primers of these 12 loci were 5′ labelled with one of three fluorescent dyes, 6-FAM (GeneWorks), VIC or NED (Applied Biosystems) and tested for polymorphism on 20 individuals from each of two populations of *A. nummularia* using the previously described PCR conditions and the appropriate concentration of $MgCl_2$ (Table 1). Loci were amplified individually and panels of three differently labelled reaction products were separated on an Applied Biosystems 3730 capillary sequencer with Applied Biosystems' GeneScan 600 LIZ size standard. Results were analysed using GeneMapper version 4.0 (Applied Biosystems).

The 12 microsatellite loci revealed multibanded patterns with three to seven bands per individual (Table 1). The average number of bands per individual ranged from 1.28 (± 0.09) to 4.60 (± 0.16) for different loci. The total number of different bands observed at a locus ranged from four to 29. The reliability of banding patterns was tested for all loci in eight individuals in three separate amplifications. The multiple banding patterns were concluded to indicate multiple alleles consistent with the octoploid chromosome number of *A. nummularia*. We were not able to determine the exact number of copies of each allele because of the unknown allele dosage of partial heterozygotes, and it was therefore not possible to calculate gene frequencies or test

for Hardy–Weinberg equilibrium and linkage disequilibrium. The presence/absence of bands will be used to estimate a variety of statistics to assess intraspecific differentiation and diversity in *A. nummularia*.

References

Byrne M, Parrish TL, Moran GF (1998) Nuclear RFLP diversity in *Eucalyptus nitens*. *Heredity*, **81**, 225–232.

Engles WR (1993) Contributing software to the Internet: the Amplify program. *Trends in Biochemical Sciences*, **18**, 448–450.

Glenn TC, Schable NA (2005) Isolating microsatellite DNA loci. *Methods in Enzymology*, **395**, 202–222.

Nobs MA (1980) Chromosome numbers in Australian species of *Atriplex*. *Carnegie Institute Yearbook*, **79**, 164–169.

Rozen S, Skaletsky HJ (2000) Primer 3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, New Jersey.