American Journal of Botany

American Journal of Botany: e99–e101. 2010.

AJB Primer Notes & Protocols in the Plant Sciences

Characterization and multiplexing of EST-SSR primers in *Cynodon* (Poaceae) species[1]

Margaret C. Jewell[2,3], Celine H. Frere[2], Peter J. Prentis[2], Christopher J. Lambrides[2], and Ian D. Godwin[2]

[2]School of Land, Crop and Food Sciences, The University of Queensland, Brisbane, QLD, Australia, 4072

- *Premise of the study: Cynodon* species are multiple-use grasses that display varying levels of adaptation to biotic and abiotic stress. Previously identified EST-SSR primers were characterized and multiplexed to assess the level of genetic diversity present within a collection of almost 1200 *Cynodon* accessions from across Australia.
- *Methods and Results:* Two multiplex reactions were developed comprising a total of 16 EST-SSR markers. All SSR markers amplified across different *Cynodon* species and different levels of ploidy. The number of alleles ranged from one to eight per locus and the total number of alleles for the germplasm collection was 79.
- *Conclusions:* The 16 markers show sufficient variation for the characterization of *Cynodon* core collections and analysis of population genetic diversity in *Cynodon* grasses.

Key words: Bermudagrass; diversity; grass; multiplex; polyploidy; SSR.

Cynodon species are perennial grasses with a cosmopolitan distribution. They are reported to display excellent heat and drought tolerance and, therefore, have global importance for turf, amenity, pasture, and stabilization and remediation projects in semiarid and arid regions (McCarty and Miller, 2002; Zhou et al., 2009). The *Cynodon* genus is a member of the Chloridoideae subfamily of the *Poaceae* family and comprises 9 species with a base chromosome number of x = 9 (Harlan and de Wet, 1969). Combinations of intra- and interspecies hybridizations, as well as natural meiotic chromosome duplications and pairing of unreduced gametes, have resulted in the evolution of triploid, tetraploid, pentaploid, and hexaploid varieties. There is ploidy level variation both within and among species. Tetraploid *C. dactylon* var. *dactylon* (2n = 4×=36) is the most widespread variety, but hybrid cultivars produced through crosses between *C. dactylon* and diploid species, such as *C. transvaalensis* (2n = 2×=18), are also relatively common (Taliaferro, 2003).

Most commercial *Cynodon* cultivars have been produced through selection of elite lines or through hybridization and mutagenesis based on selected elite lines (Anderson et al., 2001; Wu et al., 2007). However, the value of wild relatives in plant improvement has recently gained increased recognition (Hajjar and Hodgkin, 2007). This has led to several collections of wild *Cynodon* species with the aim of increasing adaptive genetic diversity available for *Cynodon* improvement (Kearns et al., 2009). Identification and classification of the diversity present within these collections via methods such as SSR-based DNA fingerprinting is fundamental to ensuring that maximum genetic diversity is catalogued and available for use in *Cynodon* breeding programs.

[1] Manuscript received 15 July 2010 ; revision accepted 9 August 2010.

[3] Author for correspondence: m.jewell@uq.edu.au

doi:10.3732/ajb.1000254

To date, the paucity of genome sequence information has precluded the use of SSR markers in studies of *Cynodon* genetic diversity. Although EST-SSRs are associated with gene coding regions, their high level of polymorphism is comparable to that of SSRs developed from nontranscribed genomic regions (Parida et al., 2009; Mace and Godwin, 2002). As EST-SSRs are associated with functional genes, however, it is more likely that they may have been the targets of recent selection (Oetjen and Reusch, 2007). This may confound patterns of population genetic structure at some loci. While there are some potential drawbacks associated with EST-SSRs, they are useful in diversity studies of polyploid genera because they are more readily transferable across species, and less susceptible to sequence mutations resulting in null-alleles (Ellis and Burke, 2007).

Here we characterize previously published EST-SSR primer sequences (Kim et al., 2008) to develop two multiplex reactions for detection of 16 loci in a subcollection of 1200 *Cynodon* accessions from across Australia. These markers provide consistent patterns of amplification and high-resolution differentiation of *Cynodon* germplasm.

METHODS AND RESULTS

The plant material being used in this study consists of a subset of a *Cynodon* germplasm collection which is being maintained at three locations in South East Queensland, Australia. Geographic origin and GPS co-ordinates for all genotypes used in this study are available in Appendix S1 (see Supplemental Data with the online version of this article). The primers used were identified by Kim et al. (2008) in *C. dactylon* ESTs. A total of 93 primer pairs were screened across a subset of 8 genotypes from the *Cynodon* germplasm collection (subset 1) representing variation in geographic distribution and ploidy. Subset 1 consisted of 5 commercial varieties (Tifton 10 (6×), *C. transvaalensis* (2×), Grand Prix (4×), Patriot (4×), and Santa Ana (3×)) and 3 wild accessions (Biloela, QLD (2×), Rochedale, QLD (3×), and Augusta, WA (6×)). PCR products were amplified by 74 primer pairs in at least one of the 8 accessions.

Genomic DNA was extracted from fresh leaf tissue using a modified CTAB extraction method (Rogers and Bendich, 1985) and DNA concentration was

determined using a NanoDrop™ 2000 (Thermo Fisher Scientific, Victoria, Australia). PCR reactions were conducted in a DNA Engine Thermal Cycler (Bio-Rad Laboratories, Richmond, Califiornia, USA) using a Multiplex PCR Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions with 2 ngμL^{-1} DNA and 0.5 uM forward and reverse primers. A touchdown PCR reaction was performed to account for varying annealing temperatures according to the following program: 1 cycle of 15 min at 95°C, followed by 36 cycles of 40 s at 94°C, 90 s at 68°C-0.5°C each cycle, 90 s at 72°C, and terminating with 1 cycle of 20 min at 60°C. Amplicons were sized by running 5 μL of amplification product on 8% PAGE. Two multiplex reactions (MP1 and MP2) were designed with 6 and 10 primer pairs, respectively (Table 1). Gradient PCR determined that annealing temperatures of 58°C and 62°C were appropriate for MP1 and MP2, respectively. The forward primers for markers of similar size in each multiplex mix were labeled with different fluorescent dyes (6-FAM (blue), NED (yellow), VIC (green), or PET (red; Applied Biosystems, Foster City, CA) to avoid allele size overlap (Table 1). Labeled primers were screened across subset 1 using the following PCR program: 1 cycle of 15 min at 95°C, followed by 30 cycles of 30 s at 94°C, 90 s of 58°C, 90 s at 72°C, and terminating with 1 cycle of 20 min at 72°C for MP1 and 1 cycle of 15 min at 95°C, followed by 27 cycles of 30 s at 94°C, 90 s of 62°C, 60 s at 72°C, and terminating with 1 cycle of 30 min at 60°C for MP2. Capillary electrophoresis analysis was performed using a 1200 bp standard (GS 1200 LIZ) for MP1 and a 500 bp standard (GS 500 LIZ) for MP2 (Applied Biosystems). Reaction products were diluted 1:100, run on a 3730 Genetic Analyzer, and results were analyzed using GeneMapper Software Version 3.7 (Applied Biosystems). Respective primer concentrations were increased or decreased if electropherogram peaks were found to be too small or too large. Final primer concentrations are included in Table 1.

The primers were then screened across a set of 21 mostly wild, putative triploid, genotypes from the Australian collection (subset 2). These included 10 from Queensland, 4 from New South Wales, 3 from Western Australia, 1 from the Northern Territory, 2 from South Australia, and 1 from Victoria. The triploid commercial varieties used in subset 1 were included in the analyses of heterozygosity within both subsets.

For subset 2, the maximum number of alleles produced by the markers retained in the analysis was 8 with an average of 5 (Table 2). Slightly fewer allele numbers were recorded in subset 1.

Observed (Ho) and expected heterozygosity (He) were calculated for all markers in both subsets using the equations published in Weir (1996). Due to polyploidy and the subsequent variations in allelic proportions of heterozygotes, it was not possible to calculate measures of genetic variability that are commonly used to describe diploid organisms. To overcome this, the presence or absence of allelogram peaks was used to construct a binary table enabling analysis of the data that is commonly used with dominant marker systems. Average ploidy levels were used to approximate the total allelic population size in the calculation of allele frequencies. The authors note that this approximation may result in an underestimation of heterozygosity statistics because potential null-alleles and multiple allele copies are not able to be detected in polyploids.

Excluding marker 35, which appears to be a presence/absence marker type rather than a SSR repeat length variation marker, *H*e values ranged from 0.82

TABLE 1. Characteristics of 16 SSR markers used in *Cynodon* fingerprinting. Shown for each primer pair are the previously published forward and reverse sequence, repeat type, expected size (bp), GenBank accession number and corresponding unigene number of the markers (Kim et al., 2008), as well as the fluorescent label, multiplex reactions (MP1 or MP2), annealing temperature, and final concentration of each marker used in the respective multiplex reaction.

Marker	Primer Sequence (5′–3′)	Repeat	Size (bp)	Accession No.	Unigene	Fluorescent Label	Multiplex Reaction	Ta (°C)	Conc (μM)
3	F: GGACTCAAAAATGCTCAGAAA	(CAAA)6	202	ES304263	773	NED	MP1	58	0.1
	R: TTTGCAGAGCCCGTAACTCT			ES297492					
				ES301678					
				ES296415					
8	F: CAAGGACCACATCACCATCA	(CGAT)6	204	ES294662	3808	VIC	MP1	58	0.05
	R: CGGCCATTGATTATCTGTGA								
10	F: GTTAGTCAGGATCCCAGTGAA	(TAG)7	231	ES297613	4637	PET	MP2	62	0.4
	R: GCAATTACGCAATGGACAAA								
17	F: TCCTGAGGGGTTCTCACTG	(CCA)9	176	ES303244	7639	VIC	MP2	62	0.4
	R: GGAGTAGGTGCTGCTGATT								
35B	F: TTGTGCCAGCTATCTTGGTG		159	ES294037	3525	PET	MP1	58	0.2
	R: TGCGAAGCAAGAAGAGTACG								
56	F: TGACGTCGTTGGTGTAGAGC	(TTG)10	239	ES302010	1927	VIC	MP2	62	0.2
	R: CGACTCCATCTGGTCCAACT			ES300198					
58	F: AGATCGGGGTGGGGAAGA	(CGA)6	199	ES306753	2154	6-FAM	MP1	58	0.1
	R: GTACATCTCCAGCAGCGACA			ES306918					
63	F: ATCCAATGAGTGGGACTCCA	(CAA)10	167	ES295668	4294	VIC	MP1	58	0.05
	R: CCAGCTTGCTTGGGATTAAA								
69	F: TGATGGTGATGCAATGGACT	(GAA)7	213	ES300504	6327	NED	MP2	62	0.2
	R: GCTGTCTTGGGTTCAGCTTC								
72	F: CTCGATAGGCACAAGGGAAG	(TCC)14	231	ES306759	9136	PET	MP1	58	0.2
	R: CCTTGCATATCGGAGTCCTG								
74	F: GGCAGCTCCTCTCTCCTTAAA	(CGG)8	166	ES305182	436	PET	MP2	62	0.3
	R: ACCATGACCTTGTCCTCGTC			ES292020					
				ES295381					
				ES297955					
				ES302814					
				ES300915					
76	F: ACATGTCTCCGTCCCATCA	CCG	167	ES306865	2224	6-FAM	MP2	62	0.2
	R: ATGAGTCGGTCCTTCTTGG			ES296960					
79	F: TCTGGCAAACCCTTGAATC	CGA	218	ES294286	3660	6-FAM	MP2	62	0.3
	R: CTCCTCCAGCTCCAAGTCCT								
87	F: AGGGGAAGAAGGGTAAGCAG	CAG	189	ES300599	6446	NED	MP2	62	0.2
	R: CACCAAATCCACCAAAGGAG								
88	F: CATGCGCTTTGAGTTTGAG	TTA	177	ES297067	6807	PET	MP2	62	0.3
	R: AACACCACAACGACCTTCG								
91	F: TCCGTTGCCTATACGGTTG	CGG	225	ES303889	7897	6-FAM	MP2	62	0.3
	R: GGAACCGATAATCACTCCA								

Table 2. Results of initial primer screen in triploid and polyploidy populations of *Cynodon* grasses. Shown for each marker are the number of alleles (A), and mean values of expected (He) and observed (Ho) heterozygosity. Astericks indicate that the value was calculated from the equation for detecting He from multilocus data (Weir, 1996).

	Subset 2 ($N = 21$)			Subset 1 ($N = 8$)		
Marker	Alleles	He	Ho	Alleles	He	Ho
63	8	0.92	0.33	5	0.97	0.38
69	3	0.88	0.29	3	0.95	0.25
72	5	0.94	0.33	4	0.96	0.38
58	4	0.87	0.33	4	0.91	0.88
76	3	0.84	0.79	3	0.93	0.75
10	5	0.87	0.67	4	0.94	0.75
87	2	0.88	0.38	2	0.95	0.25
35	1	0.96	0.00	1	0.00	0.00
79	5	0.86	0.63	4	0.94	0.63
8	6	0.88	0.75	5	0.96	0.63
3	5	0.92	0.54	4	0.98	0.38
91	8	0.91	0.63	8	0.94	0.75
17	5	0.90	0.96	5	0.96	0.50
56	6	0.82	0.96	6	0.92	0.75
74	2	0.87	0.46	2	0.94	0.00
88	5	0.91	0.17	5	0.96	0.13
Average	5	0.83*	0.51	4	0.89*	0.46

to 0.94 for subset 2 and 0.91 to 0.98 for subset 1 with multilocus averages of 0.83 and 0.89, respectively. The maximum number of alleles observed at any particular locus for an individual from subset 2 was 3, which validates the inclusion of these individuals as triploids. In subset 1, the maximum number of alleles observed for each individual was consistent with its putative ploidy.

CONCLUSIONS

Results indicate that the selected EST-SSR markers amplify across a range of *Cynodon* species that vary for geographic origin and ploidy level. The EST-SSR loci characterized in this study are sufficient for genetic fingerprinting of *Cynodon* germplasm for purposes such as cultivar identification, analysis of genetic integrity, and core collection development. EST-SSR data generated from these loci can also be used to analyze patterns of population genetic diversity of *Cynodon* species. Although the use of EST-SSR markers can potentially confound studies of population genetic structure because they may have been the target of selection, this is advantageous for studies examining heterogeneous genome divergence associated with selection at particular loci.

LITERATURE CITED

Anderson, M. P., C. M. Taliaferro, D. L. Martin, and C. S. Anderson. 2001. Comparitive DNA Profiling of U-3 Turf Bermudagrass Strains. *Crop Science* 41: 1184–1189.

Ellis, J. R., and J. M. Burke. 2007. EST-SSRs as a resource for population genetic analyses. *Heredity* 99: 125–132.

Hajjar, R., and T. Hodgkin. 2007. The use of wild relatives in crop improvement: A survey of developments over the last 20 years. *Euphytica* 156: 1–13.

Harlan, J. R., and J. M. J. de Wet. 1969. Sources of Variation in *Cynodon dactylon* (L). Pers. *Crop Science* 9: 774–778.

Kearns, R., Y. Zhou, S. Fukai, C. Ye, D. Loch, I. Godwin, T. Holton, et al. 2009. Eco-turf: Water use efficient turfgrasses from Australian biodiversity. *Acta Horticulturae* 829: 113–118.

Kim, C., C. S. Jang, T. L. Kamps, J. S. Robertson, F. A. Feltus, and A. H. Paterson. 2008. Transcriptome analysis of leaf tissue from Bermudagrass (*Cynodon dactylon*) using a normalised cDNA library. *Functional Plant Biology* 35: 585–594.

Mace, E. S., and I. D. Godwin. 2002. Development and characterisation of polymorphic microsatellite markers in taro, *Colocasia esculenta* (L.) Schott. *Genome* 45: 823–832.

McCarty, L.B. and G. Miller. (2002). Managing Bermudagrass Turf: Selection Construction, Cultural Practices, and Pest Management Strategies. Ann Arbor Press, Chelsea, USA.

Oetjen, K., and T. B. Reusch. 2007. Genome scans detect consistent divergent selection among subtidal vs. intertidal populations of the marine angiosperm *Zostera marina. Molecular Ecology* 16: 5156–5167.

Parida, S. K., V. Dalal, A. K. Singh, N. K. Singh, and T. Mohapatra. 2009. Genic non-coding microsatellites in the rice genome: characterization, marker design and use in assessing genetic and evolutionary relationships among domesticated groups. *BMC Genomics* 10: 140–160.

Rogers, S. O., and A. J. Bendich. 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant-tissues. *Plant Molecular Biology* 5: 69–76.

Taliaferro, C. M. 2003. Bermudagrass. In: M. D. Casler and R.R. Duncan [eds] Turfgrass Biology, Genetics, and Breeding, 235-256. John Wiley and Sons, Hoboken, New Jersey, USA.

Weir, B. S. 1996. Genetic Data Analysis II. Sinauer Inc., Sunderland, Massachusetts, USA.

Wu, Y. Q., C. M. Taliaferro, D. L. Martin, J. A. Anderson, and M. P. Anderson. 2007. Genetic variability and relationships for adaptive, morphological, and biomass traits in Chinese Bermudagrass accessions. *Crop Science* 47: 1985–1994.

Zhou, Y., C. Lambrides, R. Kearns, C. Ye, N. Cao, and S. Fukai. 2009. Selecting for drought tolerance among Australian green couch grasses (*Cynodon* spp.). *Crop and Pasture Science* 60: 1175–1183.