Plant Breeding **130**, 104—108 (2011)
doi:10.1111/j.1439-0523.2010.01779.x

Short Communication

Development of microsatellite markers in Guineagrass (*Panicum maximum* Jacq.) and their transferability to other tropical forage grass species

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With 1 figure and 1 table

Received November 10, 2009/Accepted February 04, 2010
Communicated by S. Korban

Abstract

The Guineagrass (*Panicum maximum* Jacq.) is one of the most important tropical forage grasses, but genetic knowledge and tools regarding this species are still limited. Therefore, 20 novel polymorphic microsatellite markers were developed, validated, and employed in estimating genetic relationships among 25 *P. maximum* genotypes selected from a Brazilian germplasm collection. In addition, they were tested for cross-species amplification in four other forage grass species. The number of alleles observed for each locus ranged from 4 to 12 (average 6.7). The values of polymorphism information content (PIC) varied from 0.41 to 0.83 (average 0.61) and the discriminating power (D) ranged from 0.53 to 0.98 (average 0.72). Cross-amplification demonstrated the potential transferability of these microsatellites to four tropical forage grass species. Cluster analysis based on the unweighted pair group method revealed three distinct groups: two clusters consisted of *P. maximum* genotypes and a third cluster, consisted of the other tropical forage grass species. The data demonstrated that the microsatellites developed herein have potential for germplasm characterization and genetic diversity analysis in *P. maximum* and other forage grass species.

Key words: Guineagrass — simple sequence repeat — molecular marker — genetic diversity

Guineagrass (*Panicum maximum* Jacq.) is a tropical forage grass native to Africa. Genetic diversity is high among *P. maximum* populations of tropical, subtropical, and warm temperate regions of the world, where it has become an important forage grass. The *P. maximum* species belongs to the family *Poaceae*, subfamily *Panicoideae*, tribe *Paniceae,* and forms an agamic complex with the botanical species *P. infestum* Anders and *P. trichocladum* K. Schum. Aposporous apomixis with pseudogamy is the propagation mode of *P. maximum*. This asexual mode of propagation enables cloning of plants through the seed and can be used for fixing desirable genotypes, as F_1 hybrids, and simplifying commercial hybrid seed production (Savidan 2000). In *P. maximum,* apomictic plants are tetraploid and have $2n = 4x = 32$ chromosomes (Savidan 1982).

The breeding of tropical forage grasses consists mainly of the evaluation and selection of natural ecotypes by exploiting the variability in centres of origin (Jank et al. 2005a). This procedure is being successfully used in *P. maximum* and several *Brachiaria* species for improved livestock performance in Brazil. Guineagrass is a high yielding and nutritious forage adapted to soils of medium to high fertility and regions receiving over 600 mm of rainfall (Pereira et al. 2001). *Panicum maximum* is the most productive seed-propagated tropical forage grass and the second most cultivated grass in Brazil.

The limited genetic knowledge of guineagrass has restricted breeding programmes. Genomic tools, such as microsatellite markers, are required for *P. maximum* improvement. Microsatellites are short tandem repeat sequence motifs, consisting of repeat units of 1–6 bp that represent highly polymorphic DNA markers with discrete loci and co-dominant alleles (Tautz and Schlotterer 1994). They are effective for genetic diversity and genetic relationship estimation, as well as for the prediction of genetic value in selected candidates derived from intraspecific crosses and the performance of their hybrid progenies (Gupta and Varshney 2000, Varshney et al. 2005, Ebina et al. 2007). Therefore, the development of microsatellite markers for *P. maximum* could be extremely important in exploiting genetic diversity of this species in breeding programmes.

This study reports the isolation and characterization of 20 polymorphic microsatellite markers for *P. maximum* and cross-species amplifications in other four tropical forage grass species.

Plant material and DNA extraction: Microsatellites were isolated from the genotype *P. maximum* cv. 'Tanzânia' (ORSTOM T58) and characterized in 25 *P. maximum* tetraploid apomictic genotypes from the EMBRAPA Beef Cattle germplasm collection at MS, Campo Grande, Brazil. The genotypes were collected and introduced in Brazil by the Institut Français de Recherche Scientifique pour le Développement en Coopération (ORSTOM), France and the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Brazil, and were designated as: ORSTOM-T21 (MASSAI), ORSTOM-T110, ORSTOM-T24, ORSTOM-G21, ORSTOM-T84, ORSTOM-K214, ORSTOM-T46, ORSTOM-T62, ORSTOM-K191, ORSTOM-K193, ORSTOM-K64, ORSTOM-K68, ORSTOM-K190A (MOMB – MOMBAÇA),

ORSTOM-T58 (TANZ – TANZÂNIA), ORSTOM-K217, ORSTOM-KK8, ORSTOM-T60, ORSTOM-T72, ORSTOM-T91, ORSTOM-T95, ORSTOM-KK33, ORSTOM-T97, ORSTOM-T65, ORSTOM-K249, and ORSTOM-T77. In addition, cross-amplification tests were performed using DNA from other tropical forage grass species in order to investigate the transferability of the microsatellites developed herein. The genera *Brachiaria* and *Paspalum* were selected because of their impact in the Brazilian forage market and their important contribution to the development of tropical pastures. The following species were evaluated: *Brachiaria humidicola*-CIAT-26149 (BH16) and *B. brizantha*-CIAT-16114 (B105) from the EMBRAPA Beef Cattle germplasm collection, and *Paspalum regnelli* (PR-019186) and *Pa. notatum* (PN-023523) from the EMBRAPA Pecuária Sudeste germplasm collection in São Carlos, SP, Brazil.

Genomic DNA was extracted from freeze-dried leaf samples following the CTAB method of Doyle and Doyle (1990). DNA samples were evaluated on a 1% agarose gel and quantified by comparison to known quantities of λ phage uncut DNA (Invitrogen, Carlsbad, CA, USA).

Library construction and screening for microsatellite repeat sequences: A genomic-enriched library was constructed for two dinucleotide repeat sequences following the protocol described by Billotte et al. (1999). Genomic DNA (20 μg) was digested with the *Rsa*I restriction enzyme (Invitrogen) and the resulting fragments were linked to *Rsa*I adapters. Dinucleotide (CT)8 and (GT)8 biotinylated probes were used for selection of *Rsa*I restricted fragments linked to *Rsa*I adapters which contained microsatellites. The selected DNA fragments were recovered with Streptavidin magneshere paramagnetic particles (Promega, Fitchburg, WI, USA) using a biotinylated probe, followed by magnetic selection as described by the manufacturer. Selected fragments were PCR amplified using primer sequences complementary to the adapters and ligated into the pGEM-T vector (Promega). *Escherichia coli* XL-1 Blue cells (Stratagene, La Jolla, CA, USA) were transformed with the recombinant plasmids and cultivated on Luria-Bertani agar plates containing 100 μg/ml ampicillin (Sigma, Germany), 50 μg/ml X-galactosidase, isopropyl **β**-D-1-thiogalactopyranoside (IPTG) (MBI Fermentas, Glen Burnie, MD, USA). Single white colonies were transferred onto microplates for long-term storage at −80°C.

DNA sequencing, primer designing, and microsatellite polymorphisms: Of the 576 recombinant colonies that were initially selected, 48 were sequenced on an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) using the Big Dye terminator version 3.1 version Cycle Sequencing Kit (Applied Biosystems). Primers were designed employing LaserGene software, version 5.03 (DNAStar Inc., Madison, WI, USA) using the following PCR conditions: amplification size products from 150 to 300 bp; GC content between 40% and 60%; annealing temperature (T_a) between 45 and 60°C; primer length between 18 and 22 bp and with no hairpins or dimmers. The simple sequence repeat identification tool (SSRIT) (http://www.gramene.org/db/markers/ssrtool) was used to identify microsatellites present in the non-redundant sequences (Temnykh et al. 2001). PCR amplifications were performed in 25 μl final volume containing 10 ng of DNA template, 0.8 μM of each forward and reverse primers, 100 μM of each dNTP (MBI Fermentas), 1.5 mM MgCl2, 10 mM Tris–HCl, 50 mM KCl, and 0.5 U Taq DNA Polymerase (Invitrogen). PCRs were performed using the following conditions: 94°C for 1 min followed by 30 cycles of 94°C for 1 min, specific Ta for 1 min, 72°C for 1 min, and a final extension of 72°C for 5 min. Amplification products were verified by electrophoresis on 3% agarose gels containing 0.1 mg ethidium bromide/ml in 1 × TBE buffer (89 mM Tris–borate, 2 mM EDTA, pH 8.0) and 6% denaturing polyacrylamide gels in 1 × TBE buffer, using a 10 bp ladder (Invitrogen) as a standard size. The DNA fragments were visualized by silver staining according to Creste et al. (2001).

Data analysis: The polymorphism information content (PIC) values were calculated for estimates of marker informativeness (Cordeiro et al. 2003). In order to compare marker efficiencies in varietal identification, a discriminating power (D) was estimated for each primer (Tessier et al. 1999). Genetic similarity for all the pairwise combinations were calculated according to Jaccard's similarity coefficient (Jaccard 1908) and clustered using the unweighted pair group method employing arithmetic averages (UPGMA) using the NTSYSpc v.2.1 software (Rohlf 2000). The reliability of the generated dendrogram was tested by bootstrap analysis using the BooD software with 1000 interactions (Coelho 2002).

Results and Discussion

The 20 novel microsatellite loci were polymorphic in *P. maximum* and in the *Brachiaria* and *Paspalum* species examined (Table 1). A total of 135 putative alleles were obtained from the 20 microsatellite markers and used to assess genetic relationships among the 25 *P. maximum* genotypes and four tropical forage grass species examined. The number of alleles ranged from 4 to 12, with an average of 6.7 alleles per putative locus. The PIC values of the 20 microsatellite loci ranged from 0.41 to 0.83 (average 0.61). The calculated D values ranged from 0.53 to 0.98 (average 0.72). The highest D value was found in 2PMc222.1 (0.91) and 1PMs11b (0.98), which were loci with the highest PIC values. The polyploidy nature of *P. maximum* does not allow for estimation of the exact copy number of each allele. Therefore, standard tests for deviation from the Hardy–Weinberg equilibrium and linkage disequilibrium were not applied.

All polymorphic markers were tested for cross-amplification in *B. humidicola, B. brizantha, P. notatum*, and *P. regnelli*. Eleven microsatellite loci amplified in these four species, but with different levels of transferability. Four loci (2PMc236, 1PMs11b, 2PMc226, and 2PMc144) amplified successfully in all species, indicating their absolute (100%) transferability. The markers 2PMc252 and 2PMc287 cross-amplified only in *Paspalum* ssp. and five markers (2PMc259, 2PMc308, 2PMc373, 2PM198.1, and 2PMc37) amplified only in *Brachiaria* ssp. Moreover, non-specific amplification of the loci 1PMc61, 2PMc342, 2PMc125, 1PMc48, 2PMc239.2, 2PMc191, 2PMc224, 1PMc48.16, and 2PMc222.1 was observed in the four species examined. These results suggest considerable sequence conservation within the primer regions flanking microsatellite loci.

Genetic analysis of 25 genotypes of *P. maximum* revealed three distinct clusters (Fig. 1). The 25 examine genotypes were differentiated based on similarity coefficients. The *P. maximum* apomictic tetraploid genotypes were separated into two clusters (I and II). Cluster I consisted of two subclusters: IA (T21 – MASSAI, and T110) and IB (T24, T46, T60, T62, T65, T72, T84, K64, K68, K191, K193, K214, K217, K249, KK8, KK33, G21, MOMBAÇA, TANZANIA, T97, T91, and T95). Cluster II contained only the T77 genotype. The T21, T110, and T77 genotypes are interspecific natural hybrids derived from crosses between *P. maximum* and *P. infestum*. The subclusters IA and IB contained three commercial varieties (Massai-T21, Tanzania and Mombaça), which were collected in Dar a Bagamoyo and Korogwe, Tanzania. The *P. maximum* genotypes that are preceded by T were collected in Tanzania (1969), KK in Kenya (1969), K in Kenya or Tanzania (1967), and G by African research institutions (seeds and seedlings). The data confirmed that the Kenya and Tanzania genotypes

Table 1: Description of 20 microsatellite markers developed for *Panicum maximum* Jacq. and cross-species amplification in four forage grass species

SSR locus	GenBank accession no.	Repeat motif	T_a (°C)	Primer sequences (5′–3′)	n	Characteristics in *P. maximum*			Transferability in tropical forage grass species			
						Size range (bp)	PIC	D	*Brachiaria humidicola*	*Brachiaria brizantha*	*Paspalum regnelli*	*Paspalum notatum*
1PMc61	FJ853735	(CA)5	60	F: ACACACCAGTCCAGTCAT R: GCCACGCTCCACAG	11	100–115	0.77	0.82	–	–	–	–
2PMc259	FJ853736	(GT)8	60	F: GGGCCGGGGGAGTAGGTTAG R: GGTTCAGGGGGAGAGCAGAGTG	9	207–216	0.62	0.76	+	+	–	–
2PMc342	FJ853737	(TG)7	53.3	F: GGGATCCGGTATGGTCA R: GTCGGCTGCGTGCTA	4	240–253	0.41	0.53	–	–	–	–
2PMc125	FJ853738	(TGT)9	55	F: CTTTGCCATCTCTTATTCCACA R: GCTGCCCATTTCTTAGTCCA	4	230–246	0.61	0.72	–	–	–	–
2PMc308	FJ853739	(CT)10(T)9	51.0	F: GGAACCGTGTAACTAAATGAT R: TGATGAATGAACCAAAAGTCT	6	219–240	0.58	0.70	+	+	–	–
1PMc48	FJ853740	(CA)6	60	F: GAAATCTGGTGCATCATCCT R: ATCCGGTTGGCAGAGTATC	4	240–253	0.51	0.63	–	–	–	–
2PMc239.2	FJ853741	(GT)6	53.0	F: CAAACAATTCAAGCACAAACTA R: CTATGGAGAAATTAAGCAACCT	4	251–262	0.53	0.69	–	–	–	–
2PMc191	FJ853742	(GT)5	49.2	F: ACACTCAACTGGCAACCT R: TTCACAATTTCTTTTCACAC	9	125–136	0.57	0.68	–	–	–	–
2PMc236	FJ853743	(TG)7	53	F: AACTGTCACATATTTTCACTTT R: GGATCCCCTCTTCATTAG	4	302–316	0.42	0.55	+	+	+	+
1PMs11b	FJ853744	(CA)9	60	F: CTGCTGCCGCCGTAGGAAT R: TTAAATGCGTCAACCCCAACC	12	345–358	0.83	0.98	+	+	+	+
2PMc226	FJ853745	(AC)5	53	F: TCGAATATGCAGGAGAAC R: GTAAAGTGTAAACCCCAGTG	4	340–349	0.61	0.73	+	+	+	+
2PMc144	FJ853746	(TG)14	55	F: CAACCTCTCGTGTCTGCTC R: CTGTCCCTGCCTGAAGTT	10	155–174	0.72	0.80	+	+	+	+
2PMc224	FJ853747	(CA)5	52	F: AAGGGGTGGGGTTCC R: TGGTCATGGTGGGTTCA	10	207–236	0.68	0.79	–	–	–	–
2PMc373	FJ853748	(AG)9	53.3	F: CAGGCGAATGAGAGCAAAAA R: TTAAAGGCATTCGGGTGTCA	12	244–253	0.79	0.86	+	–	–	–
2PM198.1	FJ853749	(CT)7(TC)6	56.5	F: CAGAAAGAAGGAAGGAAAGGAA R: TCTAGCTGCATGCATAAACACT	4	232–248	0.60	0.72	+	+	–	–
2PMc37	FJ853750	(AG)7	50	F: GATCTGTCATATTCCTTCTC R: CAAAATCGTGTATAAACTCTA	4	219–246	0.58	0.70	+	+	–	–
2PMc252	FJ853751	(CA)6	45	F: GTAAAGCGCAAAAACT R: ATCCGTATCTTAGGTGA	4	250–263	0.49	0.63	–	–	+	+
2PMc287	FJ853752	(GT)14	53.2	F: CCCTGCACAGAGAAAAC R: GCACCTGAAGTCCTGAA	4	261–292	0.57	0.69	–	–	+	+
1PMc48.16	FJ853753	(CA)6	60	F: ′GAAATGTGGTGCATCATCCT R: ATCCGGTTGGCAGAGTATC	4	135–146	0.49	0.60	–	–	–	–
2PMc222.1	FJ853754	(CT)3(CT)4	50	F: TAATCCCTTTGCCTTTTC R: CCCATTCATCTGCTGCTA	12	237–254	0.81	0.91	–	–	–	–

T_a, annealing temperature; n, number of alleles; PIC, polymorphism information content; D, discriminating power; +, successful amplification; –, unsuccessful amplification.

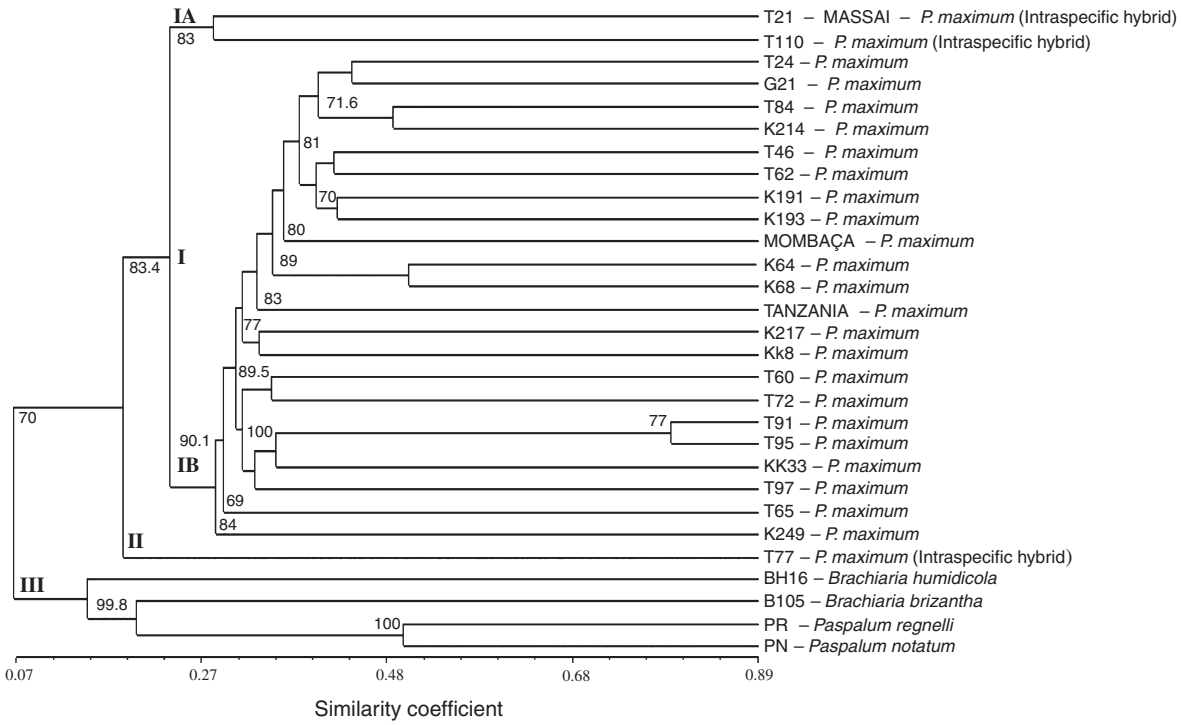

Fig. 1: Cluster grouping of 25 genotypes of *Panicum maximum* Jacq. and four species of forage grass based on Jaccard's similarity coefficient. Bootstrap values (>65) are indicated as % at major and critical subnodes

are closely related. The forage grass species *B. humidicola, B. brizantha, P. notatum,* and *P. regnelli* were grouped separately from *P. maximum*, forming a third grouping (Cluster III). Moderately bootstrap values (>65) at the main nodes indicate the relative consistency at cluster nodes.

Breeding has been largely responsible for advances in agriculture, including the development of superior cultivars either by an increase in productivity or improvement of adaptation to harsh environments. The success of a breeding programme can be accelerated by the effective use of molecular markers. The *P. maximum* microsatellite loci described herein have potential utility for genetic studies on population structure and could contribute to the development of strategies for germplasm conservation and breeding in this species and possibly in other related forage species.

Acknowledgements

The authors are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial support (Project No. 05/51010) and a graduate fellowship (2006/52953-8) to A.C.B. Sousa, and to Conselho Nacional de Pesquisa (CNPq) for financial support and, a graduate fellowship to T. Campos L., undergraduate scholarships to L. Boaventura, and a research fellowship to A.P. Souza.

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