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NOTE

Development of microsatellite panels for molecular fingerprinting of Napier grass (*Cenchrus purpureus*) cultivars

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Abstract: *Napier grass is a perennial tropical forage that is used in beef and dairy production systems. Despite its significance in animal nutrition, molecular information available, such as microsatellite or simple sequence repeat (SSR) or single nucleotide polymorphism (SNP) markers, is limited. Using an assembled transcriptome, 50 novel SSR markers were developed, of which 21 were found to be polymorphic. These polymorphic markers were tested for DNA fingerprinting of Embrapa cultivars, five of which revealed distinct allele patterns for cultivar identification. SSR markers 05, 17, and 44 identified a unique pattern in the BRS Kurumi cultivar. The BRS Capiaçu cultivar was identified using SSR markers 17, 43, and 44. The Pioneiro cultivar exhibited a rare fragment amplification pattern using SSR marker 46, while SSR marker 44 revealed a distinct allele in the BRS Canará cultivar. SSR marker panels could be utilized as DNA fingerprinting tools to assist in cultivar identification.*

Keywords: *breeding program; elephant grass; SSR*

INTRODUCTION

Napier grass (*Cenchrus purpureus* (Schumach.) Morrone syn. *Pennisetum purpureum* Schumach.), also known as elephant grass, is a perennial allotetraploid (2n = 4x = 28, genome A'A'BB) (Hanna 1981, Jauhar 1981) forage grass in the *Poaceae* family. It is one of the most important perennial tropical C4 grasses (Coombs et al. 1973, Pereira et al. 2016). It occurs naturally in a vast region of East Africa (Cavalcante and Lira 2010) and reproduces sexually, although the majority of its propagation is vegetative (Pereira et al. 2010). This plant species is used as forage in tropical and subtropical beef and dairy cattle systems owing to its excellent quality, palatability, and dry matter production (Souza Sobrinho et al. 2005, Orodho 2006). Likewise, because of its high dry biomass output, Napier grass has great bioenergy production potential (Lima et al. 2011, Morais et al. 2012, Rengsirikul et al. 2013, Fontoura et al. 2015, Rocha et al. 2017, Tsai et al. 2018, Kongkeitkajorn et al. 2020).

Since 1998, the Embrapa Dairy Cattle Research Center has coordinated a Napier grass breeding program in response to the market demand for dairy products in the tropics and the significance of this grass species (Pereira et al.

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2010). The breeding program has developed cultivars with high forage yield, tolerance to low-fertility soils, and other desirable traits (Pereira et al. 2003, Pereira et al. 2010).

Although scarce, molecular information on Napier grass germplasm accessions and cultivars could serve as a powerful tool in routine breeding programs. Recently, two genomic assemblies of Napier grass have been released, and this information should aid in the development of novel tools for use in breeding programs (Yan et al. 2020). In addition, genome-wide association study analyses have been used to reveal differences in high biomass yield among *C. purpureus* genotypes (Habte et al. 2020), and Muktar et al. (2021) identified quantitative trait loci regions associated with forage biomass yield, water usage efficiency, and feed quality traits. Azevedo et al. (2012) evaluated microsatellite or simple sequence repeat (SSR) markers discovered in pearl millet (*Cenchrus americanus*) and found that 30 SSR markers were successfully cross-amplified in Napier grass. These markers assisted in assessing the genetic diversity at the Embrapa Germplasm Bank but were insufficient to identify cultivar-specific alleles. Identifying a cultivar based on morphological characteristics alone can be challenging because of environmental interference and the prolonged time periods required to assess trait expression, for example, when identification is dependent on reproductive characteristics. Therefore, molecular identification could be extremely beneficial because there is no environmental influence, and it is feasible to screen early. DNA fingerprinting information of Embrapa cultivars, such as BRS Canará, BRS Capiaçu, BRS Kurumi, and Pioneiro, could aid the forage industry in avoiding issues such as biopiracy by authenticating the origin of these cultivars. Furthermore, DNA fingerprinting could address marketing difficulties, such as cultivars sold under multiple names in various locations (Karaagac et al. 2014).

This study aimed to develop new microsatellite markers for Napier grass and identify unique markers specific to Embrapa cultivars (BRS Canará, BRS Capiaçu, BRS Kurumi, and Pioneiro), constituting the most widely marketed forage cultivars of Napier grass in Brazil.

MATERIAL AND METHODS

Microsatellite regions were derived from a Napier grass transcriptome assembled by our research team. This transcriptome was used to identify genes associated with lignin production (unpublished data), and all sequencing data were obtained from the NCBI database (BioProject accession number PRJNA731177). The microsatellites were detected using the MISA v 1.0 web server (Beier et al. 2017) with default parameters (SSR motif length min no. of repetitions: 1-10/2-6/3-5/4-5/5-5/6-5; max_difference_between_2_SSRs: 100; GFF: true). Fifty primer sets were designed using the Primer3 v 2.3.4 web-based program (Untergrasser et al. 2012). Primers with 18 to 25 base pairs (bp) in length and amplicon products with 100 to 400 bp predominantly tandemly repeated tri-nucleotide motifs (5 di-, 43 tri-, and 2 tetra-nucleotide motifs) were selected.

DNA was extracted from young leaves using the cetyltrimethylammonium bromide method (Doyle and Doyle 1987). Polymerase chain reaction (PCR) analysis was used to evaluate the selected primers in four Napier grass samples as follows:1X GoTaq reaction buffer, 0.5 µM of each forward and reverse primer, 3 mM $MgCl_2$, 0.4 mM dNTP (Promega, Madison, WI, USA), 1 U GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA), and 45 ng genomic DNA in a final volume of 20 µL. PCR was conducted in a thermocycler (Thermo Scientific, Waltham, Massachusetts, USA), using the following cycling profile: initial denaturation at 95 °C (15 min); 5 cycles at 94 °C (30 s), annealing temperature at 57 °C (90 s) and 72 °C (1 min), with a 1 °C decrease per cycle; 25 cycles at 94 °C (30 s), annealing temperature at 52 °C (90 s) and 72 °C (1 min); and a final extension cycle at 60 °C (60 min). The amplification products were subjected to 2% agarose gel electrophoresis for 2 h and 30 min at 120V. Gels were stained for 30 min using ethidium bromide, and DNA fragments were detected using ultraviolet light via the EagleEye photo-documentation system (Stratagene, San Diego, California, USA).

Twenty-one microsatellite markers with polymorphic loci and good amplification patterns in at least three samples were selected to develop a unique marker panel for each Embrapa cultivar (BRS Canará, BRS Capiaçu, BRS Kurumi, and Pioneiro). Twenty samples, comprising cultivars and accessions from the Napier Grass Active Germplasm Bank (BAGCE 1, 2, 7, 18, 30, 53, 56, 57, 8, 60, 67, 68, 70, 71, 103, 105, BRS Canará, BRS Kurumi, Pioneiro, and BRS Capiaçu) were selected for this purpose. The accessions were selected based on a prior evaluation of genetic diversity (Azevedo et al. 2012) and represented the maximum diversity discovered in the germplasm bank. PCR was performed under the same conditions as described above, and the amplified products were loaded onto 12% native polyacrylamide gel electrophoresis for 5 hours at 500V and stained with silver nitrate (Bassan et al. 1991). Gel scoring was performed using GelAnalyzer 19.1 (www.gelanalyzer.com), and the results were exported to a Microsoft Excel spreadsheet where the presence of an allele was represented by 1 and its absence by 0 because heterozygotes could not be identified.

Diversity analyses were performed in NTSYs software (Rohlf 2009) utilizing the Jaccard coefficient to determine genetic similarity and the unweighted pair group method arithmetic averages (UPGMA) method to construct a dendrogram.

RESULTS AND DISCUSSION

Of the 50 SSR markers identified and tested, 47 (94%) were successfully amplified in Napier grass (Table 1). We identified 94 alleles from four samples in our initial PCR tests (Supplementary Figure 1), and the best markers (i.e., good amplification in at least three samples) were chosen for the following phase. This novel set of molecular markers should be of great assistance in assessing genetic diversity to maximize the advantages of crossing in situations where inbreeding depression is a concern. It could also be used to develop specific molecular marker panels for cultivar identification and protection. Previous SSR marker-based diversity studies in Napier grass used markers established in other species, such as pearl millet (Azevedo et al. 2012, Kawube et al. 2015); therefore, these markers were expected to be located in conserved regions with less polymorphism. In this study, SSR markers were identified in the transcriptome of Napier grass that had the best potential to have additional alleles.

A polymorphic SSR panel is essential for DNA fingerprinting that is useful in many species, such as pearl millet (Ambawat et al. 2021, Makwana et al. 2021) and sugarcane (Singh et al. 2019). DNA fingerprinting enables precise, objective, and rapid cultivar identification and has proven to be an efficient tool for crop germplasm characterization, collection, and management (Zhu et al. 2012). Cultivar discrimination must be quick, accurate, and exact to guarantee the protection of intellectual property associated with cultivars (Scarano et al. 2015, Le et al. 2016).

Following initial PCR primer screening, 21 polymorphic SSR markers were utilized to detect unique marker patterns in four Embrapa commercial cultivars (BRS Capiaçu, BRS Canará, BRS Kurumi, and Pioneiro). To ensure the distinctiveness of these marker panels, these cultivars were molecularly compared with 16 Napier grass accessions from the Embrapa Germplasm Bank that were selected for their high genetic diversity (Azevedo et al. 2012). Thus, fewer samples were required to establish a cost-effective and time-efficient high-resolution molecular panel (Table 2). Among the selected accessions, two BRS Capiaçu parentals (BAG 57 and BAG 60) and a BRS Kurumi parental (BRS 57) were genotyped.

Five SSR markers revealed a distinct allele pattern for one or more Embrapa cultivars (Table 2). Previous studies have shown that it is possible to differentiate cultivars using only four to six markers (McGregor et al. 2000, Moisan-Thiery et al. 2005, Reid and Kerr 2007). A protocol was established to identify cultivars using polyacrylamide gel, despite its limited resolution compared to that of capillary electrophoresis. The intention was to provide a rapid, cost-effective, and suitable protocol for laboratories equipped with basic facilities for molecular assays.

A panel consisting of three SSR markers was selected to identify the BRS Kurumi cultivar (Supplementary Table 3 and Figure 1). RNA-CE 05 exhibited a unique pattern with three alleles (275/280/295bp), RNA-CE 17 identified a 265 bp rare allele, and RNA-CE 44 revealed five alleles (150/154/162/198/210bp). Some SSR markers shared alleles across all samples; therefore, they could be used as positive controls for SSR PCR analysis. All samples contained the alleles 275 bp (RNA-CE 05), 150 bp (RNA-CE 44), and 128 bp (RNA-CE 46) (Supplementary Figure 2).

Three SSR markers (RNA-CE 17, RNA-CE 43, and RNA-CE 44) were identified as informative markers for identifying the BRS Capiaçu cultivar. RNA-CE 17 amplified a rare segment of 265bp, whereas BRS Capiaçu differentiation through RNA-CE 43 was because of a lack of amplification. RNA-CE 43 was tested under various conditions in BRS Capiaçu samples, and no amplification was detected. RNA-CE 44 exhibited a rare allele pattern (150/162 bp) in BRS Capiaçu, and a combination of these three SSR markers would be useful in identifying the cultivar.

The BRS Canará cultivar was identified using RNA-CE 44, where three alleles were detected (146/150/158bp). The 158 bp allele was exclusively amplified in this cultivar. The best SSR marker for identifying Pioneiro cultivars was RNA-CE 46, which generated a unique pattern by amplifying a rare 130 bp fragment.

Diversity analysis was performed to assess the genetic variability of the 16 samples using these five SSR markers. Three groups were formed with a diversity coefficient of 0.50: one with BRS Capiaçu, one with BRS Kurumi, and one

Table 1. List of 47 microsatellite markers successfully amplified in four samples of *Cenchrus purpureus*, forward and reverse sequences, repeat motif, predicted product size, and selected primers tested in cultivar identification

Marker name	Forward primer	Reverse primer	Repeat motif	Predicted product size (bp)	Primer selected
RNA-CE 01	TTGCGATGCACCACAACTTG	GGCAGCAGGTGAATCTTCCT	$(GAT)_5$	188	Yes
RNA-CE 02	TACACCACCTCCTTAGCCGA	TGGTTGATAGCCGTCCATCG	$(TGC)_5$	360	Yes
RNA-CE 04	GGCCTCTCCTTTCGCTCTTT	TTTGGCCGGTTGCTAGGATT	$(TG)_7$	267	Yes
RNA-CE 05	GGTCTAATGCCGGATCAGGG	GCAATGCCCATGCTAGATGC	$(GCC)_5$	125	Yes
RNA-CE 06	CGGCGACATGAAGTCCTTCT	GAAGGGATGAACGCGATTGC	$(GCG)_7$	399	No
RNA-CE 07	TTCTCACATCAGCTCGCTGG	CATTGGAGAGACGGAGCGAG	$(CCG)_7$	221	No
RNA-CE 08	TCCTCCCGCTTTACCCAAAC	TTCTCGGCATCTGCAACACT	$(CGG)_7$	304	No
RNA-CE 09	GTCTACAACACCTTCGGCGA	GTCGACCATCCGCTTGTACT	$(GAC)_5$	324	No
RNA-CE 10	TCCTCCTCTCCCTCTCAAGC	CACATCACCAGCCAAGGAGT	$(CTC)_6$	196	No
RNA-CE 11	AGCAGGGGAGGAGAGGAAAT	GAGCACCACGAACAGGATCA	$(CAG)_7$	119	No
RNA-CE 12	GAGTGGATGTTGAGGCAGCT	AAACAGGCACGCTCTAGCTT	$(GAT)_5$	246	Yes
RNA-CE 13	GTATGCACGCCAATTGCCAT	ACCACACAACAGCCGAGAAA	$(TG)_7$	367	No
RNA-CE 14	AGGTGTTCGTGAAGAGCAGG	GAACCGACAACCAAAAGCCC	$(AGG)_5$	220	Yes
RNA-CE 15	TTCTTTCCTGACCGACCGTG	GCCACCATCACCACCAAAAC	$(ATCC)_8$	117	No
RNA-CE 16	ATCTCCTCCTCCACCTCACC	CATCAGCTTGGACCTACGCA	$(GCG)_6$	294	No
RNA-CE 17	TGGTGGTGCTTTGTTCAGGT	GCTTCTCCAAACGCCACATC	$(AGA)_9$	271	Yes
RNA-CE 18	TGGATGATCCACGGTGCAAA	ATTGTAGCAAAGCCCGCCTT	$(TTG)_5$	371	Yes
RNA-CE 19	ACTAGTCACACACACAGGCG	CCCACCATGGCTTGTCTTCT	$(GGAT)_5$	195	Yes
RNA-CE 20	GATGACGACGACGATGACGA	TACCCCTCCAGCTTCTCCAG	$(CGA)_5$	146	No
RNA-CE 21	CCGTGTTGAATTGCTCCGTG	ATGTTCTTGGAGAGGCAGGC	$(GCG)_5$	143	No
RNA-CE 22	AAAGAGGAGAGGGGCTAGGG	TGTTGGTGGCCTGGTCAAAT	$(GGC)_5$	256	Yes
RNA-CE 23	CCCTCATCTCCACGCTCAAG	GGATGAGGAGGCTGAGGTTG	$(CGT)_5$	158	Yes
RNA-CE 24	ACGATCAAGGACAAGTCGCC	GCCTCTAGTTCTCGAAGGCC	$(GCA)_5$	121	No
RNA-CE 25	TCCTCCCTCTCTCTGTGCTC	TACCCCTGTCGGATCTTCGT	$(GCG)_5$	309	Yes
RNA-CE 26	CTGCAGAGCTCCACAGAACA	CCTGCAGGATCGTGTAGTCC	$(GCC)_5$	124	No
RNA-CE 27	TCACAGGAGGAGACCGATGT	CCTGTCTGCGAAGTTCACCT	$(CAC)_5$	363	No
RNA-CE 28	CTCTCTCCTCCATCCTCCCC	GGGGAAGGAGGAGAGGATGT	$(TCT)_{12}$	217	No
RNA-CE 29	CAGCCAGGTCATCCTCAGTC	TTTAGCAAAACAAGCCGCCG	$(GGC)_5$	362	No
RNA-CE 32	GTCGGGGTCGTTCAAGAAGT	GCATCCACGTCCTCGAAGAA	$(GGC)_5$	132	No
RNA-CE 33	AGGCGCAAGGGATGAATGAA	CCTATCTCGCCGTCTCACAC	$(GGC)_5$	291	No
RNA-CE 34	CTTCCCCTCATCACACCACC	GGCTGAGAAGAGGGTGTTCC	$(GCT)_6$	261	Yes
RNA-CE 35	CTTCTCCTTCGCCTCATCCC	TAAGAAGGGGATGAGGCGGA	$(CCT)_5$	121	No
RNA-CE 36	TGAGTCCCAAGAAGCAGCAG	TGCTGTTTGGCTCGATCCAT	$(CGG)_6$	356	Yes
RNA-CE 37	TTAATGCCGCTGCGATGTTG	CATCTAGCCACAGGTGCACA	$(TAG)_5$	389	No
RNA-CE 38	CTAGCTTTGCTTGCCACTGC	GCACAGCAGACATGGATCCT	$(CA)_6$	243	No
RNA-CE 39	ATCACAGCAAGAGGAGCCAC	TGTAGTGCTCGGGATCCTCA	$(CAC)_5$	345	Yes
RNA-CE 40	CCGCAAATCCTCAGAACCCT	GTGCGGTGGATTTTGCTTGT	$(CCG)_5$	267	No
RNA-CE 41	AGACCCCTACACGAGCTTCT	CCGGGTACTGATGATGAGGC	$(CGC)_7$	164	No
RNA-CE 42	GCTGCTCTGTCTCCACTTGT	GCTTACGAGGTTCCGGAACA	$(GCG)_5$	276	Yes
RNA-CE 43	AATACTCTCCCCTCCCCCAC	CGCTCTCGTACTACCAGCAG	$(AG)_8$	149	Yes
RNA-CE 44	GTGCGAGAGGGAAACACAGA	TCGGTGTGCTTGTAGTGGAC	$(GCG)_5$	155	Yes
RNA-CE 45	TCCTAGCTGACCGGACTACC	AGGCTTTAGCAACCGAAGCT	$(CGG)_6$	365	No
RNA-CE 46	GAGAGCGAGAGACATGAGGC	ACAGGCCAAGCAAGAGGTAC	$(GGC)_7$	137	Yes
RNA-CE 47	TGCCGAGGACAGAAGAAGTG	CAGACGTGCTCATCACCTCA	$(AGG)_5$	381	No
RNA-CE 48	TATACATGCCCAGCGACGAC	TCCTAGCCTTCTCTGCGTCT	$(GT)_8$	383	Yes
RNA-CE 49	GACATCCTCGTCGTCGTCTC	CCTAGTTCTTACCGGTGGCG	$(CGA)_5$	232	Yes
RNA-CE 50	AAGGGGAAGAAGTGCTACGC	GATCACCCAAGAACGTTGCG	$(GGC)_5$	117	No

Table 2. Alleles identified in each SSR marker used to differentiate *Cenchrus purpureus* cultivars. The alleles were labeled based on fragment size in the base pair (bp)

Cultivars	SSR (bp)				
	RNA-CE 05	**RNA-CE 17**	**RNA-CE 43**	**RNA-CE 44**	**RNA-CE 46**
BRS Capiaçu	275^{C}	270^{R}	-	$150^{C}/162^{R}$	128^{C}
BRS Canará	275^{C}	-	165	$145^{R}/150^{C}/158^{U}$	128^{C}
BRS Kurumi	$275^{C}/280^{R}/295^{R}$	270^{R}	155	$150^{C}/154/162^{R}$	128^{C}
Pioneiro	275^{C}	265	165	$150^{C}/154$	$128^{C}/130^{R}$

^{C} common allele (present in all samples)
^{U} unique allele (present in only one sample)
^{R} rare allele (present in a maximum of three samples)

with a parental of both cultivars, BAG 57, which could not be distinguished from BAG 105 (Supplementary Figure 3). Another group contained two other cultivars (BRS Canará and Pioneiro) with a similarity coefficient of 0.44. Although it was impossible to distinguish all samples using only these five markers, the dendrogram allowed for the differentiation of all cultivars. As expected, the cultivar pair with the highest similarity coefficient was BRS Capiaçu and BRS Kurumi (0.55) because they share a common parental line. The accessions that could not be distinguished in this study were identified and arranged with a higher similarity coefficient by Azevedo et al. (2012).

The development of a molecular marker set for Napier grass is crucial for breeding programs. This panel would aid in protecting intellectual property rights regarding cultivar products and could be used as an additional descriptor for registering and protecting a cultivar (Ercisli et al. 2011, Rauscher and Simko 2013, Scarano et al. 2015). Moreover, its unique molecular profile would facilitate the differentiation of kinship-related genotypes with similar phenotypic traits.

The molecular marker panel of the five SSR markers developed in this study is a reliable and cost-effective tool for identifying Napier grass. This test would assist breeders, germplasm collection curators, propagators, and growers in verifying the trueness-to-type information of cultivars.

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