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Marker-assisted verification of Kinggrass (*Pennisetum purpureum* Schumach. x *Pennisetum glaucum* [L.] R. Br.)

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Abstract

Perennial grasses currently targeted for large-scale deployment as biofuel feedstocks have not been definitively characterized regarding their potential weediness from either or both seed- and rhizome-derived propagules. Kinggrass (napiergrass [*Pennisetum purpureum* Schumach.] x pearl millet [*Pennisetum glaucum* {L.} R. Br.]) is unique as a seed-sterile, non-rhizomatous, perennial crop that is capable of high biomass production during the establishment year. For this study, controlled crosses were made between napiergrass and pearl millet to produce novel Kinggrass genotypes for potential biofuel crop production. Hybrids could not be confirmed by observing morphological traits because all progeny resembled napiergrass. Consequently, a DNA marker survey of napiergrass and pearl millet was conducted to validate purported napiergrass x pearl millet interspecific hybrids with Expressed Sequence Tag-Simple Sequence Repeats (EST-SSR). Three paternal, pearl millet-specific markers, as well as six codominant markers, were identified and used to screen putative Kinggrass hybrids. All paternal-specific EST-SSRs were present in each of the F_1 individuals, and the codominant EST-SSR markers fit within the expected transmission ratios. These EST-SSRs confirmed that all individuals analyzed were true Kinggrass hybrids, and they provide valuable molecular tools towards more rapid development of elite biofuel Kinggrass feedstocks.

Keywords: Marker-assisted breeding; microsatellites; biofuels; napiergrass x pearl millet; pearl millet; napiergrass; interspecific hybrid; comparative genomics; Kinggrass.
Abbreviations: EST_ expressed sequence tag; SSR_ simple sequence repeat; bp_base pairs.

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Introduction

Demand for agricultural land resources used to produce food and feed crops is of concern and will continue as the world's population increases. In recent years, this concern has been accentuated by the utilization of grain crops, primarily corn, to produce biofuels. Such prospects of bioenergy crops on productive agricultural land have resulted in substantial debates on impacts to food security. However, these concerns could be largely alleviated through cultivation of perennial grasses on marginal cropland and abandoned grasslands. Such perennial grass cropping systems on marginal lands are estimated to possess the capacity to produce as many as 377 million tons of biomass in the U.S. alone (Perlack et al., 2006) while simultaneously reducing soil erosion, fertilizer use, and herbicide use in comparison to annual crops. Transitioning to more sustainable biofuel strategies would further satisfy increasing occurrences of policy mandates, including those of the Environmental Protection Agency (EPA) for reducing greenhouse gas emissions by the sequestration of soil carbon (Costanza et al., 1997; Kort et al., 1998; McLaughlin and Walsh, 1998; Lewandowski et al., 2003; Khanna et al., 2010). Biofuel feedstock producers would also benefit economically from growing perennial grasses because they would not require yearly replanting, as is the case with annual crops.

Several perennial grasses with high-biomass production have been investigated for potential as alternative energy sources, and napiergrass (*Pennisetum purpureum* Schumach.) is one of the most promising (Samson et al., 2005). Napiergrass originated in tropical Africa and over time has become naturalized throughout the tropics worldwide (Hanna et al., 2004a). Some genotypes have sufficient cold tolerance to survive and persist in sub-tropical regions of the world, including the southern U.S. (Bogdan, 1977). This leafy, robust grass grows to a height from two to six meters and produces numerous tillers. Napiergrass has been reported to produce yields that are among the highest in candidate biofuel feedstocks and has one of the fastest growth rates of higher plants (Orodho, 2006; Renard et al., 2011; Rengsirikul et al., 2013). It can withstand extended periods of drought, and with the onset of rainfall, it recovers and grows rapidly (Bogdan, 1977). Napiergrass produces large dense clumps of basal meristems and short, compact rhizomes. It produces little or no seed and is usually established vegetatively. The species is predominately cross-pollinated because of its protogynous flowering behavior, which results in high heterozygosity and provides extensive genetic diversity that can be utilized in breeding programs.

Pearl millet (*Pennisetum glaucum* [L.] R. Br.) putatively

originated in regions of northern Africa extending from western Sudan to Senegal (Harlan and de Wet, 1971). It is an annual grass that is grown for both grain and forage production. In India, Pakistan, and Africa, pearl millet is grown primarily as a grain for human consumption; whereas, in the USA, Australia, and parts of South America, it is grown almost exclusively as forage for livestock (Hanna et al., 2004b). However, in the USA, there is increasing interest in growing the grass as a grain crop to provide feed for poultry and livestock (Hanna et al., 2004b). Pearl millet will grow and produce grain on poor, droughty, infertile soils, but it will respond to supplemental fertility and moisture. It tolerates low rainfall conditions and is grown for grain in the Sahel region of western Africa with an annual precipitation of 250 to 300 mm (Bogdan, 1977). Pearl millet is also productive on acid soils (Hanna et al., 2004b). Like napiergrass, pearl millet is predominately cross-pollinated because of its protogynous flowering behavior and is a highly diverse, heterozygous species (Bogdan, 1977; Hanna et al., 2004b).

The cytogenetics of pearl millet, napiergrass, and their interspecific hybrids is well-established (Burton, 1944; Jauhar, 1981; Jauhar and Hanna, 1998; and Barbosa et al., 2003). Pearl millet and napiergrass belong to the primary and secondary gene pools, respectively, of the genus *Pennisetum* (Harlan and de Wet, 1971; Martel et al., 1997). Pearl millet is a diploid (2n=2x=14) with the genome composition AA. Napiergrass is an allotetraploid (2n=4x=28) with a genome formula A'A'BB. Hybrids between these two species are normally triploids (2n=3x=21) with the genome formula AA'B. During meiosis in the hybrids, some members of the A' genome from napiergrass associate with members of the A genome from pearl millet and as many as seven bivalents occur (Muldoon and Pearson, 1979; Jauhar, 1981; Dujardin and Hanna, 1985). Jauhar (1981) concluded that that A' genome is predominantly homoeologous with the A genome. The A' chromosomes in napiergrass are smaller than the A chromosomes of pearl millet and the differences in chromosome size may account for the reduction in chromosome pairing in the triploid hybrids. Members of the A and B genomes do not pair with one another (Jahuar, 1981; Pantulu and Rao, 1982; Jahuar & Hanna, 1998). Members of the B genome remain as univalents, they lag behind the other chromosomes during anaphase I and II, and not all are included in the developing nuclei. This creates sterility in the F_1 hybrids that provides a mechanism to alleviate concerns of invasiveness from seed.

Pearl millet and napiergrass are readily interfertile regardless of which species is used as the maternal and paternal parent (Burton, 1944; Powell and Burton, 1966). The napiergrass x pearl millet hybrids are often referred to as 'Kinggrass' (Rodríguez et al., 1989; Burns et al., 1993; Wadi et al., 2004); whereas, pearl millet x napiergrass hybrids are called 'PMN' hybrids (Hanna et al., 1989; Cuomo et al., 1996; Osgood et al., 1997; Dowling et al., 2013). Regardless of which species is used as the maternal or paternal parent, the interspecific hybrids more closely resemble napiergrass than pearl millet (Gonzalez and Hanna, 1984). Because of the high amount of heterozygosity in napiergrass and pearl millet, both Kinggrass and PMN hybrids exhibit a high level of heterosis. For example, both hybrids produce more biomass than either parent (Burton, 1944).

Kinggrass possesses some unique attributes that delineate it from both parental species and PMN as a high biomass feedstock. These are higher biomass production and forage quality. Nutritive values, traditionally measured in terms of forage and silage with compatibility towards biofuel metrics, have been reported for Kinggrass that are superior to napiergrass (Burton and Powell, 1968; Woodard et al., 1991) and pearl millet (Gupta, 1977). Beyond the seed sterility present in Kinggrass and PMN hybrids that minimizes weediness potential in both crops, Kinggrass also has a complete absence of rhizomes in reported literature. PMN, in contrast, has been characterized to have the potential for rhizomes (Macoon et al., 2002) which is a greater invasiveness risk. Most notable in regards to biofuel traits, Kinggrass has also been found to have greater biomass yields than PMN hybrids (Burton, 1944; Gupta, 1977; Wadi et al., 2004).

Significant comparative genomic information exists for major cereal crops within the Poaceae, including completely sequenced genomes for sorghum (Paterson et al., 2009) corn (Schnabel et al., 2009), and rice (Yang et al., 2013) (Phytozome, http://www.phytozome.org/; Gramene, http://www.gramene.org/; MaizeGDB, www.maizegdb.org/). Genomic resources are also available within pearl millet because of its importance as a grain crop, but there are very limited intraspecific molecular data available for napiergrass. The genetic diversity of napiergrass has been characterized using RAPDs, AFLPs, and isozymes (Lowe et al., 2003; Bhandari et al., 2006; Harris et al., 2009). Findings from these studies revealed possible heterotic groups that could be useful in future breeding efforts (Harris et al., 2009). Azevedo et al. (2012) also reported approximately 50% cross-species amplification of pearl millet microsatellite markers in napiergrass, indicating the suitability of using microsatellites as a tool for molecular characterization, parental-species identification, and hybrid verification in progeny between these two species. The framework genetic linkage map of pearl millet (Qi et al., 2004) further provides an anchor from which to extrapolate comparative genomic data to it and napiergrass.

A similar comparative genomics example utilized paternal-specific microsatellite markers derived from related species to identify and confirm genomic introgression in progeny from the reciprocal cross of Kinggrass, pearl millet x napiergrass (Dowling et al., 2013). The paternal-specific microsatellite markers derived from Dowling et al. (2013) are unique to napiergrass. In contrast, the paternal-specific markers derived from pearl millet can be used to identify hybrids from napiergrass x pearl millet crosses (Kinggrass). The objectives of this study were to (1) produce a population of Kinggrass hybrids; (2) develop Simple Sequence Repeats from expressed sequence tags (EST-SSRs) specific to the parental pearl millet genotype; and (3) assess the developed EST-SSRs for efficacy as tools for marker-assisted verification of Kinggrass hybrids.

Results and Discussion

Barriers to delineation of Kinggrass hybrids

None of the F_1 Kinggrass hybrids produced in this study could be definitively identified as interspecific hybrids based solely on vegetative traits. Neither Burton (1944) nor van de Wouw et al. (1999) could distinguish Kinggrass phenotypes from pearl millet or napiergrass. Kinggrass is a seed sterile plant due to its triploid nature with the genomic composition of AA'B (data not shown). This genetically derived, seed sterility eliminates the concern of invasiveness by wind-blown dispersal of seed as that reported for napiergrass (Burton, 1944). This combined with its subsequent higher

Table 1. Summary of EST-SSRs in Merkeron and PEGL09TX04.

	Number of EST-SSRs					
Species	Total	Amplified	Polymorphic	Codominant	Parental-Specific	Polmorphic (%)
Merkeron (*P. purpureum*)	130	103	49	8	23	37.7
PEGL09TX04 (*Pennisetum glaucum*)	130	95	25	11	3	19.2

Table 2. Primer sequences for selected PCAR* EST-SSRs utilized for Kinggrass hybrid verification.

PCAR Marker	GenBank Identification	Forward and Reverse Primer Sequences	Segregation
80	EB657730.1	F: TGGTAGAAACCTGACTGACTG R: CAAGTGCTGCTGAGAGAGA	Codominant
193	EB671438.1	F: GAGGAGAAGGCGGTGTTT R: TACTCGTTGGTCTGATGGTC	Codominant
235	EB672552.1	F: TTGCTGTATGTCTCCTTGAAC R: CTTCGCTTCTCCTCCTCC	Codominant
245	EB665711.1	F: CGCTTTCCCTTTCTCACT R: AGATAACGACGAGCAGCA	Codominant
279	EB661335.1	F: CAGGAAGTCAAGAAGAACAGA R: GCAGAACGGAGAGGAGGG	Codominant
310	EB657678.1	F: CCTCCTCTCCAAGTCTCC R: CCGCTGCTTCCGTCATTT	Codominant
5	EB673083.1	F: CCACATCATCAAACAACAAA R: TATGGAGGAGGAGAACATCA	Paternal-specific
263	EB663720.1	F: CTCTTCCTCTCCTGCTCC R: CATCCCGAATCCCACGCT	Paternal-specific
299	EB658472.1	F: GCTAATGGGTGTATGTGTGT R: CATCTCAGCATCCGCACT	Paternal-specific

*Pennisetum Ciliare Apomictic Repeat

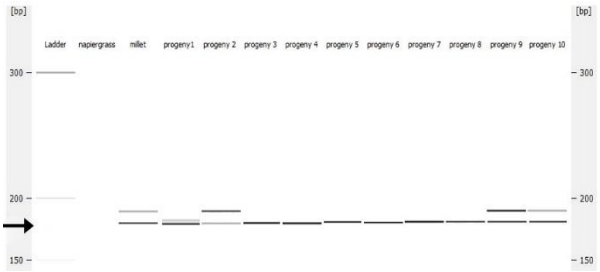

Fig 1. Electropherogram of napiergrass (Merkeron), pearl millet (PEGL09TX04), and 10 progeny. The lanes (left to right) include 1, 50bp DNA ladder; 2, Merkeron; 3, PEGL09TX04; 4 to 13, the progeny. An arrow at 184 bp denotes the paternal-specific PCAR 5 allele of interest.

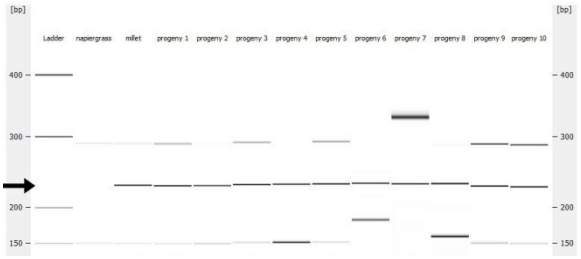

Fig 2. Electropherogram of napiergrass (Merkeron), pearl millet (PEGL09TX04), and 10 progeny. The lanes (left to right) include 1, 50 bp DNA ladder; 2, Merkeron; 3, PEGL09TX04; 4 to 13, the progeny. An arrow at 232 bp denotes the paternal-specific PCAR 263 allele of interest.

yield potential as compared to napiergrass and PMN results in a valuable bioenergy feedstock, and the prevention of invasiveness and the data tracking requirements of napiergrass in bioenergy production fields can be circumvented with the commercialization of Kinggrass and PMN seed production.

The primary objective of this research was the development of molecular tools capable of rapidly confirming Kinggrass hybrids. Time-consuming assays to confirm seed sterility require Kinggrass hybrids to grow to full maturity. In addition, cytological determinations of chromosome number were not performed due to it being labor intensive (Burton, 1944; Barbosa et al., 2003; Techio et al., 2006). Moreover, flow cytometry was not pursued in this experiment because the nuclear DNA contents of the parental species are essentially the same (Martel et al., 1997), and flow cytometry could not distinguish F_1 PMN hybrids from either pearl millet or napiergrass in previous experiments (Dowling et al., 2013). The 2C DNA content of pearl millet and napiergrass as reported by Martel et al. (1997) was 4.71 and 4.59 picograms, respectively. This provides a fitting explanation as to why neither species could be separated from the putative PMN hybrids using flow cytometry by Dowling et al. (2013). However, as a tetraploid species, napiergrass would intuitively need to have approximately twice the DNA content of the diploid pearl millet, and the DNA content of the Kinggrass and PMN hybrids would be intermediates between the two parental species because pearl millet is a diploid with 14 chromosomes and napiergrass is a tetraploid with 28 chromosomes. In addition, reported measurements of mean chromosome length for napiergrass and pearl millet further elucidate the similarities in DNA content. The mean chromosome length of pearl millet is 4.02 µm and 2.00 µm for napiergrass while the two chromosomes of pearl millet are distinctly larger morphologically (Martel et al., 1997). All of this collective information provides ample justification towards the similar DNA quantities of napiergrass and pearl millet and further supports the need for markers to delineate hybrids from self-pollinated progeny and parental genotypes.

Development of pearl millet-specific EST-SSRs

A total of 130 PCAR (Pennisetum Ciliare Apomictic Repeat) EST-SSR markers were screened in the parental accessions (napiergrass cultivar 'Merkeron' and the pearl millet genotype PEGL09TX04) utilized in this study. Details of the survey are summarized in Table 1. PCR amplification was successful with 103 and 95 of the EST-SSRs in Merkeron and PEGL09TX04, respectively. Merkeron demonstrated greater genetic heterogeneity, with 49 of the EST-SSRs identifying polymorphic loci versus 25 in PEGL09TX04. Within the polymorphic loci, 8 and 11 codominant, single-copy markers in Merkeron and PEGL09TX04, respectively, were identified and suitable for Kinggrass hybrid confirmation. Several other markers were identified with more than two allele bands and probably represent multiple-copy or repetitive genes in napiergrass and pearl millet. Because linkage analyses have not been reported in napiergrass to confirm preferential versus non-preferential chromosome pairing at the gene level, these markers were not utilized for hybrid confirmation.

Numerous parental-specific EST-SSRs were also identified. Merkeron possessed 23 maternal-specific EST-SSR markers, but these were not further analyzed due to their lack of utility in confirming plants resulting from successful hybridization with the intended pearl millet pollen donor. These markers are novel and distinct from the 12 EST-SSRs specific to Merkeron reported by Dowling et al. (2013), therefore providing additional resources for hybrid verification in the reciprocal pearl millet x napiergrass crosses. These 35 Merkeron-specific markers have potential for molecular investigations targeting the B genome in napiergrass.

Three paternal-specific EST-SSRs were identified in PEGL09TX04, and these were selected as highly informative, homozygous markers for evaluating successful transmission of the pearl millet genome in putative Kinggrass hybrids. The higher rates of both polymorphism (37.7% versus 19.2%) and parent-specificity (17.7% versus 2.3%) observed in Merkeron and PEGL09TX04, respectively, were expected outcomes. These results are largely explained by napiergrass being a tetraploid species possessing an additional genome (B), which represents a secondary gene pool unique to napiergrass within *Pennisetum* (Martel et al., 1997) and does not exhibit homeology with the A genome of pearl millet (Barbosa et al., 2003). From the polymorphic EST-SSRs, the three homozygous PEGL09TX04-specific markers and six codominant markers were chosen for Kinggrass hybrid confirmation. PCR oligonucleotide sequences for these nine EST-SSRs are listed in Table 2. Upon screening these markers across the 10 putative F_1 Kinggrass hybrids, all paternal-specific PEGL09TX04 EST-SSRs were confirmed in the 10 F_1 progeny and fit the expected 1:0 ratio upon chi-square analyses (Table 3). Similarly, all six codominant markers fit the 1:1 ratio upon chi-square analyses across the 10 F_1 progeny. The three paternal-specific markers, PCAR 5, PCAR 263, and PCAR 299, were amplified in all 10 progeny for the polymorphisms of interest (Fig. 1-3, respectively). As an example of the

Table 3. Summary of markers tested across progeny and verified Kinggrass hybrids.

Hybridization	Codominant	Paternal-Specific	Hybrids Confirmed
Merkeron x PEGL09TX04	6	3	10/10[a]

[a]Chi-square goodness of fit values determined and compared to $\chi^2_{0.05} = 3.84$ for one degree of freedom

codominant markers utilized, PCAR 279 is shown in Fig. 4. Combined, these EST-SSRs confirmed all 10 F_1 progeny to be true Kinggrass hybrids. These results are similar to those previously reported using molecular markers for hybrid confirmation in other perennial grass species (Jessup, et al., 2002; Renganayaki et al., 2005; Genovesi et al., 2009; Jessup et al., 2011; Dowling et al., 2013).

Genomic applications of EST-SSRs

Our results demonstrate that markers such as EST-SSRs can be utilized as applied molecular breeding tools to rapidly confirm Kinggrass hybrids, as well as provide novel comparative genomic resources for PMN, napiergrass, and pearl millet breeding programs. SSRs have been previously reported to transcend genera within plant families and accurately delineate species, half-sibs, full-sibs, cultivars, hybrids and genotypes (Scott et al., 2000; Decroocq et al., 2003; Takrama et al., 2005; Brunings et al., 2010; Dongre et al., 2011; Sartie and Asiedu 2011; Dowling et al. 2013). Majorities of EST-SSRs are polymorphic and have utility as genomic tools, particularly in species with sparse genomic information available (Pashley et al., 2006). EST-SSRs also offer potential towards dissecting synteny between the A and A' genomes in *Pennisetum*. Targeted cytogenetic methods, such as fiber-FISH (cf. Ersfeld, 2004), could also assess both the order and physical distances between genes along orthologous chromosomes. The evolutionary divergence of the A and A' genomes could then be distinguished either as the result of chromosome reduction of the A' genome in napiergrass (Martel et al., 1997; Martel et al., 2004) or transposable element driven expansion of the A genome chromosomes in pearl millet similar to that documented in wheat and corn (cf. Mach, 2010). As effective molecular tools to delineate Kinggrass from PMN, the identified parent-specific markers also offer the potential to investigate maternal effects in these reciprocal hybridizations. Employing established methodologies (Hayman, 1954; Durrant, 1965), maternal effects have been previously reported to affect seed production in pearl millet (Burton, 1952; Burton et al., 1980) as well as reciprocal interspecific hybrids among higher plants (Burgess and Husband, 2004; Iida et al., 2007, 2013). Following confirmation of reciprocal Kinggrass and PMN hybrids via parent-specific EST-SSRs, reciprocal maternal effects could be quantified and characterized for biomass yield and other valuable traits. Epigenetic modifications of genes identified by parent-specific EST-SSRs could further be characterized for potentially altered methylation patterns (Adams et al., 2000; Bushell et al., 2003) and resulting gene expression in Kinggrass towards discovery of novel transcriptomes that result in greater biofuel production and improved genotype development.

Materials and Methods

Plant materials and hybridizations

The napiergrass cultivar Merkeron (Burton, 1989) and a high-biomass, fertile pearl millet accession (PEGL09TX04)

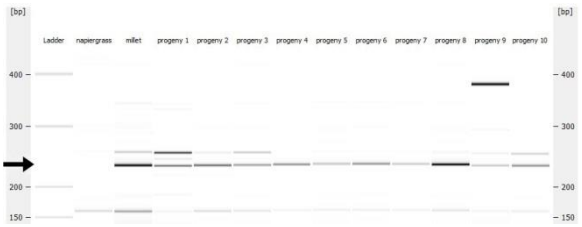

Fig 3. Electropherogram of napiergrass (Merkeron), pearl millet (PEGL09TX04), and 10 progeny. The lanes (left to right) include 1, 50bp DNA ladder; 2, Merkeron; 3, PEGL09TX04; 4 to 13, the progeny. An arrow at 236 bp denotes the paternal-specific PCAR 299 allele of interest.

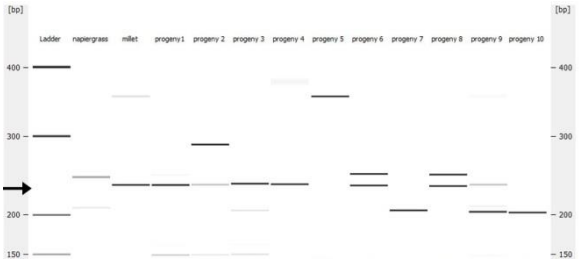

Fig 4. Electropherogram of napiergrass (Merkeron), pearl millet (PEGL09TX04), and 10 progeny. The lanes (left to right) include 1, 50bp DNA ladder; 2, Merkeron; 3, PEGL09TX04; 4 to 13, the progeny. An arrow at 238 bp denotes the paternal-specific PCAR 279 allele of interest.

from the Perennial Grass Breeding Program at Texas A&M University were crossed during the winter of 2011. Because napiergrass has a protogynous flowering behavior, its florets did not require emasculation when pollinated with pearl millet. Pearl millet pollen was collected by gently tapping inflorescences with recently exerted anthers just above an opened 150 mm diameter glass petri dish, which was covered and immediately transported to a flowering napiergrass plant. The napiergrass inflorescence was gently rolled in the pollen in the bottom of the petri dish. Following pollination, all involucres on the inflorescences containing florets that had not exerted stigmas were removed with forceps. The pollinated inflorescences were then enclosed in glassine bags and remained in the bags until the seed matured. After approximately 30 days, the inflorescences were removed from the bags and the florets were threshed and cleaned. All the seed were germinated and the seedlings were planted into pots and kept in a greenhouse. These plants were planted into a space-planted field nursery at a distance of 1 m between plants within a row and 1 m between rows during the first week of June 2012 near College Station, TX.

EST-SSR development

In the absence of publicly available sequence data for napiergrass, a total of 21,745 full-length complementary DNA (cDNA) sequences from apomictic buffelgrass

[*Pennisetum ciliare* (L.) Link syn. *Cenchrus ciliaris* L.] pistils were downloaded from GenBank (Jessup et al., 2002). Simple sequence repeats were identified and primer sequences were designed using the SSRLocator software package (SSRLocator). Selected SSRs contained at least 10 di-nucleotide or 5 tri-, tetra-, or penta-nucleotide repeats. Primer design criteria included 50% guanine-cytosine content, minimum melting temperature of 50°C, absence of secondary structure, length of 20–27 nucleotides, and amplified polymerase chain reaction (PCR) product range of 100–400 base pairs (bp) in length. The derived EST-SSRs were labeled 'PCAR'. A subset of 130 PCAR markers between 100-300 bp were selected and utilized in DNA analyses of Merkeron and PEGL09TX04.

DNA isolation

Genomic DNA was isolated using a modified rapid salt extraction protocol described by Aljanabi and Martinez (1997). The plant tissue was pulverized for 1 to 2 minutes until adequately pulped in 1.7 mL microtubes. Samples were centrifuged at 12,000 rpm for 10 minutes. The supernatant was transferred into new tubes, the samples were centrifuged again at 12,000 rpm for 10 minutes, and supernatant was transferred into new tubes without disturbing the remaining pellets. Samples were centrifuged at 10,000 rpm for 5 minutes, and the supernatant was removed. Then 500 µL of cold 70% ethanol was added to each tube. The samples were centrifuged at 10,000 rpm for 5 minutes, and the supernatant was removed. Microtubes containing DNA were inverted until dry, and the DNA was re-suspended in 200 µL of sterile deionized H_2O.

Identification of parental-specific EST-SSRs

Polymerase chain reactions were performed in a total volume of 20 µL using 11.8 µL PCR H_2O, 1 µL of 50 ng μL^{-1} DNA, 1 µL of 4mM deoxynucleoside triphosphates (dNTPs), 2 µL of 1X Promega $MgCl_2$-free PCR buffer, 2 µL of 2.5 mM $MgCl_2$, 0.2 µL of Taq polymerase and 1 µL each of the forward and reverse primers. The reactions were conducted in 96-well plates and temperature cycling was carried out using a PTC-220 Dyad Thermal Cycler (MJ Research Inc., Waltham, MA). The PCR began with an initial denaturation at 95°C for 3 minutes; followed by 40 touchdown decrement cycles at 95°C for 25 seconds, 55°C for 25 seconds, and 70°C for 45 seconds; and concluded with an elongation stage of 72°C for 10 minutes. The final hold was at 4°C indefinitely. Visualization of the PCR products was completed using polyacrylamide gel electrophoresis (PAGE) on a MEGA-GEL (C.B.S. Scientific, Del Mar, CA) high-throughput unit and nondenaturing gels as described by Wang et al. (2003). The gel visualization and documentation of PCR amplification was completed as described by Washburn et al. (2013). Gels were scored for the presence or absence of allele bands according to the procedure set forth by Rodríguez et al. (2001). Standard PCR and PAGE methods were verified under more stringent conditions utilizing Phusion® HF DNA polymerase (2,000 units per mL) (New England Biolabs, Inc., Ipswich, MA) and with the Agilent 2100 Bioanalyzer (Panaro et al., 2000) for three replications. Electropherogram results were transformed into gel images using the 2100 Bioanalyzer software.

The female parent, putative male parent, and 10 potential hybrid progeny were analyzed with the subset of 130 markers selected for hybrid determination. Chi-square goodness of fit values were calculated and compared to the probability value of $\chi^2_{0.05} = 3.84$ for one degree of freedom. The method for determining hybrids was adopted from Genovesi et al. (2009) where values were calculated for paternal-specific markers using both a 1:0 expected transmission ratio for homozygous markers and a 1:1 expected transmission ratio for heterozygous markers. Transmission of at least 50% of paternal-specific markers with acceptable chi-square values was considered evidence that a plant was a hybrid.

Conclusion

Molecular markers are essential in increasing the efficiency of wide-hybridization breeding programs by identifying true interspecific hybrids, and they are also becoming more accessible and frequently used by plant breeders to characterize genetic diversity, investigate phylogenetic relationships, and identify polymorphic regions within heterotic groups. The lack of currently available genomic resources emphasizes the need to develop strategies for marker-assisted breeding in napiergrass, pearl millet, and Kinggrass. The identification of informative EST-SSRs and their utilization in confirming true Kinggrass hybrids in this study provide novel molecular tools with utility across Kinggrass, PMN, napiergrass, and pearl millet breeding programs.

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