Journal of Environmental Biology
© Triveni Enterprises, Lucknow (India)
Free paper downloaded from: www. jeb. co. in

November 2010, 31(6) 965-968 (2010)
For personal use only
commercial distribution of this copy is illegal

Use of degenerate primers in rapid generation of microsatellite markers in *Panicum maximum*

Kapil Kumar Tiwari*[1] and Amaresh Chandra[2]

[1]National Research Centre on Plant Biotechnology, IARI, New Delhi - 110 012, India
[2]Crop Improvement Division, Indian Grassland and Fodder Research Institute, Jhansi - 284 003, India

(Received: May 25, 2009; Revised received: February 15, 2010; Accepted: February 26, 2010)

Abstract: *Guineagrass (Panicum maximum Jacq.) is an important forage grass of tropical and semi-tropical regions, largely apomictic and predominantly exist in tetraploid form. For molecular breeding work, it is prerequisite to develop and design molecular markers for characterization of genotypes, development of linkage map and marker assisted selection. Hence, it is an important researchable issue to develop molecular markers in those crops where such information is scanty. Among many molecular markers, microsatellites or simple sequence repeat (SSR) markers are preferred markers in plant breeding. Degenerate primers bearing simple sequence repeat as anchor motifs can be utilized in rapid development of SSR markers; however selection of suitable degenerate primers is a prerequisite for such procedure so that SSR enriched genomic library can be made rapidly. In the present study seven degenerated primers namely* $KKVRVRV(AG)_{10,}$ $KKVRVRV(GGT)_{5,}$ $KKVRVRV(CT)_{10}$, $KKVRVRV(AAT)_{6}$, $KKVRVRV(GTG)_{6}$, $KKVRVRV(GACA)_{5}$ *and* $KKVRVRV(CAA)_{6}$ *were used in amplification of Panicum maximum genomic DNA. Primers with repeat motifs* $(GGT)_{5}$ *and* $(AAT)_{6}$ *have not reacted whereas* $(AG)_{10}$, $(GACA)_{5}$ *and* $(CAA)_{6}$ *highly informative as they have generated many DNA fragments ranging from 250 to 1600 bps as revealed from the results obtained with restriction digestion of recombinant plasmids. Primer with* $(CT)_{10}$ *anchor repeat, amplified fragments of high molecular weight where as* $(GTG)_{6}$ *primer generated only six bands with low concentration indicating less suitability of these primer in SSR markers development in P. maximum.*

Key words: *Degenerate primers, Microsatellite markers, Panicum maximum*
PDF of full length paper is available online

Introduction

Guineagrass (*Panicum maximum* Jacq.) belongs to the family Poaceae, subfamily Panicoideae and tribe Paniceae. It forms an agamic complex with other two species, *Panicum infestum* Anders and *Panicum trichocladum* K. Schum. (Muir and Jank, 2004). These three species, having the same chromosome number (2n = 4x = 32), are inter-crossable and morphologically similar, and intermediate type are found in the natural population of East Africa (Savidan and Pernes, 1982). Therefore, no definite taxonomy can be recognized (Clayton and Renvoize, 1982). Guineagrass is one of the major forage grass species in tropical and semitropical regions and is characterized as an apomictic species (gametophytic aposporous type) (Warmke, 1954, Jain *et al*., 2006). Most of guineagrass accessions are autotetraploid (2n = 4x = 32), and reproduce through facultatative aposporous procedure. Some completely sexual guineagrass accessions were found in East Africa, and these sexual plants are identified as diploid (2n = 2x = 16) or tetraploid (Nakajima *et al.,* 1979). In the natural population, 7% of genotypes are diploid and reproduce sexually. It was considered that these diploid sexual forms were intercrossed with *P. maximum*, *P. infestum* and *P. trichocladum* by spontaneous haploidization and recurrent tetraploidization (Savidan and Pernes, 1982).

* **Corresponding author:** kapil21282@gmail.com

At present in India there is a deficit of 62% of green fodder, hence there is a need of over production of quality fodder especially the range grasses which could rejuvenate the fast degrading grasslands. In order to improve the productivity, adaptability and quality of guineagrass, it is important to understand the genetic diversity that exists in the population which also helps in their conservation and germplasm management. Though isozyme markers have been used to distinguish homozygous from heterozygous individual and to determine degree of genetic variability in plant population, its use is restricted because of low number of loci that are available as well as change in its patterns with plant development (Tanksley, 1993). Diversity based on isozyme markers have been reported in guineagrass accessions (Jain *et al.,* 2006). Polymerase chain reactions (PCR) based markers like random amplified polymorphic DNA (RAPD) is relatively simple and rapid but have some limitations such as dominant behavior and its non-reproducibility. Under the category of co-dominant markers, microsatellites or simple sequence repeat (SSR) markers have been reported a highly reproducible and utilised for various molecular breeding program (Liu *et al*., 1996; Smith and Helentjaris, 1996; Xiao *et al*., 1996). The development of microsatellite markers in plant species includes utilization of the sequences in public databases to generate expressed sequence tags (EST) based SSR and the construction and screening of SSR enriched libraries (Wang *et al.,* 1994). Markers developed based on EST sequences are largely

less polymorphic while enriched procedure provides polymorphic SSR markers but its production is time consuming and costly. In recent past degenerate primers have been utilized in developing the SSR markers in less time, which is also cheaper as only one primer is required to be synthesized for the amplification of desired DNA (Fisher *et al.,*1996; Shokeen *et al*., 2005). However, there is a prerequisite to select suitable degenerate primers having anchor repeat motifs of di, tri or tetra nucleotides. Appropriate size of DNA fragments are needed to be amplified with these primers with genomic DNA of *P. maximum* which later can be inserted in desired plasmid. Therefore selection of repeat motifs is also important and its selection depends on repeats found in genomic DNA of targeted plant species. Being guineagrass an important component of Indian grasslands and further the concerted efforts made at Indian Grassland and Fodder Research Institute, Jhansi in collection of germplasm, a reasonable number of SSR markers are required to study the genetic relationships and phylogenetic analysis among the collected accessions. In total 13 SSR markers including 8 from genomic clones and 5 from EST have been reported by Ebina *et al*. (2007). Recently, Chandra and Tiwari (2010) have reported another set of 15 SSR markers in guineagrass. Nevertheless, concerted efforts must be continued to generate sufficient markers for efficient breeding program. The objective of the present study is to identify suitable degenerate primers which can be utilized in development of SSR markers in *P. maximum*.

Materials and Methods

DNA extraction: Genomic DNA was isolated from 1 month old plant of *P. maximum* following the N-Cetyl-N,N,N-trimethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980) with some modifications. Approximately 1 g tissue from young leaves of a single plant was ground to fine powder in liquid nitrogen and mixed with preheated 4.0 ml CTAB DNA extraction buffer [CTAB 2% w/v, NaCl 1.4%, Tris-HCl 100 mM (pH 8.0), EDTA 20 mM and 2-mercaptoethanol 100 mM, added freshly] and incubated at 65°C for 1 hr with gentle mixing at 15 min intervals. After several standard steps, the pellet was washed with 70% ethanol and dissolved in 500 µl of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Quantity of isolated DNA was assessed by running aliquot of DNA on 0.8% agarose gel in 1X TBE buffer using uncut λ DNA (50 ng μl^{-1}). After electrophoresis, gel containing 0.5 µg ml^{-1} of ethidium bromide was visualized under UV light. Quality of DNA was also checked spectrophotometrically by calculating A_{260}/A_{280} ratio.

Amplification of genomic DNA of *P. maximum* with degenerate primers: To amplify genomic DNA of *Panicum maximum* a protocol based on the polymerase chain reaction (PCR) was carried out with 5'-anchored degenerate primers (Fisher *et al.,* 1996). Seven degenerate primers namely KKVRVRV$(AG)_{10}$, KKVRVRV$(GGT)_5$, KKVRVRV$(CT)_{10}$, KKVRVRV$(AAT)_6$, KKVRVRV$(GTG)_6$, KKVRVRV$(GACA)_5$, KKVRVRV$(CAA)_6$ where K = G/T, V = G/C/A, R = G/A were used (Fisher *et al.,* 1996). Amplification was carried out in 20 µl reaction mixture containing 25 ng genomic DNA, 1X PCR buffer (50 mM KCl, 20 mM Tris-HCl pH 8.4), 1.5 mM $MgCl_2$, 0.125 mM each dNTPs, 0.5 µM degenerate primer (Sigma-Aldrich, USA) and 0.5 U of *Taq* DNA polymerase (Bangalore Genei, India). Amplification profile consisted of an initial denaturation at 94°C for 2 min followed by 39 cycles of denaturation for 10 sec at 94°C, annealing for 10 sec at 57°C and extension for 10 sec at 72°C. A final extension step was carried out at 72°C for 7 min.

Ligation and restriction digestion of recombinant plasmids: The amplified DNA fragments were purified using Montage® PCR centrifugal filters devices (Millipore, USA). Ligation reaction was performed with 32 ng of purified amplification products and 50 ng of plasmid DNA (vector : insert = 1 : 4) using pDrive cloning vector kit (QIAGEN, Germany) with additional one unit of T4 DNA ligase per reaction (Bangalore Genei, India) in a total volume of 10 µl at 4°C over night. *E. coli* (DH5α) competent cells were transformed with ligated products and transformants were plated on LB/ ampicillin plates with X-gal and IPTG for blue white screening. The plasmid DNA was isolated from white colonies following standard procedure (Sambrook *et al.,* 1989). These plasmids DNA were checked on 1.2% agarose gel and further digested with ECoR1 restriction enzyme using ECoR1 standard buffer. Plasmid DNA along with buffer and restriction enzyme was incubated at 37°C over night. The digested DNA was resolved on 1.5% agarose gel along with molecular weight marker. Gels were stained with ethidium bromide and analyzed using the gel documentation system (Alpha Imager 2200, Alpha Innotech Corp., USA).

Results and Discussion

Polymerase chain reactions carried out at 57°C annealing temperature, genomic DNA of *P. maximum* reacted and showed DNA fragments of variable sizes with five degenerate primers namely KKVRVRV $(AG)_{10}$, KKVRVRV $(CT)_{10}$, KKVRVRV $(GTG)_6$,

Table - 1: Amplification details of *P. maximum* genomic DNA obtained with degenerate primers

Primers name	Status of amplified products	Range of the sizes of amplified products (bps)	Intensity of amplified DNA bands
KKVRVRV$(AG)_{10}$	Multiple bands with background	250 to 1500	High
KKVRVRV$(GGT)_5$	No amplification	-	-
KKVRVRV$(CT)_{10}$	More than ten bands	350 to 2500	High
KKVRVRV$(AAT)_6$	No amplification	-	-
KKVRVRV$(GTG)_6$	Seven bands with light background	500 to 1500	Low
KKVRVRV$(GACA)_5$	Multiple bands with background	300 to 1500	High
KKVRVRV$(CAA)_6$	Multiple bands with background	450 to 1600	High

- = Indicate no reaction

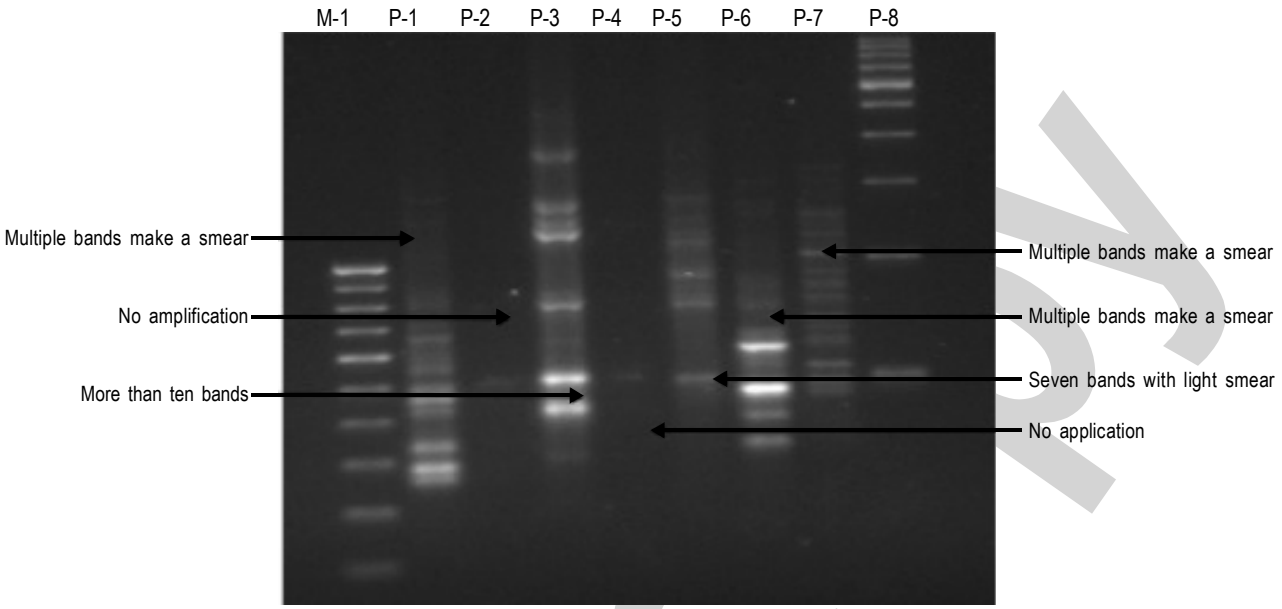

Fig. 1: Ethidium bromide stained agarose gel depicting amplification of *P. maximum* genomic DNA with degenerate primers. Lane 1 = Molecular weight marker M_1 (100 to 1000 bp DNA ladder), Lane 2 = amplification with primer KKVRVRV$(AG)_{10}$, Lane 3 = amplification with primer KKVRVRV$(GGT)_5$, Lane 4 = amplification with KKVRVRV $(CT)_{10}$, Lane 5 = amplification with primer KKVRVRV$(AAT)_6$, Lane 6 = amplification with primer KKVRVRV$(GTG)_6$, Lane 7 = amplification with primer KKVRVRV$(GACA)_5$, Lane 8 = amplification with KKVRVRV$(CAA)_6$ and Lane 9 = molecular weight marker M_2 (500 to 5000 bp DNA ladder)

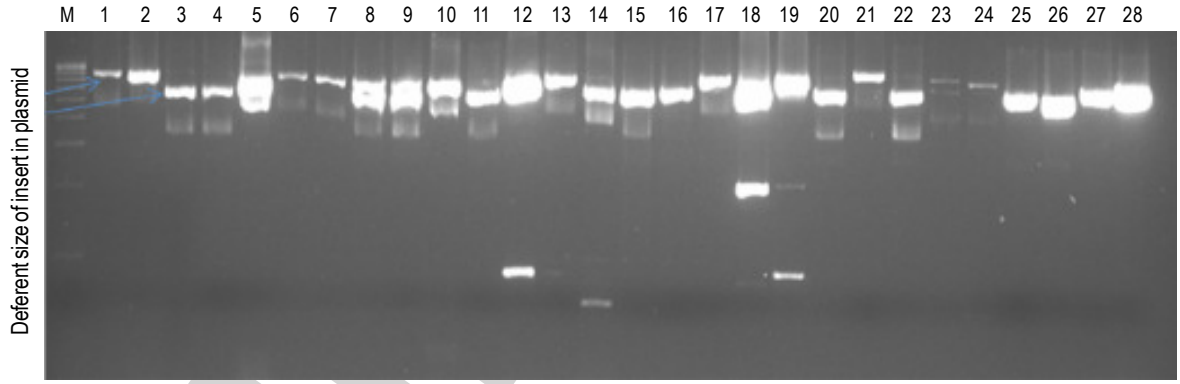

Fig. 2: Recombinant plasmids containing degenerate amplified products. M : 500 to 5000 bp marker, Lanes 1 to 28 EcoR1 digested recombinant plasmids DNA

KKVRVRV $(GACA)_5$ and KKVRVRV $(CAA)_6$. As these primers have shown reactions, it can be presumed that *Panicum maximum* genomic DNA possessed compatible repeat motifs (Table 1). Since annealing temperature during polymerase chain reactions was as high as 57°C, a reasonable level of sequence homology would be presumed between primers and genomic sequences of guineagrass which eventually generated DNA fragments of variable sizes. Primers KKVRVRV $(AG)_{10}$, KKVRVRV $(GACA)_5$ and KKVRVRV $(CAA)_6$ amplified multiple DNA fragments seen as background in agarose gel with intense DNA bands of the sizes of 250 to 1600 bps (Fig. 1). The intensity of the amplified product was also high indicating better suitability of this primer over others for the construction of SSR enriched library for the development of SSR markers. As the size of the amplified DNA products with primer KKVRVRV $(CT)_{10}$ was high (350 to 2500 bps), indicating less suitability of high size fragments for cloning and sequencing, but at the same time the intensity of the DNA products was high, the DNA fragments was used for cloning, thus showed partial suitability of this primer. Primer KKVRVRV $(GTG)_6$ generated only seven bands with less concentration indicating less utility of this primer (Fig. 1). When all these fragments after standard purification were cloned, a total around 500 *E. coli* colonies were obtained. Of these, 250 clear and isolated white recombinant clones were used for plasmid extraction. The recombinant plasmid DNA was digested with ECoR1 restriction enzyme where most of the DNA fragments of respective sizes as seen in PCR products were observed in the recombinant clones (Fig. 2). Thus the study revealed the primers bearing microsatellite repeat motifs $(AG)_{10,}$ $(CT)_{10,}$ $(GACA)_5$, $(CAA)_6$ were highly useful in the development of SSR marker in *Panicum maximum*. Since the amplified DNA fragments were successfully cloned, it can be presumed that the fragments would have repeat sequences as per the sequence composition of degenerate primers. The flanking

sequences of these repeats can be utilized for the construction of primer pairs (Fisher *et al.,* 1996; Gupta and Varshney, 2000). Simple sequence repeat makers have been developed successfully in several crops utilizing such approach (Fisher *et al.,* 1996; Shokeen *et al.,* 2005).

In conclusion, the degenerate primers having variable repeat unit generated clonable DNA fragments of variable sizes with guineagrass demonstrated the utility of such approach in this crop also. Further, the number of recombinant clones obtained as well as less time and cost involved in the present method can be taken as added advantage in generating SSR markers in less explored crop like guineagrass.

Acknowledgments

Authors are thankful to the Director of the Institute and Head, Crop Improvement Division for providing the necessary facilities to carry out the reported work. Authors are grateful to the two anonymous reviewers who have helped in improving the manuscript.

References

Chandra, A. and K.K. Tiwari: Isolation and characterization of microsatellite markers from guineagrass (*Panicum maximum*) for genetic diversity estimate and cross-species amplification. *Plant Breeding*, **129**, 120-124 (2010).

Clayton, W.D. and S.A. Renvoize: Graminae (Part 3). *In*: Flora of Tropical East Africa. pp. 1-898 (1982).

Ebina, M., K. Kouki, S.I. Tsuruta, R. Akashi, T. Yamamoto, M. Takara, M. Takahara, M. Inafuku, K. Okumura, H. Nakagawa and K. Nakajima: Genetic relationship estimation in guineagrass (*Paicum maximum* Jccq.) assessed on the basis of simple sequence repeat markers. *Japa. Soci. Grassl. Sci.*, **53**, 155-164 (2007).

Fisher, P.J., R.C. Gardner and T.E. Richardson: Single locus microsatellites isolated using 5' anchored PCR. *Nucl. Acids Res*., **24**, 4369-4371 (1996).

Gupta, P.K. and R.K. Varshney: The development and use of microsatellite markers for genetic analysis and plant breeding with special emphasis on bread wheat. *Euphytica*, **113**, 163-185 (2000).

Jain, A., A.K. Roy, P. Kaushal, D.R. Malaviya and S.N. Zadoo: Isozyme banding pattern and estimation of genetic diversity among guineagrass germplasm. *Genet. Resour. Crop Evol*., **53**, 339-347 (2006).

Liu, Z.W., R.M. Biyashev and M.A. Saghai Maroof: Development of simple sequence repeat markers and their integration into a barley linkage map. *Theor. Appl. Genet*., **10**, 869-876 (1996).

Murray, M.G. and W.F. Thompson: Rapid isolation of high-molecular-weight plant DNA. *Nucl. Acids Res*., **8**, 4321-4325 (1980).

Muir, J.P. and L. Jank: Guineagrass. *In*: Warm-Season (C4) Grasses (*Eds*.: L.E. Moser, B.L. Burson and L.E. Sollenberger). Agronomy monograph no. 45. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI. pp. 589-621 (2004).

Nakajima, K., N. Komatsu, N. Mochizuki and S. Suzuki: Isolation of diploid and tetraploid sexual plants in guineagrass (*Panicum maximum* Jacq.). *Jpn. J. Breed*., **29**, 228-238 (1979).

Sambrook, J., E.F. Fritsch and T. Maniatis: Molecular cloning. 2nd Edn. Cold Spring Harbor Laboratory Press, New York (1989).

Savidan, Y.H. and J. Pernes: Diploid-tetraploid-dihaploid cycles and the evolution of *Panicum maximum* Jacq. *Evolution*, **36**, 596-600 (1982).

Smith, S. and T. Helentjaris: DNA fingerprinting and plant variety protection. *In*: Genome mapping in plants (*Ed*.: A.H. Paterson). New York. pp. 95-110 (1996).

Shokeen, B., N.K. Sethy, S. Choudhary and S. Bhatia: Development of STMS markers from the medicinal plant Madagascar periwinkle [*Catharanthus roseus* (L.)]. *Mol. Ecol. Notes*, **5**, 818-820 (2005).

Tanksley, S.D.: Mapping polygenes. *Ann. Rev. Genet.,* **27**, 205-233 (1993).

Wang, Z., J.L. Weber, S.D. Tanksley: Survey of plant short tandem repeats. *Theor. Appl. Genet*., **88**, 1-6 (1994).

Warmke, H.E.: Apomixis in *Panicum maximum*. *Am. J. Bot.,* **41**, 5-11 (1954).

Xiao, J., J. Li, L. Yuan, S.R. Mc Couch and S.D. Tanksley: Genetic diversity and its relationship to hybrid performance and heterosis in rice as revealed by PCR-based markers. *Theor. Appl. Genet*., **92**, 637-643 (1996).