12-2007

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Publication Info

Published in *Journal of SAT Agricultural Research*, ed. Dave Hoisington, Volume 3, Issue 1, 2007, pages 34-37.

Development of new simple sequence repeat markers for pearl millet

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Introduction

Availability of good numbers of polymerase chain reaction (PCR)-compatible markers is a prerequisite for their application in marker assisted breeding (MAB) for the genetic improvement of agricultural crops. Simple sequence repeats (SSRs) or microsatellites, which consist of a variable number of tandem repeats of a simple motif sequence, typically a mono-, di-, tri- or tetranucleotide repeat, are very useful markers for a number of plant species. The main advantages of SSRs are that they are abundant, reproducible, co-dominant, widely distributed in crop genomes and require a small quantity of DNA for PCR to detect polymorphisms. Because of these properties, SSRs are suitable markers for use in breeding programs, which essentially require high throughput markers as breeders need to screen a large number of breeding lines and segregating progenies.

Like other crops, MAB has potential application in pearl millet (*Pennisetum glaucum*), which is a very important grain crop in the arid and semi-arid parts of Africa and South Asia. Pearl millet is confronted by a number of biotic (mainly diseases) and abiotic (drought, heat, salinity and low nutrient supply) constraints to its production. Selecting this crop for better tolerance to these stresses has been an important objective in its breeding programs. Marker assisted selection (MAS) offers a considerable advantage over conventional breeding methods in terms of precise and quick improvement in hard-to-screen traits like drought tolerance and downy mildew resistance (Hash and Witcombe 2002).

Recently, there has been a tremendous progress in developing SSR markers for a number of grain crops like rice (*Oryza sativa*) (McCouch et al. 2002), wheat (*Triticum aestivum*) (Gupta and Varshney 2000), barley (*Hordeum vulgare*) (Varshney et al. 2007) and sorghum (*Sorghum bicolor*) (Menz et al. 2004). Compared to these crops, however, the number of available SSR markers in pearl millet is much lower and many more are required to produce a linkage map with high marker density to facilitate MAS.

Previously, approximately 150 SSRs have been developed for use in pearl millet (Allouis et al. 2001, Qi et al. 2001, 2004, Budak et al. 2003, Senthilvel et al. 2004, Mariac et al. 2006). Development of many of these SSRs was very costly as it involved library construction, hybridization screening and clone sequencing. Currently, large public expressed sequence tags (EST) databases exist for several grass species which can be utilized for identification and development of SSR for orphan crops like pearl millet. Senthilvel et al. (2004) and Mariac et al. (2006) used this approach to design SSR markers. We report here designing and development of additional SSR markers for pearl millet.

Materials and methods

We downloaded the nearly 6800 *Pennisetum* sp EST sequences available in the National Center for Biotechnology Information (NCBI) database (as of December 13, 2006) and assessed them for the presence of SSR motifs using the Simple Sequence Repeat Identification Tool (SSRIT) (Temnykh et al. 2001) with the preset parameters to look for motifs including all di- to pentanucleotides and repeat numbers. We then identified sequences that had not been used before to develop SSR markers by comparing our set of SSR containing sequences to those that had already been used for this purpose (based on the literature and personal communications), and used Primer 3 (Rozen and Skaletsky 2000) to design primers only for those that had not been used before. Parameters for primer design were as follows: the melting temperature (T_m) was kept between 55 and 62°C with forward and reverse primers having a difference of 1–2°C in their T_m; the length of primer was 18–25 bp; and the length of the resulting PCR product was 150–200 bp. We designed primers to amplify small fragments so that polymorphisms could be more readily detected on agarose gels.

The primers were tested for amplification on pearl millet genotype Tift 23A and then validated on seven additional lines (RIB 335/74, J 28, J 35, ICMB 841-P3, 863B-P2, 81B-P6 and ICMP 451-P8) that are parental lines of four existing mapping populations in pearl millet and represent a range of diversity in terms of the geographic origin and phenotype. Thus, SSR primers were assayed with eight parental lines. The PCR reaction mixture (20 µl

total volume) consisted of 1X buffer (Promega GoTaq with green loading dye), dNTP (0.33 mM each), 5 p mol primer (both forward and reverse), 20 ng of template DNA, 1 µl of dimethyl sulfoxide (DMSO) and 0.5 units of Taq DNA polymerase (GoTaq Promega). Amplifications were performed at a MJ Research Tetrad thermocycler programmed for 35 cycles of 20 s at 95°C, 20 s at 55°C, 1.5 min at 72°C and ending with 10 min at 72°C after an initial denaturation for 3 min at 95°C.

The PCR products were fractioned on 2.5% agarose gels consisting of 3.75 g of agarose (MP Biomedical Inc, USA) and 3.0 µl of ethidium bromide in 150 ml TBE (Tris boric acid, EDTA) buffer. Electrophoresis was performed at 120 volts for 1.5 h at room temperature. Gels were photographed using a Kodak UV light camera. Primers that amplified SSR loci in the test DNA (Tift 23A) were then screened for polymorphisms in seven additional lines using 4% EXCLUSIVE (The Nest Group Inc, USA) high resolution agarose gels.

Results and discussions

Of the 6788 sequences retrieved from database, for a total sequence length of 3716878 bp, 162 sequences contained SSRs. From these, we eliminated sequences from which millet SSRs had already been developed. In total, we were able to design primers to amplify 19 new SSR loci (Table 1). To summarize, the new SSRs consisted of eight dinucleotide and eight trinucleotide repeat motifs and three tetranucleotide motifs. The dinucleotide motifs 'ag/tc' and 'ac/tg' were detected at equal frequencies (four times each). The length of repeat motifs varied from 5 in loci EB411079 and BM084420 to 32 in locus BM084913 (Table 1).

When tested in Tift 23A (our DNA standard), alleles were amplified in 11 of 19 SSR loci and eight loci failed to amplify (Table 1). Reasons for amplification failure include: primer sequence mismatch in the test DNA; errors in the DNA sequence deposited in the database; or one or both primers may flank an intron (intronic sequences are not present in ESTs). Because intron lengths may vary from a few hundred to thousands of nucleotides, the PCR conditions used may not be appropriate for amplifying fairly large DNA fragments.

When fractioned on high resolution agarose gels, four of the 11 loci that amplified in the test DNA, viz, EB411043, CD726723, BM084758 and BM084922, were polymorphic between the parents of at least one of four mapping populations. More specifically, loci EB411043 and CD726723 were polymorphic between ICMB 841-P3 and 863B-P2; and between 81B-P6 and ICMP 451-P8, while BM084922 and BM084758 varied between RIB and J 28 (Fig. 1), and CD726723 was polymorphic between RIB and J 35. We should also note that the seven monomorphic loci may show variation among our mapping parents if assayed using platforms with higher resolving power than agarose (ie, acrylamide gels or capillary systems).

These new SSR loci can now be added to existing maps of pearl millet (Qi et al. 2004) and also be used, in addition to those available earlier, in assessing genetic diversity in pearl millet. Availability of additional SSRs for pearl millet will help towards facilitating MAS in this orphan crop in which MAS has already been demonstrated to be successful in terms of delivering to farmers an

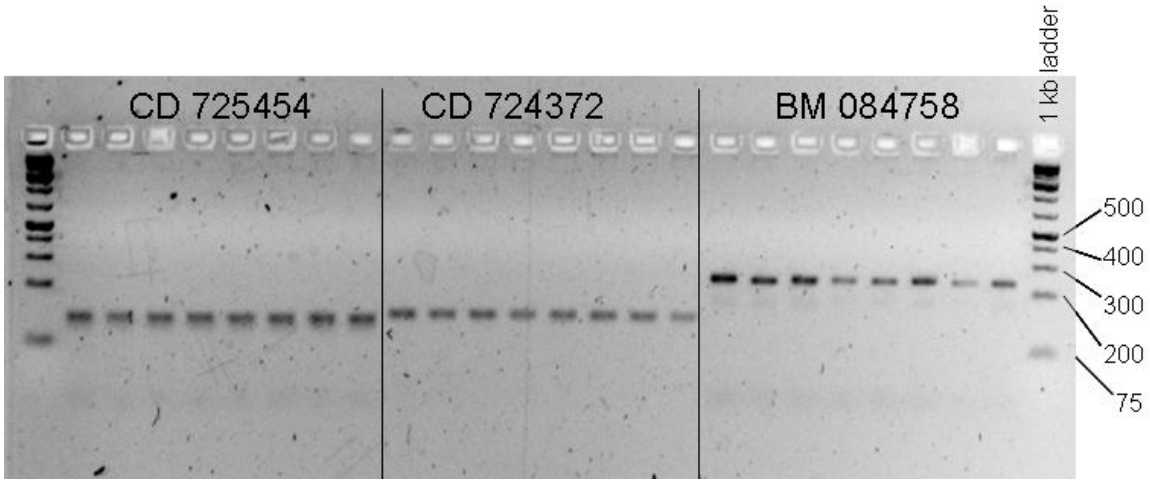

Figure 1. PCR products of three EST-SSR primer pairs corresponding to pearl millet ESTs CD725454, CD724372 and BM084758 amplified in eight pearl millet genotypes (lane 1 = RIB 335/74, lane 2 = J 28, lane 3 = J 35, lane 4 = Tift 23A, lane 5 = ICMB 841-P3, lane 6 = 863B-P2, lane 7 = 81B-P6 and lane 8 = ICMP 451-P8). The numbers on the right hand side of the figure indicate size in base pairs of known DNA fragments.

Table 1. Simple sequence repeat (SSR) loci developed including primer sequences, repeat type and length, melting temperature (T_m), expected size of amplicon and GC content.

Locus	EST	Primer[1]	DNA sequence (5'-3')	Repeat length and motif	Expected amplicon size (bp)	T_m (°C)	GC content (%)
Xcump001	DQ875457[2]	F	GCACGAGGCTTATCTGTGTTTC	$(ag)_9$	157	60.3	50.0
		R	CAACTCTTGCCTTTCTTGGCCT			60.3	50.0
Xcump002	EB411079[2]	F	GCACGAGGCAAAATATAAAGGTG	$(gtgc)_5$	198	58.9	43.5
		R	ACGTAGACTTGCACCACCAGA			59.8	52.4
Xcump003	EB411043[2]	F	CATGCGACGTGGTCTATCTG	$(tcc)_8$	118	59.4	55.0
		R	GAGAGAGAACCAGCAGCACC			61.4	60.0
Xcump004	EB410932	F	CACGAGGCTCACTAGGGTTT	$(cgg)_7$	113	59.4	55.0
		R	ACCCGGGTCTGGTTAGACTT			59.4	55.0
Xcump005	CD726723[2]	F	GCACGAGGGCCAGATTCTAGAA	$(ctg)_9$	164	62.1	54.5
		R	CACGGTGATGACACGACATGGT			62.1	54.5
Xcump006	CD726532	F	GAAATCGGCAGAGGGCAT	$(tatg)_9$	100	56.0	55.6
		R	CAATGAGTATGTGCACGCTGCA			60.3	50.0
Xcump007	CD725454[2]	F	GAGGGATTCCAGGCGGTTC	$(ag)_{11}$	201	61.0	63.2
		R	GCGAGGAGCACATTCGATGAA			59.8	52.4
Xcump008	CD724372[2]	F	GTTGACTACCACTATTATGCTCC	$(ctc)_6$	175	58.9	43.5
		R	GACCAAGAACTTCATACAATTCAG			57.6	37.5
Xcump009	CD724352[2]	F	ATCTGATCGTGAGGCCTCAAC	$(ctc)_6$	225	59.8	52.4
		R	GCCGACCAAGAACTTCATACAAT			58.9	43.5
Xcump010	BM084922[2]	F	GCTGAACTATTCTGTAAACTTAAC	$(ca)_{20}$	173	55.9	33.3
		R	TATCGAAACGGTACTAAAATCATG			55.9	33.3
Xcump011	BM084913	F	TGATGGGAACCGAGAGCATGA	$(ac)_{32}$	196	59.8	52.4
		R	TAGCACAGCAATAACATGGCATC			58.9	43.5
Xcump012	BM084896	F	TGTGATCTGTGGTCTCAGGC	$(ac)_{22}$	165	59.4	55.0
		R	CGTGAAAGCTCTCCAGGACT			59.4	55.0
Xcump013	BM084883	F	ACCGACAGCAACAAATCCTCC	$(ct)_{10}$	194	59.8	52.4
		R	GCTCTTGTGTGTAGTTGTGCTT			58.4	45.5
Xcump014	BM084862	F	CTGACCTCTCCTCTCCTTCG	$(ccg)_6$	185	61.4	60.0
		R	GAGCAGATCCTTGGCCTTCTTG			62.1	54.5
Xcump015	BM084822	F	GAAGCATAGGAGAGGAGGG	$(ag)_9$	158	58.8	57.9
		R	CTTGCTGCTCGGACTTCTCT			59.4	55.0
Xcump016	BM084758[2]	F	CATTTCTCTCGCCAGTGCTC	$(ct)_9$	250	59.4	55.0
		R	ATCTCCAGAACCGAGCGCA			58.8	57.9
Xcump017	BM084738[2]	F	ATAGCTGGGTGTTGTCTGGC	$(aag)_7$	124	59.4	55.0
		R	CCCTGGCGCTTAATTGTAAA			55.3	45.0
Xcump018	BM084569[2]	F	TGCTTTCTTCCCAACCAGTGG	$(gca)_7$	264	59.8	52.4
		R	TGCTGAGTGGGGTGCTGCT			61.0	63.2
Xcump019	BM084420	F	GGCCTAACTCTCTGTTCTTCTTC	$(agcg)_5$	212	60.6	47.8
		R	GAGAAGCTAACATTTGGGGCCTA			60.6	47.8

1. F = Forward; R = Reverse.
2. Locus amplified in Tift 23A.

improved downy mildew resistant version of an extra-early maturing single cross hybrid.

Acknowledgments. This work was supported by the Department of Biotechnology, Indian Ministry of Science and Technology (Overseas Fellowship award to the first author). We acknowledge excellent technical help of Charlotte B Acharya during the course of this investigation.

References

Allouis S, Qi X, Lindup S, Gale MD and **Devos KM.** 2001. Construction of a BAC library of pearl millet, *Pennisetum glaucum*. Theoretical and Applied Genetics 102:1200–1205.

Budak H, Pedraza F, Cregan PB, Baenziger PS and **Dweikat I.** 2003. Development and utilization of SSRs to estimate the degree of genetic relationships in a collection of pearl millet germplasm. Crop Science 43:2284–2290.

Gupta PK and **Varshney RK.** 2000. The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis on bread wheat. Euphytica 113:163–165.

Hash CT and **Witcombe JR.** 2002. Gene management and breeding for downy mildew resistance. Pages 27–36 *in* Sorghum and millets diseases (Leslie JF, ed.). USA: Iowa State Press.

Mariac C, Luong V, Kapran I, Mamadou A, Sagnard F, Deu M, Chantereau J, Gerard B, Jupiter Ndjeunga, Bezançon G, Pham J and **Vigouroux Y.** 2006. Diversity of wild and cultivated pearl millet accessions (*Pennisetum glaucum* [L.] R. Br.) in Niger assessed by microsatellite markers. Theoretical and Applied Genetics 114:49–58.

McCouch SR, Teytelman L, Xu Yunbi, Lobos KB, Clare K, Walton M, Fu B, Maghirang R, Li Z, Xing Y, Zhang Q, Kono I, Yano M, Fjellstrom R, DeClerck G, Schneider D, Cartinhour S, Ware D and **Stein L.** 2002. Development and mapping of 2240 new SSR markers for rice (*Oryza sativa* L.). DNA Research 9:199–207.

Menz MA, Klein RR, Unruh NC, Rooney WL, Klein PE and **Mullet JE.** 2004. Genetic diversity of public inbreds of sorghum using mapped AFLP and SSR markers. Crop Science 44:1236–1244.

Qi X, Lindup S, Pittaway TS, Allouis S, Gale MD and **Devos KM.** 2001. Development of simple sequence repeat markers from bacterial artificial chromosomes without subcloning. BioTechniques 31:355–361.

Qi X, Pittaway TS, Lindup S, Liu H, Waterman E, Padi FK, Hash CT, Zhu J, Gale MD and **Devos KM.** 2004. An integrated genetic map of pearl millet, *Pennisetum glaucum*. Theoretical and Applied Genetics 109:1485–1493.

Rozen S and **Skaletsky H.** 2000. Primer3 on the WWW for general users and for biologist programmers. Pages 365–386 *in* Bioinformatics methods and protocols: Methods in molecular biology (Krawetz S, Misener S, eds.). Totowa, New Jersey, USA: Humana Press.

Senthilvel S, Mahalakshmi V, Sathish Kumar P, Reddy AR, Markandeya G, Reddy MK, Misra R and **Hash CT.** 2004. New SSR markers for pearl millet from data mining of Expressed Sequence Tags. *In* Proceedings of the 4th International Crop Science Congress held at Brisbane, Australia from 26 September to 1 October 2004. Published on CD (available at http// www.cropscience.com.au).

Temnykh S, DeClerck G, Lukashova-A, Lipovich L, Cartinhour S and **McCouch S.** 2001. Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.): frequency, length variation, transposon associations, and genetic marker potential. Genome Research 11:1441–1452.

Varshney RK, Marcel TC , Ramsay L, Russell J, Röder MS, Stein N, Waugh R, Langridge P, Niks RE and **Graner A.** 2007. A high density barley microsatellite consensus map with 775 SSR loci. Theoretical and Applied Genetics 114:1091–1103.