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Efficient Microsatellite Enrichment in Finger Millet (*Eleusine coracana* (L.) Gaertn) – An Improved Procedure to Develop Microsatellite Markers

I. N. Bheema Lingeswara Reddy[1*] • D. Srinivas Reddy[2] • V. Prathap Reddy[3] • M. Lakshmi Narasu[1] • S. Sivaramakrishnan[3]

[1] Centre for Biotechnology, Jawaharlal Nehru Technological University, Hyderabad- 500 085 (A.P), India
[2] Genetic Transformation laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad - 502 324 (A.P), India
[3] Department of Agricultural Biotechnology, Acharya N.G. Ranga Agricultural University (ANGRAU), Rajendranager, Hyderabad - 500 030 (A.P), India

Corresponding author: * **bheeminja@rediffmail.com**

ABSTRACT

One of the most recent advances in molecular genetics has been the introduction of microsatellite markers to investigate the genetic structuring of natural populations. To facilitate simple sequence repeat (SSR) discovery in finger millet (*Eleusine coracana* (L.) Gaertn), a simplified protocol is proposed for using multi-enzyme digestion/ligation, biotin-labelled probes and a streptavidin-coated magnetic beads hybridization capture strategy. The procedure was carried out with trinucleotide repeats and the analysis showed more enriched microsatellites. More than 200 SSR motives were detected (SSR motifs ≥3 repeat units or 9 bp). Considering SSRs with 4 or more repeat units, the level of enrichment was 83.3%. 27 primers were designed. This method is an improvement over the current repeat-enrichment strategies which usually give fewer clones with trinucleotide repeats, resulting in representative small-insert libraries with a very high proportion of positive clones. Development and utilization of markers such as SSRs is a valuable asset for estimating genetic diversity and analyzing the evolutionary and historical development of cultivars at the genomic level in finger millet breeding programs.

Keywords: magnetic beads hybridization capture, polymerase chain reaction, simple sequence repeats, primer design

INTRODUCTION

Finger millet (*Eleusine coracana* (L.) Gaertn) spp. *coracana* belongs to the Poaceae family, Eragrostideae tribe. Finger millet is an important cereal crop for subsistence agriculture in dry areas of Eastern Africa, India and Sri Lanka and has an excellent nutritional value as its seeds contain protein ranging from 7 to 14% being particularly rich in methionine, iron and calcium (Barbeau and Hilu 1993; Vadivoo *et al.* 1998). Finger millet also contains two polyunsaturated fatty acids that are essential for humans, linoleic acid and α-linolenic acid (Fernández *et al.* 2003). Finger millet is a popular food among diabetic patients. Its slow digestion indicates low blood sugar levels after a finger millet diet thereby reacting as a safer food for diabetics (DOASL 2006).

One of the most recent advances in molecular genetics has been the introduction of microsatellite markers to investigate the genetic structuring of natural populations. Microsatellites are the class of repetitive DNA sequences present in both eukaryotes (Morgante *et al.* 2002) and prokaryotes (Gur *et al.* 2000). They consist of tandemly arranged repeats, usually 2-6 nucleotides, which are distributed throughout the whole genome and their distribution varies in different species and are flanked by highly conserved sequences (Chambers *et al.* 2000). The most frequent microsatellites in plants are dinucleotide motifs, usually (AT)n and (GT)n, whereas (AC)n repeats are common in animals (Morgante *et al.* 2002). Some types of microsatellites seem to be specific to a certain group of plants, e.g. CCG/CGG repeats are much more abundant in rice than in other cereals or dicotyledonous plants (Morgante *et al.* 1993).

Several enrichment protocols have been reported (Karagyzov *et al.* 1993; Edwards *et al.* 1996; Hamilton *et al.* 1999; Zane *et al.* 2002; Mathews *et al.* 2007). The efficiency of these protocols is estimated based on the number of clones that contain microsatellite motifs. The estimates range from 10-95%.

For regular plant breeding applications, PCR-compatible markers based on microsatellites or simple sequence repeats (SSRs) are considered to be the most appropriate. SSRs typically provide single-locus markers, which are often co-dominantly inherited and characterized by hypervariability, abundance and reproducibility. To date, collections of 82 SSR markers (Mathews *et al.* 2007) and 31 EST (expressed sequence tag) SSR markers (Arya *et al.* 2009) have been reported in finger millet. This is too little compared to other crops like rice where the number of SSRs exceed 19,000, etc. (www.gramene.org; Graminae site). Therefore, development of additional SSR markers is a valuable objective for the finger millet research community. To facilitate SSR marker development in finger millet, we present a highly simplified protocol. We used an enrichment strategy reported by Hamilton *et al.* (1999) with modifications for the isolation of microsatellites. This method is based on capture of single-stranded restriction fragments annealed to biotinylated microsatellite oligonucleotides and subsequent magnetic separation.

MATERIALS AND METHODS

Total genomic DNA of finger millet was extracted from flushing young leaves (10-day old) by using a using a modified cetyl trimethyl ammonium bromide (CTAB) method (Porebski *et al.* 1997) and quantified. All the reagents required for CTAB buffer preparation were obtained from Sigma-Aldrich (St. Louis, MI, USA). Combinations of restriction enzymes (*Alu*I, *Hae*II, *Rsa*I, *Nhe*I, *Sau*3A, and *Xmn*I) (New England Biolabs, MA, USA) were used to digest DNA. Genomic DNA (5 µg) was digested in a reaction volume of 50 µl having a final concentration of 1X buffer

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(compatible with all the restriction enzymes), 10 U/µl of enzyme, and incubated at 37°C overnight and then heated at 65°C for 20 min to inactivate restriction enzymes. Five units of Mung bean nuclease (MBN) (New England Biolabs) were added directly to digest genomic DNA and incubated at 30°C for 30 min. Genomic digests were run on 1.5% agarose gel (Sigma-Aldrich). Fragments ranging from 300-1000 bp were cut and eluted using QIAquick Purification Columns (Qiagen, Valencia, USA) and eluted in 50 µl of Elusion buffer provided with the columns. Genomic DNA ends were then dephosphorylated by adding 6 µL NEB buffer 2 (New England Biolabs), 3 µL sterile water and 1 µl (10 U) calf intestinal phosphatase (CIP) to 50 µL of digested, MBN-treated genomic DNA and incubated for 2 h at 37°C. Genomic DNA was purified with a QIAquick PCR column (Qiagen) and eluting the DNA in 30 µL of elution buffer. Digested, MBN-treated and dephosphorylated genomic DNA was ligated to double-stranded SNX linkers using DNA ligase (New England Biolabs). 5 µL of linker, 3 µl of NEB buffer 2, 1 µl of ligase, 0.3 µl of BSA (bovine serum albumin), 1 µl of *Xmn*I, 10 µl of eluted DNA after CIP treatment and made up to 30 µl using Millipore Water was ligated at 14°C overnight. Ligation of linkers to digested genomic DNA fragments was confirmed by setting three PCR reactions with SNX forward, three different volumes of linker-ligated DNA as template in separate reactions, 1.0, 2.0 and 4.0 µl in a reaction volume of 20 µl containing, 1X PCR buffer (Invitrogen, CA, USA), 1.5 mM $MgCl_2$ (Invitrogen), 2 mM dNTPs (Invitrogen), 0.5 ρmol of SNXF primer, 1 U of *Taq* polymerase (Invitrogen) along with one negative control with no linker-ligated DNA and were run on a thermal cycler ep gradient S (Eppendorf, Germany) with an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1.30 min and final extension at 72°C for 10 min. 10 µl of PCR product was run on a 1.5% agarose gel (Sigma-Aldrich) along with a negative control (only ligation mix without PCR) and 50-bp marker (NEB) to check the amplification and for further use in enrichment. Repeat enrichment of genomic DNA fragments was done using biotinylated repeat oligos [$(AGG)_7$, $(CAC)_7$, $(CAG)_7$, $(ATC)_7$]. The left over 10 µl of PCR product was mixed with biotinylated repeat probes in 6X SSC with 0.1% SDS (sodium dodecyl sulfate), denatured at 95°C for 5 min, and hybridized at 60°C for 1 h. After hybridization, 50.0 µl of streptavidin-coated paramagnetic beads equilibrated in 6X SSC was added, and the suspension was mixed continuously for 30 min to capture the biotin-containing hybrids. The beads were concentrated in the magnetic stand obtained along in the kit, washed in 6X SSC/0.1% SDS (twice at room temperature, and three times at 60 min followed by two washes in 6X SSC at room temperature. The captured DNA strands were eluted with 100 µl TE (repeat-enriched DNA).

Amplification of repeat-enriched DNA

A total of 5 PCR reactions for each of the six eluted samples were carried out in a reaction volume of 20 µl containing 8.0 µl of eluted DNA, 1 x PCR buffer (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 0.1 mM dNTPs (Invitrogen), 0.5 p moles of SNX primer, 0.5 U *Taq* polymerase (Invitrogen) and were run on a thermal cycler (Eppendorf) with an initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 2.0 min and final extension at 72°C for 10 min. 2 µl of PCR product was run on a 1.5% agarose gel at 90 V.

Ligation of enriched PCR products

A ligation reaction was performed individually for all the repeat enriched DNA fragments obtained by using biotinylated repeat oligos. The gel-eluted PCR fragments were cloned in pGEM®-T Easy vector. The ligation reaction was set up for a 10 µl reaction vol-ume in 5 µl rapid ligation buffer [30 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 5% polyethylene glycol (MW 8000)] with 0.5 µl of 50 ng of pGEM®-T Easy vector, 3.5 µl gel-purified PCR product and 1 µl of T4 DNA ligase. Ligation was carried out by incubating for 16 h at 14°C.

Transformation of DH10B cells with ligation mix

Competent cells of *E. coli* DH10B strain (Invitrogen) were prepared using 10% glycerol (Sambrook 1989) and bacterial transformation was carried by electroporation. Colony PCR was carried out for the confirmation of DNA inserts in the selected putative recombinant clones. PCR was performed in a total reaction volume of 30 µl with M13 universal forward and reverse primers. PCR was carried out in a PTC-100™ thermal cycler (MJ Research®, USA) and the parameters were: initial denaturation step of 95°C for 5 min followed by 30 thermal cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, with a final extension step of 10 min. PCR products were verified for cloned inserts by resolving the DNA fragments on a 1.5% TAE (*TAE* buffer is a buffer solution containing a mixture of Tris base, acetic acid and EDTA) agarose gel, at 90 V.

Sequencing and analysis

Initially, 5 plasmids from each repeat were isolated by alkaline lysis using the PEG precipitation method (Sambrook and Russell 2001) checked with restriction digestion and were sequenced. Repeats were checked using "SSRIT" software (Temnykh *et al.* 2001) present in Gramineae website (www.gramene.org/db/markers/ssrtool).

Dot blot hybridization of SSR clones was carried out to select more clones for sequencing as per the method of Sambrook *et al.* (1989). 96 clones were used for dot blot analysis from each ligation event and SSR clones of the same ligation event were used as probes for hybridization in the dot blots. Additional clones were identified based on the intensity of hybridization in the dot blot and were sequenced.

Designing of primers for microsatellite flanking regions

The sequences containing microsatellites with sufficient flanking regions on either side were submitted in primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), for primers of 18-22 base length and product sizes of 150-250 bp.

RESULTS AND DISCUSSION

The conventional procedure to identify microsatellite sequences is to construct and screen genomic DNA libraries. Although this strategy has been used for the development of SSR markers in several crop species, the procedure has drawbacks especially in case of trinucleotides; besides being labor intensive, time consuming, and expensive only fewer clones with repeats were generally obtained. To improve the efficiency of SSR capture, several methods were developed for the SSR enrichment of libraries that used physical (e.g., sonication) or enzymatic (e.g., restriction digestion) means to fragment DNA followed by capture with repeat oligos (Powell *et al.* 1996).

To minimize some of the shortcomings in the above-mentioned methods and to improve enrichment efficiencies, we have developed an improved procedure for the construction of genomic libraries. 5 µg of genomic DNA was used after quantification (**Fig. 1**) in combination with a few restriction enzymes; we included *Nhe*I because any genomic fragments containing this site would be cut during ligation into vector (**Fig. 2**). SNX linkers were used as they will provide the primer binding site for subsequent PCR steps. They also provide sites to ease cloning of fragments into the vectors that will subsequently be used. The single enzyme digested and linker ligated DNA fragments usually yielded lesser number of fragments in the expected range compared to multiple enzymes digested DNA, the colonies obtained were mostly in range of 300-900 in case of multi-enzyme digestions and that can be used directly for library construction. Use of a single restriction enzyme for digestion (Fischer *et al.* 1998) is a limitation since the restriction enzyme creates a complementary overhang which yields a small proportion of the genome as fragments of lesser than 1000

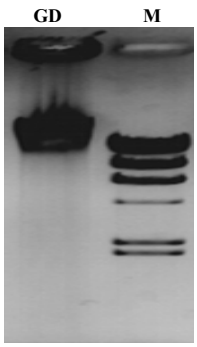

Fig. 1 Quantification of genomic DNA of Finger millet (*Eleusine coracana*). Lane GD: 1 µl of genomic DNA (UR762), Lane M: Lambda *Hind*III digest marker (200 ng).

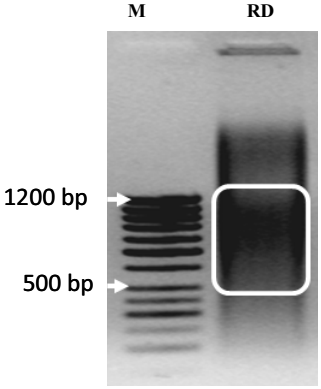

Fig. 2 Agarose gel electrophoresis pattern of restriction enzyme digested finger millet genomic DNA. Lane M: 100 bp marker (NEB), Lane RD: Finger millet genomic DNA digested with multiple restriction enzymes.

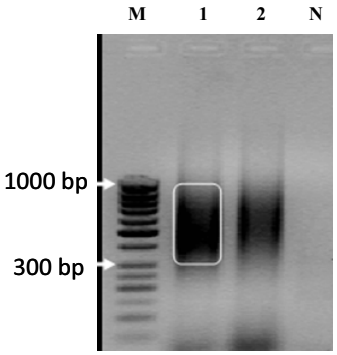

Fig. 3 Amplification pattern of linker ligated DNA. Lane M: 50-bp marker (NEB), Lane 1: Amplification profile when multi enzyme digested linker ligated DNA as template, Lane 2: Amplification profile when *Sau*3A digested linker ligated DNA as template, Lane N: Negative control without linker ligated DNA.

bp. Single restriction enzyme size-selection can effectively result in the discarding of potential microsatellite loci with fragments > 1000 bp and in libraries from a limited samples of a genome (Armour *et al.* 1994). Initially the restriction digested fragments in the range of 300-1000 bp were eluted from gel as the fragments higher than 1000 bp usually do not have microsatellites in them. This size range was chosen to increase the probability of finding a microsatellite repeat toward the center of the sequence thereby increasing the success rate of primer design and also to reduce sequencing costs because sequences for most fragments (300-700 bp) can be obtained in a single reaction. The single enzyme digested and linker ligated DNA fragments usually yielded lesser number of fragments compared to multiple enzyme digested and linker ligated DNA (**Fig. 3**). MBN was added to elute digested genomic DNA to remove single-stranded

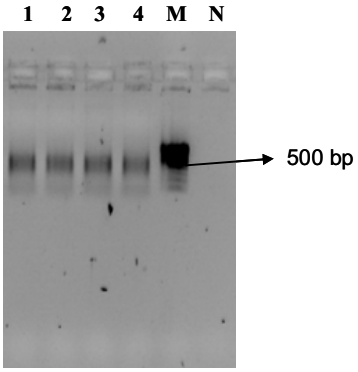

Fig. 4 Amplification pattern of DNA fragments enriched with repeat oligos. Lane M: 100-bp marker, Lanes 1-4: DNA fragments enriched with 4 different repeat oligos [$(AGG)_7$, $(CAC)_7$, $(CAG)_7$, $(ATC)_7$], Lane N: Negative control without DNA.

overhangs and leave all genomic fragments with blunt ends. Survey of the literature on available trinucleotide SSR motifs in monocots and dicots revealed that in addition to the AGA, ACA, CAT, and CTA repeat motifs used by Connell *et al.* (1998), several dicots contain repeat motif such as AAT (T) (Wang *et al.* 1994). We constructed libraries for a total of different SSR repeats using 4 repeats (CAC, CAG, AGG and ATC) (**Fig. 4**). Digestion with multiple restriction enzymes, linker ligation and capture with streptavidin magnetic beads yielded a large proportion of fragments within the 300–800-bp size range after colony PCR (**Fig. 5**) that can be used directly for library construction. Few of the fragments that were > 1000 bp were discarded. Plasmids were isolated and restriction digested to check for inserts (**Fig. 6**). Initially 5 clones from each repeat were sent for sequencing and then Dot blot was done to further confirm the presence of SSR in the inserts (**Fig. 7**). Dot blot was done to further confirm the presence of SSR in the inserts. Dot blot too showed the same results of sequencing yields of initially sent samples i.e. most of the multi-enzymes digested DNA fragments had most SSR inserts in them. So single enzyme digested DNA fragments were not included for further sequencing.

A total of 60 clones with an average length of 450 bp (±100 bp) were sequenced. A search for SSR motifs was performed with SSRIT. We looked for microsatellites composed of tandemly repeated units repeated at least 3 times, more than 200 SSR motifs were detected (SSR motifs ≥3 repeat units or 9 bp) which included multiple repeats in a sequence, 90% of the clones contained SSR motifs. With regard to SSRs having 4 or more repeat units and a minimum length of 12 bp, 50 of them showed repeats, the level of enrichment was 83.3%. Nearly 80% of all the detected motifs were from (CAC)n and (GCA)n repeats. (CAC)6 is the most abundant repeat of all the repeats. Of the clones sent for sequencing from each repeat set 10 out of 15 sequences were (CAC) and (GCA) group of repeats suggesting that these might be the most abundant repeats in finger millet. CAG and TCT were the most abundant trinucleotide repeat motif in foxtail millet (Jia *et al.* 2007). Of the 60 sequences 50 had more than 4 repeats and 27 primers were designed (**Table 1**). Though all most all of the clones showed positive in Dot blot hybridization not all clones had SSRs in them. The difference in number of clones that contained SSRs based on hybridization assays and those identified as suitable for marker development can be explained by a number of factors. Clones that contained multiple SSR motifs and duplicate clones were counted only once. Sequences that consisted of strings of SSRs and SSR-like repeats were considered unsuitable for primer design and were also discounted. Some SSRs might have been present in the un-sequenced clones as all the clones were not sent for sequencing. The percentage of clones sequenced to the number of primer designed based on the flanking sequences varied. In case of previous report on SSR isolation in finger

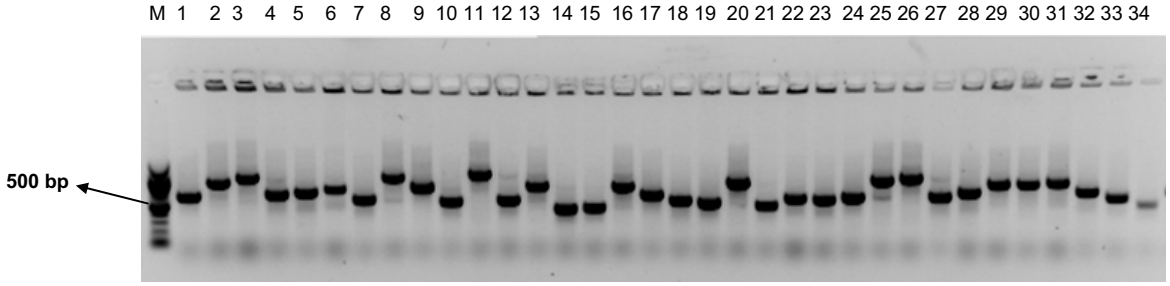

Fig. 5 Amplification profile of colony PCR. Lane M: 100-bp marker, Lanes 1-34: transformed colonies with inserts.

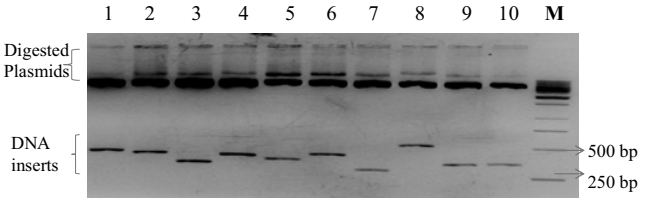

Fig. 6 Restriction digestion of cloned plasmids with *Eco*RI. Lane M: 1-kb marker, Lanes 1-10: Digested plasmids with inserts.

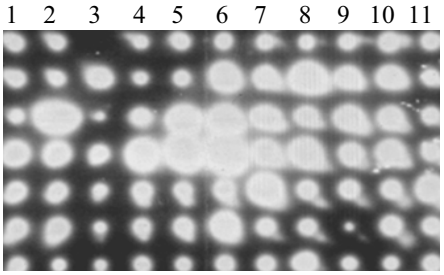

Fig. 7 Dot blot hybridization. Rows 1-11: Plasmids taken for dot blot.

millet by Mathews *et al.* (2007) the percentage of clones having SSRs varied with RE digestion (1.95, 2.57, and 1.80% for *Hin*dIII, *Sal*I, and *Pst*I, respectively). The ratio of non-redundant SSR inserts was 43.7%, 123 primer pairs were designed (Chuan *et al.* 2007) for peanut (*Arachis hypogaea* L.). Gao *et al.* (2003) identified 14 (5.5%) unique SSR-containing sequences in 256 clones in peanut. He *et al.* (2003) sequenced 401 randomly picked clones from AFLP pre-amplification based protocol in which 83 (20.7%) were unique SSRs, and 56 (14.0%) primer pairs were designed in cultivated peanut. Ferguson *et al.* (2004) identified 348 (21.3%) SSRs by sequencing 1,627 clones of which only 226 (13.9%) primers could be designed in peanut. Wang *et al.* (2004) isolated SSR markers from azuki bean (*Vigna angularis*), 1,536 clones tested, 588 (38.3%) showed a positive signal when hybridized with the (AG)12 oligonucleotide probes. Of the 277 positive clones sequenced, 273 (98.6%) had at least one (AG)n motif while the other four had no motif. 240 clones contained unique sequences flanking the motif. Ritschel *et al.* (2004) isolated microsatellite markers from melon (*Cucumis melo* L.). A *Tsp*-AG/TC genomic library was constructed and a total of 700 positive colonies were identified as a result of the hybridization of 1600 clones with (AG/TC)13 probes, most of them containing microsatellite repeat sequences. PCR reactions confirmed microsatellite presence in 450 of the 700 clones and allowed the identification of the size, orientation and position of microsatellite sequences in these clones. 144 primer pairs designed and synthesized. In the case of microsatellite capture from pumpkin (*Cucurbita moschata* L.) by Watcharawongpaiboon *et al.* (2007), 133 colonies were randomly picked from the primary transformation plates and 31 posi-

Table 1 List of primers designed.

Repeat motif	Forward primer	Reverse primer
(GCG)4	CCAAAAACCGAAGAGGCTTT	AAAAGAACCAGGGGGTTCAC
(TGA)4	GACGCTCCTGCAAATTCAGT	GAATCCTCCCAGCTGTTGTC
(GAA)4	GCAGAATCGCTTCAAGTGTG	TCACATGGTGGCTGATGATT
(CTA)4	TTAAGAACCCACCGCAAAAC	TGTGGAATTGTGAGCGGATA
(CCA)4	CATGACTGGATGGACAGCAA	CTTTCGGGCTTTGTTAGCAG
(TGC)4	TGGATGTCGATGCTGTTTGT	AGCAGCAGCAGTCACAGAAA
(AGG)5	AGTGATTAGCAGCAGCAGCA	AGCAGAATCGGAGCTTCATC
(TGC)5	GTGGATGTCGATGCTGTTTG	GAGTGTCACGCAGGGAATCT
(ACC)5	GTATCGGCTGGACACGAAAT	CCCAAGGGGGTTATGCTAGT
(CAC)6	TACTGGTGGACAGCAAATGG	CTTTCGGGCTTTGTTAGCAG
(CAC)6	ATCGAGCTCCAGCATACGAC	CCCCAAGGGGTTATGCTAGT
(CAC)6	TAGCATGACTGGTGGACAGC	CTTTCGGGCTTTGTTAGCAG
(CAC)6	TAGCATGACTGGTGGGACAG	CCCCAAGGGGTTATGCTAGT
(CAC)6	ACATGACTGGTGGACAGCAA	CTTTCGGGCTTTGTTAGCAG
(CAC)6	TACGATGGTGGACAAGCAAA	CCCCAAGGGGTTTAATGTCT
(CAC)6	GCAGGAGCATCAGATGGAAT	CCAATGCTCAGCTTCCTTTC
(CAC)6	ATGATGGTGGACAGCAAATG	AAGAACCCCGGTTGTTAAGG
(CAC)6	TAGCATGACTGGTGGACAGC	CTTTCGGGCTTTGTTAGCAG
(AGC)7	CGGCCGCGAATTCACTA	GTTTTCCCAGTCACGACGTT
(AGC)7	TTTCAACTATGCATGCTCACG	TGTGGATGTCGATGCTGTTT
(AGC)7	GCGAATACACTAGTAGATATAGCAGCA	GCCAACTCGAGCAAGCTTAT
(AGC)7	AAAATTCAGCTATGCATCAACG	GTCTCCTCCTCCTCCTCCTG
(CTG)8	TGTTGCTGCTGCTAATCCTG	GTGCTGCAAGGCGATTAAGT
(TGC)8	TGGATGTCGATGCTGTTTGT	GTTTTCCCAGTCACGACGTT
(CTG)8	CGGTGCTGTTGTTGTTTCTG	AGGGAAGAAAGCGAAAGGAC
(AGC)11	AAATCAGCATGCTCAACG	ATCCCTGATTTGGGTGATGA
(CCAC)6	ACTCCGACACTTCGACGACT	ACTGCAGCTGTGCTCTGTGT

tive clones (23.31%) were sequence identified. Among these microsatellite-containing fragments, 9 (29.03%) primer pairs were designed. We have designed 27 primers from 60 clones sent for sequencing suggesting that this is a very efficient technique in isolation of microsatellite markers.

The ability to digest a large proportion of genomic DNA into the size range that can be sequenced entirely provides several advantages; the amplification product was generally between 300-700 bp. Too short DNA sequences in the latter case may increase the possibility of lack of adequate flanking sequences. Genomic library prepared with this method will yield microsatellite loci representing the majority of the genome and obviates the step of construction of genomic library. The markers developed by this method might aid in the construction of genetic linkage map (where markers are needed from all regions of a genome), population genetic studies (which depend on randomly located genetic markers to infer subdivision and phylogenetic relationships). In conclusion microsatellites have become a must for many genetic studies, but some issues about their isolation are still open. The situation at present is such that a careful evaluation of the experimental strategy has to be carried out case by case and necessary modifications made according to the crop selected for microsatellite isolation.

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