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Screening of SSR marker for sugar and sugar related traits

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ABSTRACT

Saccharum is a complex genome characterized by high polity levels and composed of six distinct species S. officinarum, S. barberi, S. sinensi, S. spontaneum, S. robustum and S. edule. The commercial sugarcane is no longer pure Saccharum officinarum but a species hybrid, complex polyploidy with a large number of chromosomes. Sugarcane cultivars are characterized by a high polyploidy level with more than eight homologous copies of each basic chromosome of Saccharum officinarum and several copies of homologous chromosomes from S. spontaneum. This work is on screening and characterization of microsatellite or simple sequence repeat (SSR) markers which can be used as both a mapping tool, and a method for varietal identification and pedigree control in sugarcane. A molecular marker is an identifiable DNA region whose inheritance can be followed along generations. Microsatellites, also known as simple sequence repeats (SSR) or short tandem repeats (STR), are repeated sequence motifs, whose unit of repetition is between 1 and 6 bp. SSRs marker have broad range of applications, such as genome mapping and characterization, phenotype mapping, marker assisted selection of crop plants and a range of molecular biology and diversity studies. The optimum size for the primers is 18 bases, with a maximum of 24 bases. The optimum melting temperature is 55 °C, with a minimum of 50 °C and a maximum of 70 °C. The optimum GC content is set to 50%, with a minimum of 30% and a maximum of 70%. Screening of these SSR primers are under process for tagging of sugar trait gene among Co86011 (high sugar) and UP9530 (low sugar) parents.

Key words: Sugarcane, SSR, PCR, Microsatellite Markers, gene mapping, AFLP.

INTRODUCTION

Sugarcane is a member of the Andropogoneae tribe of the Gramineae and belongs to the genus Saccharum. Saccharum is associated with four other genera, Erianthus (sect. Ripidium), Miscanthus (sect. Diantra), Sclerostachya and Narenga, to constitute the "Saccharum complex", a closely related interbreeding group suggested to be involved in the origin of sugarcane (1). Increasing importance will be given to the production and utilization of renewable forms of energy, especially biomass. Breeders aim to select superior sugarcane clones with desired traits such as high sugar content, high tonnage, and high juice purity. However, actual yields of sugarcane cultivars are much lower than their yield potential. Sugarcane scientists attribute this to the narrowness of the genetic base of sugarcane cultivars during domestication in which loss of some wonderful characteristics has occurred (2-5). Sugarcane breeders attempt to expand the genetic base of sugarcane cultivars by introducing agriculturally desirable traits from related wild species through introgression or basic breeding (6-8).

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However, the efficiency of introgression in sugarcane has been low due to the low efficiency and technical difficulties in crossing and selection processes. Sugarcane is an open-pollinated crop that, without strict pollen control, is able to self-pollinate, resulting its cross progeny being a mixture of self's and hybrids. Since visual selection for promising hybrids among these progeny populations is unreliable (8-10), there has been an increasing usage of both species- and trait-specific DNA markers in sugarcane breeding (11-15). Microsatellite DNA markers, also known as simple sequence repeats (SSRs), are one of the most powerful genetic marker classes. They are ubiquitous and highly polymorphic, owing to the mutation affecting the number of repeat units. The hyper variability of SSRs among related organisms makes them an informative and excellent choice of markers for a wide range of applications in sugarcane, which include high-density genetic mapping (16), molecular tagging of genes (17), genotype identification (18), genetic analysis of diversity (19,20), paternity determination (21), and phenotype mapping and marker assisted selection (22,23).

Distribution

For optimum growth sugarcane require temperature between 25° C to 30° C and moisture requirement ranges between 77%-88% of field moisture capacity and good illuminization i.e., bright sunshine and water availability. In this contest sugarcane can be grown in areas lies between 0° equator to $23 \frac{1}{2}^{\circ}$ North and south latitude i.e., the countries lies between tropics. In India North Indian sugarcane belt consisting of Punjab, Haryana, U. P., M. P., etc., gives 64% of total cane production and occupies 76% of the total acre under the crop. The south Indian can belt consisting of Andhra Pradesh, Tamil Nadu and Kerala, etc.

SSR (Simple Sequence Repeat) or STR (Short Tandem Repeat)

Simple sequence repeats (SSR), also called microsatellites, are becoming the most important molecular markers in both animals and plants. SSR are stretches of 1 to 6 nucleotide units repeated in tandem and randomly spread in Eukaryotic genomes. SSR are very polymorphic due to the high mutation rate affecting the number of repeat units. Such length-polymorphisms can be easily detected on high resolution gels (e. g. sequencing gels), by running PCR amplified fragments obtained using a unique pair of primers flanking the repeat (24). SSR have several advantages over other molecular markers. For example, (i) microsatellites allow the identification of many alleles at a single locus, (ii) they are evenly distributed all over the genome, (iii) they are co-dominant, (iv) little DNA is required and (v) the analysis can be semi-automated and performed without the need of radioactivity. SSR are characterized in limited number of crop species so for, Sequence information of SSR primer is available in public domain for several commercially important crop plants (25, 26).

Microsatellites consist of randomly repeated units, each between one and 10 base-pairs in length, such as (TG) n or (AAT) n (27). They are widely dispersed through eukaryotic genomes and are often highly polymorphic. These markers are one of the molecular tools of choice for biodiversity studies because of their high information content (28). PCR amplification protocols used for microsatellites employ either unlabelled primer pairs or primer pairs with one of the primers being radio labeled or fluoro labeled. Electrophoresis of unlabelled PCR products can be carried out on smaller vertical poly acrylamide gels or on horizontal agarose gels.

Microsatellites, like mini satellites, represent tandem repeats, but their repeat motifs are shorter (1–6 base pairs). If nucleotide sequences in the flanking regions of the microsatellite are known, specific primers (generally 20–25 bp) can be designed to amplify the microsatellite by PCR. Microsatellites and their flanking sequences can be identified by constructing a small-insert genomic library, screening the library with a synthetically labeled oligo nucleotide repeat and sequencing the positive. Alternatively, microsatellites may be identified by screening sequence databases for microsatellite sequence motifs from which adjacent primers may then be designed. In addition, primers may be used that have already been designed for closely related species. Polymerase slippage during DNA replication, or slipped strand miss-pairing, is considered to be the main cause of variation in the number of repeat units of a microsatellite, resulting in length polymorphisms that can be detected by gel electrophoresis. Other causes have also been reported (29).

Microsatellites are co-dominant markers and the data generated are similar to those of allozymes, except that the number of alleles and hetero zygosity revealed is almost always higher. Population genetic, parentage relatedness analysis can then be carried out. SLATKIN (1995) and GOLDSTEIN *et al.* (1995) took advantage of our knowledge of the predominant mode of microsatellite evolution (i.e. stepwise mutation) to derive the measures of population subdivision and average genetic distance (30, 31). The strengths of microsatellites include the co-dominance of alleles, their high genomic abundance in eukaryotes and their random distribution throughout the genome, with

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preferential association in low-copy regions (32). Because the technique is PCR-based, only low quantities of template DNA (10–100 ng per reaction) are required. Due to the use of long PCR primers, the reproducibility of microsatellites is high and analyses do not require high quality DNA. Although microsatellite analysis is, in principle, a single-locus technique, multiple microsatellites may be multiplexed during PCR or gel electrophoresis if the size ranges of the alleles of different loci do not overlap. This decreases significantly the analytical costs. Furthermore, the screening of microsatellite variation can be automated, if the use of automatic sequencers is an option (33).

One of the main drawbacks of microsatellites is that high development costs are involved if adequate primer sequences for the species of interest are unavailable, making them difficult to apply to unstudied groups. Although microsatellites are in principle codominant markers, mutations in the primer annealing sites may result in the occurrence of null alleles (no amplification of the intended PCR product), which may lead to errors in genotype scoring. The potential presence of null alleles increases with the use of microsatellite primers generated from germplasm unrelated to the species used to generate the microsatellite primers (poor "cross-species amplification"). Null alleles may result in a biased estimate of the allelic and genotypic frequencies and an underestimation of heterozygosity. Furthermore, the underlying mutation model of microsatellites (infinite allele model or stepwise mutation model) is still under debate. Homoplasy may occur at microsatellite loci due to different forward and backward mutations, which may cause underestimation of genetic divergence. A very common observation in microsatellite analysis is the appearance of stutter bands that are artifacts in the technique that occur by DNA slippage during PCR amplification. These can complicate the interpretation of the band profiles because size determination of the fragments is more difficult and hetero-zygotes may be confused with homo-zygotes. However, the interpretation may be clarified by including appropriate reference genotypes of known band sizes in the experiment. In general, microsatellites show a high level of polymorphism. As a consequence, they are very informative markers that can be used for many population genetics studies, ranging from the individual level (e.g. clone and strain identification) to that of closely related species. Conversely, their high mutation rate makes them unsuitable for studies involving higher taxonomic levels. Microsatellites are also considered ideal markers in gene mapping studies .Genetic segregation in Saccharum officinarum and S. spontaneum by microsatellite markers has been studied (34).

Materials and Methods: Plant Materials:

Different varieties of *Saccharum officinarum* available in UPCSR research farm were used in this study. The germplasm accessions are listed below:

A: Saccharum species	Genotype	
Saccharum officinarum L.	Azul de Caza, Paunda	
Saccharum barberi, Jeswiet	Pansahi, Pathri	
Saccharum sinense, Roxb	Saretha, Nargoni	
Saccharum spontaneum L.	Pusa-7, WS-18	

B: Commercial Cultivars:

1: <u>Early Maturing groups:</u> CoS95255, CoS96268, CoS8436, CoJ64, CoS97261, CoSe98231, CoS96268, CoS8436, Cos99259.

2: <u>Mid Maturing groups:</u> UP9530, Co997, Co453, CoS97264, CoS8432, Co86011, Co 331, Co7717, CoS96275, CoSe92423, CoC671, Co975, Co312, CoS96259, CoS97264.

Isolation of Plant DNA from fresh tissue: Grind 0.5 gm of leaf material in liquid nitrogen to fine powder using pre-chilled mortar & pestle. Transfer the powder to a 15 Ml polypropylene centrifuge tube containing 5.0 ml of pre warmed extraction buffer use spatula to disperse the material completely. Incubate samples at 60°C for 1 hour with occasional mixing by gentle swirling. Add 3 ml Chloroform, isoamyl alcohol and Mix by inversion to emulsify. Spin at 15,000 rpm for 10 min. at room temp. Remove the aqueous phase with a wide bore pipette, transfer to a clean tube and add 2/3 volume of iso-propanol and mix by quick gentle inversion. Spool DNA using a bent Pasteur pipette and transfer to another tube. Alternatively, if the DNA appears flocculent, centrifuge at 5000 rpm for 2 min and gently pour off the supernatant. Wash the DNA pellet in 70% ethanol (5-10 ml) for 20 min. dry the pellet and dissolute in 100- 500 μ l tris buffer (35).

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Purification of DNA: Add 5 μ l of RNase (stock 10 mg/ ml.) DNA sample and incubate 37°C in water bath for 1 hour. Add equal volume of phenol: Chloroform: Isoamyl alcohol (25:24:1) and mix. Spin at 15000 rpm for 15 min. at room temp. Take out the aqueous phase and transfer to a fresh micro tube. Extract twice with equal volume of Phenol: Chloroform: isoamylalcohol (25:24:1), Centrifuge and take out the aqueous phase. Add 0.1 Volume of 3M sodium acetate (pH 4.8) and mix properly. Add 2.5 times absolute alcohol mix by quick gentle diversion to precipitate the DNA. Pellet the DNA by Centrifugation at 15,000 rpm for 15min. in a microfuge (36). Decant Supernatant carefully, wash the pellet with 70% ethanol & air dry and dissolve DNA in 50-100 μ l tris Buffer. (0.1N HCl).

Quantification of DNA (Gel Analysis): Cast agarose gel 0.8% in 0.5 X TBE (Tris Borate EDTA) Buffer. Load 2-5µl of DNA sample. Load a known amount of uncut phage DNA as control in the adjacent well. Run the gel at 60 V for 1 hour. Stain the gel with ethidium bromide Solution (0.5µg/ml) for 10 minutes wash with distilled, water and visualize under UV light. Judge the DNA quality. Presence of a single compact band at corresponding position to phage. Estimate the quantity of DNA in the sample is its comparison with the control either by eye Judgment or by densitometry measurements (37).

Procedure: The protocol available and developed in the laboratory at UPCSR was employed for PCR amplification (38). PCR amplification is carried in 10 μ l volume using 0.2 ml thin walled PCR tubes. The reaction was setup as follows.

- Reaction mixture was prepared for 45 samples excluding DNA template.
- The reaction was distributed in 45 tubes.
- 2 µl of each DNA sample was added to respective tubes.
- The PCR tubes containing reaction were placed in thermal cycler.

Table1: Details about PCR reaction

Reagents	Stock Concentration	Final Concentration	Volume/reaction (10µl)
Genomic DNA	200 ng	25 ng	1.0 µl
Taq Buffer	10 X	1X	1 µl
dNTPs	10 Mm	2.5 Mm	0.2 µl
Forward primer	-	5 Pico mole	1.0 µl
Reverse primer	-	5 Pico mole	1.0 µl
Taq DNA Polymerase	3U/ µl	0.21U	0.073 µl
DDW	-	To make up final volume	5.73 μl

RESULTS AND DISCUSSION

Plant material consists of young leaves of high and low sugar verities eg. Co86011 (high sugar), CoH 70(low sugar) & their progenies eg. Co86011x CoH 70. DNA was extracted from immature leaves and then purified by RNAse enzyme after that we checked the yield of DNA on 0.8 % agarose gel through agarose gel electrophoresis. The yield of DNA was observed in the concentration of 200, 150, 100, 50 ng. the high yielding DNA was diluted by dilution formula (N1V1= N2V2) 10 mM tris HCl solution and again checked the final concentration of 0.8 % agarose gel. Thus, 25ng DNA was ready for PCR amplification with SSR primer. Polymerization of PCR product was detected on polyacrylamide gel electrophoresis (PAGE) and visualized in alpha innotech gel documentation system. Few gel photographs are given here with the size of amplified fragment (bp).



Figure 1. Diluted DNA of saccharum parent and their progenies



Microsatellite markers are important for sugarcane research because they are PCR markers and are easy to perform; also they are the product of specific primers and are more stable than those generated by random primers such as random amplified Polymorphic DNA (RAPD) markers. Another advantage is that, unlike Amplified Length Polymorphism (AFLP) markers, they are locus-specific and are transferable across genotypes within the species, which is an important factor for mapping purposes. Another aspect of the complex sugarcane genome that can be addressed using the information obtained from repetitive DNA is polyploid genome evolution. This occurs in allopolyploid cotton, where repetitive DNA accounts for about half of the genome size differences between the 2

diploid progenitors. Continued application of molecular markers, such as EST-SSRs, to sugarcane genome analysis holds great promise for producing lasting insight into processes by which novel genotypes are generated.

Computational Study of SSR: A molecular marker is an identifiable DNA region whose inheritance can be followed along generations. Molecular markers have become a powerful tool in many areas of biology. Microsatellites, also known as simple sequence repeats (SSR) or short tandem repeats (STR), are repeated sequence motifs, whose unit of repetition is between 1 and 6 bp. SSR markers are the marker of choice for most genetic and breeding applications due to their widespread distribution, high levels of polymorphism, high reproducibility and co dominant mode of inheritance in S. Officinarum. They are highly abundant in the genomes of eukaryotes, polymorphic and usually co-dominant and transferable between different mapping populations. Microsatellite markers can also be used in automated genotyping techniques. SSRs or microsatellites have been shown to be one of the most powerful genetic markers in biology. Defined as runs of tandem repeated DNA, they exhibit a high degree of polymorphism due to the mutation affecting the number of repeat units. This hyper variability among related organisms makes them excellent markers for genotype identification, analysis of genetic diversity, phenotype mapping and marker assisted selection of crop plants. SSRs marker have broad range of applications, such as genome mapping and characterization, phenotype mapping, marker assisted selection of crop plants and a range of molecular biology and diversity studies. The optimum size for the primers is 18 bases, with a maximum of 24 bases. The optimum melting temperature is 55 °C, with a minimum of 50 °C and a maximum of 70 °C. The optimum GC content is set to 50%, with a minimum of 30% and a maximum of 70%. Screening of these SSR primers are under process for tagging of sugar trait gene among Co86011 (high sugar) and UP9530 (low sugar) parents.

Computational Study of Length and GC content for all available SSR primers: It is generally accepted that the optimal length of PCR primers is 18-22 bp. This length is long enough for adequate specificity, and short enough for primers to bind easily to the template at the annealing temperature. All the sequences of forward and reverse SSR primers were putted properly in the readable form for MACHINE. Computational study of their length and GC content distribution has been performed by my won written programs in MATLAB. GC-content (or guanine-cytosine content), in molecular biology, is the percentage of nitrogenous bases on a DNA molecule which are either guanine or cytosine. The GC content (the number of G's and C's in the primer as a percentage of the total bases) of primer should be 40-60%. Result of analysis of distribution of length and GC content for all 614 primers are as following

- > The optimum size for the primers is 18 bases, with a maximum of 24 bases, with an average length of 20.7.
- > The optimum melting temperature is 55°C, with a minimum of 50°C and a maximum of 65°C.
- > The optimum GC content is set to 50%, with a minimum of 30% and a maximum of 70%.



Figure5 : Plot of the histogram of length of SSR primers



Figure6: Plot of Length for f-sequence SSR primers

CONCLUSION

This project work relates Saccharum spp. microsatellites and their flanking sequences, there polymorphic survey. These polymorphic primers can be used for fingerprinting purpose and that may be used for applications such as: identification of Saccharum complex accessions, management of germplasm collections, selection of progenitors, distinction between varieties, identification of hybrids, introgression of desirable genomic sequences, paternity determination, mapping of genes and quantitative trait loci (QTLs) and development of MAS (marker-assisted selection) procedures. Clear polymorphic band were observed for sugar and sugar related traits in the Bulk DNA of high sugar and low sugar varieties. SSRs markers derived from grape ESTs showed that the 3 untranslated regions was the most polymorphic. The potential utility of SSRs as a molecular profiling technology to aid in sugarcane research and product development remains to be evaluated, although the design of PCR primer pairs for the generation of sugarcane microsatellite markers reported in this work can be used to assess the usefulness of sugarcane EST-SSR markers. High sugar and low sugar commercial hybrids were chosen for primer screening because they are the parents of a population currently being used for linkage mapping and quantitative trait loci (QTL) tagging, it is planned that the EST-SSR primers showing polymorphism will be mapped in this population. Most of the primers studied amplified larger fragments than expected, reflecting the possible presence of introns within the genomic DNA sequence, and suggests that the presence of long introns between the sequences homologous to the primers in the genomic DNA may explain the lack of amplification in some of the primers that did not amplify.

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REFERENCES

[1] Li W., Wolfe KH, and Sharp PM. Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proceedings of the National Academy of Sciences USA*, **1987**, **84**:9054–9058. [2] Arcenaux G. *Proc Int Soc Sugarcane Technol*, **1967**, 12: 844–854.

[3] D'Hont A, Rao PS, Feldmann P, Grivet L, Islam-Faridi N, Taylor P, Glaszmann JC. *Theor Appl Genet*, **1995**, 91: 320-326.

[4] Roach BT, Proc Aust Soc Sugar Cane Technol, 1989, 11: 34-47.

[5] Tew TL., World sugarcane variety census-Year 2000. Sugar Cane Intl March/April, 2003, 12-18.

[6] Burner DM, Legendre BL, Sugar Cane, **1993**, 3: 5-10.

[7] Legendre BL, Breaux RD, The USDA basic sugarcane breeding program in Louisiana. Proc Inter-Amer Sugar Cane Sem: Varieties and Breeding III, **1983**, 96-98.

[8] Tai PYP. Progress and problems of intergeneric hybridization in sugarcane breeding. *Proc Inter-Amer Sugar Cane Sem I*, **1989**, 391-395.

[9] Divinagracia NS, Proc Intl Sugar Cane Technol, 1980, 17: 1287-1295.

[10] Heinz DJ, Tew TL, Hybridization procedures. In: Sugarcane Improvement through Breeding. D.J. Heinz (eds.), Elsevier, Amsterdam, **1987**, pp 313-342.

[11] Maccheroni W, Jordao H, De Gaspari R, De Moura GL, Matsuoka S, Sugar Cane Intl., 2009, 27(2):47-52.

[12] Oliveira KM, Pinto LR, Marconi TG, Mollinari M, Ulian EC, Chabregas SM, Falco MC, Burnquist W, Garcia AAF, Souza AP, *Genome*, **2009**, 52:191-209.

[13] Pan YB. Sugar Cane Intl, 2001, 14-17.

[14] Pan YB, Sugar Tech, 2006, 8(4): 246-256.

[15] Selvi A, Nair NV, Noyer JL, Singh NK, Balasundaram N, Bansal KC, Koundal KR, Mohapatra T, *Genet Res Crop Evol*, **2006**, 53: 831-842.

[16] Chen C, Yu Q, Hou S, Li YJ, Eustice M, Skelton RL, Veatch O, Herdes RE, Diebold L, Saw J, Feng Y, Qian W, Bynum L, Wang L, Moore PH, Paull RE, Alam M, Ming R, *Genetics*, **2007**, 177:2481–2491.

[17] Singh RK, Singh P, Misra P, Singh SP, Singh SB, Sugar Tech, 2005, 7(2&3):74-76.

[18] Pan YB, Scheffler BS, Richard JEP., Sugar Tech. 2007, 9:176-181.

[19] Cordeiro GM, Pan YB, Henry RJ., Plant Sci, 2003, 165: 181-189.

[20] Brown J.S., Schnell RJ, Power EJ, Douglas SL, Kuhn DN., Genet Resour Crop Evol., 2007, 54:627-648.

[21] Tew TL, Pan YB., Microsatellite-based paternity analysis of a seven-parent sugarcane polycross [abstract]. In: 8th International Society of Sugar Cane Technologists Breeding and Germplasm Workshop Abstract Book, May 1-5, **2006**, Guayaquil-Ecuador. p. 5.

[22] Powell W, Machray GC, Provan J., Trends Plant Sci, 1996, 1: 215-222.

[23] Awadalla P, Ritland K., Mol Biol Evol, 1997, 14: 1023-1034.

[24] Weber JL and May PE, Am J Hum Genet, 1989, Mar;44(3):388-96

[25] Gupta M, Chyi Y-S, Romero-Severson J and Owen JL. Theoretical and Applied Genetics, 1994, 89:998–1006.

[26] Powell, L. D., Paneerselvam, K., Vij, R., Diaz, S., Manzi, A., Buist, N., Freeze, H., Varki, A. Carbohydratedeficient glycoprotein syndrome: not an N-linked oligosaccharide processing defect, but an abnormality in lipidlinked oligosaccharide biosynthesis? J. Clin. Invest. 94: **1901-1909**, **1994**. [PubMed: 7962535, related citations] [Full Text: Journal of Clinical Investigation].

[27] Bruford M.W., Wayne R.K., Curr. Opin. Genet. Develop., 1993, 3: 939–943.

[28] Morin P.A. Woodruff D.S., Noninvasive genotyping for vertebrate conservation. In: Wayne R.K., Smith T.B. (eds): *Molecular Genetic Approaches in Conservation*, Oxford University Press, **1996**, New York: 298–313.

[29] Matsuoka Y, Mitchell SE, Kresovich S, Goodman M and Doebley J. *Theoretical and Applied Genetics*, **2002**, **104:**436–450.

[30] Slatkin M., Genetics, 1995, 139: 463–471.

[31] Goldstein D.B., Linares A.R., Cavalli-Sforza L.L., Feldamn M.W., Genetics, 1995, 139: 463-471.

[32] Morgante M, Hanafey H and Powell W. Nature Genetics, 2002, 30:194–200.

[33] Ghislain M, Spooner DM, Rodríguez F, Villamon F, Núñez C, Vásquez C and Bonierbale M. *Theoretical and Applied Genetics*, **2004**, **108**:881–890.

[34] Edme SJ, Miller JD, Glaz B, Tai PY-P, Comstock JC. Crop Science 2005;45:92-97.

[35] Edward K, Johnstone C, Thomson C., Nucleic Acids Res., 1991, 19: 1349.

[36] Reddy OUK, Pepper AE, Abdurakhmonov I, Saha S, Jennin JN. J. Cotton Sci., 2001, 5: 103-113

[37] Hoisington D, Laboratory Protocol. CIMMYT applied molecular genetics laboratory. CIMMYT. Maxico, D.F., **1992**, CIMMYT.

[38] Parida SK, Rajkumar KA, Dalal V, Singh NK, Mohapatra T, Unigene *Theor Application Genet*, **2006**, 112:808-817.