Construction of enriched genomic library for development of microsatellite markers in *Luffa cylindrica*

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Geneconserve 14 (55): 30-41

ABSTRACT:
Microsatellites or simple sequence repeats (SSRs) consist of tandem repeats of di- to penta-nucleotide motifs with conserved flanking sequences. These conserved sequences have been used for designing suitable primers for amplification of the SSR loci using PCR and reflects polymorphism in the form of differences in the length of amplified product. Microsatellite markers were developed in *Luffa cylindrica* using the biotin-streptavidin enrichment approach. Plasmid DNA was isolated from 100 white colonies and out of which 50 colonies were selected for sequencing. The sequenced fragments were checked for vector contamination using VecScreen software and most of them were found contaminated. Only 11 were without contamination but no microsatellite repeats were found within these sequences, when analyzed with WebSat software. This paper reports the first attempt of isolation of microsatellite markers in *Luffa* through enrichment method.

**Key words:** *Luffa cylindrica*, microsatellite markers, biotin-streptavidin, enrichment.

**INTRODUCTION**

*L. cylindrica* is a cross-pollinated (2n = 2x = 26) (Joshi et al. 2010) monoecious crop. It is an annual herbaceous vine with tendrils and its fruit are edible and used as a vegetable when young (Okusanya et al. 1981). It is cultivated in India during the summer season (Oboh and Alyor 2009). Its young fruits are edible and on maturity, its fiber used for cleaning purposes, as a bath sponge, a component of shock absorbers, as a sound proof lining, as a utensils cleaning sponge, as packing materials, for making crafts, as filters in factories and as a part of soles of shoes (Bal et al. 2004).

There is a lack of information on morphology, reproductive biology, agronomy and genetic structure of sponge gourd. Thus, urgent efforts are required to improve yield and production of
Luffa using conventional and biotechnological approaches. Lack of such information could be considered as a major obstacle to develop and popularize this nutritionally as well as industrially important crop in the country.

SSRs because of their simplicity, effectiveness, abundance, hypervariability, reproducibility, codominant inheritance, extensive genomic coverage (Powell et al. 1996) have wide applications in plant research including genome mapping, cultivar identification, marker-assisted selection, genetic diversity studies, phylogenetic relationships and population studies (Wang et al. 2009).

Thus, in this study we had made an attempt to construct SSR markers through genomic library method enriched for L. cylindrica.

MATERIAL AND METHODS

Genomic DNA extraction

High quality genomic DNA (>100ng/μl) was extracted from fresh leaves of accession DSG-6 which is resistant to tomato leaf curl New Delhi virus (Saha et al. 2013) using CTAB method (Saghai-Maroof et al. 1984). The genomic library was constructed according to Bloor et al. (2001) protocol.

Digestion and adapter ligation

The extracted genomic DNA was digested with partially digested with 40 Unit (U) of two different sets of restriction enzymes (RE); one sticky end producer (Sau3AI) and other blunt end producers (HaeIII, RsaI, AluI) (New England BioLabs, USA) for 3 hrs at 37°C. The digestion was checked on 2% agarose gel containing 0.5 μg/ml ethidium bromide (Sigma). Afterwards, 25 pmol/μl adaptor (MWC-Biotech), 1 U T4 DNA ligase 1X buffer and 10 mM rATP were added and incubated at 4°C overnight.
**Elution and amplification of ligated DNA**
The adaptor-ligated DNA was run alongside a 100 bp ladder in a 1.8% NuSieve GTG agarose (FMC Bioproducts) gel and fragments between 400-1000 bp were quickly excised which served as a suitable insert size for the pGEM-T Easy vector system (Promega Corp., USA) shown. The eluted bands were purified by the MinElute gel extraction kit (Qiagen Inc. USA).

To test the success of ligation of adaptors to the digested DNA, PCR was performed. 25 µl reaction mixture contained 5 ng of DNA, 1.25 µl of 200 pmol/µl oligonucleotide (FMC Bioproducts) corresponding to the sequence of the adaptor, 1X Taq buffer, 10 mM dNTP, 1.5 mM MgCl₂, and 1 U of Taq polymerase. The PCR profile was as follows: initial denaturation at 95°C for 5 min; 95°C for 50 sec, 56°C for 1 min, 72°C for 2 min and final extension at 72°C for 10 min of 30 cycles. The successful ligation showed smear between 400-1000 bp when 5 µl of sample was run on 2% agarose gel.

**Capturing of repetitive sequences with biotinylated oligonucleotide**
3'-biotinylated oligonucleotide probe (MWC-Biotech) of sequence (AAC)₈ was used to capture DNA fragments containing repetitive sequences. The streptavidin coated Dynal bead M280 (Dynal, Norway) (10 mg/ml) was added to capture biotinylated oligonucleotide probes (100 µM) which bind the SSR containing DNA fragments. The bound complex was kept in the magnetic separation stands (Promaga inc. USA), where DNA fragments were washed several times. PCR was performed to increase the quantity of enriched DNA in 50 µl reaction volume as follows: 40 µg of beads solution, 200 pmol/µl oligonucleotide probe as a primer, 1X Taq buffer, 10 mM dNTP, 1.5 mM MgCl₂, and 1 U Taq polymerase. The PCR profile was 94°C for 30 sec, 56°C for 1 min, and 72°C for 1 min for 35 cycles. The PCR product was run on 2% agarose gel at 100 V for 15-20 min and a smear was obtained between 400-1000 bp indicate successful capture. Finally, the PCR amplified products were used for the genomic DNA library construction.

**Genomic DNA Library construction**
The enriched DNA fragments were then ligated to the pGEM-T Easy vector system (2X
ligation buffer, 50 ng pGEM-T Easy vector, 1U T₄ ligase, 1µl rATP and 150 ng PCR product) and incubated at 4°C overnight (Promega, USA) and transformed into the competent *E. coli* DH5α cells by electroporation. The transformants were selected on LB agar plates containing 100 μg/ml ampicillin with 40 μg/ml X-Gal and 0.1 mM IPTG. The resulting DNA library was selected through blue white colony screening. Single white colonies were randomly picked for plasmid isolation and restricted so that vector and desired DNA fragments appeared as 2 bands of different sizes on 2% agarose gel. Clones containing an insert of more than 500 bp were selected and sent for custom sequencing (Sanger sequencing). After sequencing, sequences were checked for vector contamination using VecScreen software of NCBI. Microsatellite repeats were searched by analyzing with WebSat software (Martins et al. 2009).

**RESULTS AND DISCUSSION**

SSR markers are preferred over other markers due to their simplicity, effectiveness, abundance, codominant nature, ease of assay, widely distributed throughout the genome, and reproducibility (Powell et al. 1996). These are regarded as good markers for effective characterization of germplasm, phylogenetic studies and population structure analysis (Slatkin et al. 1995). The enrichment of DNA fragments is a simple, efficient cost effective and most popular approach (Billotte et al. 2001) for SSR isolation and has been successfully applied to a number of plant genomes (Hirata et al. 2006; Lendvay et al. 2013). Therefore, we have employed this approach for isolation of SSRs from *Luffa*.

For this, first total genomic DNA was isolated by CTAB method which provides high quality DNA suitable for restriction. It was restricted with both blunt end and staggered end producer enzymes (fig. 1), adaptor-ligated and fragments were eluted in the size range of 400-1000 bp (fig. 2). The eluted fragments were purified, ligated to p-GEMT Easy vector, transformed and plated for blue-white colonies selection (fig. 3). Around 100 colonies were picked and plasmid
DNA was isolated, restricted for confirmation of insert presence (fig. 4 & 5) and a total of 50 colonies were selected for sequencing. The sequenced fragments were checked for vector contamination using VecScreen software and most of them were found contaminated. Only 11 were without contamination but no microsatellite repeats were found within these sequences, when analyzed with WebSat software. Previously, only one effort was made for microsatellite development in *Luffa* that was based on the cross species transferability method in which total 52 primers of cucumber and melon were used (Jun et al. 2010). The development of enriched library has been used in a number of plants including *Phalaris canariensis* L. (Li et al. 2011), *Lupinus luteus* (Gonzalez et al. 2011), and *Paeonia lactiflora* (Sun et al. 2011).

This study build a framework for further studies as very few microsatellite markers are available for *Luffa*. Therefore, it is necessary to develop this potential marker system for its exploitation in the characterization of the *Luffa* genome and crop improvement.

**ACKNOWLEDGMENTS**

We are grateful to the Head, Division of Genomic Resources and Director, National Bureau of Plant Genetic Resources for permitting access to the facilities.

**REFERENCES**


Figure 1. Restriction digestion product of accession DSG-6 of *L. cylindrica* on 2% agarose gel; lane 1-*Sau*3AI digested, lane 2-*Alu*I digested, lane 3-*Rsa*I digested, lane 4-*Hae*III digested and lane M-50 bp ladder.

Figure 2. PCR products of adaptor-ligated DNA on 2% agarose gel lane M-ladder 100bp, lane1 and 2-PCR products of *Sau*3AI, lane 3 and 4-PCR products of *Alu*I, lane 5 and 6-PCR products of *Rsa*I, lane 7 and 8-PCR products of *Hae*III and lane M-100 bp ladder.
Figure 3. Blue-white colonies for selection of recombinants.

Figure 4. Representative figure showing plasmids isolated from different recombinants colonies (lane 1-12) and lane M-1Kb ladder.
Figure 5. Restriction digestion of isolated plasmids from different clones (lane 1-18), and lane M-1 Kb ladder.