

Chapter 2

Screening of Genomic Libraries

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Abstract

Microsatellites, or simple sequence repeats (SSRs), have proven to be an important molecular marker in plant genetics and breeding research. The main strategies to obtain these markers can be through genomic DNA and from expressed sequence tags (ESTs) from mRNA/cDNA libraries. Genetic studies using microsatellite markers have increased rapidly because they can be highly polymorphic, codominant markers and they show heterozygous conserved sequences. Here, we describe a methodology to obtain microsatellite using the enrichment library of DNA genomic sequences. This method is highly efficient to development microsatellite markers especially in plants that do not have available ESTs or genome databases. This methodology has been used to enrich SSR marker libraries in *Citrus* spp., an important tool to genotype germplasm, to select zygotic hybrids, and to saturate genetic maps in breeding programs.

Key words Microsatellites, Molecular markers, Enrichment methods

1 Introduction

Microsatellites, or simple sequence repeats (SSR), are arrays of hypervariable short (1–5 bp) repeat motifs that can be found in both coding and noncoding DNA sequence of organisms. These single-locus markers are mainly characterized by high frequency, Mendelian inheritance, and co-dominance. Microsatellites have proven to be important molecular markers in plant genetics and breeding, because of their variability, detection based on DNA amplification, accessibility of detection, and reproducibility (1). Microsatellites are polymerase chain reaction (PCR) based, requiring previous sequence identification, primer designing for the conserved flanking regions, and amplification of the target repeat (2). The availability of microsatellite markers has been limited in a great number of species. Construction of genomic libraries for microsatellite markers' development has been an effective way to obtain polymorphic markers very useful to characterize germplasm

collections or as molecular tool for genetic mapping in breeding programs. Genomic libraries allow the screening of an entire genome (or a collection of genomes) by digesting genomic DNA (gDNA), cloning into vectors, and transforming bacterial cells that can be screened for a desired phenotype, i.e., clones containing DNA fragments with repeat motifs (3).

Despite their great utility for a broad range of plant species, especially in economically important crops, the number of polymorphic markers obtained using this strategy has been limited, requiring an intensive labor to generate an appropriate set of useful markers.

In the last 20 years, the number of methods and strategies to development genomic libraries for microsatellite isolation has evolved considerably, from traditional library screening and development of enriched libraries (reviewed by 1) to mining genomic and EST databases (4), and high-throughput identification from next-generation sequencing data (5). These strategies were able to identify and obtain a great number of markers rapidly and cost-effective, including high-quality genetic markers in non-model and understudied plant species (6).

2 Materials and Methods

Here we describe the main steps for the development and selection of microsatellite from genomic sequences from citrus DNA using the procedure of library enrichment. The first step is to obtain a DNA with high quality and purity, followed by digestion with restriction enzymes. *Sau3AI* has been chosen for citrus SSR library and the restriction fragments were ligated by corresponding adapters and amplified. The biotinylated SSR probes were used to hybridize the denatured pre-amplified fragments. The hybridized mixture was added to streptavidin-coated paramagnetic beads. The DNA-probe hybrids were incubated at room temperature, and a magnetic field was applied to precipitate the beads, which were attached by fragments containing SSR that hybridized to biotinylated probes. The SSR-enriched fragments were amplified by polymerase chain reaction (PCR); products were cloned into the pGEM®-T Easy Vector Systems, transformed into competent *Escherichia coli*, and plated onto Luria-Bertani medium (LB medium) with antibiotic selection. Single colonies were selected and they were grown overnight in LB. Plasmids were purified and the insert sequenced. All the steps for construction of genomic libraries of citrus are detailed below (Fig. 1).

2.1 DNA Extraction

Adapted from Murray and Thompson (7): Grind the sample (1 g) with liquid nitrogen to a powder. Transfer the sample to tube, add 20 mL CTAB–Sarkosyl Buffer (1 M Tris–HCl pH 7.5, 0.5 M EDTA, 5 M NaCl, 5 % CTAB, 10 % Sarkosyl, 140 mM

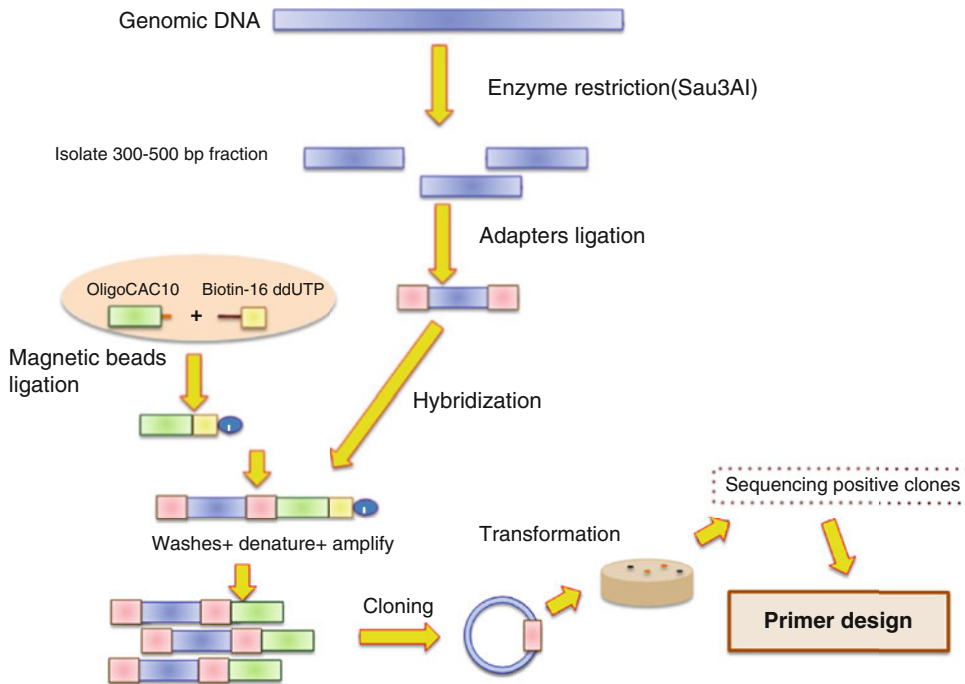


Fig. 1 Schematic representation for microsatellites' isolation from enriched genomic libraries

β -mercaptoethanol), homogenize and incubate at 60 °C for 10 min. Add 1 volume chloroform:isoamyl alcohol (24:1), mix by gentle inversion, and centrifuge at $1,900 \times g$ for 8 min. Carefully remove the aqueous (top) layer to a fresh tube. Add 0.1 volume of a solution of 10 % CTAB, 5 M NaCl and mix carefully. Extract with an equal volume of chloroform:isoamyl alcohol (24:1), mix by gentle inversion, and centrifuge at $1,900 \times g$ for 8 min. Transfer 15 mL of the aqueous (top) layer to a fresh tube, add 1 volume of precipitation buffer CTAB (1 % CTAB, 1 M Tris-HCl pH 7.5, 0.5 M EDTA), mix gently, and incubate at room temperature for 30 min. Centrifuge at $9,600 \times g$ for 5 min. Discard the supernatant and dissolve the pellet in 4 mL of TE high salt (1 M Tris-HCl pH 7.5, 0.5 M EDTA pH 8.0, 5 M NaCl), and incubate at 65 °C for 10 min to total dissolution. Precipitate DNA by adding 2 volumes of cold (-20 °C) absolute ethanol and mix by gentle inversion and centrifuge at $3,500 \times g$ for 6 min. Discard the supernatant and wash the pellet with 7 mL of cold (-20 °C) 70 % ethanol and centrifuge at $3,500 \times g$ for 6 min. Remove the supernatant, add 4 mL of cold (-20 °C) absolute ethanol, and centrifuge at $3,500 \times g$ for 6 min. Carefully remove the supernatant and incubate at room temperature for 20 min or until DNA is completely dry. Dissolve the pellet in 100 μ L of TE 1/10 plus RNase and incubate at 37 °C for 2 h, and after estimate the DNA concentration.

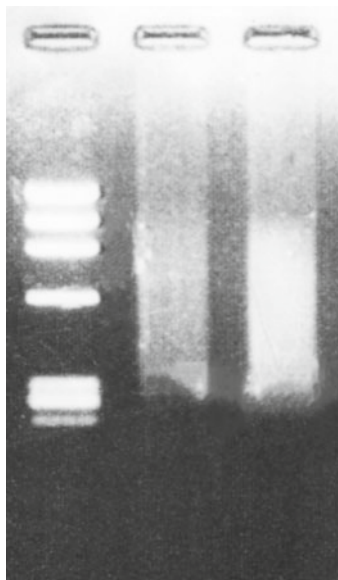


Fig. 2 Electrophoresis of fragment DNA obtained from digestion by Sau3AI

2.2 Genomic DNA Digestion

Digest the total genomic DNA (50 μg) using the blunt end-generating restriction endonuclease Sau3AI (250 U), Sau3AI buffer 10 \times (20 μL) and add water to a volume of 200 μL . Incubate at 37 °C overnight. Check digestion quality using the digested DNA (10 μL) and ΦX174 (50 ng/ μL) as molecular weight standard, by electrophoresis through a 1.5 % agarose gel in 1 \times TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA).

2.3 Gel-Fractionate to Isolate DNA Fragments

Perform electrophoresis through a 0.8 % low melting point agarose in 1 \times TAE buffer, using 190 μL of DNA digestion. Excise the 234–872 bp fraction from the gel and transfer to 1.5 mL microcentrifuge tube (Fig. 2). Add 3 volumes of TE buffer, and incubate at 65 °C for 5 min until complete agarose dissolution. Add an equal volume of TE-saturated phenol to the DNA sample, mix by vortex, and centrifuge. Remove about 90 % of the upper aqueous layer to a clean tube, carefully avoiding proteins at the aqueous:phenol interface. Extract a second time with an equal volume of 1:1 TE-saturated phenol:chloroform:isoamyl alcohol, centrifuge at 3,500 $\times g$ for 5 min, and repeat the extraction using just chloroform. Transfer the supernatant and precipitate in 1:10 acetate sodium (3 M) and 3 volumes of absolute ethanol; incubate at –20 °C overnight. Carefully mix and centrifuge at 3,500 $\times g$ for 30 min. Remove the supernatant, add 500 μL of 70 % ethanol, and centrifuge at 3,500 $\times g$ for 20 min. Discard the supernatant, dry the precipitate for 5 min, and suspend in 50 μL water. Estimate the DNA concentration.

2.4 Ligation of Adapters

After confirming digestion on agarose gel electrophoresis and excise the 234–872 bp fraction from the gel, the *Sau*3AI adapters are ligated to the genomic fragments. Fragments of genomic DNA (10 μ g) were ligated to adapters (200 μ M) using T4 DNA ligase (400 U/ μ L) at 16 °C overnight. The adapters oligo sequences used were shorter adapter (5'CAG CCT AGA GCC GAA TTC ACC3') and longer adapter (5'GAT CG GTG AAT TCG GCT CTA GGC TG3').

2.5 Biotin-Labeled Oligonucleotide

Mix 100 pmol/ μ L of oligoprobe (for example, CAC₁₀), 5 \times terminal transferase buffer, Biotin-16 ddUTP (2 μ L), terminal transferase (30 U), and water to 40 μ L final volume. Incubate at 37 °C for 30 min and then add 0.5 M EDTA (4 μ L) to enzyme inactivation. Precipitate with 2.5 \times volume of 100 % ETOH (\pm 110 μ L) incubating at –20 °C overnight. Centrifuge at 4 °C for 30 min at 13,800 $\times g$, washing twice with 100 μ L 70 % ETOH, centrifuge at 4 °C for 10 min at 13,800 $\times g$, drying under vacuum and suspend in 30 μ L of water. Incubate at refrigerator.

2.6 Preparation of Magnetic Beads

Use 1 mg Beads (Beads Streptavidin—Dynal S/A) (100 μ g/ μ L) for each hybridization. Take out 100 μ L and wash twice in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM NaH₂PO₄, 2 mM KH₂PO₄, HCl to pH 7.4) plus 1 % BSA (400 μ L). Place tube in magnet stand for 1–2 min to allow beads to migrate to the side of the tube. Remove supernatant by aspiration with a pipette. Remove tube from magnet stand. Wash once in 400 μ L 1 \times BEW buffer (1 M Tris–HCl pH 7.5, 100 mM EDTA pH 7.6, 5 M NaCl). Repeat the magnetic separation. Suspend gently in 2 \times BEW (200 μ L), add 170 μ L of H₂O and 30 μ L of the biotin-labeled oligonucleotide. Shake at room temperature for 60 min. Wash twice in 1 \times BEW (400 μ L) and wash once in 5 \times SSPE (20 \times SSPE=0.2 M NaH₂PO₄, 3.0 M NaCl, 0.02 M EDTA, NaOH to pH 7.4) plus 400 μ L 0.1 % SDS. Suspend in 10 \times SSPE+0.2 % SDS (150 μ L) pre-warmed at 65 °C. Save at 65 °C until hybridization.

2.7 Hybridization

To allow the biotinylated probe to hybridize to the target DNA, denature DNA plus adaptors heating at 95 °C for 10 min in 150 μ L (before this, make the 1:1,000 dilution in water and save to carry out the PCR control). Transfer the tube to ice. Then, add the DNA+adaptors to the beads incubating at 65 °C for 1 h and 30 min and shake in each 10 min. After that, recover the hybridization solution in a new tube. To capture the fragments hybridized to the probe, it was used the affinity of the biotin in the probe for the streptavidin-coated magnetic beads. Then proceed washes: (a) twice in 2 \times SSPE+0.1 % SDS (400 μ L) for 5 min at room temperature, (b) once in 2 \times SSPE+0.1 % SDS (400 μ L) for 15 min at 65 °C, and (c) rinse the beads in 2 \times SSPE solution (400 μ L). Suspend the beads in 200 μ L water. Recover solutions after each wash.

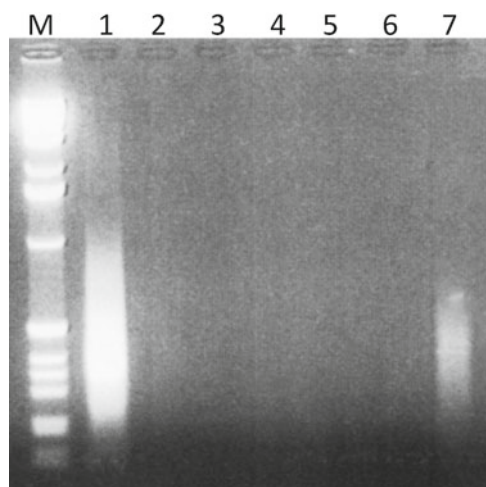


Fig. 3 Electrophoresis of products from enrichment procedure. M = ladder 1 kb, 1 = DNA + adaptor (1:1,000), 2 = hybridization solutions, 3–6 = wash solutions, 7 = DNA adsorbed to beads

2.8 PCR Control for Enrichment Procedure and Chemiluminescent Probe Detection

Mix 10× PCR buffer (2.5 μ L), 2 mM dNTPs, 0.01 M adaptor primer, 1.5 U Taq DNA polymerase, and 3 μ L of sample* and complete at 25 μ L final volume [*samples of DNA-adaptor (1:1,000), hybridization solutions, wash solutions (first to fourth), and DNA adsorbed to beads]. PCR conditions of 95 °C for 3 min (hot start), 94 °C for 2 min, before 25 cycles of 94 °C for 45 s, 56 °C for 45 s, and 72 °C for 2 min followed by 7 min at 72 °C for final extension.

2.9 Hybridization

PCR products (25 μ L) are electrophoresed in 2 % agarose using TAE buffer, stained with ethidium bromide (Fig. 3). After electrophoresis, treat gel with denature solution (NaOH 0.5 M + NaCl 1.5 M) with constant agitation for 30 min. Rinse in deionized water, add neutralization solution (NaCl 0.5 M + 0.5 M Tris-HCl pH 8.0), and shake for 30 min. Transfer the DNA by capillary using Hybond-N⁺ membrane and allow the transfer for 8–12 h. Expose the membrane to a source of UV irradiation (254 nm) and neutralize by washing, twice for 5 min each, in 2× SSPE and pre-hybridize at 65 °C for 3 h. Then, discard the pre-hybridization solution, add the probe (3 μ g/ μ L) previously denatured in 2× SSPE (95 °C for 20 min), and incubate overnight. Remove unbound biotinylated probe by washing 2× for 5 min each in 2× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate) + 0.1 % SDS followed by 15 min in 0.1 % SSC + 0.1 % SDS. After rinse, detect the site of biotinylated probe by chemiluminescence using a digoxigenin-labeled nucleic acids and CSPD substrate.

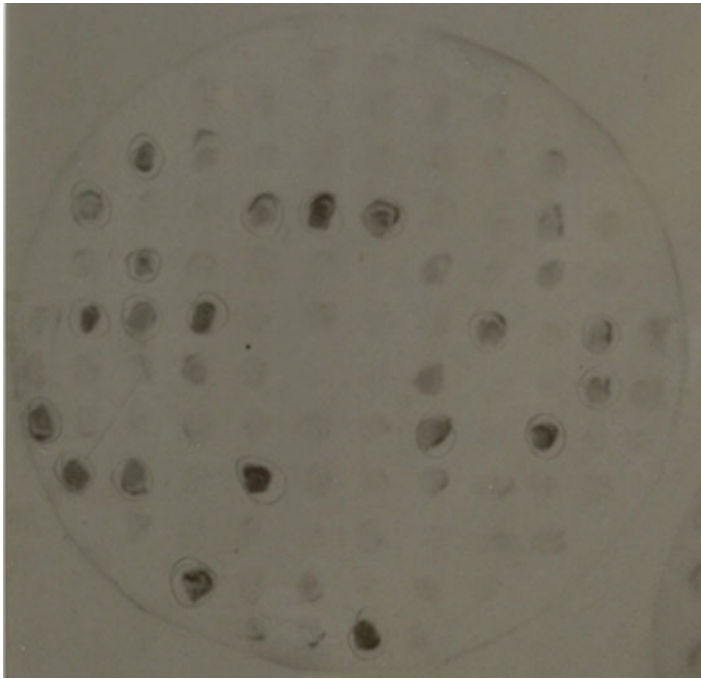


Fig. 4 Positive clones to microsatellite sequences (GT) after enrichment procedure

2.10 Cloning of PCR Fragments

The DNA recovered from the enriched library is PCR-amplified in a 25 μ L reaction with ten replicates. Purify the PCR products are purified using a Gel and PCR Clean-Up System. Then, cloning the fragments into a vector using 10 \times T4 Buffer (1 μ L), vector (1 μ L), T4 DNA ligase (1 U); complete the volume with water to 10 μ L, and incubate overnight at 4 $^{\circ}$ C. Transform recombinants into competent Dh 5 α or JM109 *E. coli* cells. Transfer the resulting colonies to Hybond-N⁺ membrane. Expose the membrane to a source of UV irradiation (254 nm) and neutralize by washing 2 \times 5 min each in 2 \times SSPE and pre-hybridize at 65 $^{\circ}$ C for 3 h. Then, discard the pre-hybridization solution, add the biotinylated probe (3 μ g/ μ L) previously denatured in 2 \times SSPE (95 $^{\circ}$ C for 20 min), and incubate overnight. Remove unbound biotinylated probe by washing 2 \times for 5 min each in 2 \times SSC (1 \times SSC = 150 mM NaCl, 15 mM sodium citrate) + 0.1 % SDS followed by 15 min in 0.1 % SSC + 0.1 % SDS. After rinse, detect the site of the biotinylated probe by chemiluminescence using a digoxigenin-labeled nucleic acids and CSPD substrate (Fig. 4).

In the next and last step, plasmid DNA is isolated from the selected positive clones and they are sequenced using standard protocol and primers flanking the repeated sequences are designed.

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