Short Technical Reports

Microsatellite Enrichment in Organisms with Large Genomes (*Allium cepa* L.)

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ABSTRACT

To exploit the polymorphism of repeat numbers in short tandem repeat (STR) sequences (microsatellites) as molecular markers, STRs must be isolated and PCR primers must be developed in flanking sequences. In species with large genomes such as Allium cepa *L. (onion and shallot), an efficient selection procedure for genomic fragments containing STRs is a crucial step. Here we describe a nonradioactive method for microsatellite isolation based on affinity capture of single-stranded restriction fragments annealed to biotinylated microsatellite oligonucleotides* $(CA)_{10}$, $(GAA)_8$ *and* $(AAC)_8$ *followed by adapter-mediated genomic PCR. Cloning of the products in* E. coli *and plasmid sequencing revealed more than 60% positive clones. Primers were designed in STR-flanking regions, and one or two bands were amplified in 13 diploid onion and five shallot accessions. Allelism of the bands was confirmed by product sequencing.*

INTRODUCTION

Short tandem repeats (STR), also referred to as microsatellites, are abundant sequences dispersed throughout most eukaryotic nuclear genomes. In plants, they have been shown to be polymorphic at an intraspecific level in several cases (10,12,15). The isolation of microsatellites by standard techniques (screening plasmid or phage libraries by colony hybridization with radioactively labeled oligonucleotide probes) turned out to be a cumbersome and non-cost-effective procedure. Polymerase chain reaction (PCR)-based enrichment of previously cloned inserts (7,9), novel PCR-based techniques (e.g., Reference 4) and techniques based on hybridization selection of restriction fragments before cloning (2,6) have proven to be more powerful tools in microsatellite marker development in both plants and animals. The first approach to obtain microsatellite sequences in onion by hybridizing immobilized DNA from random-amplified polymorphic DNA (RAPD) gels to microsatellite oligonucleotides (3) and sequencing of positive bands failed. Microsatellite enrichment using microsatellite oligonucleotides cross-linked to nylon filter pieces as a probe, according to Reference 2, was not successful in onion.

Here we describe the nonradioactive establishment of a genomic *Rsa*I library highly enriched for three micro-

satellite motifs—$(CA)_n$, $(GAA)_n$ and $(CAA)_n$—known to exist in the onion genome (13).

MATERIALS AND METHODS

Chromatin was extracted (14) from leaves of vegetatively propagated plants of a common onion ('Kaba', accession ALL0917; Genebank Gatersleben, Germany). DNA was isolated from this chromatin fraction using a standard cetyltrimethylammonium bromide (CTAB) procedure and subsequently purified from residual polysaccharides and RNA (11). Six micrograms of genomic DNA were digested by blunt-end-generating restriction endonuclease *Rsa*I (6 U/µg DNA). Thereafter, the

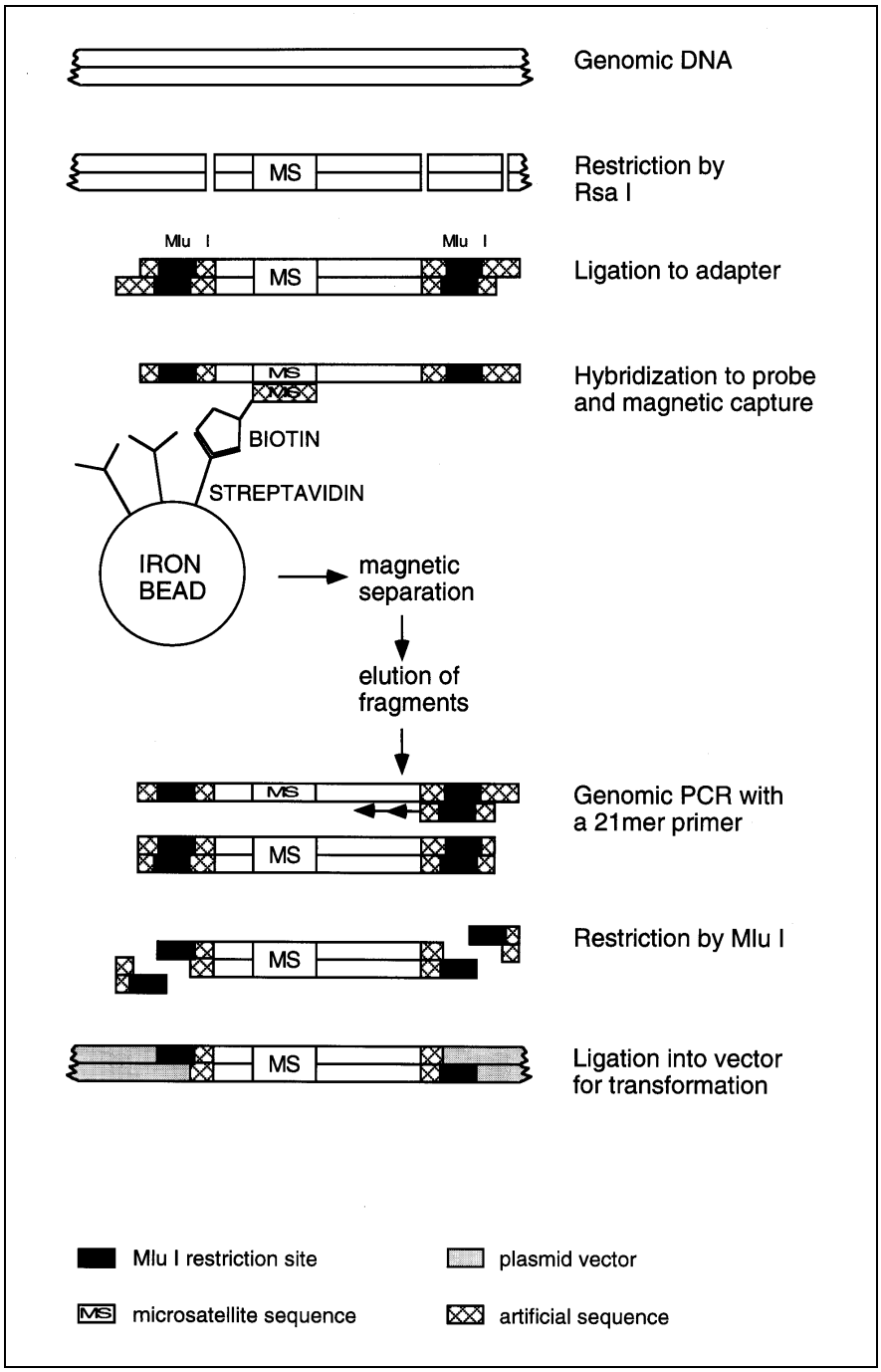

Figure 1. Schematic representation of the microsatellite enrichment method described in the text.

hybrid oligonucleotides from a 5′-phosphorylated 25-mer and a 21-mer with overlapping complementary sequence containing an *Mlu*I site were ligated to the blunt termini of the restriction fragments using 50 ng adapter-to-primer hybrid per 1 µg of genomic DNA (2).

Restriction by *Mlu*I and subsequent ligation were performed in one tube at 37°C using 0.5× universal potassium glutamate buffer (8). After 2 h, 1 U of T4 DNA Ligase (Amersham Pharmacia Biotech, Little Chalfont, Bucks, England, UK) per µg DNA was added, followed by an additional 2-h incubation at 37°C to allow recutting of restriction fragment multimers generated during ligation. The constructs were then heat-denatured and allowed to hybridize to biotinylated microsatellite oligonucleotides (150 nM solution of each oligonucleotide, 6× standard saline citrate [SSC]) of sequence types listed above (6,13). These hybrids were subsequently bound to streptavidin-coated magnetic beads (Dynabeads®; Dynal GmbH, Hamburg, Germany) (350 µg beads in 100 µL 6× SSC final vol). Beads were then washed twice at low-stringency conditions (25°C in 2× SSC, 0.1% sodium dodecyl sulfate [SDS], 5 min each), four times at high-stringency conditions (twice in 1× SSC at 25°C, 5 min each, and twice in 1× SSC at melting temperature [T_m] -5°C for 2 and 5 min, respectively) and eluted as single-stranded fragments in triple-distilled water.

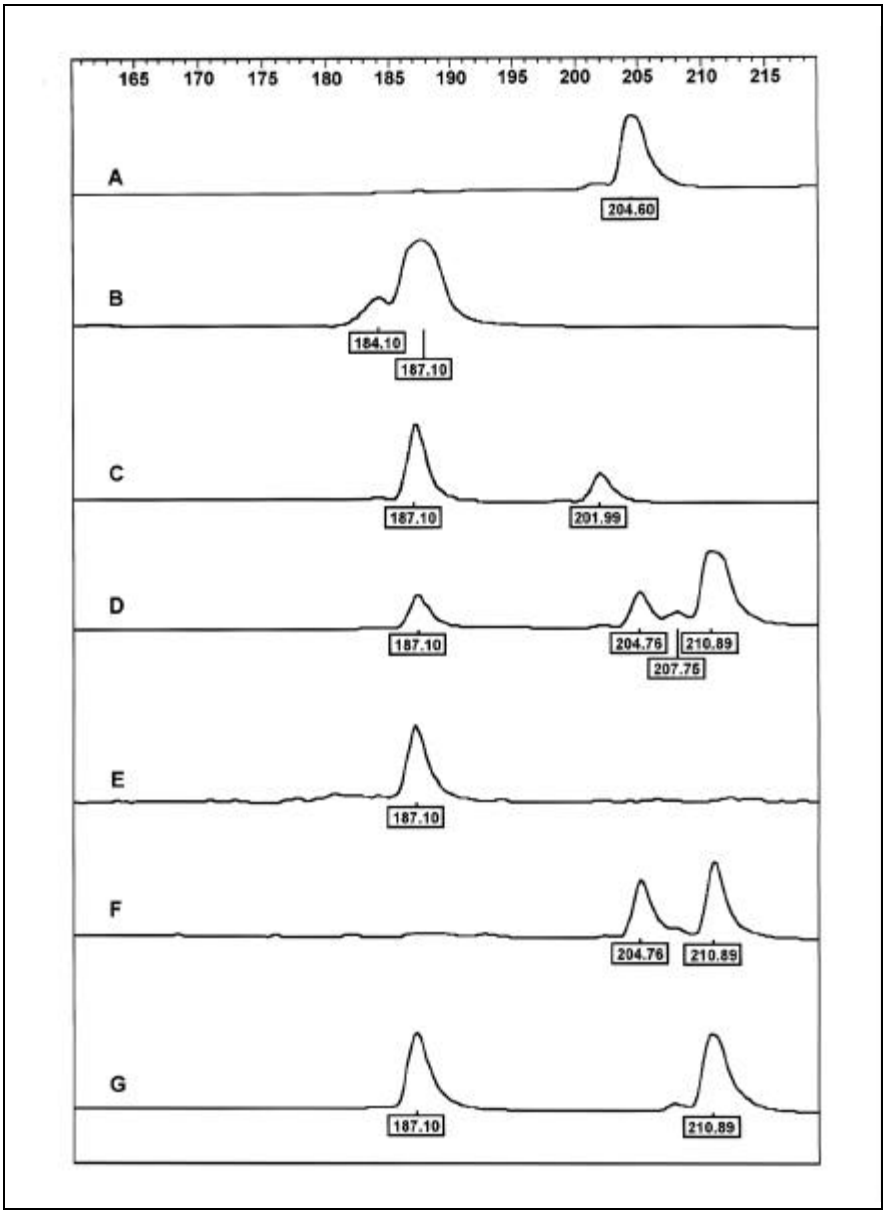

Figure 2. Seven fluorograms showing electrophoretic analysis of PCR fragments obtained with one primer fluorescently labeled at microsatellite locus AMS08 in onions (*Allium cepa* L.). (A–E) Common onions. (F and G) Shallots. (A) Accession 'KaBa', ALL0917 from which microsatellite loci have been isolated. Fragment sizes given in bp.

The obtained DNA solution (0.2–1.0 µL) served as template for PCR (28–30 cycles with 56°C annealing temperature) using the 21-mer oligonucleotide as primer. The amplification products were *Mlu*I-digested to obtain vector-compatible, sticky-ended fragments. Restriction fragments selected in this way were ligated in the presence of *Mlu*I into a plasmid vector (pCR-Script™ Amp$^+$; Stratagene, La Jolla, CA, USA), which had been linearized with *Bss*HII and dephosphorylated, thus creating a site that cannot be recut by *Mlu*I (Figure 1). This was necessary to prevent religation of the inserts. T4 DNA Ligase and *Mlu*I (both from Amersham Pharmacia Biotech) performed together very well in 0.5× potassium glutamate buffer (8).

The ligated vector fragments were transformed into chemically competent *E. coli* XL1-Blue Strain Cells (Stratagene) and plated onto LB agar containing 2.67×10^{-4} M isopropyl β-D-thiogalactopyranoside (IPTG), 3.18×10^{-4} M 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 30 mg/L carbenicillin. Plasmid DNA was isolated from white colonies using the QIAGEN® Plasmid Mini Kit (Qiagen GmbH, Hilden, Germany). Colony PCR was performed under reported PCR conditions (16) using cells from single colonies adhering to a 200-µL pipet tip washed into 30 µL of master mixture.

Most of the recombinant clones contained inserts of suitable length (from about 400 up to 1000 bp) and were sequenced on a Model 377 Fluorescent Sequencer (PE Applied Biosystems, Foster City, CA, USA) without further screening. Primer pairs were designed in the microsatellite flanking regions using the DesignerPCR™ computer program (Research Genetics, Huntsville, AL, USA), and PCR was per-

formed with these primers, one of each being 5′-fluorescein-labeled, using genomic DNA from 14 onions and five shallots as templates. PCR products were checked on 3% agarose gels (45 mM Tris-borate, 1 mM EDTA) and then analyzed on a Model 377 Fluorescent Sequencer (15-cm electrophoresis glass plates) following the protocol provided by the manufacturer. Resulting data were processed with GENESCAN™ and GENOTYPER™ Fragment Analysis Software (PE Applied Biosystems).

RESULTS

Twenty-nine of forty-eight readable sequences contained microsatellites, in most cases corresponding to the oligonucleotides used as probes. No duplicate sequences were observed. This indicates a high degree of microsatellite enrichment. So far, primer pairs in the flanking regions of 16 microsatellite loci (AMS01–AMS16) have been developed, six of which amplify one or two bands from the diploid genomes in 18 of the 19 onion genotypes. Using fluorescent fragment analysis, six alleles (showing sizes of 184, 187, 202, 205, 208 and 211 bp) could be distinguished at locus AMS08 in the 19 genotypes mentioned above (examples shown in Figure 2).

Sequencing some of the AMS08 amplification products showed that the length differences were due to various numbers of a trinucleotide repeat. These primer pairs therefore reveal polymorphic single-locus genotypes.

DISCUSSION

We have obtained polymorphic, codominant, single-locus markers with our approach. Homology between putative alleles was verified by spot-check sequencing. As an additional advantage, some of the expense and time-consuming procedures of conventional microsatellite marker development could be avoided (e.g., radioisotopes, large amounts of probe oligonucleotides and hybridization screening). The onion genome consists of 17.4×10^9 bp (1). Studies on large genomes giving spurious hybridization signals with microsatellites raise the necessity of new methods in marker development (5, 6,9). The method of Kandpal et al. (5) requires PCR before enrichment selection, which initially reduces the population of fragments, whereas the method of Ostrander et al. (9) starts with bacterial cloning, which hampers selection by elimination or at least shortening of microsatellite loci during bacterial growth. Enrichment methods based on immobilization and fixation of genomic fragments using oligonucleotide probes (2) turned out to be difficult to optimize. Calculated hybridization temperatures often failed, probably because there is no control of the apparent probe length actually available for hybridization. For instance, a cross-linked $(CT)_{12}$ probe may contribute between 0 and 10 core motifs to hybridization, which means a theoretical hybridization temperature interval between <15° and 77°C. This makes a stringent selection much more difficult than the selection by annealing in liquid medium and subsequent retention by the high-affinity mechanism of streptavidin-biotin bonding. Independence of microsatellite selection and capture of the complex improves control over selection parameters without major influence on the yield. Previously published methods achieved a maximum enrichment rate of 50%, whereas our approach achieves a slightly higher efficiency (>60%) with a simpler procedure.

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