

# Benchmarks

## Microsatellite Libraries Enriched for Several Microsatellite Sequences in Plants

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Simple sequence repeats (SSRs), or microsatellites, have been isolated and characterized from numerous animal and plant species (6,7). These sequences have been shown to be highly polymorphic between individuals within populations or closely related genotypes. This, together with the fact that they can be converted into a simple polymerase chain reaction (PCR)-based assay, has led to their widespread use as molecular markers of genetic diversity (6). The use of microsatellites has been limited in plants, however, by the costs involved in isolating large numbers from the target species. Recently, a number of papers have reported the isolation of microsatellite clones by an enrichment procedure (1,2,4,5). These reports have all resulted in the isolation of a single type of microsatellite. However, as little is known about the level of polymorphism of individual microsatellite sequences, it is possible that this approach will result in markers that do not detect the level of polymorphism often required in diversity studies. Furthermore, the targeting of a single microsatellite species may produce only a small number of clones necessitating the construction of further libraries in the future. To eliminate these problems, we have significantly modified the enrichment technique (2,5) to yield clones that contain a variety of microsatellites. These clones can then be characterized by either direct sequencing or colony hybridization using specific microsatellite oligonucleotides. Interestingly, the technique also yields a high percentage of clones containing more than one type of microsatellite. Results to date suggest that the clones produced by this procedure are sufficiently polymorphic to be used in population genetics and/or breeding studies.

**Preparation of reagents for microsatellite enrichment.** The following oligonucleotides can be used to en-

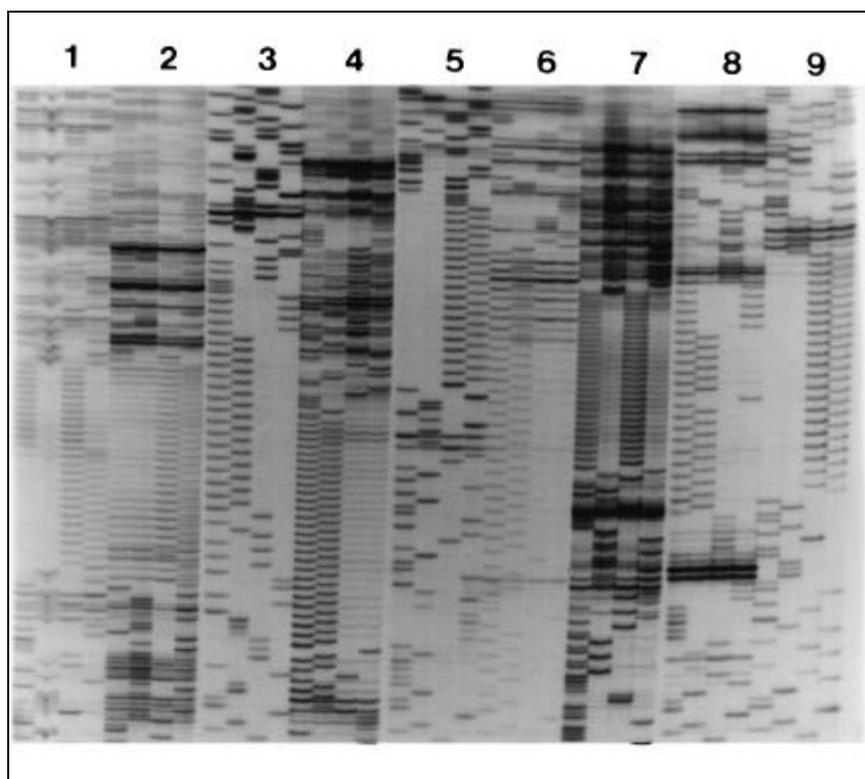
Table 1. Frequency of Different Microsatellite Sequences in the Multiplex Libraries

Type of microsatellite	Frequency of microsatellite as a % of the total number of clones containing a microsatellite	Average repeat length
GT	30	18
GA	20	20
CGG	10	10
CCA	5	8
CATA	5	30
Compound	30	25

rich for DNA fragments containing microsatellites: [GA]<sub>15</sub>, [GT]<sub>15</sub>, [AT]<sub>15</sub>, [GC]<sub>15</sub>, [CAA]<sub>10</sub>, [CATA]<sub>10</sub>, [ATT]<sub>10</sub>, [GATA]<sub>10</sub>, [GCC]<sub>10</sub> and [ATAG]<sub>10</sub>. Fifty nanograms of each oligonucleotide in 3× SSC (45 mM sodium citrate, pH 7.0 and 450 mM NaCl) are pooled in a total volume of 80 μL, spotted onto a 0.5-cm<sup>2</sup> piece of Hybond<sup>®</sup> N<sup>+</sup> (Amersham, Arlington Heights, IL, USA) and air-dried for 1 h. The dry membrane is UV-treated for 30 s using a 260-nm transilluminator. Weakly

bound oligonucleotides are washed off the membrane by washing twice in 10 mL of hybridization buffer (50% formamide, 3× standard saline citrate [SSC], 25 mM Na-phosphate, pH 7.0, and 0.5% sodium dodecyl sulfate [SDS]) at 45°C for 2 days. The membranes are then stored at -20°C until required.

**Preparation of genomic DNA.** One microgram of genomic DNA is digested with 2 units of *RsaI* in a volume of 20 μL for 1 h at 37°C. Fifty nanograms



**Figure 1. Sequence analysis of nine microsatellites clones.** Nine clones were chosen at random from a number of plant DNA libraries enriched for microsatellites and sequenced using the universal forward primer. Autoradiography was performed on the dried sequencing gel for 18 h at room temperature. Samples were loaded onto the gel in the order ACGT.

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of an *MluI* adaptor (consisting of a 21-mer: 5'CTCTTGCTTACGCGTGGA-CTA3' and a phosphorylated 25-mer: 5'pTAGTCCACGCGTAAGCAAGA-GCACAA3') together with 2  $\mu$ L 10 mM ATP are added to the digestion mixture along with 1 unit of  $T_4$  DNA ligase. Ligation is then allowed to proceed for 2 h at 37°C. Finally, the ligated DNA is denatured by boiling for 5 min.

**Enrichment of microsatellites.** Enrichment for microsatellites is carried out using 100 ng of the ligated, denatured DNA in 500  $\mu$ L of hybridization buffer containing 2  $\mu$ g of the 21-mer oligonucleotide (5'CTCTTGCTTACG-CGTGGACTA3') using a single Hybond N<sup>+</sup> filter with bound oligonucleotides for 24 h at 37°C. Following hybridization, the filter is washed 20 $\times$  (5 min per wash) in 0.5 $\times$  SSC at 65°C. Bound DNA is then eluted in 200  $\mu$ L sterile distilled water by boiling for 5 min. One microliter of the eluted DNA is amplified by PCR using the 21-mer adaptor primer in 25  $\mu$ L of 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M each dATP, dCTP, dGTP and TTP, 1 $\times$  reaction buffer and 1 unit *Taq* DNA Polymerase (Life Technologies, Gaithersburg MD, USA). Amplification is allowed to proceed for 20 cycles (94°C for 40 s, 60°C for 60 s and 72°C for 120 s) in a Stratagene Robocycler™ (Stratagene, La Jolla, CA, USA), after which the enrichment step and amplification are repeated once more using all of the material generated from the previous step.

**Characterization of enriched microsatellites.** Approximately 2.5 ng of enriched DNA were taken directly from the final amplification, digested with 1 unit of *MluI* and ligated into 10 ng of a modified pUC19 vector (pJV1) containing a *Bss*HI site (K.J. Edwards, unpublished results). Inclusion of *MluI* in the ligation ensures that each plasmid contains only a single insert. Plasmids are transformed into DH10B™ (Life Technologies) and plated onto L-agar plates containing 100  $\mu$ g/mL ampicillin. Following incubation overnight at 37°C, colonies are transferred into microplates for long-term storage. Plasmids from individual colonies are prepared using the Promega Wizard™ kit (Madison, WI, USA) and sequenced using Sequenase® Version 2 (United States Biochemical, Cleveland, OH, USA).

This protocol was developed as part of an EC-funded collaborative project (3) in which the facile construction of microsatellite-enriched libraries from a variety of plant species was desired. To date, the procedure has been used successfully to prepare enriched libraries for DNA from maize, willow, rhododendron, barley, sunflower, sugarbeet, wheat and diploid wheat. The procedure is quick and relatively inexpensive in that it does not require the biotinylation of the oligonucleotides used to select the microsatellites. It also results in the production of large numbers of clones. These clones can be organized as an ordered array at high density using a Biomek® workstation (Beckman Instruments, Fullerton, CA, USA) and probed with the individual microsatellite oligonucleotides. This procedure would allow the operator to identify all the clones containing a particular type of microsatellite for further characterization. Sequencing of at least 10 clones chosen at random from each of the libraries (140 clones in total) has confirmed that they are highly enriched for microsatellite sequences with between 50% and 70% of the clones containing at least one microsatellite (Figure 1). Sequencing has also shown that the insert size ranges from 250 bp to 900 bp. To date, sequence comparison of the enriched clones from the various libraries has shown that there is no evidence for a selective enrichment of specific sequences. Detailed characterization of 15 clones containing microsatellites including the synthesis of flanking primers and amplification from a selection of genotypes has shown that in every case a microsatellite containing amplification product of the expected size is produced. Furthermore, 13 of the 15 primer pairs used produced polymorphic bands in several of the genotypes tested (K.J. Edwards, unpublished results). Interestingly, a significant proportion of the clones contain two or more different microsatellite sequences. We have identified clones containing all of the oligonucleotides bound to the Hybond N<sup>+</sup> (Table 1) with the exception of the ATAG oligonucleotide. This last observation suggests that many more different microsatellite sequences probably exist within the genome of most plant

and animal species. Taken together, the results suggest that the clones produced by this procedure are both representative of the original genomic sequences and sufficiently polymorphic to be used in population genetics and/or breeding studies.

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