INCREASING THE EFFICIENCY OF MICROSATELLITE DISCOVERY FROM ENRICHED LIBRARIES IN CONIFEROUS FOREST SPECIES

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INTRODUCTION

Microsatellites or SSR (simple sequence repeats) are usually single locus, highly polymorphic and robust markers. Microsatellites are increasingly the marker of choice for genome analysis, mapping and diversity studies in plants (Gupta et al. 1996, Westman and Kresovicch 1997) and forest trees (for example Bradshaw et al. 1994, Brondani et al. 1998, Devey et al. 1996). The isolation and development of microsatellite markers, however, is often costly and problematic, especially in organisms with large genomes (Fisher and Bachmann 1998), such as conifers. For example, from our own experience, enriched microsatellite libraries for seven angiosperms *(Eucalyptus pilularus, E. globulus, E. grandis, E. cloeziana, Grevillia robusta, Corymbia variegata, Toona ciliata)* and three gymnosperm *(Araucaria cunninghamii, Pinus elliottii, P. caribaea),* using the method of Edwards et al. 1996, yielded enrichment of up to 50% in the angiosperm species but below 10% for all the gymnosperm species. Here we outline a library screening method which recovered high levels of microsatellite containing clones from libraries of coniferous species.

Keywords: Microsatellite, enrichment, *Pinus, Araucaria*

METHODS

The technique utilises the DIG detection system (Roche, Basel, Switzerland) and is based on their protocol (van Miltenburg et al. 1995). Oligo probes used to enrich the libraries ($(CA)_{20}$, $(CAT)_{14}$, $(AGC)_{14}$, $(GAC)_{14}$, $(AGA)_{14}$, $(ACA)_{14}$, $(CTT)_{14}$, $(CTA)_{14}$, $(CAG)_{10}$, $(CT)_{15}$, $(CAA)_{14}$, $(CTG)_{10}$, $(ACT)_{14}$) were 3'-end labelled with Digoxigenin-11-ddUTP (van Miltenburg et al. 1995). The libraries were transformed into DH5ct cells and colonies containing inserts identified by blue white screening. These colonies were grided out (5 mm grid, 200 colonies per 82 mm plate) on LB agar with 50 µg/mL ampicillin and grown at 37°C overnight. Nylon membranes were carefully overlaid on the cooled plates for 30 sec and the orientation of the membrane was marked before it was gently removed. The plates were incubated at 37°C for several hours to allow the colonies to recover.

The membranes were processed to lyse the DNA and fix the nucleic acid as described in van Miltenburg et al.(1995). Membranes were incubated in hybridisation oven for 60 min at 65°C in pre-hybridisation buffer (5x SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% blocking reagent (Roche)). The membranes were then incubated overnight at 65°C in pre-hybridisation buffer containing 20ng/mL of each labelled oligo probe, then washed twice at low stringency (2x SSC, 0.1% SDS at 60°C) for 5 min and twice at higher stringency (0.5x SSC, 0.1% SDS, 60°C) for 5 min. Positive colonies were identified by simple colorimetric detection using NBT and BCIP (van Miltenburg et al. 1995) then used to spike a PCR containing 10 mM Tris-HCI (pH8.3), l.5 mM $MgCl_2$, 50 mM KCI, 200 µM of each dNTP, 400 nM of puc+ (GTTTTCCCAGTCACGACG) and puc- (CAGGAAACAGCTATGACC) primers (Pacific Oligos, Lismore, NSW, Australia) and 0.05 U/µL *Taq* DNA polymerase (Roche). PCR was completed on a 9700 thermal cycler (PE, Foster City, CA) with 35 cycles of 92°C denaturation for 10 sec, 55°C

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annealing for 30 sec and 75°C extension for 60 sec, followed by a 5 min extension. PCR products were purified by precipitation with sodium chloride and ethanol and sequenced with ABI big dye terminator chemistry (PE, Norwalk, CT) by the Australian Genome Research Facility (Brisbane, Queensland, Australia).

RESULTS AND DISCUSSION

A strategy of recovering microsatellites from the poorly enriched libraries using DIG non-radioactive screening proved extremely efficient (Table 1). This approach required minimal specialised equipment and effectively identified colonies containing microsatellite sequences within three days. The recovery of useful microsatellite sequences from *A. cunninghamii* and *P. elliottii* increased from 1.5% to 98%, and from 3% to 100%, respectively. Redundancy levels varied between the two libraries, however, it appeared overall high compared with experience with angiosperms. In the *A. cunninghamii* library, only 16 sequences were unique out of 49 and in the *P. elliottii* library, 10% of sequences were redundant. Only a low level (<1%) of redundancy was evident in a large sample of clones sequenced from a library for a *Melaleuca* sp generated by the same enrichment methodology (Rossetto et al 1999). High levels of redundancy may result from poor enrichment procedures or as a consequence of high levels of repetitive DNA in the genome. High levels of repetitive DNA have been reported for libraries from other conifers (Smith and Devey 1994). The repetitive nature of the conifer genome appears to have contributed to levels of redundancy in our experience. Nonetheless, useful numbers of unique microsatellites have been in identified in both of our libraries. Out of 15 primer pairs designed to *A. cunninghamii* sequence, 13 apparently amplify single locus regions whereas 3 out of 5 designed from *P. elliottii* sequence amplify single loci from *P. elliottii* and *P. caribaea* templates.

Table 1. Detection of microsatellites from poorly enriched libraries by DIG screening

	Araucaria cunninghamii				***Pinus elliottii***			
Efficiency before screening	1/70				1/20			
No. positive colonies	50				43			
No. containing microsatellite	49				43			
No. unique microsatellites	16				43			
Simple repeat type	GA	CA	GT	TTC	GA	TGC	GAA	GAAAAT
No. of repeats	14-33	9	12	10	10-26	6	6	3
No. of microsatellites	10	1	1	1	39	2	1	1
Compound repeat type	TG-GA	GT-GA	CA-TA					
No. of repeats	24	19	21					
No. of microsatellites	1	1	1					

This technique compares favourably with other high throughput methods that require radioactively labelled probes and robotic workstations (for example Connell et al. 1998). It has proved very effective in recovering microsatellite sequences from libraries of extremely low enrichment efficiency and could

be readily applied directly to genomic or cDNA libraries. The technique is cost effective, rapid, utilising basic techniques and equipment that are widely available in laboratories equipped for cloning.

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