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TECHNICAL NOTE

Use of SSCP to improve the efficiency of microsatellite identification from microsatellite-enriched libraries

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Abstract

An inefficient aspect of marker identification from microsatellite-enriched libraries is the proportion of clones with identical sequences. This can substantially increase the number of clones that need to be sequenced in order to identify a sufficient number of microsatellite loci. We propose the use of single-stranded conformation polymorphism (SSCP) analysis to identify unique clones prior to sequencing. We used this approach prior to sequencing from microsatellite-enriched libraries for three marine invertebrate species and were able to obtain a given number of unique clone sequences for only 28% of the sequencing effort that would have been required without SSCP screening.

Keywords: clones, microsatellite enrichment, SSCP

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Microsatellite enrichment protocols are commonly used for the isolation and characterization of microsatellite loci. A variety of protocols and modifications have been described (Ostrander *et al*. 1992; Fischer & Bachmann 1998; Paetkau 1999; Zane *et al*. 2002; Saltonstall 2003). Commonly, the enrichment procedure involves digestion of genomic DNA using restriction enzymes followed by ligation of the fragments to oligo adapters. The DNA is then annealed to biotinylated oligo probes (e.g. CA_n or GA_n), and the sequences that anneal to the probes are isolated by a streptavidin magnetic beads protocol. The microsatellite-enriched DNA is then amplified by the polymerase chain reaction (PCR) using an oligo adapter as a primer. The DNA is purified before ligation to a vector and transformation. Microsatellite loci are identified by sequencing of the purified plasmid DNA from positive colonies.

One of the problems we and others (Paetkau 1999; Zane *et al*. 2002; Squirrell *et al*. 2003) have encountered with the enrichment method is the redundancy of a proportion of clones. If a large proportion of clones have identical sequences, this can substantially increase the time and cost required to identify sufficient microsatellite loci. We have found this to be a problem during microsatellite development, with between 8% and 63% of our sequenced clones being redundant in libraries for a range of vertebrate and invertebrate species (Table 1).

To overcome the inefficiency caused by selecting clones with the same sequence, we have used single-stranded conformation polymorphism (SSCP) to identify clones with identical sequences prior to sequencing. SSCP is an accurate method for detecting nucleotide differences among PCR products (Lessa & Appelbaum 1993; Sunnucks *et al*. 2000). While the resolving power of SSCP decreases for sequences larger than 500 bp (Sunnucks *et al*. 2000), we expected to be able to distinguish different insert sequences as large as 1000 bp, due to the high proportion of nucleotide differences between the sequences at different microsatellite loci. We used the SSCP screening approach when sequencing from libraries for three marine invertebrate species, the sea urchin *Centrostephanus rodgersii*, the rock oyster *Saccostrea glomerata* and the ascidian *Pyura stolonifera*. We used 10 μL PCRs containing 4 pmol of the M13 (–20) primers, 0.5 U of *Taq* polymerase (Promega), 200 μM of each dNTP, 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100 and 0.05 μL [α^{33}P] dATP at 1000 Ci/mmol and template DNA picked directly from a single bacteria colony. PCR amplifications consisted of 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s; 55 °C for 30 s and 72 °C for 1 min, with a final extension step of 72 °C for 5 min. The SSCP protocol

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Table 1 Efficiency of sequencing from microsatellite-enriched libraries for a range of vertebrate and invertebrate species to obtain unique clone sequences for microsatellite identification. All libraries were developed in the molecular ecology laboratory at Macquarie University using the same modified protocol of Saltonstall (2003)

Species	No. of colonies sequenced	No. of unique sequences	Highest number of times a single clone was sequenced	Reference
Carnegiella marthae (hatchetfish)	32	19	7	Beheregaray *et al*. (2006)
Paracheirodon axelrodi (cardinal tetra)	24	20	2	Beheregaray *et al*. (2004a)
Hemigrammus bleheri (rummy nose tetra)	39	36	2	Beheregaray *et al*. (2005)
Nannostomus unifasciatus (one-lined pencilfish)	39	15	16	Beheregaray *et al*. (2004b)
Macquaria novemaculeata (Australian bass)	21	16	3	Schwartz *et al*. (2005)
Orectolobus maculatus (wobbegong shark)	66	42	10	Schwartz *et al*. unpublished
Ciulfina (praying mantid)	47	27	14	Holwell *et al*. unpublished
Centrostephanus rodgersii (sea urchin)	18	10	11	Banks *et al*. (2006)
Saccostrea glomerata (Sydney rock oyster)	41	16	7	Banks *et al*. (2006)
Pyura stolonifera (cunjevoi)	27	10	10	Piggott *et al*. unpublished

followed Sunnucks *et al*. (2000). We used 8% polyacrylamide gels run in 0.5× TBE buffer for 5 h at 12 W at 4 °C.

From the first SSCP gels for each species, we sequenced at least two samples representing each banding pattern to check whether clones with similar banding patterns could have different sequences. Of 43 sequences (representing clones with 20 distinct banding patterns across the three species) compared in this way, we detected no sequence differences among clones with similar SSCP banding patterns. This evidence supports our expectation that clones with identical SSCP banding patterns would have identical sequences. Clones that yielded identical SSCP banding patterns were therefore considered to have the same insert sequence (Fig. 1). To increase the efficiency of sequencing unique clones, one representative clone for each SSCP banding pattern was amplified in a 40 μL PCR in which reagent concentrations were as previously described except for the exclusion of [α^{33}P] dATP and dNTPs at 100 μM. The PCR products were purified and sequenced on an ABI 377 automated DNA sequencer (PE Applied Biosystems) using dye terminator chemistry.

The SSCP screening approach considerably reduced the amount of sequencing required to obtain the desired number of microsatellite loci. Following SSCP, we identified 56 unique sequences from *C. rodgersii*, *S. glomerata* and *P. stolonifera*. These sequences were identified among a total of 344 colonies picked from the libraries for these species. However, because of the SSCP screening of these 344 clones prior to sequencing, we only needed to sequence 97 clones to obtain these sequences, corresponding to 28% of the sequencing

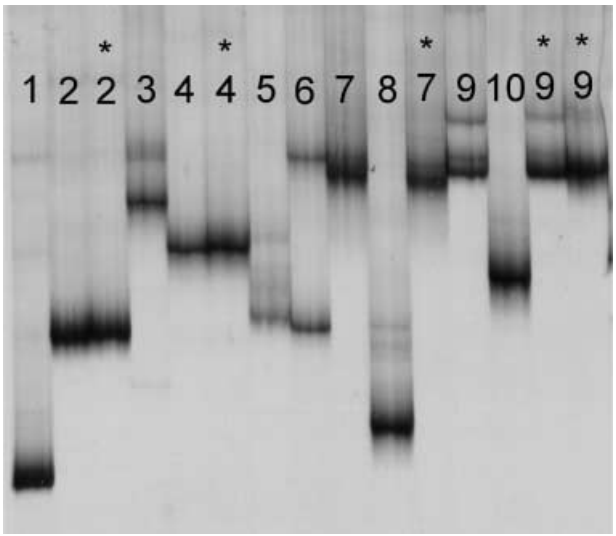

Fig. 1 Single-stranded conformation polymorphism autoradiograph of 15 *Centrostephanus rodgersii* clones. Unique insert sequences have been labelled 1–10 and redundant inserts are labelled with an asterisk.

effort that would have been required without SSCP. The sequencing efficiency could have been increased if we were less conservative in matching SSCP banding patterns, but due to the low numbers of microsatellites obtained from these species compared to other libraries developed in our laboratory (Table 1), we did not want to risk ignoring potentially unique microsatellite sequences. To maximize the efficiency of this process, we suggest that researchers only sequence clones that have distinctly different banding patterns, as the large sequence difference between distinct clones results in very different SSCP banding patterns.

A less sensitive alternative to the SSCP protocol presented here is to run the radioactively labelled PCR products on denaturing polyacrylamide gels rather than SSCP gels. The denaturing polyacrylamide gels can only distinguish size variation and not sequence variation among clones. However, different clone sequences are unlikely to be of exactly identical size, and we found that by running the PCR products on 6% polyacrylamide gels for approximately 4 h, we could identify size differences among over 90% of the clones that could be distinguished with SSCP.

The SSCP screening approach presented here provides a simple solution to an inefficient aspect of the commonly used microsatellite enrichment procedure. Middleton *et al*. (2004) also presented a protocol for identifying differences among clones using denaturing gradient gel electrophoresis (DGGE). While the DGGE method was not presented specifically for microsatellite libraries, both methods (SSCP and DGGE) are applicable to libraries for microsatellites and other types of DNA sequences, where prior knowledge of similarities or differences among clones would increase the efficiency of sequencing. Nevertheless, compared to DGGE, SSCP requires less development, optimization and calibration (Lessa & Appelbaum 1993; Sunnucks *et al*. 2000) and is a relatively more accessible technique for molecular ecology laboratories.

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