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TECHNICAL NOTES

An efficient method for PCR-based isolation of microsatellite arrays (PIMA)

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Polymorphic microsatellite repeat arrays have become some of the most valuable DNA markers for a range of applications including genomic mapping, pedigree analysis and investigations of the genetic structure of populations (Jarne & Lagoda 1996). Use of microsatellites has been compromised to some extent, however, by the time, expense and difficulty of isolating these repeats and their flanking sequences from the genome. Several methods which produce genomic libraries enriched for microsatellite repeats have been reported (see O'Connell & Wright 1997). However, enrichment protocols are a subset of isolation procedures, and do not characterize the microsatellite flanking sequences. Therefore, although they can increase microsatellite yield, they do not make the procedure routine and a substantial effort may be required for repeat isolation.

PCR-based methodologies can be used to isolate many kinds of genomic components, although the lack of flanking sequence information has precluded many of these for microsatellite isolation. Here we describe the development of PCR isolation of microsatellite arrays (PIMA), an approach to isolate and characterize microsatellite flanking sequences from small quantities of genomic DNA. This approach builds on previously described random amplified polymorphic DNA (RAPD) enrichment procedures (Ender *et al*. 1996) but develops the use of repeat-specific PCR to detect microsatellite arrays in contrast to standard radioactive hybridization techniques. The protocol is cheap and efficient, with the advantage that it requires a minimum of specialized equipment, removes the need to carry out radionucleotide hybridization techniques, and produces template suitable for sequencing both flanks with universal vector primers.

Here we attempt to assess the utility of PIMA by comparing its efficiency in isolating microsatellites (from a library of cod, *Gadus morhua*, RAPD fragments) to that of the standard technique of radioactive hybridization. We chose a TG-repeat primer which is reported to be the most frequent dinucleotide repeat in many vertebrates (Jarne & Lagoda 1996). The isolation strategy described in this study is outlined in Fig. 1. RAPD reactions were carried out using 10-mer primers and standard conditions. The three reactions showing the greatest number of equally intense bands were selected for subsequent stages in order to maximize the diversity of templates cloned. These were produced with primers A1 5'-CAGGCCCTTC-3'; A2 5'-TGCCGAGCTG-3'; A4 5'-AATCGGGCTG-3'. Aliquots of PCR reactions were cleaned using a Wizard-PCR spin column (Promega) and cloned into pCR2.1 T-vector (Invitrogen). Recombinant colonies were transferred to replica plates suitable for a multichannel pipette, which was used for all subsequent stages. Colony PCR was performed in duplicate reactions which contained (a) M13 forward and M13 reverse vector primers, or (b) both vector primers plus a repeat-specific primer (5'-TGTGGCGGCCGC(TG)$_8$V-3'). Once reactions with an extra amplification product were identified (see Fig. 1E), 1 μL of PCR reaction (a) was used as template for a cycle sequencing reaction (ThermoSequenase kit, Amersham/Pharmacia) and resolved on an ALF*express* sequencer (Pharmacia). Repeat arrays of less than four units were not scored as microsatellites. Hybridization was carried out using standard conditions (Sambrook *et al*. 1989) with the TG-repeat primer ^{32}P-labelled (hybridization at 47 °C in 5× SSC, 0.1× SDS; washes in 2× SSC, 0.1% SDS at 55 °C). Membranes were exposed to film for 18 h at – 80 °C with intensifying screens.

The combination of RAPD enrichment and repeat-specific PCR isolation has a number of advantages in addition to avoiding radioactive hybridization. RAPD techniques have previously been identified as a valuable source of specific genomic components (Mitchelmore *et al*. 1991; Williamson *et al*. 1994) and are also known to be a rich source of microsatellites and other repetitive elements, probably because they must necessarily include inverted repeats which are themselves associated with repeat duplication processes (Ender *et al*. 1996). Cloning PCR products (Mead *et al*. 1991) into commercially available T-vectors is a rapid and reliable technique, with amplified DNA ligated into the vector and *Escherichia coli* transformed in ≈ 24 h. This cloning approach allows the DNA to be assayed in robust PCR reactions with universal primers located on the vector. This rapid processing of samples enables one person to effectively screen 192 colonies (4 microtitre plates) per day in a moderately well-equipped laboratory. The use of a multichannel pipette for colony lifting, PCR preparation and gel loading, was a critical factor in ensuring high throughput for this approach. Additionally, the PCR reaction from positive colonies can be used directly as template for a cycle-sequencing reaction, negating the need to return to the plate, isolate the corresponding colony, culture overnight and isolate plasmid DNA.

PIMA and radioactive hybridization were used to screen the same 336 bacterial colonies with the TG-oligonucleotide. Fourteen strongly positive colonies were detected by hybridization and comparisons were restricted to these and the first 14 positives obtained by PIMA. Although the data comparing the two techniques are limited (Table 1), certain inferences can be made. When the 14 positives from each technique were sequenced, PIMA was seen to have identified twice as many microsatellites (12) as hybridization (6). Of the

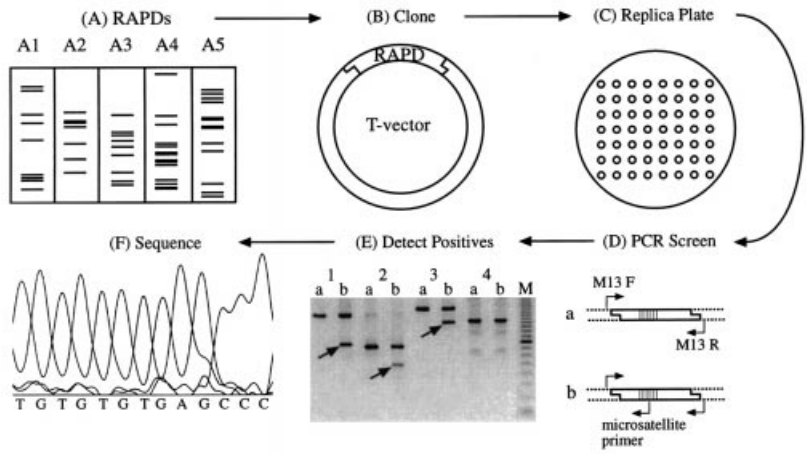

Fig. 1 (A) Amplify genomic DNA in separate reactions with 1–5 RAPD primers. (B) Ligate PCR reactions into a T-vector and transform *Escherichia coli*. (C) Pick transformants onto a replica plate with spacing suitable for a multichannel pipette. (D) Carry out duplicate colony-PCR using (a) two vector primers (b) both vector primers plus a microsatellite-specific primer. (E) Choose colonies whose PCR shows an additional smaller band in PCR reaction (b) as arrowed. M denotes 100 bp ladder. (F) Use 1 μL from PCR reaction (a) as template for a cycle sequencing reaction.

Microsatellites detected	By both methods	By one method	Total microsatellites	False positives	Total colonies
Hybridization	4	2	6	8	14
PIMA	4	8	12	2	14

Table 1 A comparison of the 14 strongest positives identified by radioactive hybridization and PIMA

remaining eight colonies identified by hybridization, five contained microsatellites composed of only three repeat arrays, which may account for the hybridization of the probe to this insert. The ability to detect fewer false positives may, if confirmed in other studies, be of particular importance because sequencing represents the principal financial investment in any isolation strategy.

It is difficult to compare the costs of different techniques objectively as they vary depending upon experimental parameters such as scale and laboratory setup. In our laboratory PIMA was at least as cost effective as hybridization and could be significantly cheaper in a laboratory which is not regularly performing hybridization techniques. We suggest that costs are viewed as comparable for the scale of screening typically undertaken.

In some organisms microsatellites appear to be at very low abundance in the genome (Estoup & Angers 1998). Although in our studies we have readily detected microsatellites, in certain species it may be necessary to screen many thousands of colonies. In such circumstances initial rapid screening by PIMA could be followed up by a large-scale hybridization approach as the initial enrichment and cloning are common to both.

A similar strategy using repeat-specific and vector primers has been described previously (Grist *et al*. 1993; Cooper *et al*. 1997). This approach does not transform bacteria but amplifies directly from a ligation mix of (unenriched) whole genomic DNA. We favour the PIMA technique outlined here because it has the ability to isolate both flanking regions simultaneously. PCR from bacterial colonies also allows robust amplification of products with significantly less optimization. Although we have considered several other variations on this strategy, the benefits of obtaining both flanks simultaneously, the lack of a need for specific sequencing primers, the ease of handling and robust amplification from bacterial colonies persuades us of its high potential. We are as yet still to assess the relative merits of multiplex PCR using several repeat primers simultaneously. Although this approach will increase screening speed, it may also increase the percentage of false positives.

We have found that many distinct microsatellite loci are present in cod RAPD profiles (12 for primer A1), and because the number of unique cloned products can exceed the number of bands visible, the cloning and screening stages seem suitably sensitive. Sub-visible microsatellite-containing RAPDs have also been reported previously (Ramser *et al*. 1997). This repetitive element component may have important implications for the analysis of RAPD band polymorphisms, which are in many cases assumed to change by point substitutions at primer-binding sites (Clark & Lanigan 1993).

In summary, we have outlined a straightforward technique, PIMA, for isolating microsatellites which avoids handling radioactivity, and utilizes the reagents, experience and equipment already present in a laboratory equipped for PCR analysis. The costs and efficiency of this technique are at least comparable with those typically incurred using radioactive hybridization procedures. Furthermore, this study indicates that PIMA may produce fewer false positives than hybridization, which could significantly reduce sequencing, and total isolation expenses.

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A nondestructive technique for the recovery of DNA from dried fish otoliths for subsequent molecular genetic analysis

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The advent of the polymerase chain reaction (PCR) has given access to a wealth of molecular genetic information from archived tissue samples stored in museums and scientific research institutes. Yet, although many of these archives are well documented, samples have rarely been collected as part of a systematic spatial and temporal sampling regime. One of the few such cases includes national marine and freshwater research institutes where extensive sampling of scales and otoliths has been used to monitor the state of exploited fish stocks since the 1900s, thereby providing a resource for geographical and long-term temporal genetic analyses.

Research, hitherto limited to scales and formalin-preserved samples, has used recovered DNA to investigate, e.g. past effective population sizes (Miller & Kapuscinski 1997), interdecadal heterogeneity in mtDNA (Purcell *et al*. 1996), the impact of hatchery escapees on wild populations (Williams *et al*. 1996), and temporal levels of genetic diversity (Nielsen *et al*. 1997). Despite the reported interest in utilizing otoliths for molecular genetic analyses (Ward & Grewe 1994; ICES 1997; Rivers & Ardren 1998), publication of a suitable technique for recovering DNA has been slow, primarily due to large variations in DNA recovery and PCR amplification. In this study we outline a robust procedure for extracting DNA from the surface of intact otoliths.

Otoliths are acellular calcareous deposits contained within a fluid-filled chamber, the labyrinth, situated in the cranial bones. Recoverable DNA is present only on the surface of the otoliths, due to their acellular formation, the quantity of which will depend upon the nature of preparation during their collection.

The samples, provided by CEFAS, Lowestoft, UK, were collected from Atlantic cod (*Gadus morhua*) caught by commercial fishing boats along the northeast coast of England, and ranged in age from 2- to 6-years old. Pairs of otoliths were air-dried in individual paper envelopes. Batches of 10 otoliths were taken from eight separate market sampling events dating from 1955 to 1960.

The otoliths were placed in 1.5-mL microfuge tubes and incubated for 5 h at 55 °C in 500 μL of extraction solution containing 100 mM Tris (pH 8.0), 100 mM NaCl, 10 mM EDTA, 2% SDS, and 200 μg/mL proteinase K. The samples were vortexed briefly and then centrifuged at 13 000 rpm for 10 s prior to the removal of the otolith. The otolith was rinsed thoroughly at this stage to remove traces of extraction solution. A second centrifugation at 13 000 rpm for 2 min pelletted any remaining debris, and the supernatant was subsequently transferred to a fresh 1.5-mL microfuge tube. Volumes of 40 μL of 5 M NaCl, 10 μL of 1 M MgCl, and 1 mL of chilled absolute ethanol were added and mixed thoroughly. The samples were then stored overnight at – 20 °C.

The precipitated DNA was then pelleted by centrifugation at 13 000 rpm for 1 h at 0 °C. The ethanol was removed, taking care to avoid the DNA pellet, and a further 500 μL of chilled 70% ethanol added. The samples were then centrifuged at 13 000 rpm for 3 min. The ethanol was removed once again and the remaining DNA allowed to air dry for 15–20 min at room temperature. The DNA was re-suspended in 50 μL of double-distilled water.

The DNA was PCR amplified using primers for the cod microsatellite loci *Gmo2* and *Gmo132* (Brooker *et al*. 1994). The reactions for both loci contained 3.5–5 μL of DNA extract,

250 pM labelled forward primer, 250 pM reverse primer, 1× NH_4 reaction buffer (Bioline), 4 mM $MgCl_2$, 50 mM KCl, 500 μM dNTPs, 100 μg/mL BSA, and 1.125 units of *Taq* polymerase (Bioline) in a 30-μL volume. Amplification was completed in a Hybaid Omnigene thermal cycler using 1 cycle of 95 °C for 90 s; 5 cycles of 94 °C for 30 s, 49 °C for 45 s, and 72 °C for 30 s; and 35 cycles of 94 °C for 30 s, 51 °C for 45 s, and 72 °C for 30 s. One μL of the reaction was electrophoresed on a 6% polyacrylamide gel using a Pharmacia ALFexpress™ instrument.

All sample extractions and subsequent preparation of PCR reactions were completed within a dedicated isolated ancient DNA laboratory located on a separate floor to the laboratory in which the PCR products were analysed.

Of the 80 otoliths extracted, 78 yielded DNA which successfully PCR amplified using primers to target the *Gmo2* and *Gmo132* microsatellite loci. Increasing the quantity of template DNA in the PCR reactions did not alter the scoring of alleles, confirming that genotyping errors (false heterozygotes and homozygotes) were not occurring through insufficient DNA copy numbers (Goossens *et al*. 1998).

During the original market sampling of the otoliths, no precautions were taken against the transfer of DNA among samples. Consequently, a quarter of the samples showed faint evidence of multiple genotypes, with the presence of additional weakly amplified products. The 80 otoliths tested had been selected from eight separate sampling events, and the majority of the samples containing multiple genotypes could be traced to two specific batches. As all the extraction and PCR negative controls were clean, and there was no evidence of cross-contamination of samples following the recovery of the DNA from the otoliths, it is acceptable to conclude that the contamination occurred solely during market sampling. Of the samples showing evidence of multiple genotypes, all but eight could be resolved by discarding the substantially weaker alleles originating from the contaminant tissue. Consequently, of the 80 samples tested, 70 were successfully extracted and genotyped using the two microsatellite loci. This illustrates that otolith archives represent a significant and readily accessible source of DNA for the study of past populations, and for assessing the genetic impacts of anthropogenic and environmental change.

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