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# Rapid and Non-radioactive Detection Method of Microsatellites in *Mystus nemurus*: A Refined Technique

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## ABSTRAK

*Satu kaedah mudah dan cepat untuk pemencilan mikrosatelit DNA berdasarkan teknik PCR 'Random Amplified Microsatellites' (RAMs) telah digunakan dalam kajian ini. Kajian ini adalah sebahagian usaha yang berterusan untuk memperbaiki dan menyempurnakan teknik tersebut supaya lebih cepat, efektif dan mencapai produktiviti optimum dalam membangunkan penanda mikrosatelit lokus tunggal bagi ikan Baung,* Mystus nemurus. *Protokol pemencilan mikrosatelit yang telah diperbaiki ini berkebolehan mengesan sebanyak 135 bahagian mikrosatelit yang menghasilkan 42 jujukan genom unik yang telah diserahkan ke GenBank. Teknik yang diperbaiki ini dapat mengurangkan jumlah masa yang diperlukan dari pengklonan PCR hingga penjujukan bahagian mikrosatelit yang spesifik kepada kurang dari tiga setengah bulan.*

## ABSTRACT

*A simple and rapid method of DNA microsatellite isolation based on the Random Amplified Microsatellites (RAMs) PCR technique was used in this study. The work presented here is part of a continuous effort in refining and perfecting the technique for more rapid, effective and optimum productivity in single locus microsatellite marker development for the River catfish,* Mystus nemurus. *The current refined protocol for microsatellite isolation was able to detect a total of 135 microsatellite regions resulting in 42 unique genomic sequences being submitted to GenBank. This refined technique is able to reduce the total time required from PCR cloning till sequencing specific microsatellite regions to less than three and a half months.*

## INTRODUCTION

The importance of microsatellites in genetic studies has been greatly acknowledged over the years (Chambers and MacAvoy 2000). This is due to microsatellite markers being a codominant marker system which is more informative than dominant markers such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphisms (AFLP) and Direct Amplification of Length Polymorphisms (DALP). Another added advantage of microsatellite markers is that they amplify regions of repetitive elements with simple repeat motifs of one to six nucleotides which show high levels of allelic variations in the number of repeat units. All these make microsatellites a popular and effective marker system that is useful for various genetic studies such as population, linkage and phylogenetic studies and also for quantitative traits loci (QTL) studies.

However the conventional method used for microsatellite detection is far from being cost effective as it is a laborious and time-consuming process (Tan 2002). In recent years, researchers around the world especially in developing countries had developed different methods for detecting microsatellites. The main objectives were targeted on saving cost and time, increasing effectiveness and productivity. Generally, most methods use the library-enrichment approach which can later be divided into several categories such as probe hybridisation, streps-avidin capture of microsatellites and conversion of multi-locus

microsatellite banding patterns to single locus microsatellite markers. In this paper, the method described was used to achieve the last of the categories previously mentioned. This approach is the most direct and straightforward method, giving rapid results. The concept behind this method is the use of a multi-locus microsatellite marker system namely Random Amplified Microsatellites (RAMs) with the amplified microsatellite bands later being converted to a marker amplifying a single microsatellite locus through a series of steps.

## MATERIALS AND METHODS

*DNA Extraction and PCR Amplification by RAMs Primers*

Genomic DNA was extracted from a single *Mystus nemurus* blood sample following the method of Taggart *et al.* (1992) for microsatellite isolation. A total of 8 RAMs primers (Table 1) were selected for PCR amplification which were cloned in the later part of this work. Amplification was done in a total reaction volume of 10 $\mu$L containing 1.75 mM of magnesium chloride, 1X of Promega reaction buffer, 0.4 mM each of dATP, dCTP, dGTP and dTTP, 3 units of Promega *Taq* polymerase, 50 $\rho$mol of RAMs primer and 0.3 $\mu$L of template DNA ($\approx$ 50 ng). The general PCR profile consisted of 3 minutes of pre-denaturation at 95°C and 35 cycles each consisting of 20 seconds of denaturation, 20 seconds of annealing at an optimised temperature (Table 1), 20 seconds for elongation at 68°C and final elongation at 68°C for 5 minutes. This was followed by electrophoresis of the PCR product on a 2% agarose gel to detect the presence of bands when viewed under transilluminator after ethidium bromide staining.

*Cloning of RAMs Primer*

Fresh PCR amplicon of RAMs primer were cloned into pCR®2.1-TOPO® vector using TOPO TA Cloning Kit (Invitrogen, USA) following the manufacturer's instructions. Positive clones that contained inserted PCR amplicon were identified as white colonies in LB agar (Pronasida, USA) plates containing ampicillin (50 $\mu$g/mL) and X-gal (40 mg/mL). All the positive clones were selected and grown in another LB agar plate.

*Storage of Clones Containing Inserts of Microsatellite Sequences*

All the identified positive clones were cultured separately in LB medium (5 mL) containing ampicillin (50 $\mu$g/mL) in a 15 mL Falcon tube. The culture was incubated overnight at 37°C with vigorous shaking at a speed of 250 rpm. In the morning, approximately 0.85 mL of each culture was mixed with 0.15 mL of sterile glycerol and transferred into a 1.5 mL microcentrifuge tube. The mixture was vortexed for a few seconds and stored at -80°C. The stored clones could be retrieved at any time in the future for further analysis.

*Preparation of Crude Plasmid DNA*

The remaining culture left in the Falcon tube (approximately 4.15 mL) in the previous section was centrifuged at 15,300 x g for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 100 $\mu$L of de-ionised distilled water by vortexing. The mixture was then transferred

TABLE 1
List of the 7 RAMs primers used in this study

| No. | Name | Sequences | Targeted repeat motifs | Annealing temperature (°C) |
|---|---|---|---|---|
| 1 | SC1 | KRKRDKDKDKDK$(CA)_6$ | CA | 52 |
| 2 | SC2 | $N_5$MMHYHYHYH$(GA)_6$ | GA | 52 |
| 3 | SC6 | $N_2$MMBRBRBRB$(GA)_{10}$ | GA | 50 |
| 4 | BP3 | $N_3$YYHMHMHMH$(TG)_6$ | TG | 58 |
| 5 | BP9 | WWWVYVYVYV$(AG)_8$ | AG | 58 |
| 6 | BP10 | KKDRDRD$(TC)_{10}$ | TC | 58 |
| 7 | BP13 | KKBSBSBSB$(CT)_6$ | CT | 58 |

(*Note:* B=C/G/T, D=A/G/T, H=A/C/T, K=G/T, M=A/C, N=A/C/G/T, R=A/G, S=C/G, V=A/C/G, W=A/T, and Y=T/C)

into a 0.5 mL PCR tube followed by heating at 99°C by using a thermocycler for 20 minutes. This was followed by centrifugation at 14,000 rpm for 5 minutes to pellet down the bacterial cell debris. The supernatant containing crude plasmid DNA was ready for the next step of the PCR work.

*M13 PCR Amplification*

This step was to analyse and estimate the insertion size of all the positive clones stored in glycerol by using M13 Forward (-20) and M13 Reverse primer. The PCR amplification was done in a reaction mixture containing 2.0 mM of magnesium chloride, 1X of Promega reaction buffer, 0.25 mM each of dATP, dCTP, dGTP and dTTP, 1 unit of Promega *Taq* polymerase, 50 rmol of each M13 Forward (-20) and M13 Reverse primer and 1.0 $\mu$L of crude plasmid DNA. De-ionised distilled water was added to a final volume of 10 $\mu$L. The PCR profile consisted of a pre-denaturation step of 95°C for 3 minutes followed by 35 cycles at 91°C for 20 seconds, at 55°C for 20 seconds and at 72°C for 30 seconds with a final elongation step at 72°C for 5 minutes. The PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide and visualised under UV light (*Fig. 1*).

*Identifying Positive Clones with Different Insert Size for DNA Sequencing*

After the M13 PCR amplification, the single band that appeared on the 2% agarose gel for each of the positive clones showed that the insertion size of the RAMs amplicon were successfully cloned. A band that appeared in the region around 200 bp indicated that no PCR amplicon was successfully cloned into the vector. This is because the region flanking the PCR inserted by the M13 Forward (-20) to M13 Reverse primer is 202 bp in length. Any length of PCR amplicon inserted in between the flanking region after cloning will add to the 202 bp resulting in a band which appears in the gel with a molecular weight of more than 202 bp. This step is essential to allow the researchers to select the 'real' positive clones and clones that contained different PCR insertion sizes to be sent for sequencing. This is crucial to ensure that not a single RAMs amplicons cloned is left out of the sequencing process. In this study, three positive clones having the same insertion size were selected for plasmid extraction followed by sequencing. The rest of the positive clones of the same insertion size were considered as a redundancy of the same PCR insert.

*Plasmids DNA Extraction and Automated DNA Sequencing*

Once the positive clones with the estimated PCR insertion size were identified for sequencing, the desired clones were re-grown in LB agar (Pronasida, USA) plates retrieved from the previously stored clones. The plasmid DNA was extracted according to Sambrook *et al.* (1989). Automated sequencing of the plasmid DNA was carried out at the Institute of Bioscience (IBS), Universiti Putra Malaysia by using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA).

*Submission of DNA Sequences to GenBank*

Unique DNA sequences containing microsatellites were submitted online to GenBank. This was done by using the online tool BankIT provided by the GenBank website, *http://www.ncbi.nlm.nih.gov/*.

## RESULTS AND DISCUSSION

The method described in this study was able to detect a total of 135 microsatellite regions by utilising only seven RAMs primers within three and a half months.

The novel idea of introducing the M13 PCR amplification step in this refined technique for rapid and enhanced efficiency in detecting microsatellites was stressed in this study. The aim of this refined technique is to solve some of the limitations encountered by the previously published protocols (Teh *et al.* 2003; Kumar *et al.* 2002a; Kumar *et al.* 2002b) using the RAMs method.

The limitation of using the RAMs method in the previously described protocols is the inability to handle the large number of positive clones produced after the cloning process. In studies reported by Teh *et al.* (2003), Kumar *et al.* (2002a) and Kumar *et al.* (2002b), no protocol was suggested for screening the large numbers of clones produced. The studies, instead, used the approach of randomly picking up clones for sequencing. The shortcoming of this approach is that not all the PCR amplicons with different sizes that had been cloned are sequenced. Without guidance from the M13 amplification analysis, the probability of maximising the

potential of the RAMs technique for detecting more microsatellites is greatly reduced. The M13 PCR amplification enhanced the method's efficiency by allowing researchers to determine the size of the PCR insert of each positive clone thus helping in choosing the right clones for sequencing. Another advantage is the ability to distinguish between a false positive clone (one without a PCR insert in the vector) from a 'real' positive clone. This advantage served as a safer alternative by avoiding the use of radioactive components (Chenuil *et al.* 2003; Watanabe *et al.* 2001; Kawai *et al.* 2001; Miller *et al.* 2001) and the toxic chemicals of fluorescein-labeled oligonucleotides (Wimberger *et al.* 1999) for screening of 'real' positive clones.

More researchers are encouraged to be involved in isolation of microsatellites efforts for the species of their interest since microsatellites is becoming the popular choice of marker systems. The perception that isolating single locus microsatellite markers is time consuming, technically demanding and cost ineffective should be reconsidered with an open mind given the availability of the approaches used in this study.

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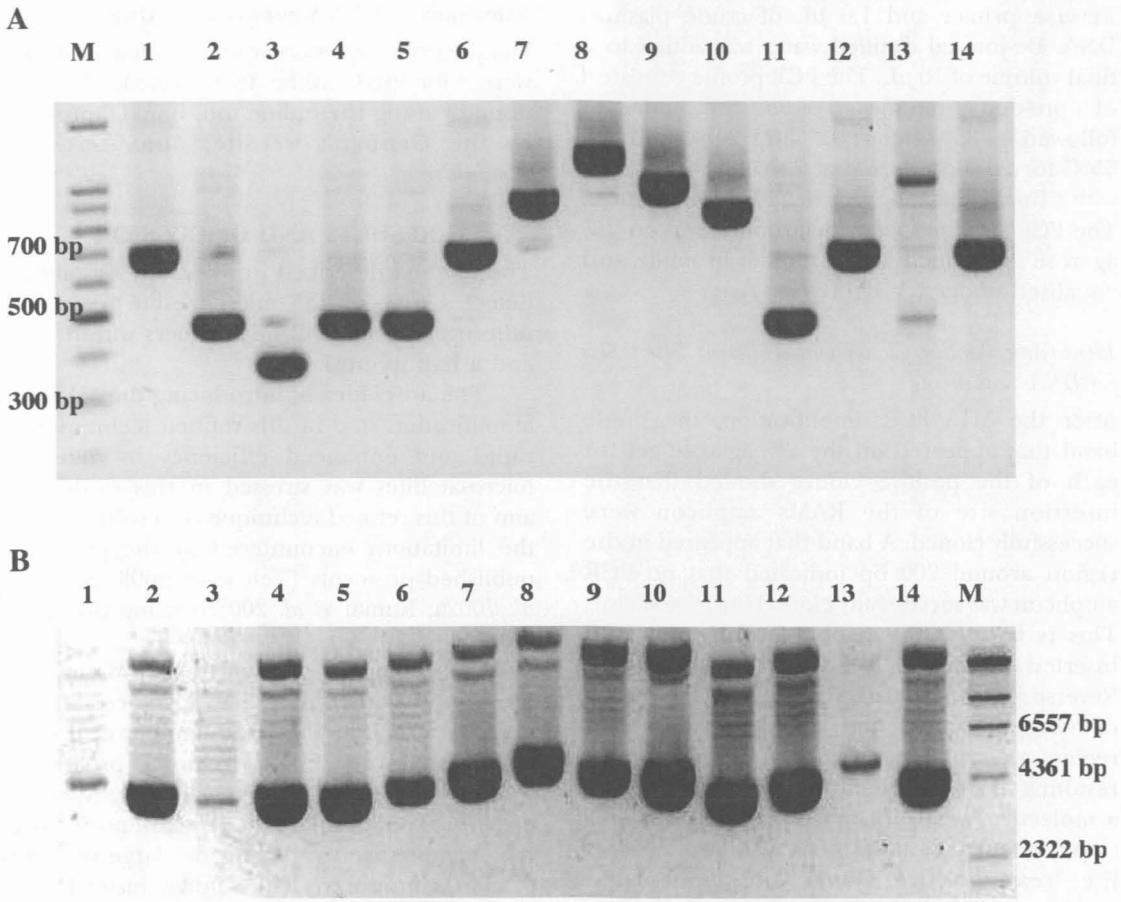


*Fig. 1. Comparison of M13 PCR amplification banding profiles corresponding to the banding profile of the extracted plasmid DNA for RAMs primer BP3. (A) Banding profile of M13 PCR amplification for positive clones of BP3. Lane M: 100 bp ladder; lanes 1-14: band profile of positive clones 1-14. Amplified PCR products were electrophoresed on a 2% agarose gel in 1X TBE buffer at 70 V (B) Banding profiles of plasmid DNA for positive clones of BP3. Lanes 1-14: banding profile of plasmid DNA for positive clones 1-14; lane M: Lambda Hind III marker. Plasmids DNA were electrophoresed on a 1% agarose gel in 1X TBE buffer at 70 V*

TABLE 2
List of GenBank accession number for the unique genomic sequences submitted and the number of microsatellite regions detected by using RAMs primers

| No. | RAMs primer | Total no. of micro-satellite regions isolated | Total no. of unique genomic sequences produced | GenBank accession no. |
|---|---|---|---|---|
| 1 | SC1 | 14 | 4 | AY845085-AY845088 |
| 2 | SC2 | 31 | 7 | AY845089-AY845095 |
| 3 | SC6 | 26 | 6 | AY845096-AY845101 |
| 4 | BP3 | 14 | 9 | AY845102-AY845110 |
| 5 | BP9 | 21 | 7 | AY845111-AY845117 |
| 6 | BP10 | 15 | 4 | AY845118-AY845121 |
| 7 | BP13 | 14 | 5 | AY845122-AY845126 |

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