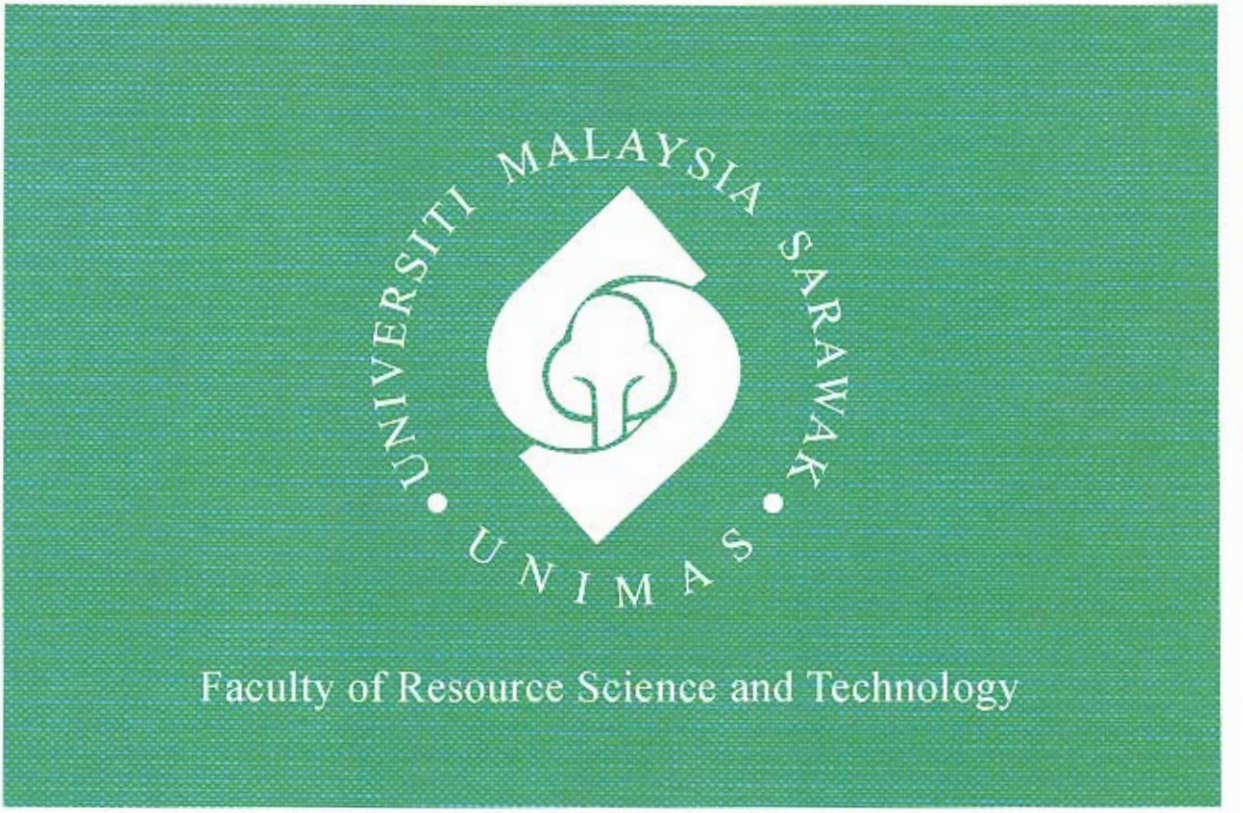

CLONING OF MICROSATELLITE LOCI FROM IKAN TENGADAK *(Barbonymus schwanenfeldii)* USING RAMs TECHNIQUE

Mimi Nadhira Binti Mustapha @ Harun

Bachelor of Science with Honours
(Resource Biotechnology)
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Cloning of Microsatellite Loci from Ikan Tengadak (*Barbonymus schwanenfeldii*) Using RAMs Technique

Mimi Nadhira Binti Mustapha @ Harun

This project is submitted in partial fulfillment of the requirements for the degree of

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DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualitification of this or any other university or institution of higher learning.

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LIST OF ABBREVIATIONS

°C	Degree Celcius
µl	Microlitre
bp	Base pair
CTAB	Cetyltrimethylammonium bromide
EDTA	Ethylenediaminetetraacetic acid
hr	Hour
kb	Kilobase
$MgCl_2$	Magnesium chloride
min	Minutes
ml	Mililitre
mM	Millimole
pH	Potential of hydrogen
Rpm	Revolutions per minutes
s	Second
SDS	Sodium dodecyl sulphate
TAE	Tris-acetate-EDTA
V	Volt

Cloning of Microsatellite Loci from Ikan Tengadak (*Barbonymus schwanenfeldii*) Using RAMs Technique

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ABSTRACT

A study on cloning of the microsatellite loci from Ikan Tengadak; *Barbonymus schwanenfeldii* was done by using random amplified microsatellites (RAMs) or 5'-anchored PCR technique. The methodologies used in this study include DNA extraction using CTAB method, followed by DNA amplification of microsatellite repeats using 5'-anchored or RAMs primers. Nine primers were tested but only four primers produced amplification products; VJ1 (5'-NNNNNNNKKVRVRV$(CT)_{10}$-3'), PCT4 (5'-KKVRVRV$(CT)_6$-3'), Primer 3 (5'-CHY$(GA)_7$-3') and Primer 6 (5'-KKRVRVR$(TC)_6$-3') where R=A/G; Y=C/T; K=G/T; H=A/C/T; V=A/C/G and N=A/C/G/T. However, only Primer 6 was selected for cloning due to the presence of a clear single band PCR products. The PCR products were cloned by using the pGEM-t vector and a Cloning Kit, followed by transformation on the LAIX plates. Then, the identification of positive clone by using the blue/white colony screening and finally plasmid extraction. However the plasmid was failed to be extracted.

Key words: Microsatellite loci, *Barbonymus schwanenfeldii*, Random Amplified Microsatellites (RAMs) or 5'-anchored PCR technique, clone.

ABSTRAK

Kajian tentang pengklonan lokus mikrosatelit dari Ikan Tengadak; Barbonymus schwanenfeldii (Lampam sungai) telah dijalankan dengan menggunakan kaedah amplifikasi mikrosatelit secara rawak (RAM) atau teknik 5'- berpaut PCR. Kaedah-kaedah yang digunakan dalam kajian ini meliputi pengekstrakan DNA menggunakan kaedah CTAB, diikuti dengan mengamplifikasi DNA mikrosatelit dengan menggunakan teknik 5'-berpaut atau primer RAM. Sembilan jujukan diuji tetapi hanya empat primer menghasilkan produk amplifikasi; VJ1 5'-NNNNNNNKKVRVRV (CT) 10-3 '), PCT4 (5'-KKVRVRV (CT) 6-3'), Primer 3 (5 '-chy (GA) 7-3 ') dan Primer 6 (5'-KKRVRVR (TC) 6-3') di mana R = A / G; Y = C / T; K = G / T, H = A / C / T, V = A / C / G dan N = A / C / G / T. Walaubagaimanapun, hanya Primer 6 dipilih untuk pengklonan kerana kehadiran jalur produk PCR yang tunggal dan jelas. Produk PCR diklon dengan menggunakan vektor pGEM-t dan Kloning Kit, diikuti dengan transformasi di piring LAIX. Seterusnya, identifikasi klon positif dengan menggunakan kaedah penyaringan koloni biru/putih dan akhir sekali pengekstrakan plasmid. Walaubagaimanapun plasmid gagal diekstrak.

Kata kunci: Lokus mikrosatelit, Barbonymus schwanenfeldii, mikrosatelit secara rawak (RAM) atau teknik 5'-berpaut PCR, klon.

1.0 INTRODUCTION

Family Cyprinidae is the largest family of freshwater in terms of its abundant genera and species throughout the world. About one-third of all freshwater fishes in western Borneo belong to this family (Robert, 1989). Tropical cyprinids are very beautiful in colour and hence are very important as an aquarium fishes.

Barbonymus schwanenfeldii is one of the important cyprinids fishes in Malaysia. *B. schwanenfeldii* Bleeker (Tinfoil barb) is locally known as Lampam Sungai in Peninsular Malaysia, or Tengadak fish in Sarawak (Mohsin and Ambak, 1983 and Litis *et al.*, 1997). The dorsal fin in living specimens of *Barbonymus schwanenfeldii* is reddish in colour, with a large black bloth anteriorly while the caudal fin is orange with a black band on each lobe. The upper part of the eye is orange. The standard length of this species is 60 to 200 mm (Mohsin and Ambak, 1983). The species is widely distributed in all rivers and lakes in Peninsular Malaysia particularly in Pahang, Perak, Kelantan, Terengganu and Selangor (Mohsin and Ambak, 1991) and indigenous to upper mid-zone of Rejang River system, as well as Limbang and Batang Ai Rivers (Litis *et al.*, 1997).

One three decades, molecular techniques have become available to screen large number of individual animals for examining their genetic variability at the molecular level (Amos and Hoelzel, 1992). The development of molecular techniques provides opportunity to conduct studies on the genetic variation, population genetics and phylogenetic of freshwater fishes (Amos and Hoelzel, 1992; Vrijenhoek, 1998 and Stepien and Koacher, 1997).

Microsatellite is defined as loci or region within DNA sequences, where short sequences of DNA nucleotides are repeated in tandem arrays. Microsatellites as a new class of

polymorphic DNA markers became available only after the discovery of multilocus fingerprinting with minisatellites by Jeffreys *et al.* (1985) and the invention of the PCR by Saiki *et al.* (1988). These markers also called simple sequence repeats (SSR) or short tandem repeats (STR), typically contain 10-50 copies of a short repeat motif (1-10 base pairs, usually 1-5 bp) (Tautz, 1989; Rosenbaum & Deinard, 1998; Scribner & Pearce, 2000). Microsatellite alleles are the result of mutations, causing changes in repeat number during DNA slippage, an intramolecular mutation mechanism. Normally, these mutations are recognized and repaired system (Schlötterer & Pemberton, 1998). Repeat number can very between individuals of the same species and the markers are considered to be neutral, highly polymorphic and codominant (Schlötterer, 1998; Fagerberg *et al.*, 2001). Microsatellites have therefore become a very useful marker system for genome mapping, paternity and kinship analysis and for studying the genetic structure of natural populations (Schlötterer & Pemberton, 1998; Scribner & Pearce, 2000 and Fagerberg *et al.*, 2001).

In this study, the Random Amplified Microsatellites (RAMs) or 5'-anchored PCR technique was used to clone of microsatellite primer from *Barbonymus schwanenfeldii*. The development of a 5'-anchoring produced that consistently anchors PCR primers at the 5' ends of microsatellites, amplifying two close and inverted simple sequence repeats and the region between them. This technique offers a number of advantages compared with conventional cloning method of microsatellites. Firstly, amplification of genomic DNA yields PCR products containing at least two microsatellites (one on each end of the amplicon), which retain their original repeat length. The multi-locus profiles produced from 5'-anchored primers should be more polymorphic than those from non-anchored primers. Secondly, this technique can be used to produce libraries which are highly enriched for single locus microsatellite.

Lastly many of these individual loci can be amplified with one locus-specific primer which further reduces the cost of microsatellite discovery.

The PCR products then were cloned into the replication of DNA fragments by the use of a self-replicating genetic material. These fragments are then inserted into cloning vectors, such as bacterial plasmids or bacteriophages, which transfer the recombinant DNA to suitable host cells, such as the bacterium *E. coli*. Inside the host cell the recombinant DNA undergoes replication, thus, a bacterial host will give rise to a colony of cells containing the cloned target gene. Various screening methods may be used to identify such colonies, enabling them to be selected and cultured such as Blue/White Colony Screening and PCR colony.

OBJECTIVES

1) To amplify microsatellite repeats using RAMs or 5'-anchored PCR technique.

2) To clone the microsatellite repeats.

2.0 LITERATURE REVIEW

2.1 Lampam Sungai Fish (*Barbonymus schwanenfeldii*)

Barbonymus schwanenfeldii is one of the species in the family Cyprinidae. This species was originally described as *Barbus schwanenfeldii* by Pieter Bleeker (1853). *Barbonymus schwanenfeldii* have life span of 8 to 10 years, and its natural habitat is in flooded fields, rivers and streams with a 6.5–7.0 pH and temperature range 22-25°C. Cyprinidae is one of the most diverse freshwater fish groups in the world that achieve their maximal diversity in Asiatic waters (Fu *et al.*, 2003). Family Cyprinidae is currently the largest family of freshwater fish in terms of its abundant genera and species throughout Malaysia. According to Mohsin & Ambak (1983), the Malaysian region is considered as the southern centre for distribution of primary freshwater fish.

2.2 Microsatellite Loci

Microsatellite loci are often highly polymorphic due to a high rate of mutation through replication slippage, resulting in the gain or loss repeat units. In addition, microsatellite loci are as vulnerable to point mutations as the rest of the genome, which tends to divide longer repeat stretches into smaller units, and hence decrease the rate at which slippage occurs (Bell and Jurka, 1997; Kruglyak *et al.*, 1998). This slippage or point-mutation theory suggests that the frequency distribution of microsatellite lengths is a balance of expansion due to slippage and contraction due to point mutation (Sibly *et al.*, 2003).

Microsatellites can be simply and rapidly detected by the Polymerase Chain Reaction (PCR) using two unique oligonucleotide primers that flank the microsatellite and hence define the microsatellite locus. Because of their multi-allelic nature, codominant inheritance, small length, extensive genome coverage and relative abundance, microsatellites have been successfully applied in wide variety of research field and practical disciplines (Powel *et al.*, 1996).

2.3 RAMs Technique / 5'-anchored Technique

The 5'-anchored PCR technique was reported by Fisher and Gardner (1996). This technique is simple, rapid and has been successfully used and reported in previous studies. This is the first report that microsatellite markers were isolated and characterized for *Perna viridis* where they were designed seven microsatellite primer pairs based on the *Perna viridis* DNA sequences obtained from a modified 5' anchored PCR technique.

The study from Latipp *et al.* (2010) showed that 5'anchored PCR technique was suitable and efficient for isolating single locus DNA microsatellites for *Helopeltis theivora*. The totals of 6 polymorphic microsatellite markers were isolated successfully and their study is important in the quest to provide a sufficient number of codominant genetic markers for studying the population genetic structure of *H. theivora* in Asia.

In Shou *et al.* (2008) study of isolating microsatellite loci in blunt snout bream, the microsatellites were isolated using an enrichment procedure based on the 5'-anchored PCR technique described by Fisher *et al.* (1996), Brachet *et al.* (1999) and Kumar *et al.* (2002). Meanwhile, in a study to develop long repeats microsatellite markers from Tartary buckwheat, 5'-anchored PCR and Dynabeads purifying technology were used for development of long repeats microsatellite markers (Yanqin Li *et al.*, 2006). Their results obtained the sequence

analysis of 5 of these clones revealed that each contained a $(gT)_n$, microsatellite at both ends of the inserted.

Hantula *et al.* (1996) has demonstrated that RAMS is applicable in studying genetic variation in fungi. In this study, RAPD and RAMS analyses were used to determine the genetic relationship within and between *G. boninense* isolates from infected oil palm and *Ganoderma* sp. from coconut stumps.

According to Zane *et al.* (2002), the success rate of DNA isolation microsatellite markers usually ranges from 12% to less than 0.04%. Here they reported on the development of microsatellites from *P. viridis* using a modification technique of the 5'-anchored PCR technique of Fisher *et al.* (1996).

3.0 MATERIALS AND METHODS

3.1 Sample Collection

Samples of *B. schwanenfeldii* (Figure 4.1) were collected from Terengganu. All of the samples were stored in -20 °C freezer until used for DNA extraction. For long-term storage, the samples were stored at -80 °C.

Sample Description

ORDER: Cypriniformes

FAMILY: Cyprinidae

GENUS: Barbonymus

SPECIES: *Barbonymus schwanenfeldii*

LOCAL NAME: Lampam Sungai (Peninsular Malaysia) / Tengadak (Sarawak)

Tinfoil Barb

Figure 3.1: *Barbonymus schwanenfeldii* Bleeker (Lampam Sungai).

3.2 DNA Extraction

Total DNA was extracted from tissue muscle using a modified CTAB method (Grewe *et al.*, 1993) with the presence of Proteinase K. DNA pellet was dissolved in 60 µl sterile distilled water. Quality and approximate yield was determined by electrophoresis in a 1% agarose gel containing ethidium bromide at 90V for 40 min. the isolated genomic DNA was used for 5'-anchored PCR analysis.

3.3 Polymerase Chain Reaction (PCR) using RAMs Primers

The PCR technique involve denature of double helix of DNA into its two strands, annealing of primers to each original strand for new strand synthesis and extraction of the new DNA stands from the primers. Thermal cycle amplification was performed using Swift^{TM} MiniPro Thermal Cycle machine in a reaction volume containing sterilized distilled water, *Taq* DNA polymerase (Promega), reaction buffer (Promega), dNTPs (Promega), magnesium chloride (Promega) and nine different types of RAMs primers. The DNA preparation was subsequently added into the PCR mix.

5'-anchored PCR technique is where the PCR primers anchored at the 5' ends of microsatellites, amplifying two close and inverted simple sequence repeats and the region between them. The successful of 5'-anchored PCR technique is primarily dependent on careful consideration in preparation of reagent, template DNA, cycling times and temperatures, visualization of PCR products and scoring of data (Scott and Graham, 2001).

Amplification of DNA was performed in a 10 µl reaction mixture as shown in Table 1. The 5'-anchored PCR was carried out in PCR machine with the following conditions in Table 2. One negative control (absence of template DNA) was performed for each set of amplifications. The nine random amplified microsatellites (RAMs) primers were tested and listed in the Table 3.3.

Table 3.1 : Cocktails for reaction mixture used in 5'-anchored PCR technique.

Component	**1 X**
ddH_2O	5.0 µl
5 x reaction buffer	2.0 µl
dNTP mix (10 mM)	0.3 µl
RAM Primer (10µM)	0.5 µl
$MgCl_2$ (15 mM)	1.0 µl
Template DNA (10 ng/µl)	1.0 µl
Taq polymerase (5 units/µl)	0.2 µl
Total of Reaction	**10.0 µl**

Table 3.2 : The 5'-anchored PCR conditions using PCR machine.

Step	Temperature	Time	No. Cycles
Denaturation	94 °C	3 min	1
Denaturation	94 °C	30 s	
Annealing	55 °C	30 s	
Extension	72 °C	1 min	35
Extension	72 °C	5 min	
Soak	10 °C		1

Table 3.3 : Lists of random amplified microsatellite (RAM) primers.

Primer	Sequence
VJ1	5'-NNNNNNNKKVRVRV$(CT)_{10}$-3'
PCT4	5'-KKVRVRV$(CT)_6$-3'
Primer 1	5'-KKVMVMV$(GT)_6$-3'
Primer 2	5'-BDB$(CA)_7$-3'
Primer 3	5'-CHY$(GA)_7$-3'
Primer 4	5'-KKVHVHV$(GT)_6$-3'
Primer 5	5'-KKRYRYY$(AC)_6$-3'
Primer 6	5'-KKRVRVR$(TC)_6$-3'
Primer 7	5'-KKHVHVH$(TG)_6$-3'

Where R=A/G; Y=C/T; K=G/T; H=A/C/T; B=C/G/T; V=A/C/G; M=C/A, D=A/G/T and N=A/C/G/T

3.4 Electrophoresis and Visualization

The 5'-anchored PCR products were then separated by electrophoresis on 1% agarose gel in 1X TAE buffer. Agarose gel electrophoresis was used to separate DNA molecules by size. The negatively charged DNA molecules migrate in an electrical field from the negatively charged anode to the positively charged cathode, with smaller molecules through the pores of the gel matrix. The concentration and quality determine pore size in the agarose gel. Electrophoresis was performed in a horizontal apparatus for 90 min with a constant power of 90 V. The gel was mixed with 1 µl of ethidium bromide and visualized on a UV transilluminator. Molecular sizes of the amplification products were estimated by using MassRuler^{TM} Ladder.

3.5 Clone PCR Product using pGEM-t Easy Vector and Cloning Kit

One positive amplification product was selected and cloned using pGEM-t Easy vector. The ligation reactions were set up as following in the Table 3.4.

Table 3.4 : Lists of components for cloning PCR products.

Component	Volume
2X rapid ligation buffer	5 µl
pGEM-t vector	1 µl
T4 ligase	1 µl
Insert DNA	3 µl
Total of Reaction	**10 µl**

The mixtures were mixed by gentle pipetting it in 1.5 ml of autoclaved Eppendorf tubes for ligation. The sample was keep in refrigerator (4 °C) and leave for overnight incubation.

3.6 Overnight Bacteria Culture

Approximately 3µl of *E.coli* from glycerol stock was cultured overnight into 5 ml of sterile Luria Broth and incubated with shaking at 37 °C.

3.7 Preparation of Competent Cell

Approximately 5 ml of overnight cultured *E. coli* was added into 50 ml of pre-warmed Luria Broth in the Erlenmeyer flask. The bacterial cultured was grew inside the incubator shaker at 37 °C for 1 hr 20 min until the OD600 reaches approximately 0.45 – 0.50. The flask was cold on ice for 10 to 20 min. Then, the bacterial cultured was centrifuged at 3500 rpm at 4 °C for 5 min in the Falcon tube. The supernatant was discarded and the cells were washed gently by resuspending the cell pellet in 25 ml iced-cold 100 mM calcium chloride ($CaCl_2$). The cells suspension was kept on ice for 10 min and then recentrifuged again. After centrifugation, the supernatant was discarded and the cell pellet is resuspend in 2.5 ml of cold 100 mM $CaCl_2$. The competent cell was incubated on ice for 1 hr until use.

3.8 Bacteria Plasmid Transformation

Begin starting transformation, the waterbath was heated to 42 °C. The ligation reaction mixture was removed from refrigerator and equilibrates to room temperature for 1 min. The mixture was centrifuged to move all liquid to the bottom of the tube. Next, 2 µl of each ligation was added to the bottom of a sterile 5 ml Falcon round-bottomed tube that has been pre-cooled on ice. Each tube was labeled. 50 µl of competent cells were added into the Falcon round-bottomed tubes on ice using wide-bore pipette tips and gently pipette and flicked gently. The mixture was leaved on ice for 20 min. Next, the mixture was heat-shock for the cells for 45 seconds at 42 °C in the waterbath. The mixture was return to ice for 2 min. 950 µl SOC media was added to each transformation and mixed by flicking gently. Then, the tubes were closed completely and put it in an incubator-shaker at 37 °C. After 30 min, the mixture was mixed into ligation mixture. Using a sterile pipette tip, the mixture was spread into two LAIX plates (each one 150 µl). Lastly, seal plates with Parafilm strip and place in 37 °C dry oven for overnight.

3.9 Blue/White Colony Screening

After the plates have been incubated for overnight, place them in a fridge for 30 min to allow easier screening of blue/white colonies. Using sterile pipette tip, the white colonies were inoculated into bijou bottle containing 5 ml LB with 5 µl of Ampicilin. The cells were incubated overnight with shaking at 37 °C.