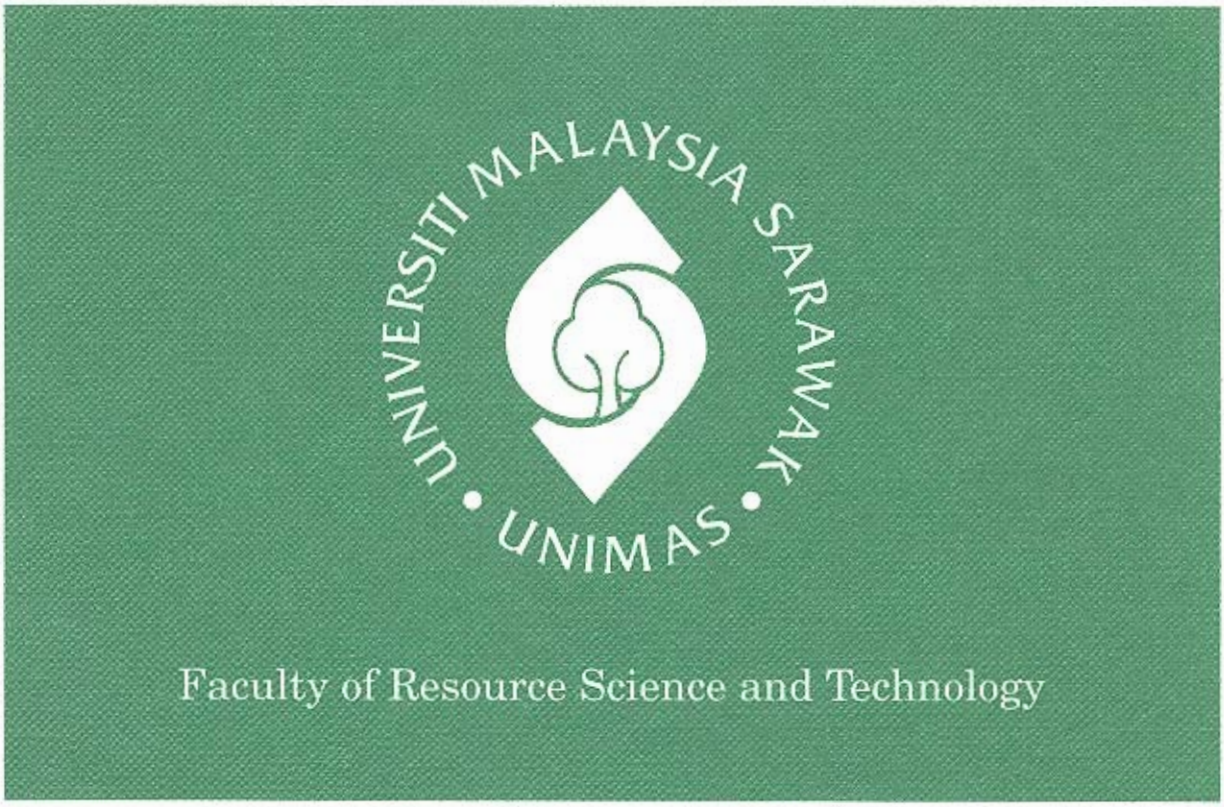

IDENTIFICATION OF GENETIC MARKERS AMONG SEVERAL INDIGENOUS FRESHWATER FISHES OF THE FAMILY CYPRINIDAEA USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) TECHNIQUE

Mohd Shahril B. Mohd Safri

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Identification of Genetic Markers among Several Indigenous Freshwater Fishes of the Family Cyprinidae using Random Amplified Polymorphic DNA (RAPD) technique.

by

Mohd Shahril bin Mohd Safri

Faculty Resource Science and Technology

UNIVERSITI MALAYSIA SARAWAK

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DECLARATION

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

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(Mohd Shahril bin Mohd Safri)
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Identification of Genetic Markers among Several Indigenous Freshwater Fishes of the Family Cyprinidae using RAPD technique.

Mohd Shahril bin Mohd Safri

Resource Biotechnology Programme
Faculty Resource Science and Technology
University Malaysia Sarawak

ABSRACT

Objective of this project is to identify potential genetic marker for species identification among several indigenous species in Cyprinids family using RAPD technique. Modified CTAB method with presence of Proteinase K has been used to extract the total genomic DNA of nine Cyprinid species, however only seven species were proceeded for further RAPD analysis. RAPD analysis was performed by applying two sets of RAPD primers kit (Operon kit D and Operon kit A) to seven species of Cyprinid. Only two primers (OPD12 and OPA4) out of 27 RAPD primers gave positive results on seven species of Cyprinids; *Puntius bramoides* (kachong), *Puntius bulu* (mengalan), *Puntius sealei* (tengadak), *Puntius binotatus* (Banga/tebal sisek), Tor *tambroides* (empurau), *Tor douronensis* (tengeh), and Nolissochilus *strachegi* (tengas). RAPD technique offers a suitable method in identification of genetic marker among seven species of Cyprinids as it produced different RAPD profile that can be use to distinguish these species.

Key words: Cyprinidae, RAPD, CTAB, RAPD profile, primer.

Abstrak

Tujuan projek ini dijalankan adalah untuk mengenalpasti penanda genetik yang berpotensi untuk pengecaman spesis didalam genus yang sama untuk ikan dalam famili Cyprinidae menggunakan teknik RAPD. DNA sembilan Cyprinid spesis telah diekstrak dengan menggunakan kaedah CTAB yang diubahsuai dengan kehadiran Proteinase K tetapi hanya tujuh spesis telah di analisis dengan RAPD. Dua set primer RAPD (Operon kit D dan Operon kit A) telah digunakan di dalam analisis RAPD terhadap tujuh spesis tersebut. Hanya dua primer (OPD12 dan OPA4) telah menunjukkan keputusan yang positif ke atas tujuh spesis dalam famili Cyprinidae; Puntius bramoides (kachong), Puntius bulu (mengalan), Puntius sealei (tengadak), Puntius binotatus (Banga/tebal sisek), Tor tambroides (empurau), Tor douronensis (tengeh), and Nolissochilus strachegi (tengas). Teknik RAPD merupakan teknik yang boleh diguna pakai untuk mengenalpasti penanda genetik yang berpotensi untuk pengecaman spesis dalam famili Cyprinidae berdasarkan profil RAPD yang terhasil. Profil yang terhasil berbeza diantara spesis, maka ia boleh dijadikan penanda genetik untuk proses pengecaman spesis.

Kata kunci : Cyprinidae, RAPD, CTAB ,profil RAPD , primer.

1.0 INTRODUCTION

Within the last decade, technological advancement has increasingly supported the use of genetics method in analysis of population diversity (Wolf 1993). Many molecular techniques are now available which allow ecologists and evolutionary biologists to determine the genetic architecture of a wide variety of closely related individuals (West 1993)

The freshwater fishes of the family Cyprinidae contribute significantly to the daily protein diet resources worldwide. Over 35,000 species of fish have been described so far; where approximately 100 new species are describe every year (Maitland, 1992). Most of the species in this family have high potential for aquaculture and aquarium industries. Fishes in this family that are usually found in Sarawak such as *Tor tambroides* (Empurau) and some of them which is *Puntius bulu* (mengalan) found in peninsular Malaysia

A number of fishes in this family Cyprinidae such as *Tor duoronensis* (semah) and *Borbonymus schwanenfeldii* (tengadak) are highly valued for their delicious flesh. However, the population size of many Cyprinids has dramatically been reduced due to environment disasters such as water pollution that comes from development and uncontrolled fishing (over fishing).

Thus, given the importance of our indigenous freshwater cyprinids as food and aquarium fishes, and the on-going depletion in their population numbers; a molecular study to identify genetic markers among these fishes is urgently required.

Indeed there are many molecular studies that already developed for genetic determination of individuals or species. This method includes isozyme analysis, restriction fragment length polymorphisms (RFLP), and random amplified polymorphic DNA (RAPD) and many others (Mulcahy *et al.* 1993). Although isozyme analysis and RFLPs are a source of readily available genetic information which is easily reproduced, they often do not show polymorphisms which are necessary to determine variation within a group of genetically similar individuals.

Random amplified polymorphic DNA, or RAPD, analysis is a widely used technique for detecting genomic polymorphisms at a number of different loci using minimal quantities of genomic DNA. Using an oligonucleotide primer of random sequence in a polymerase chain reaction (PCR), a reproducible array of strain-specific products are generated that can be analyzed using gel electrophoresis (Baillie *et al.*, 1990).

The advantages of RAPD is its simplicity, rapidity, the requirement for only a small quantity of DNA, and the ability to generate numerous polymorphisms (Cheng *et al.*, 1997). Therefore, it has been a powerful technique for genetic analysis (Chapco *et al.*, 1992; *Kiss et al.*, 1993; Landry *et al.*, 1993; Wight *et al.*, 1993; Williams *et al.*, 1990).

2.0 LITERATURE REVIEW

2.1 CYPRINID FAMILY

Fish in the family Cyprinidae is the dominant group of freshwater fishes in Southeast Asia (Roberts, 1989). Cyprinids consist of carp and minnows, which abundantly found in western Borneo such as Kapuas River. All cyprinids have toothless oral jaws but well-toothed pharyngeal jaws with 1-3 rows of large teeth (Roberts, 1989). This family comprised of about 50 genus and several hundreds species (Roberts, 1989).

Cyprinids family has been chosen for this study because of their wide distribution in west Borneo and it easy to find (Roberts, 1989). For example, *Tor douronensis* (semah) had been noted as the state fish of Sarawak and famous freshwater food fishes. They are highly prized for the taste of their flesh.

2.2 RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) PCR

Random amplified polymorphic DNA (RAPD) is a PCR base marker system. RAPD technique was developed by Williams *et al*, (1990) and had found a broad application in population genetic study. RAPD reaction basically used PCR reaction, but the difference is the target sequence to be amplified is unknown. This is the simplest of all PCR based markers whereby a genomic DNA are amplified using a single oligonucleotide primer of arbitrary sequence. This method is relatively fast and easy to obtain and yields high levels of polymorphism. A discrete PCR product

is produced when the primer binds to sites on opposite strands of the DNA that are within an amplifiable distance (less than 3000 bp usually). The presence or absence of this specific product is thought to represent mutations in the primer-binding sites of the DNA. RAPD reactions normally amplify three to ten different pieces of DNA (Andersen, 2000; Wolfe and Liston, 1998).

Advantages

The broad use of RAPD markers in molecular study is due to several factors: (1) no prior sequence knowledge is necessary; (2) small amounts of DNA can be used (25 nanograms per reaction); (3) the technique is technically simple; (4) detection is not radioactive and (5) a large number of potential markers can be generate using readily available markers (Rhodess Uni, 1999). This, in addition to its swift pace of obtaining data, makes RAPD a popular technique, for studies of interspecific hybridization, introgression, identification of clones, development of markers linked to sex determination and measurements of genetic diversity, as well as many other studies (Wolfe & Liston, 1998). The technique also reveals relatively high levels of polymorphism and has a low cost compared to other techniques, such as allozymes and RFLP (Robinson & Harris, 1999).

Disadvantages

Because of the short primers used (only 10 bp) the reannealing temperature in the PCR must be low (35-40°C) for the primer to bind. With such low temperature, however, binding is not very specific, which means that primers will also bind to sequences which are not completely complementary (Andersen, 2000).

The ability of RAPD markers to yield an accurate result depend on reliable amplification of diagnostic fragment, which have certain factors that can effect the reaction such as DNA purity, Magnesium chloride concentration, annealing temperature, primer to template ratio and different thermostable polymerases. All these factors could cause changes in binding pattern. Anomalous RAPD bands can occur if there is competition among binding sites, as well as contaminant DNA from parasites and symbiotic organisms (Wolfe and Liston, 1998).

The scoring of RAPDs is open to interpretation, making results difficult to compare as criteria for band scoring can differ from researcher to researcher (Rhodess Uni, 1999). Two studies of the same species by different researchers have resulted in a 4 fold difference in polymorphic bands obtained per primer, clearly showing differences in band scoring criteria (Wolfe & Liston, 1998).

RAPD markers tend to underestimate genetic distances between distantly related individuals, for example in inter-specific comparisons. It is wise to be cautious when using RAPD for taxonomic studies above the species level (Caetano-Anolles, 1998).

3.0 MATERIALS AND METHODS

3.1 Fish tissue collection and preservation

All the fish samples used in this study have been collected from various river systems in Sabah and Sarawak. A total of nine species of cyprinids were selected for this study. *Puntius bramoides* (Kachong), *Puntius bulu* (Mengalan), *Puntius sealei* (Tengadak), *Puntius binotatus* (Banga/Tebal sisek), Tor *tambroides* (Empurau), *Tor douronensis* (Tengeh), and Nolissochilus *strachegi* (Tengas), (Table1). All fish samples were collected and stored at -20°C freezer prior to future analysis.

Table 1: species name, location and number of genomic DNA successfully extracted.

	Species	Location	No. of sample extracted
1.	*Puntius bramoides* (Kachong)	Sg. Balung Hulu Tawau	5
2.	*Puntius bulu* (Mengalan)	Sg. Besar Sabak Bernam	4
3.	*Puntius sealei* (Tengadak)	Sg. Balung Ecor Resort	5
4.	*Puntius binototus* (Bangha/tebal sisek)	Sg. Kelalan Hulu	5
5.	*Tor tambroides* (Empurau)	Sg. Rajang	2
6.	*Neoloschilus strachegi* (Tengas)	Sg. Iti Sipitang	3
7.	*Tor dourenensis* (Semah)	Sg. Baleh Kapit	2
8.	*Cyclocheihichthys apogon* (Boeng)	Sg. Tiagau Kalabakan	2
9.	*Barbodes Coolingwoodi* (Kepiat)	Sg. Kelalan besar	2
		Total =	27

3.2 DNA Extraction

All fish samples were extracted using a modified CTAB method (Grewe *at. al*, 1993) with the presence of Proteinase K. Upper tissue/muscle of fish was taken out and then minced. Seven hundred micro liter (µl) of 2x CTAB buffer was then added into centrifuge tube, after that minced tissue was then added into centrifuge tube that contain 2x CTAB buffer. 5µl of Proteinase K was added later. Next, centrifuge tube was vortexed for 5 minutes and incubated in water bath for 2-3 hour at 60 ° C. After incubation, 600µl of isoamyl-alcohol was added and inverted gently to mix the mixture. The sample then was centrifuged for 10 minutes at 13,000 rpm.

Two aqueous layers were formed after centrifugation. Genomic materials contained in the upper layer and the cell component was in the bottom layer. Five hundred micro liter (µl) of upper layer was sucked up and transferred into a new centrifuge tube. Absolute alcohol of 500 µl was then added into the centrifuge tube and centrifuged for 10 minutes at 13,000 rpm. DNA pellet determined after centrifugation, and then the supernatant was discarded after centrifugation. Five hundred micro liter (µl) of 70 % ethanol and 25 µl of NaCl were added into the tube. Again, centrifugation was performed for 10 minute at 13,000 rpm and after that the supernatant was discarded and DNA pellet was dried using tissue paper or air dry. Finally, DNA was resolved in 50 µl of sterile water or ddH_2O at -20 ° C for storage.

3.3 RAPD-PCR

Two types of RAPD primer kits, each containing 10 base pair random primers were tested (Table 2). PCR reaction mixture was applied from according to the manufacture instruction (PROMEGA). The DNA amplification was amplified using standard polymerase chain reaction (PCR) protocols (Williams *et at.*, 1990). Approximately 14 primers of RAPD primer kit D and 12 primers from RAPD primer kit A were tested during RAPD-PCR. From RAPD kit D, only two primers (primer kit D 12 and primer kit D 18) gives repeatable and consistent result on the seven individuals at 34° C of annealing temperature. On the other hand, only seven primers out of 12 primers of kit A managed to be tested and only 1 primer (primer kit A 4) give consistent and reproducible result.

Table 2: List of RAPD primers

RAPD KIT A		RAPD KIT D	
Oligo name	Sequence (5'-3')	Oligo name	Sequence (5'-3')
OP A-01	CAGGCCCTTC	OP D-01	ACCGCGAAGG
OP A-02	TGCCGAGCTG	OP D-02	GGACCCAACC
OP A-03	AGTCAGCCAC	OP D-04	TCTGGTGAGG
OP A-04	ATTCGGGCTG	OP D-06	ACCTGAACGG
OP A-05	AGGGGTCTTC	OP D-07	TTGGCACGGG
OP A-06	GGTCCCTGAC	OP D-09	CTCTGGAGAC
OP A-07	GAAACGGGTG	OP D-10	GGTCTACACC
OP A-09	GGGTAACGCC	OP D-11	AGCGCCATTG
OP A-10	GTGATCGCAG	OP D-12	CACCGTATCC
OP A-15	TTCCGAACCC	OP D-13	GGGGTGACGA
OP A-16	AGCCAGCGAA	OP D-14	CTTCCCCAAG
OP A-20	GTTGCGATCC	OP D-15	CATCCGTGCT
		OP D-16	AGGGCGTAAG
		OP D-17	TTTCCCACGG
		OP D-18	GAGAGCCAAC

PCR-RAPD was carried out using Promega PCR mixture consist of PCR reaction buffer, Mg Cl_2, dNTPs and Taq polymerase. Reaction unit for each sample was 25µl included 0.2µl of Taq polymerase and 1µl of DNA sample. The DNA was added with PCR mixture (Table 3). Annealing temperature that has been tested was ranging from 30 to 36 ° C using PCR program that can be seen in Table 4.

Table 3: RAPD – PCR component

Component	1 reaction	10 reactions
H2O (RO, sterile)	18.3 µL	183.0 µL
10x reaction buffer	2.5 µL	25.0 µL
$MgCl_2$	1.5 µL	15.0 µL
dNTP mix (10 mM)	0.5 µL	5.0 µL
Primer (10 µM)	1.0 µL	10.0 µL
Template DNA (10 ng/µL)	1.0 µL	****
Taq polymerase (5 units/µL)	0.2 µL	2.0 µL

**** - amount of template DNA is subjected to the experiment.

Table 4: PCR cycle procedure that has been used in thermocycler.

Step	Temp.	Time.	No. Cycle.
Denaturation	94° C	2 min	
Annealing	40° C	30 s	1
Extension	72° C	2 min	
Denaturation	94° C	15 s	
Annealing	40° C	30 s	40
Extension	72° C	2 min	
Extention	72° C	5 min	
Soak	25° C	5 min	1

3.4 Electrophoresis

After extraction was successfully carried out, all samples were visualized under UV light to determine the presence of DNA. Electrophoresis using agarose gel containing ethidium bromide was performed using the following ingredient: 0.75 g of agarose powder was used and dissolved with 50 ml of 1x TAE buffer and cooked using microwave oven for 1½ minute. For the staining purpose, 0.4µl of ethidium bromide but apparently produced unclear and poor visualization under UV light. Thus, the amount of ethidium bromide was increased to 0.6 µl. Electrophoresis was performed at 90V or 80V for 30 to 45 minutes. A standard size marker (Low Range DNA ladder (80-1031bp)) was used.

4.0 RESULT AND DISCUSSION

4.1 Extraction samples

DNA extraction of nine individuals of cyprinid species was successfully achieved. However DNA extraction task has been done twice due to the decreasing of DNA quality of the primary extracted samples. The list of samples was shown in Table 1. Most of the fish samples used in this study had been preserved in ethanol and had been store for more than two years. Nevertheless genomic DNA was successfully extracted from nine cyprinid species. As mentioned before, DNA extraction task had to be done twice due to the low quality of extracted DNA obtained during the primary extraction procedure. Figure 1 showed an example of the extracted DNA of four cyprinid species, (*Puntius bramoides*, *Puntius bulu*, *Puntius sealei* and *Puntius binotatus*).

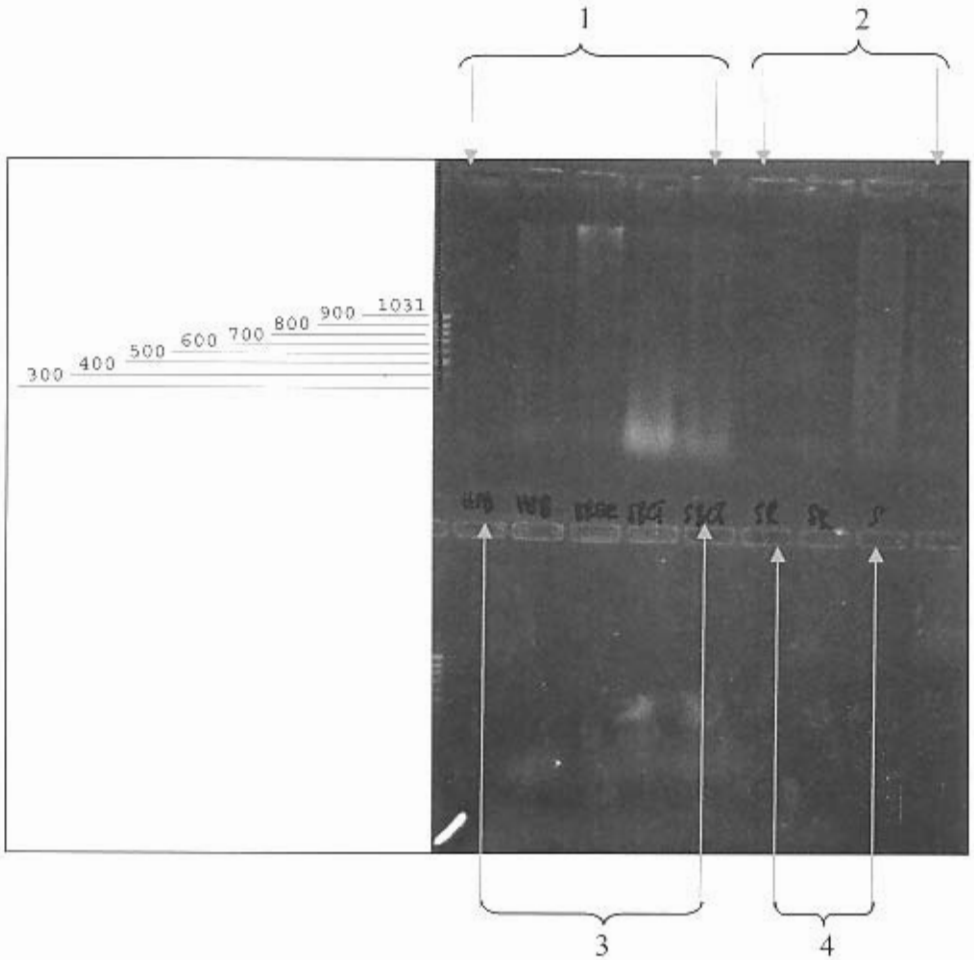

Figure 1. Electrophoresis for primary extracted samples consisted of samples number 1-4 and each replicates. 1 = *Puntius bramoides*, 2 = *Puntius bulu*, 3 = *Puntius sealei*, 4 = *Puntius binototus*.

Based on Figure 1, low molecular weight DNA was normally obtained. This shows that most of the samples have degraded DNA due to long term storage in ethanol or at -20°C freezer. Samples that were preserved in ethanol and stored at room temperature for a long period of time (3 years) would have been exposed to degradation of their DNA material.

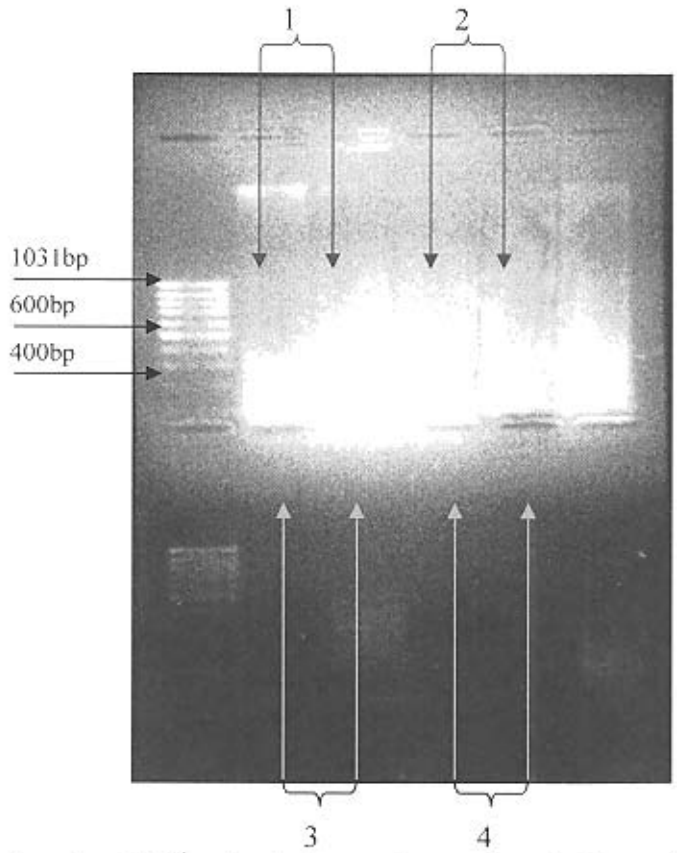

Figure 2: Electrophoresis of 2nd extracted sample, consisted of samples number 1-4 and each replicates. 1 = *Puntius bramoides*, 2 = *Puntius bulu*, 3 = *Puntius sealei* , 4 = *Puntius binotatus*.

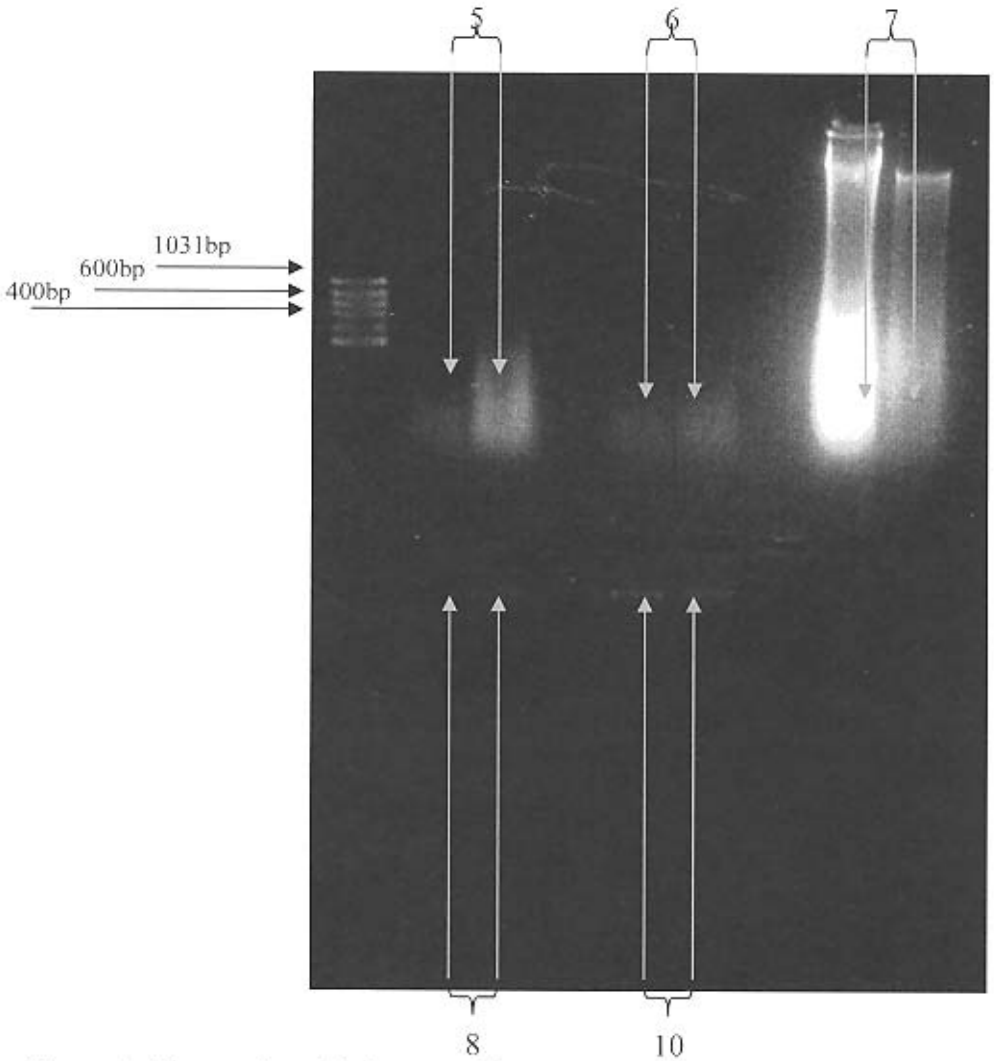

Figure 3. Electrophoresis picture of five extracted samples,5 = *Tor tambra*, 6 = *Tor douronensis*, 7 = *Nolissochillus strachgi*, 8 = *Barbodes coolingwoodi*, 10 = *Cyclocheilicthys apagon*.

Figure 3 showed the electrophoresis result of another five species of cyprinid fishes. Sample 5 and 6 produced smearing results while sample 8 and 10 showed a single DNA band and these samples was being proceeded to PCR-RAPD protocol. Unlike others, sample 7 produces a highly smearing band, possibly due to large amount of DNA extracted and/or the presence of large content of RNA.