
# PHYLOGENETIC ANALYSIS OF TWO FISH GENUS: *OSTEOCHILUS* AND *CYCLOCHEILICHTHYS*

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**(35034)**

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**(Aquatic Resource Science and Management)**
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UNIVERSITI MALAYSIA SARAWAK

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# PHYLOGENETIC ANALYSIS OF TWO FISH GENUS: *OSTEOCHILUS* AND *CYCLOCHEILICHTHYS*

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**This project report is submitted in partial fulfillment of the requirements for the Degree of Bachelor of Science with Honours**
**(Aquatic Resource Science and Management)**

**Faculty of Resource Science and Technology**
**UNIVERSITI MALAYSIA SARAWAK**
**2015**

**Declaration**

I hereby declare that no portion of the work referred in this project has been submitted in support of an application for another degree qualification of this or any other university or institution of higher learning.

**NADIA SYAHIRAH BINTI RADZALI**

Aquactic Resource Science and Management Programme

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## List of Abbreviations

| | |
|---|---|
| COI | Cytochrome C Oxidase Subunit I |
| PCR | Polymerase Chain Reaction |
| NJ | Neighbor-Joining |
| ML | Maximum-Likelihood |
| MP | Maximum-Parsimony |
| EDTA | Ethylenediaminetetraacetic acid |
| TBE | Tris-borate-EDTA |
| T | Thymine |
| G | Guanine |
| A | Adenine |
| C | Cytosine |

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**Phylogenetic Analysis of Two Fish Genus: *Osteochilus* and *Cyclocheilichthys***

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## Abstract

*Osteochilus* spp and *Cyclocheilichthys* spp are part of the Cyprinidae family. The aim of this study was to construct the phylogenetic tree of *Osteochilus* spp and *Cyclocheilichthys* spp using Cytochrome C Oxidase Subunit I (COI) gene and to study the genetic divergence of the *Osteochilus* spp and *Cyclocheilichthys* spp using COI gene. Samples were collected from Batang Kerang, Balai Ringin, Serian using three-layered gill net method. A total of three *Cyclocheilichthys* spp and one *Osteochilus* spp tissue samples were successfully processed for DNA analysis. The obtained sequences were aligned and edited using MEGA 6.06 program. The genetic divergence was analysed using Kimura 2-parameter distances. Neighbor-Joining, Maximum-Likelihood and Maximum-Parsimony trees were constructed to determine the phylogenetic relationships between species. The genetic divergence between species ranged from 0 % (between *C. apogon* individuals) to 0.163% (between *O. vittatus* and *C. armatus*). The phylogenetic analysis suggested that *Cyclocheilichthys* spp and *Osteochilus* spp are of different genus. This study was able to determine the monophyletic of *Cyclocheilichthys* spp and *Osteochilus* spp based on the COI gene.

Keywords: *Osteochilus* spp, *Cyclocheilichthys* spp, COI, phylogenetic analysis, genetic divergence

## Abstrak

*Spesis* Cyclocheilichthys *dan* Osteochilus merupakan sebahagian daripada keluarga Cyprinidae. *Objektif penyelidikan ini adalah untuk membentuk pokok filogenetik antara spesis* Cyclocheilichthys *dan* Osteochilus *dengan menggunakan gen cytochrome c oxidase I (COI) dan mengkaji jarak genetik antara* Cyclocheilichthys *dan* Osteochilus*. Sampel ikan diperolehi dari Batang Kerang, Balai Ringin, Serian menggunakan pukat tiga lapis. Tiga spesis* Cyclocheilichthys *dan satu* Osteochilus *sampel tisu telah berjaya diproses untuk analisis DNA. Penjajaran rangkaian DNA telah dilakukan menggunakan MEGA 6.06. Kimura 2-parameter telah digunakan untuk menganalisis jarak genetik antara spesis. Neighbor-Joining, Maximum-Likelihood dan Maximum-Parsimony telah digunakan untuk mengkaji hubungan filogenetik antara spesis. Jarak genetik antara spesis adalah dalam anggaran 0 % (antara individu C. apogon) dan 0.163% (antara O. vittatus dan C. armatus). Analisis filogenetik menunjukkan spesis* Cyclocheilichthys *dan* Osteochilus *merupakan dua genus berbeza. Kajian ini telah berjaya memperoleh hubungan monofiletik spesis* Cyclocheilichthys *dan* Osteochilus *berdasarkan gen COI.*

*Kata kunci: spesis* Osteochilus*, spesis* Cyclocheilichthys*, COI, analisis filogenetik, jarak genetik*

## 1.0 Introduction

The Cyprinidae family holds the largest number of freshwater fish with 200 genera and 2100 species (He *et al.*, 2008). Unfortunately the phylogenetics studies of cyprinids are poorly resolved in Malaysia (Esa *et al.,* 2012).

The two genera of the Cyprinid family that were focused on in this study are the *Osteochilus* spp and *Cyclocheilichthys* spp to construct the phylogenetic tree of *Osteochilus* spp and *Cyclocheilichthys* spp using Cytochrome C Oxidase Subunit I (COI) gene and to study the genetic divergence of the *Osteochilus* spp and *Cyclocheilichthys* spp using COI gene.

This study was conducted in Batang Kerang Floodplain, Balai Ringin, Sarawak. The fishes were collected using three-layered gill nets (12 cm, 14 cm, 14 cm mesh size). The samples were amplification of Cytochrome C Oxidase Subunit I (COI) gene with 675 bp. 3 *Cychlocheilichthys* spp (Cyclo1,Cyclo2,Cyclo3) and 1 *Osteochilus* spp (Osteo1) were successfully sequenced.

*Osteochilus* spp has compressed, oblong bodies. Their snouts are more or less pointed. Both their upper and lower lips are thick, fringed or papillate. They have either transverse, inferior or subinferior mouths. Plus, two rostral barbels. These fishes have long dorsal fin with 10-18 branched rays. Their lateral line complete and runs in the middle of the caudal fin. *Osteochilus hasselti* for example is the most common Cyprinids and are usually found in streams, ponds, mining pools and many more. *Osteochilus* spp feed on filamentous algae, detritus, plant parts, protozoa, aquatic insects and diatoms based on the study of their stomach content (Mohsin & Ambak, 1983; Hanjavanit & Sangpradub, 2012).

The *Cyclocheilicthys* spp has compressed and oblong bodies (Mohsin & Ambak, 1983). Their upper and lower lips are continuous around the corners of their mouths. They have 8 branched dorsal rays with the last simple dorsal ray denticulate on the posterior

border. They also have four, two or no barbels that are sometimes branched. Next, their lateral.

This two genus are morphologically alike and are found to be difficult to ditinguish. Hence, the use of molecular study is important to correctly distinguish them genetically. The study conducted is evidence that *Cyclocheilichthys* spp and *Osteochilus* spp are genetically different. The genetic divergence between species was low in the range of 0 % (between *C. apogon* individuals) to 0.163% (between *O. vittatus* and *C. armatus*). The distance between the two genus is low and indicates that they are closely related.

The phylogenetic analysis was done using the Neighbor-Joining, Maximum-Likelihood and Maximum-Parsimony methods. The relationships of *Cyclocheilichthys* spp and *Osteochilus* spp are confirmed as two different genus as it is supported by low bootstrap value . The outgroup used is *Channa striata* from the class Actinopterygii and family Channidae.

**2.0 Objectives**

2.1 To construct the phylogenetic tree of *Osteochilus* spp and *Cyclocheilichthys* spp using Cytochrome C Oxidase Subunit I (COI) gene.

2.2 To study the genetic divergence of the *Osteochilus* spp and *Cyclocheilichthys* spp using Cytochrome C Oxidase Subunit I (COI) gene.

## 3.0 Literature Review

### 3.1 Cyprinidae Family

Cyprinidae has the largest number of freshwater fish with 200 genera and 2100 species ( He et al., 2008). They can be found in abundance in almost every water body. In Malaysia, the fish of cyprinidae family is called many names such as *Sebarau, Temoleh, Jelawat* and *Kelah* They are utilized for food resources, fisheries, aquaculture and for ornamental purposes. Molecular studies are done on this family to better understand their relationships and to develop more accurate taxonomic classifications based on phylogeny (Yang *et al*., 2012).

The smallest species is also from this family with the longest known length of 12mm (Nelson, 2006). Furthermore, Cyprinidae contains species of extreme range of shapes, sizes, colours, behaviours and habitats (Rohde *et al*, 2009), causing it to be difficult to define. Many species exhibit sexual dimorphism (Rohde *et al*, 2009) and a variety of spawning habits (Mohsin and Ambak, 1983). Members build nests under stones, logs and ther heavy objects, where the male carries the responsibility to carry the eggs.

### 3.2 *Osteochilus* spp

Mohsin and Ambak (1983) characterized *Osteochilus* as having an oblong, compressed body. Their snout are more or less pointed and have thick upper and lower lips. They have inferior and subferior mouths (Figure 3.1).


Figure 3.1: Picture of *Osteochilus* spp

Examples of species are *Osteochilus hasselti, Osteochilus melanopleura* and *Osteochilus vittatus. Osteochilus* fishes feed on filamentous algae, detritus, plant parts, protozoa, aquatic insects and diatoms based on the study of their stomach content (Mohsin & Ambak, 1983; Hanjavanit & Sangpradub, 2012). They are locally called *Ikan Kelabau, Ikan Rong* and *Terbau. Osteochilus* spp. are distributed in Thailand, Sumatra, Borneo and Java.

In Indonesia, *Osteochilus vitatus* is important as a source of protein for the rural community and also urban. It has become a source of income for the rural people. Hence, the study of morphometric and molecular study are important for the domestication and culture (Azrita *et al.,* 2014).

### 3.3 *Cyclocheilichthys* spp

*Cyclocheilichthys* fishes have oblong, compressed body. The lower and upper lips are continuous around the corners of the mouth (Figure 3.2). Their barbels may be present or not. Lateral line of this fish ends in the mid-line of the caudal base (Mohsin and Ambak, 1983). They inhabit small streams, reservoirs, lakes, canals and ditches (Sim, 2002).

Figure 3.2: Picture of *Cychlocheilichthys* sp

### 3.4 Molecular Study

Species identifications are commonly done using morphological methods. However, the species identification and characterization of this family based on morphological characters and are sometimes found to be erroneous and environmentally affected (Malakar *et al.,* 2012). Some species may appear morphologically alike. Therefore, molecular analysis with high accuracy can verify these species as every species contains a unique gene sequence.

A phylogenetic analysis of cyprinids based on their Cytochrome C Oxidase Subunit I (COI) gene was carried out to clarify their relationships (Esa *et al.,* 2012). The reason for

this study is because cyprinids relationships are yet to be resolved. This study also includes *Cyclocheilichthys apogon.*

Molecular study of *Osteochilus* sp. was done by Azrita *et al.* (2014), which they studied the genetic variation among three *O. vittatus* populations using polymorphic DNA (RAPD) markers. The *O. vittatus* among the three populations have low genetic diversity. The low migrations of this freshwater fish was suggested to be the cause of low genetic diversity.

Most of molecular study of *Cyclocheilichthys* spp and *Osteochilus* spp are done separately. No study are found to determine the relationships of these two genus.

### 3.5 Mitochondrial DNA (mtDNA)

The mitochondria are cytoplasmic organelles found in the eukaryotic cells and they have their own DNA. which is physically separated from the cell's own DNA (Park & Moran, 1994). MtDNA is circular around 16 kb in length. There is no meiosis in the replication of mtDNA, unlike chromosomal DNA (Beaumont *et al.,* 2010). The advantages of using mtDNA in species identification or relationships are the mtDNA is inherited maternally as mitochondria are cytoplasmically inherited and there is no contribution of paternal mtDNA. Next, mtDNA evolves more quickly than most nuclear genes that allows for the identification of phylogenetic characters among even closely related species and populations (Kocher & Stepien, 1997;Park & Moran, 1994; Beaumont *et al.,* 2010).

Some of the gene sequence of mtDNA are cytochrome *b* (Cytb), Cytochrome C Oxidase Subunit I (COI) and D-loop (Beaumont *et al.,* 2010; Park & Moran, 1994). Cytochrome C Oxidase Subunit I (COI) gene is different in certain species and can be used to differentiate two morphologically alike species (Li *et al.,* 2011). COI can determine the relationships of fish species even when they are closely related.

## 4.0 Materials and Methods

### 4.1 Study Site

The samples were collected from Batang Kerang, Balai Ringin, Serian, Sarawak (Figure 4.1). The fish collection will be done in one day using the gill net collection method. Three-layered gill nets (12 cm, 14 cm, 14 cm mesh size) were placed at five stations.


Figure 4.1: Map of Batang Kerang, Balai Ringin, Sarawak

Batang Kerang has two types of water habitats which are the brown and black water. Floating vegetations such as *Hanguana malayana* and *Eichhornia crassipes* are common in the river. The river is surrounded by undisturbed riverine mixed-dipterocarp, swamp forest and marshland (A. Rahim *et al*. 2009)

The study of freshwater fishes and its composition in Batang Kerang by A. Rahim *et al*. (2009) shows that the brown water habitat is more diverse and abundant population of fishes. Results of the study show that the cyprinidae family is the most abundant.

### 4.2 Sample Collection

The fish collection were done using three layered gill nets (12 cm, 14 cm, 14 cm mesh size). The gill nets were positioned at five stations and left overnight. After approximately 14 hours, the fishes caught on the net were collected and immediately stored in a cooler box with ice. Then, the fishes were sorted out morphologically according to their species.

### 4.3 Laboratory Work

#### 4.3.1 Muscle Tissue Extraction

In the laboratory, the fishes obtained were measured for their weight, standard length and total length. Muscle tissue samples of *Osteochilus* spp and *Cyclocheilichthys* spp were extracted by cutting out a portion of the body tissue from the lateral side of the fish, wich was approximately 1cm in size. Then, the tissues were stored in a urine bottle and filled with 95% ethanol for tissue preservation. They were stored in -80 C.

### 4.3.2 Genomic DNA Extraction

The DNA extraction was performed according to the manufacturer's instructions (Wizard® Genomic DNA Purification Kit). First, 120 µl of 0.5M EDTA solution (pH 8.0) was added to 500 µl of nuclei lysis solution in a centrifuge tube and then chilled on ice. 0.5 cm of tissue was minced until fine and then transferred in a 1.5 microcentrifuge tube. 600 µl of EDTA and nuclei lysis mix was added into the tube containing the fish tissue. Next, 18 µL of 20mg/ml Proteinase K was added and the tube was incubated at 46°C until the tissue is completely digested.

At room temperature, the sample was added with 200 µl of protein precipitation solution. The sample was vigorously vortex and chilled in ice for 5 minutes. The sample was centrifuged for 10 minutes at 1350 rpm and a tight white pellet was formed. The supernatant which contains the DNA was carefully removed using a micropipette and transferred to a microcentrifuge tube containing 600 µl of room temperature isopropanol. The solution was mixed by inverting the tube several times until a white thread-like strands DNA was formed.

The sample was again centrifuged for 5 minutes 1350 rpm and a small white pellet was formed. The supernatant was removed. The DNA was washed with 600 µl of chilled 70% ethanol and centrifuged for 5 minutes at 1350 rpm at room temperature. The ethanol was removed by inverting it on an absorbent paper and left to air-dry for 15 minutes. Finally, 100 µl of DNA rehydration solution was added and incubated at 50°C for one hour.

### 4.3.3 Gel Electrophoresis

The 1% agarose gel was first prepared. 0.4 g of agarose powder was put in a beaker and added with 40 ml of 1x of Tris-borate-EDTA (TBE) buffer. The mixture was heated in a microwave for 1 minutes to dissolve the solution. The prepared agarose gel was poured into a gel tray with comb and was mixed with 1 µl of ethidium bromide (EtBr).

After the gel had solidified, the comb was removed and the tray was placed in the electrophoresis box. 1x TBE buffer was added to cover the gel. Next, 1 µl of 6X loading buffer was added to 1 µl of DNA samples. 1 Kb DNA ladder marker and 2 µl of samples was loaded on the gel. The gel was run for 1 hour at 75V. The DNA fragments were visualized under ultraviolet light with a UV transilluminator.

### 4.3.4 Polymerase Chain Reaction (PCR)

PCR was performed to amplify the target DNA sequence. The amplification of the Cytochrome c oxidase I (COI) gene was done using the following primers,

FishF1 (5′TCAACCAACCACAAAGACATTGGCAC3′)

FishR1 (5'TAGACTTCTGGGTGGCCAAAGAATCA3').

The volume of the PCR cocktails used are as follows (Table 4.1):

Table 4.1 : PCR cocktails for the amplification of COI gene of *Cyclocheilichthys* sp. and *Osteochilus* sp.

| Components | 1x reaction (µl) |
|---|---|
| Deionized Water ($ddH_2O$) | 17.55 |
| 5X Taq Buffer (Promega) | 2.25 |
| $MgCl_2$ | 1.5 |
| Mixed dNTP (10mM) (Promega) | 0.5 |
| Fish F1 Forward Primer (COI) | 1.0 |
| Fish R1 Reverse Primer (COI) | 1.0 |
| DNA Template | 1.0 |
| Taq Polymerase (Promega) | 0.2 |

The negative control and pipetting error were also included. The negative control contained the components in the PCR cocktails excluding the DNA template. The PCR was performed using the BioER Little Genius thermocycler, which the run using the PCR

profile in Figure 4.2. After the amplification, the PCR products were visualized through electrophoresis for 60 minutes at 75V.

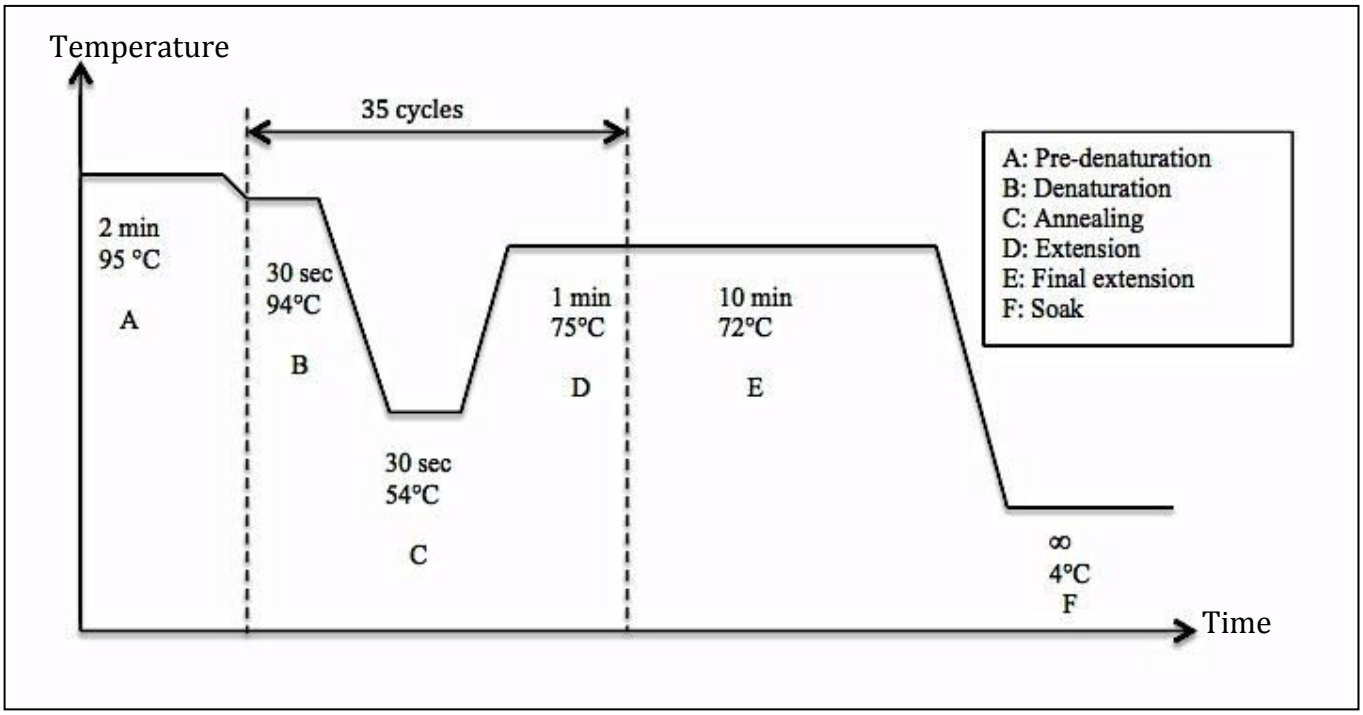


Figure 4.2: Polymerase chain reaction profile

### 4.3.5 DNA Purification

The PCR products were purified according to the manufacturer's manual of PCR Clean-Up System (Wizard® SV Gel and PCR Clean-Up System). First, the 25 µl PCR products were added with 25 µl of membrane binding solution. Next, the samples were transferred into an assembly of SV Minicolumn and collection tube. They were then incubated at room temperature at 1 minute. Then, the tubes were centrifuged at 1350 rpm for 2 minutes and the flowthrough was discarded.

700 µl of membrane wash solution (ethanol added) was added to the samples and centrifuged again at 1350 rpm for 2 minutes. The flowthrough was discarded. Washing of samples were repeated by adding 500 µl and then centrifuged for 10 minutes. Next, the

collection tubes were emptied and recentrifuged for 2 minutes to evaporate any residual ethanol.

The Minicolumns were carefully transferred to new 1.5 ml microcentrifuge tubes. 50µl of Nuclease-Free Water was added and incubated at room temperature for 1 minute. Then, the tubes were centrifuged at 1350 rpm for 2 minutes. Finally, the minicolumns were discarded and the purified DNAs were stored at -20°C.

### 4.4 Mitochondrial DNA Data Analysis

The PCR products of *Cyclocheilichthys* spp and *Osteochilus* spp were sent to 1st BASE for sequencing. The sequenced products were confirmed for their species using Basic Local Alignment Search Tool (BLAST) that is available in MEGA 6.06 (Molecular Evolutionary Genetic Analysis 6.06). The sequences were compared with the sequences from the GenBank (Refer Appendix 1). Next, CLUSTAL W was used to align the sequences.

The MEGA 6.06 program was used to determine the nucleotide composition. The genetic divergence of the samples were conducted using Kimura 2-parameter model ,which is integrated in MEGA 6.06. This program also was used to construct the Neighbour-Joining (NJ), Maximum-Likelihood, and Maximum-Parsimony (MP) tree.

## 5.0 Results

### 5.1 Genomic DNA Extraction

Figure 5.1 shows the DNA extraction of some of representatives of *Cyclocheilichthys* spp and *Osteochilus* spp. Bands in Lane 2, Lane 3 and Lane 4 and 5 are faint. Lane 6 shows a bright band. The faint bands may be caused by low concentration of DNA.

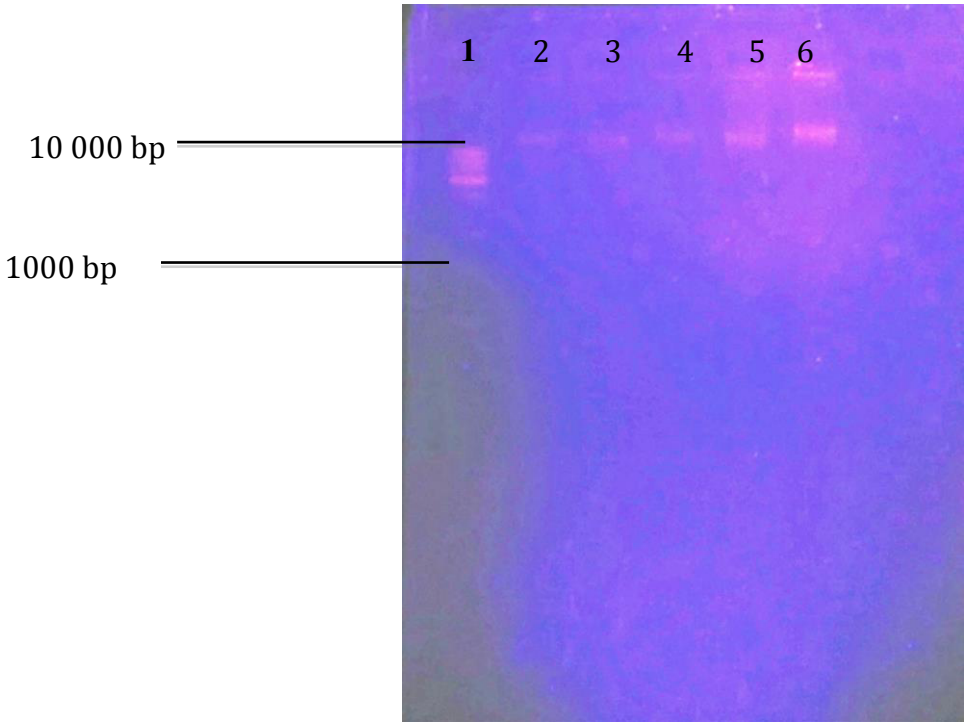


Figure 5.1: Agarose gel photograph showing genomic DNA extraction product of *Cyclocheilichthys* sp. and *Osteochilus* sp. using Lane 1-1kb ladder (Promega), Lane 2- *Cyclochelichthys* sp., Lane 3-*Cyclocheilichthys* sp., Lane 4- *Cyclochelichthys* sp., Lane 5- *Osteochilus* sp. , Lane 6- *Osteochilus* sp.