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

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Bachelor of Science with Honours
(Aquatic Resource Science and Management)
2015

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

Faculty of Resource Science and Technology

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Acknowledgement

I would like to express my gratitude to Dr Fazimah Aziz, my supervisor for guiding me during the completion of this final year project. Thank you also to Dr Khairul Adha A. Rahim as my co-supervisor. Their knowledge and guidance was tremendous help in conducting this project.

Next, I would also like to thank the Mr. Zaidi Ibrahim, Ms. Lucy and Mr. Zulkifli Ahmad for their assistance for their help during laboratory work and field work. My gratitude goes to all my helpful coursemates for their support and guidance.

My appreciation also goes to my family especially my parents for supporting me emotionally and financially. Lastly, I thank everyone that directly or indirectly contributed to the completion of this final year project.

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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.

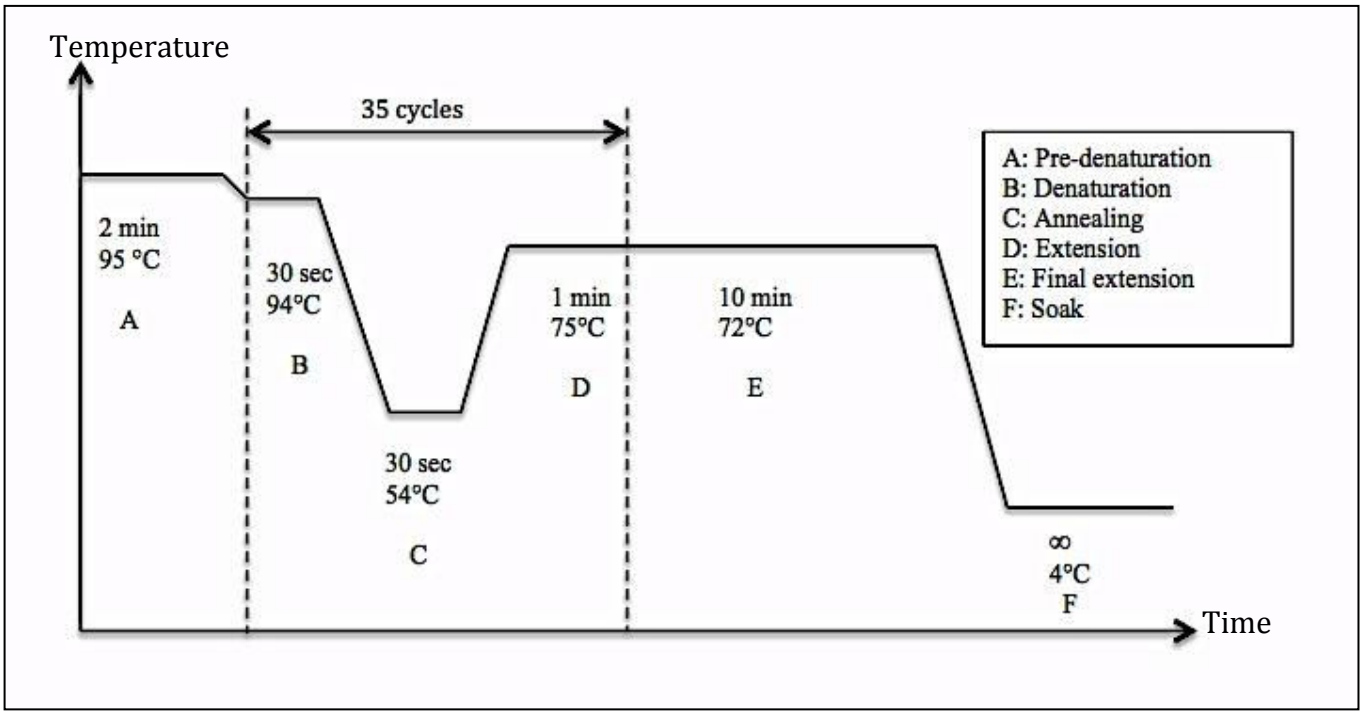

Figure 4.2: Polymerase chain reaction profile

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.

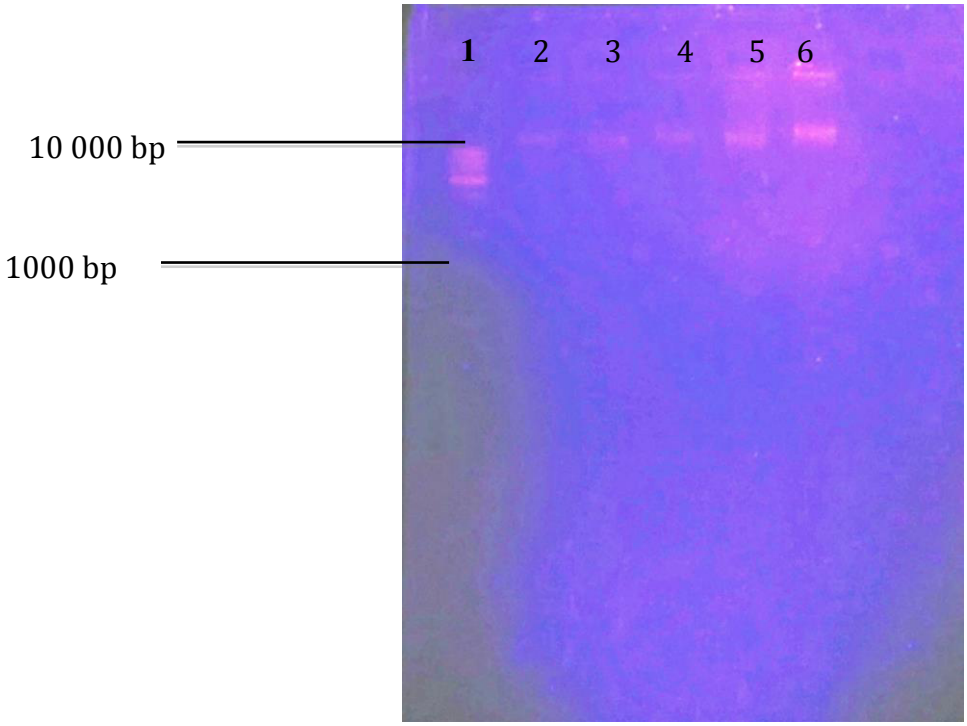

Figure 5.1: Agarose gel photograph showing genomic DNA extraction product of *Cyclocheilichthys* sp. and *Osteochilus* sp. using Lane 1-1kb ladder (Promega), Lane 2- *Cyclochelichthys* sp., Lane 3-*Cyclocheilichthys* sp., Lane 4- *Cyclochelichthys* sp., Lane 5- *Osteochilus* sp. , Lane 6- *Osteochilus*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

5.2 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) was performed to amplify Cytochrome C Oxidase Subunit I (COI) gene of the samples. PCR products showed satisfactory results with no smearing. All of the lanes show strong bands with no smearing (Figure 5.2).

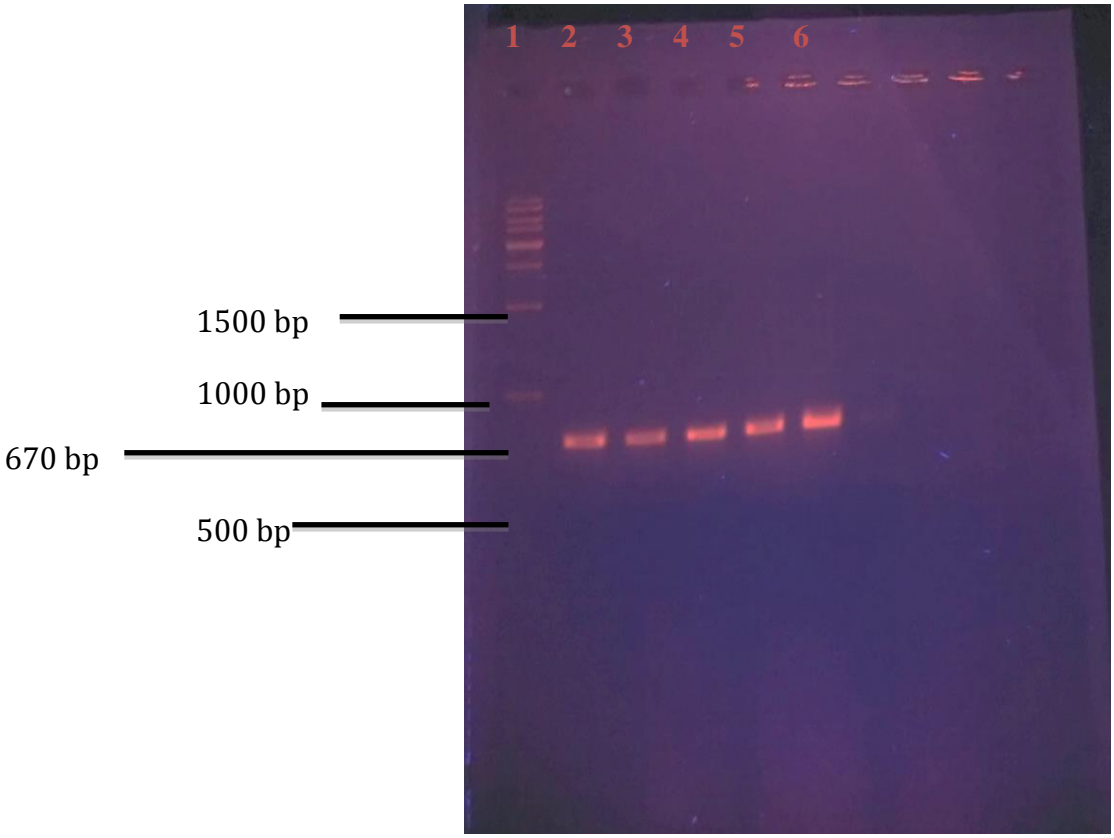

Figure 5.2: Agarose gel photograph showing polymerase chain product for tissue samples of *Cyclocheilichthys* sp. and *Osteochilus* sp.. Lane 1-1kb ladder, Lane 2- *Cyclocheilichthys* sp., Lane 3- *Cychlocheilichthys* sp., Lane 4- *Cyclocheilichthys* sp., Lane 5- *Osteochilus* sp., Lane 6-*Osteohilus* sp.

5.3 Sequence Analysis

A total of three *Cyclocheilichthys* spp and one *Osteochilus* spp were successfully sequenced. The sequence alignment of *Cyclocheilichthys* sp. produced 657 bp with conserved sites of 657 (100%), 0 variable sites, 0 parsimony-informative sites and 0 singletons.

The alignment of *Cyclocheilichthys* spp from Balai Ringin and the GenBank sequences produced 595 bp with 533 (89.6%) conserved sites, 62 (10.4%) variable sites, 12 (2.0%) parsimony-informative and 50 (8.4%) singletons. The percentage of nucleotide composition (Table 5.2) are 27.9% Thymine (T), 27.4 % Cytosine (C), 28.2% Adenine (A), 16.5% Guanine (G).

The *Osteochilus* sp. sequence from Balai Ringin has 674 bp. After the incorporation of *Osteochilus* spp GenBank sequences, the alignment produced 635 bp with 515 (81.1%) conserved sites, 120 (18.9%) variable sites, 41 (6.5%) parsimony-informative sites and 79 (12.4%) singletons. Their nucleotide composition percentage (Table 5.3) consists of 28.7% Thymine (T), 26.7% Cytosine (C), 27.6% Adenine (A) and 17.0% Guanine (G).

Furthermore, the aligned sequence of all 11 sequences produced 595 bp with 450 (75.6%) conserved sites, 145 (24.4%) variable sites, 93 (15.6%) parsimony-informative and 52 (8.7%) singletons. Based on Table 5.4, the percentage of nucleotide composition are 28% Thymine (T), 27.3% Cytosine (C), 27.9% Adenine (A) and 16.8% Guanine (G). Next, the nucleotide composition (Table 5.4) including the outgroup *Channa striata* consists of 28% Thymine (T), 27.5% Cytosine (C), 27.7% Adenine (A), and 16.8% Guanine (G).

The BLAST results (Table 5.1) of all the *Cytocheilichthys* spp showed a 99% match to the *Cyclocheilichthys apogon* from the GenBank (accession No. KP712065.1) with 89% query coverage. The *Osteochilus* spp was also confirmed to the GenBank and the BLAST result indicated a 90% match and 94% query coverage towards *Osteochilus waandersii* (accession No. KC631203.1). Sequences from the GenBank were obtained to study the relationships of the two genera more successfully.

Table 5.1: Summary of BLAST results of *Cytochelichthys* spp and *Osteochilus* spp COI gene sequence.

Samples	BLAST result	Accesion No.	Max. identical	E value
Cyclo1	*C. apogon*	KP712065.1	99%	0.0
Cyclo2	*C. apogon*	KP712065.1	99%	0.0
Cyclo3	*C. apogon*	KP712065.1	99%	0.0
Osteo1	*O. waandersii*	KC631203.1	90%	0.0

Table 5.2: Percentage of nucleotide composition of *Cyclocheilichthys* spp

No.	Species	Code name	Nucleotide composition				
			T	C	A	G	Total
1	*C. apogon*	Cyclo1	27.7	27.6	28.1	16.6	595.0
2	*C. apogon*	Cyclo2	27.7	27.6	28.1	16.6	595.0
3	*C. apogon*	Cyclo3	27.7	27.6	28.1	16.6	595.0
4	*C. apogon*	KP712065.1	28.1	27.2	28.2	16.5	595.0
5	*C. jantochir*	HM536907.1	28.1	27.2	28.4	16.3	595.0
6	*C. armatus*	HM546926.1	27.9	27.4	28.6	16.1	595.0
Average			27.9	27.4	28.2	16.5	595.0

Table 5.3: Percentage of nucleotide composition of *Osteochilus* spp

No.	Species	Code name	Nucleotide composition				
			T	C	A	G	Total
1	*O. waandersii*	Osteo1	27.9	28.2	26.9	17.0	635.0
2	*O. waandersii*	KC631203.1	28.8	26.1	28.5	16.5	635.0
3	*O. waandersii*	JX074190.1	28.7	26.3	27.9	17.2	635.0
4	*O. vittatus*	JX074144.1	29.0	26.8	26.8	17.5	635.0
5	*O. salsburyi*	HM536883.1	29.3	26.1	27.7	16.9	635.0
Average			28.7	26.7	27.6	17.0	635.0

Table 5.4: Percentage of nucleotide composition of *Cyclocheilichthys* sp. and *Osteochilus* spp

No.	Species	Code name	Nucleotide composition				
			T	C	A	G	Total
1	*C. apogon*	Cyclo1	27.7	27.6	28.1	16.6	595.0
2	*C. apogon*	Cyclo2	27.7	27.6	28.1	16.6	595.0
3	*C. apogon*	Cyclo3	27.7	27.6	28.1	16.6	595.0
4	*C. apogon*	KP712065.1	28.1	27.2	28.2	16.5	595.0
5	*C. jantochir*	HM536907.1	28.1	27.2	28.4	16.3	595.0
6	*C. armatus*	HM546926.1	27.9	27.4	28.6	16.1	595.0
7	*O. waandersii*	Osteo1	27.2	28.7	27.1	17.0	595.0
8	*O. waandersii*	KC631203.1	28.1	26.7	28.6	16.6	595.0
9	*O. waandersii*	JX074190.1	27.9	26.9	27.9	17.3	595.0
10	*O. vittatus*	JX074144.1	28.4	27.2	26.7	17.6	595.0
11	*O. salsburyi*	HM536883.1	28.7	26.6	27.6	17.1	595.0
12	*Channa striata*	KJ937450	28.7	29.4	24.9	17.0	595.0
	Average without outgroup		28.0	27.3	27.9	16.8	595.0
	Average with outgroup		28.0	27.5	27.7	16.8	595.0

5.4 Genetic Divergence

The genetic divergence analysis among six *Osteochilus* spp and five *Cyclocheilichthys* spp was conducted using the Kimura 2-parameter model to estimate the pairwise divergence based on the COI gene sequences. 12 nucleotide sequences were analysed including the outgroup, *Channa striata*

Based on Table 5.5, the highest genetic divergence is between *O. vittatus* (JX074144.1) and *C. armatus* (HM536926.1) with 16.3% divergence, followed by *O. waandersii* (Osteo1) and *C. armatus* (HM536926.1) with 16.0%. The lowest genetic divergence is between the *C. apogon* (Cyclo1, Cyclo2, Cyclo3) individuals with 0%. *C. apogon* (KP712065.1) and Cyclo1, Cyclo2 and Cyclo3 have a genetic divergence of 0.5%.

Osteo1 shows a genetic divergence of 11.2% and 11.8% with KC6313203.1 and JX074190.1 respectively. The overall average of genetic divergence is 0.129% including the outgroup, *Channa Striata*. Plus, the genetic divergence between *Osteochilus* spp and *Cyclocheilichthys* spp are 11.2% to 16.3%.

Table 5.5 : Estimates of Evolutionary Divergence between Sequences

No.	Species	1	2	3	4	5	6	7	8	9	10	11	12
1	*C. apogon* (Cyclo1)	-											
2	*C. apogon* (Cyclo2)	0.000	-										
3	*C. apogon* (Cyclo3)	0.000	0.000	-									
4	*C. apogon* (KP712065.1)	0.005	0.005	0.005	-								
5	*C. janthochir* (HM536907.1)	0.044	0.044	0.044	0.046	-							
6	*C. armatus* (HM536926.1)	0.087	0.087	0.087	0.089	0.087	-						
7	*O. waandersii* (Osteo1)	0.141	0.141	0.141	0.143	0.141	0.160	-					
8	*O. waandersii* (KC631203.1)	0.116	0.116	0.116	0.118	0.112	0.142	0.112	-				
9	*O. waandersii* (JX074190.1)	0.124	0.124	0.124	0.122	0.114	0.147	0.118	0.012	-			
10	*O. vittatus* (JX074144.1)	0.134	0.134	0.134	0.132	0.144	0.163	0.123	0.130	0.128	-		
11	*O. salsburyi* (HM536883)	0.138	0.138	0.138	0.138	0.122	0.159	0.125	0.062	0.069	0.138	-	
12	*Channa striata* (KJ937450)	0.267	0.267	0.267	0.270	0.275	0.252	0.246	0.238	0.245	0.269	0.265	-

5.5 Phylogenetic Analysis

The Neighbor-Joining (NJ) tree, Maximum-Likelihood (ML) tree and Maximum-Parsimony (MP) tree show similar tree topology of the COI gene. The *Osteochilus* spp and *Cyclocheilichthys* spp are separately grouped into two monophyletic clades.

NJ tree shows that Cyclo1, Cyclo2 and Cyclo3 with *C. apogon* (KP712065.1) with 100% bootstrap value (Figure 5.3). Maximum-Likelihood and Maximum-Parsimony tree also show high bootrap value within *C. apogon* with 90% and 99% respectively. This result confirms that the samples Cyclo1, Cyclo2, Cyclo3 and *C. apogon* (KP712065.1) are one species.

Osteo1 is paraphyletic with *O. waandersii* and *O. Salsburyi*. Osteo1 is also grouped close to the other *Osteochilus* spp than *Cyclocheilichthys* spp (Figure 5.4). The phylogenetic trees highly support the *O. salsburyi* with *O. waandersii* with bootstrap value 99% (NJ), 96% (ML and MP).

Similar to Neighbor-Joining, Maximum-Likehood and Maximum-Parsimony (Figure 5.5) show that all the species forms one large monophyletic tree. Two monophyletic group of *Osteochilus* spp and *Cyclocheilichthys* spp are formed supported by low bootstrap value (34% to 47%).

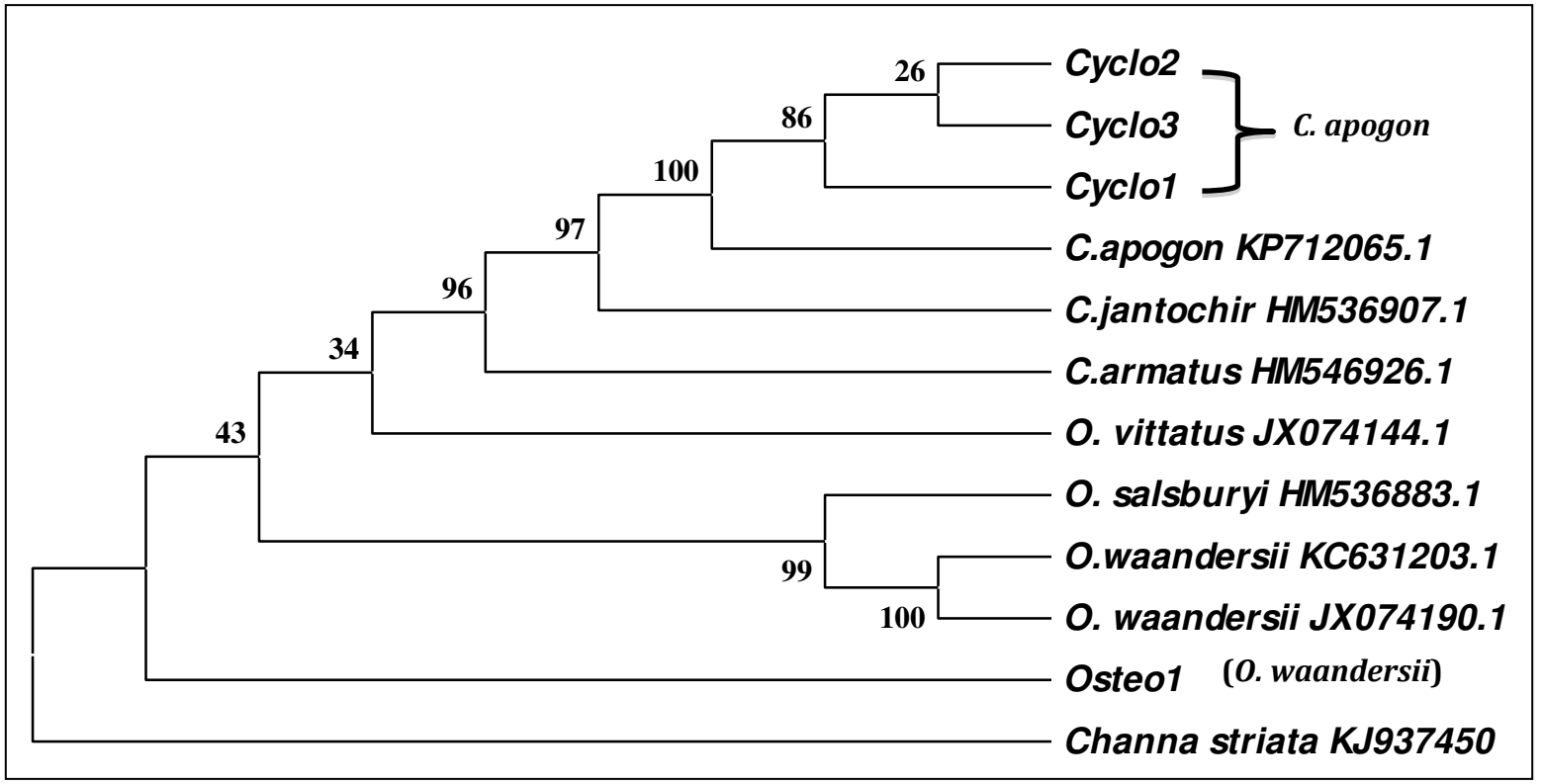

Figure 5.3: Neighbor-Joining (NJ) tree showing the relationships among cytochrome c oxidase I (COI) gene of *Osteochilus* sp. and *Cyclocheilichthys* sp. rooted with *Channa Striata.* The number on the branhes indicate bootstrap value of Neighbor-Joining based on 500 replications.

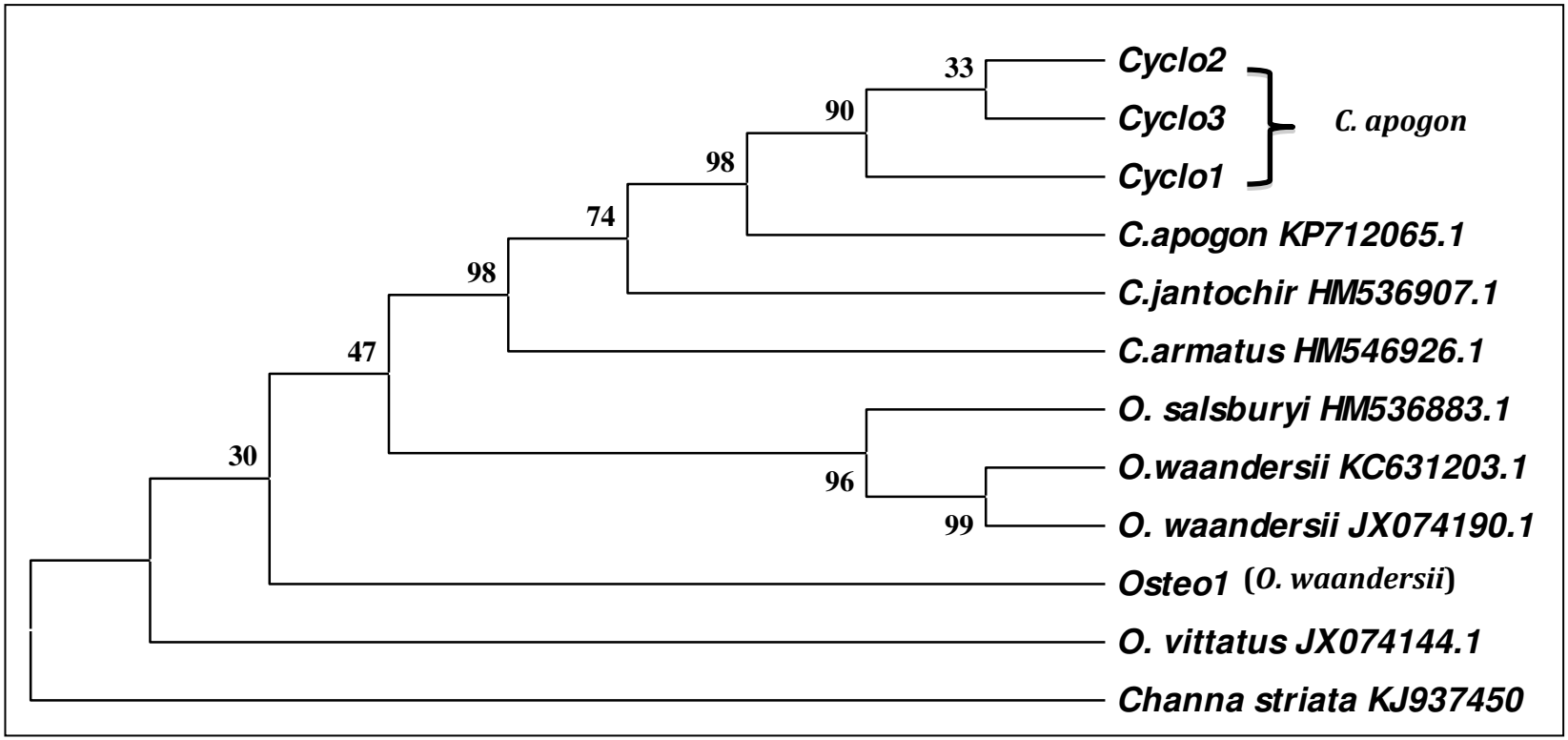

Figure 5.4: Maximum-Likelihood tree to show relationships among the COI gene of *Cyclocheilichthys* sp. and *Osteochilus* sp. rooted with *Channa striata.* the values on the branches are percentages of bootstrap value based on 500 replications.

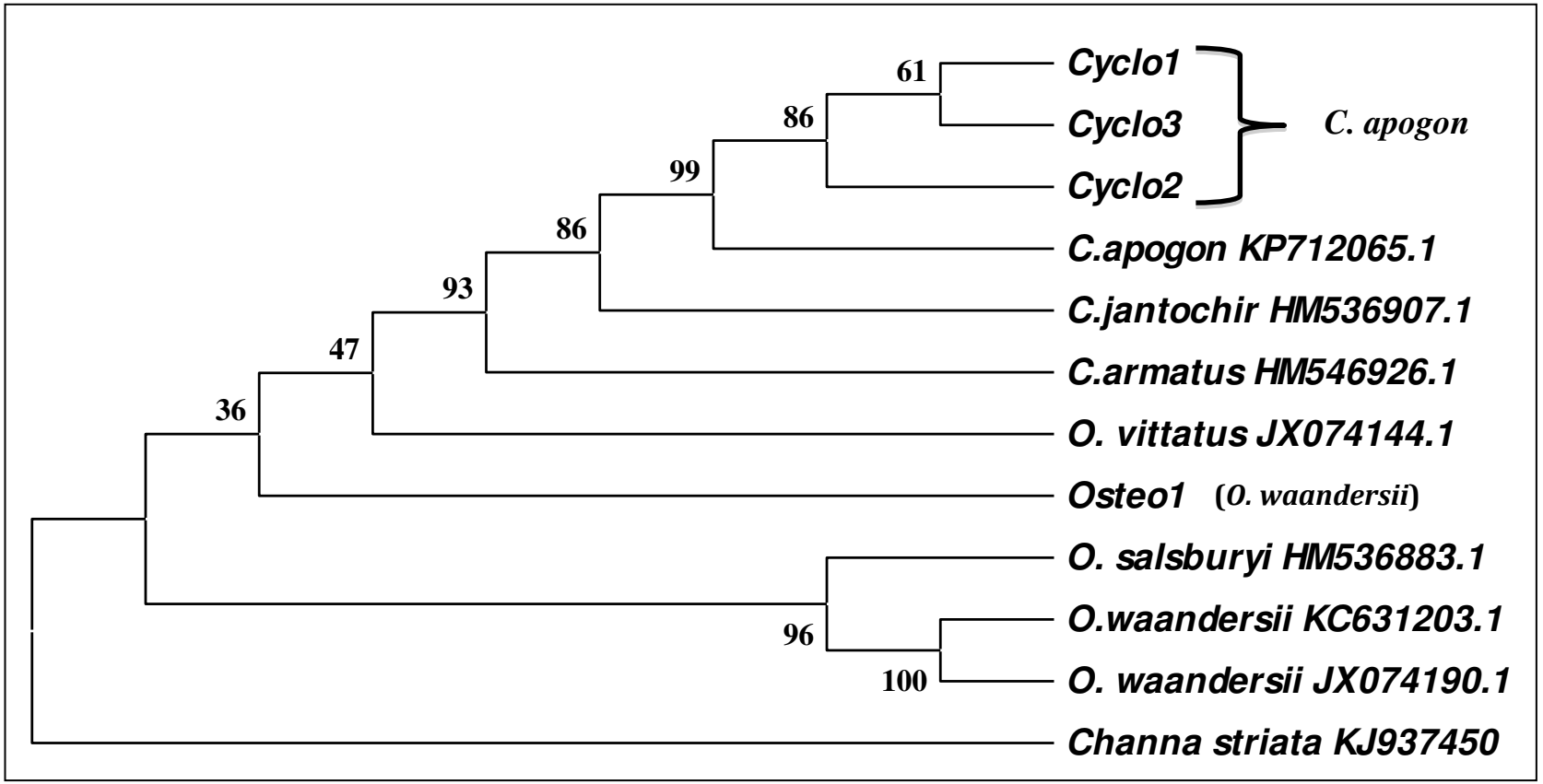

Figure 5.5: Maximum-Parsimony tree to show the relationships among COI gene of *Cyclocheilichthys* sp. and *Osteochilus* sp. rooted with *Channa striata.* The numbers on the branches shows the bootstrap values based on 500 replications.

6.0 Discussion

The faint DNA bands obtained from DNA extraction are caused by the low concentration of DNA loaded on the gel. A better band can be obtained by increasing the amount of DNA. A problem that occurred during the DNA extraction was the muscle tissue was left too long at high temperature in the waterbath, which caused the DNA to degrade.

Four tissue samples (Cyclo1, Cyclo2, Cyclo3, Osteo1) were successfully matched to the sequence in the GenBank. The Neighbour-Joining, Maximum-Likelihood and Maximum-Parsimony constructed have similar tree topology in showing the relationships of Cytochrome C Oxidase (COI) gene. There are two monophyletic groups of *Cyclocheilichthys* spp and *Osteochilus* spp. The bootstrap value between them is supported by low bootstrap value (34% to 47%). It is clear that the *Cyclocheilichthys* spp and *Osteochilus* spp are genetically different despite having similar morphology.

Based on the BLAST results (Refer Table 5.1), the *Cyclocheilichthys* spp individuals from Balai Ringin were confirmed to have a 99% match to *C. apogon* (Accession No. KP712065.1) sequence from the GenBank. Cyclo1, Cyclo2 and Cyclo3 from Balai Ringin has a slight genetic distance (0.5%) with *C. apogon* (KP712065.1). The reason may be because *C. apogon* (KP712065.1) is from a different locality. However, they are grouped in one clade, supported by 100% bootstrap value. They are one species as agreed in the Barcoding of Australia's Fish Species (Ward *et al.,* 2005) that conspecific individuals have a genetic divergence between 0 to 14% with a mean of 0.39%.

Next, Oteo1 from Balai Ringin was a match to *O. waandersii* with a match of 90%. However, the phylogenetic tree shows that *O. waandersii* is paraphyletic to *O. waandersii* and *O. salsburyi* (Refer Figure 5.3). They are from different populations and evolutionary changes may occured.

The genetic distances between individuals of the two genera are ranged from 11.2% to 16.3% with 8% mean distance. The congeneric divergence are within the range stated in Australia's Marine Waters (Ward *et al.,* 2005) with congeneric average of 9.93%.

7.0 Conclusion

The Phylogenetic tree of COI gene gave similar tree topology which *Osteochilus* spp and *Cyclocheilichthys* spp are separately grouped supported by 34% (NJ), 47% (ML) and 47% (MP) bootstrap value. Next, the result of phylogenetic tree confirmed the separation of *Osteochilus* spp and *Cyclocheilichthys* spp based on morphology. The sequence divergence between *Osteochilus* spp and *Cyclocheichthys* spp are between 11.2% to 16.3% (Refer Table 5.5).

8.0 References

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9.0 Appendix

Appendix 1 : GenBank Sequences

Accession number	Species
KP712065.1	*Cyclocheilichthys apogon*
HM536907. 1	*Cyclocheilichthys janthochir*
HM536926. 1	*Cyclocheilichthys armatus*
KC631203.1	*Osteochilus waandersii*
JX074190.1	*Osteochilus waandersii*
JX074144.1	*Osteochilus vittatus*
HM536883.1	*Osteochilus salsburyi*
KF511508.1	*Channa striata*