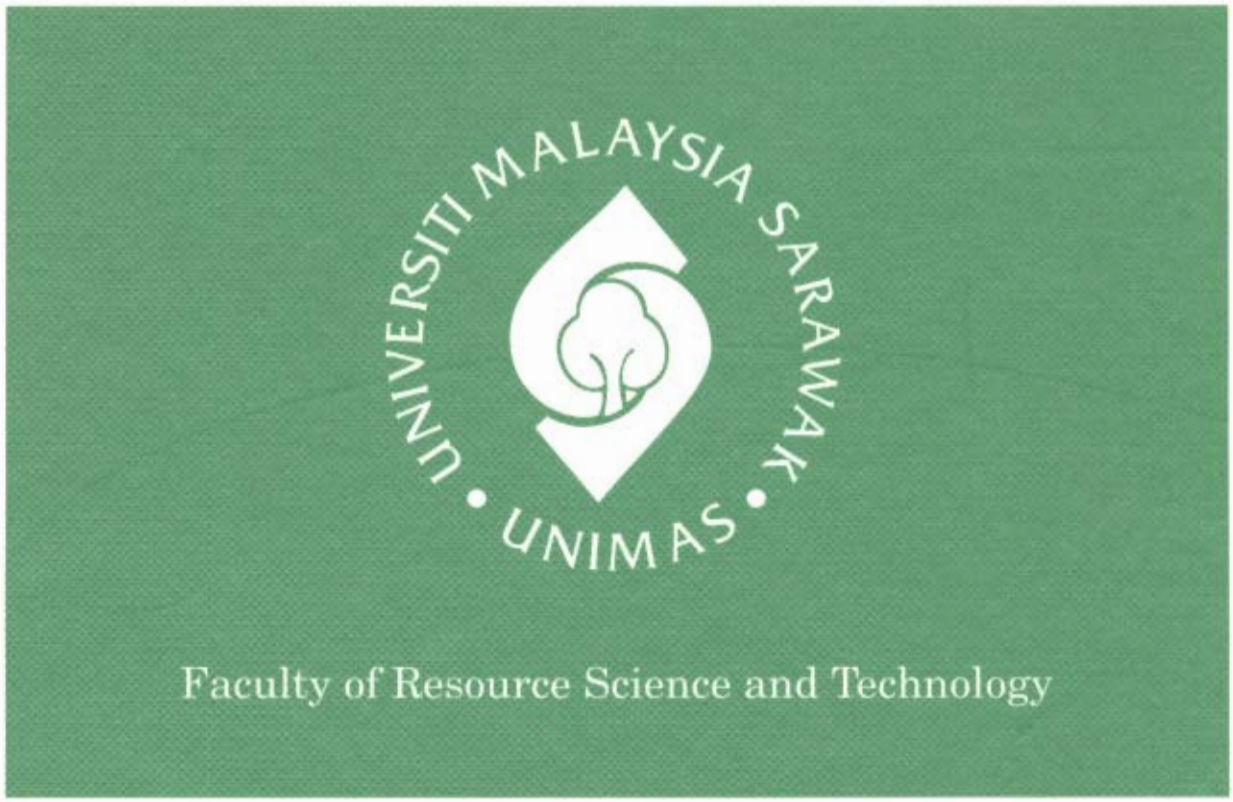

AN EXPANSION OF THE MOLECULAR PHYLOGENIES OF BABBLERS (FAMILY: TIMALIIDAE) USING MITOCHONDRIAL DNA CYTOCHROME OXIDASE I (COI) GENE

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Bachelor of Science with Honours
(Animal Resource Science and Management)
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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 qualification of this or any other university or institution of higher learning.

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An Expansion of the Molecular Phylogenies of Babblers (Family:Timaliidae) using Mitochondrial Cytochrome Oxidase I (COI) Gene

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ABSTRACT

Cytochrome Oxidase c subunit I (COI) gene of the mitochondrial DNA (mtDNA) sequences data had been used to investigate the phylogenetic relationship of the selected babbler species from the family Timaliidae. The samples were taken from two different locations in Sarawak namely Jambusan (Bau) and Kubah National Park (Kuching). Five blood and five tissue samples were obtained from both locations. Neighbor-joining (NJ) and Maximum Parsimony (MP) methods were used to construct the phylogenetic trees. Phylogenetic tree showed the selected babbler species evolved into two major clades. The first clade consisted of genus *Macronous* and *Stachyris*. The second clade consisted of genus *Malacopteran*, *Pellornuem* and *Trichastoma*. The result showed that the relationship of the selected species of babbler could be determined by using COI gene as a specific genetic marker.

Key words: Mitochondrial DNA (mtDNA), cytochrome oxidase I (COI) gene, Timaliidae and phylogenetic.

ABSTRAK

Salah satu daripada data jujukan yang terdapat dalam mitokondria DNA (mtDNA) iaitu sitokrom oksida c subunit I (COI) telah digunakan untuk mengkaji hubungan filogenetik di kalangan spesies babbler yang terpilih. Kesemua sampel telah diperolehi daripada dua tempat yang berbeza di Sarawak iaitu Jambusan (Bau) dan Taman Negara Kubah (Kuching). Sebanyak lima sampel darah dan lima sampel tisu telah diperolehi daripada kedua tempat tersebut. Pokok filogenetik telah dibina menggunakan dua kaedah iaitu kaedah 'Neighbor-joining (NJ)' dan 'Maximum Parsimony (MP)'. Pokok filogenetik di kalangan spesies babbler yang terpilih ini menunjukkan ia berevolusi kepada dua kumpulan utama. Kumpulan pertama terdiri daripada genus Macronous dan Stachyris manakala kumpulan kedua terdiri daripada genus Malacopteran, Pellornuem dan Trichastoma. Keputusan kajian ini menunjukkan bahawa hubungan di kalangan spesies babbler yang terpilih boleh ditunjukkan dengan menggunakan petunjuk genetik yang baik iaitu sitokrom oksida (COI).

Kata Kunci: Mitokondria DNA (mtDNA), gen sitokrom oksida I (COI), Timaliidae dan pokok filogenetik.

1.0 INTRODUCTION

Systematic is the comparative biology known as scientific study of the kinds and diversity of organisms (Henning, 1966; Wiley, 1981). All relationships among the organism can be classified by producing the classifications and identifications of keys (Minelli, 1993). The most basic step in systematic is comparing the similarity or differences of individual organism (Wiley, 1981). According to Wiley (1981), the consistent evidences to build biological classification is through the phylogenetic information because the evolution has occurred in the species. Evolution had been defined as a change in genetic composition of population by adaptation and constraints (Minnelli, 1993: Avise, 1994). Thus, we need to study evolution from a molecular perspective because their approaches are based on the DNA information that transmitted through the generation so that relative confidences only can be placed on molecular-based phylogenetic conclusions.

Molecular systematic is the detection, description and the explanation of molecular biological diversity (Hills *et al.*, 1996). It covers the phylogenetic relationships among group organisms and produces classifications among populations, species or higher taxa (Wiley, 1981). The approaches of molecular systematic now come in many ways without sacrificing the individuals. The molecular studies can settle many issues and confusing problems that occur in phylogeny classifications because it works based on the DNA that is the sources of the genetic information. There are three advantages in molecular methods; the size of the data sets potentially available for comparisons, the broad phylogenetic limits within which to extend the comparisons and theindependence of molecular traits from external influences (Avise, 1994). The classifications

that result from the phylogenetic analysis are important for the evolutionary studies because the result can be used to describe the relationship between the organisms (Wiley, 1981).

Phylogenetic study is used to classify the species and lead to the construction of the tree of phylogeny. From this information, the distances can be estimated through the branching of genetic changes from ancestral gene to ancestral species and species to subspecies (Hussen, 2005). According to Sibley and Alhquist (1995), the plants and animals of earth evolved from a single origin of life and had a single evolutionary or phylogeny. For phylogenetic analysis of vertebrates, the mitochondrial genome had been an important of DNA sequences data because it is important to accelerate the taxonomic classification through the comparison of sequences (Vilaca *et al.*, 2006). This is because mtDNA has a number of desirable properties that useful in the analysis (DeFilippis and Moore, 2000). Large scale of DNA barcoding can identify and discriminate known species and probably can indicate new species within previously unstudied taxa (Vilaca *et al.*, 2006). According to Scott and Graham (2001), the comparative analyses between gene sequences are a powerful means of allocating identities to organism and studying population structure and relatedness. The sequencing allows the development of diagnostic of individuals so evolutionary history can be predicted. According to Sibley and Alhquist (1995), DNA comparison has an evidence of phylogeny because the genetic information encoded in base sequences and genetic evolution recorded in the form of changes in base sequences over the time. Besides that, phylogenetics approaches also can be used in conservation biology because it is highly informative since the phylogenetic characteristic cannot prove the important differences that distinguish populations and directly concern about the biodiversity (Weaver and Hedrick, 1992).

Several approach methods in molecular systematic are by their laboratory techniques. According to Avise (1994), mitochondrial approaches dominated the molecular systematic during late 1970s and 1980s while the important part is the introduction of polymerase chain reaction (PCR) for in vitro amplifications of specific DNA fragments. Because of the PCR can amplify particular DNA segment of the tiny amount of tissues, it has extended the molecular applications to a much wider biological arena (Avise, 1994; Zhang and Hewitt, 1996).

1.1 Problem Statement

The understanding of the phylogenetic relationship between taxa is very unclear (Beecher, 1953; Seutin and Bermingham, 1997). By studying the phylogenetic relationship, it can determine the taxonomic classification (Hussen, 2005). Most of the systematic study used the morphological characteristic as an evidence for relationship but over the last 20 to 30 years, the molecular study focused toward phylogenetic researches (Miyomoto and Cracraft, 1991). The morphological characteristics are not sufficient to prove the relationship because the parameter had adapted to survive in the new environment (Sibley and Alhquist, 1995).

Babblers are one of the unclear groups that are difficult to distinguish by their morphological characteristic because they look similar (Hussen, 2005). Taxonomic classification of certain systematic relation and cryptic species of babbler cannot be explained and defined through morphological and anatomical characteristics alone. Recently, a phylogenetic study was conducted by Hussen and Rahman (2005) using cytochrome oxidase I (COI) as a specific genetic marker which focused on three species of babblers. Another study was conducted by Ursula (2005) who used the same species but with different specific genetic marker of 16S rRNA.

However, the result of COI is much better because it has a higher ability to detect the variation between species compare to 16S rRNA (Hussen and Rahman, 2005).

This study was designed to evaluate more species of babblers and therefore to further extend on the phylogenetic study of babblers using COI.

1.2 Objectives

The objectives of this study were:

- to expand the molecular phylogenies of the babbler's species.
- to determine the evolution and relationship among the selected species in family Timaliidae.

2.0 LITERATURE REVIEW

2.1 Distributions

According to Gill (1990), class aves was classified into 30 orders, 174 families, 2044 genera and 9021 species in the world. Most passerines were in small size and land-dwelling birds that eat insects, seeds, fruits or nectar (Sibley and Alhquist, 1995). In Malaysia there are 702 species while in Borneo there are 580 species of birds comprise of 12 orders, 72 families which about 300 species are typical of the evergreen rainforest (Davidson *et al.*, 1989 ; Davidson and Fook, 2001).

Babbler is classified under the family Timaliidae. There are 35 species of babblers occuring in Borneo (Smythies, 1999). According to Smythies (1981) and MacKinnon and Phillipps (1993) babblers are divided into five distinct groups; jungle-babblers (subfamily Pellorneinae; *Pellorneum, Trichastoma, Malacocincla* and *Malacopteran*), scimitar and wren-babblers (subfamily Pomatorhinae; *Pomatorhinus, Rimator, Ptilocichla, Kenopia, Napothera* and *Pneopyga*), tree-babblers and tit-babblers (subfamily Timaliinae; *Stachyris, Macronous,* and *Timalia*), song-babblers (subfamily Turdoidinae; *Garrulax, Leiothrix, Pteruthius, Alcippe, Crocias, Heterophasia* and *Yuhina*) and ground babblers (subfamily Cinclosomatinae; *Eupetes*). Babblers make a lot of noise and many of the odd sounds and chattering (Smythies, 1999). They live close to the ground and feed on insects. They are poor flyers because they have a rounded and short wing, also spend their time close to the ground by feeding insects (MacKinnon and Phillipps, 1993; Smythies, 1999).

2.2 Techniques

MacKinnon and Phillipps (1993) described that the relationships of genera and families are not only focused on their morphological identification, but also referred to the DNA analysis. This is because by using DNA hybridizations, it shows more precise results rather than morphological identifications (MacKinnon and Phillipps, 1993; Sibley and Alhquist, 1995; Sorenson *et al.*, 1999). The double stranded structure of DNA provided the general patterns of the mechanism of heredity because the composition of bases along one strand of the DNA chain provided the similar genetic information. Every single change could be passed on in subsequent cycles of DNA replication and on to future generations (Scott and Graham, 2001).

According to Sibley and Alhquist (1995), the techniques of DNA-DNA hybridization are applied to reconstruct the phylogeny of the living birds. The classifications of the birds were elaborated based on that phylogeny information. Hoelzel (1992) stated that the amplification of short sequences of mitochondrial DNA has become the basis for sequences comparison. The nucleotide sequences provided many sources of data to resolve the phylogenetic relationship (Zardoya and Meyer, 1996). By using appropriate primers, large DNA can be digested and the fragment can be analyzed and compared among ind ividuals to look for the similarity and the differences (Hoelzel, 1992). There are many sources of DNA, for examples blood and tissues. Both have different procedures in extractions and preservations. The techniques chosen will depend on the nature of samples and the circumstances by which it was obtained.

2.3 Cytochrome Oxidase I (COI) mtDNA Gene

Zardoya and Meyer (1996) and DeFilippis and Moore (2000) described that the mtDNA genome are very useful for phylogenetic analysis of vertebrates because it provides the source of DNA sequence. MtDNA are very appropriate to study in the short-term evolution because the mutation in mtDNA greater than 10 times and evolves more rapidly than nuclear DNA (Weaver and Hedrick, 1992; Hebert *et al.*, 2004; Hussen, 2005). Sibley and Alhquist (1995) described that the animal mtDNA genomes are smaller than the plants. Animal mtDNA is a small circular molecule, maternally inherited without recombination, haploidy and rapid rate evolution (Arctander, 1988; Weaver and Hedrick, 1992; Zhang and Hewitt, 1996; Zardoya and Meyer, 1996; Vilaca *et al.*, 2006). According to Arctander (1988), the avian mitochondrial is small compared to the nuclear genome (about 16500 bp). The avian mtDNA is shown in Figure 1.

DNA barcode is effective for vertebrates' identification especially birds (Hebert *et al.*, 2004). Primers are the short DNA sequences from a standardized region of the genome that is used to start the DNA synthesis (Palumbi, 1996; Hebert *et al.*, 2004). According to Sorenson *et al.* (1999), it is in various sizes, it can be short or long. The longer the primer is, the higher annealing temperature is needed for a greater specificity (Palumbi, 1996). According to Sorenson *et al.* (1999), every primer has different sequences that work on different target region on different taxa.

According to Vilaca *et al.* (2006) and Hebert *et al.* (2004), COI is very effective and efficient marker for DNA-based identification to identify the animals' species especially birds species. The COI gene is 1500 bp long and occurs in all eukaryote (DeFilippis and Moore, 2000; Vilaca *et al.*,

2006). COI is one of the specific regions in mtDNA and it is a subunit of the cytochrome c oxidase complex gene (Palumbi, 1996 and Nylander *et al.*, 1999). It is also one of the general primers for protein coding regions (Nylander *et al.*, 1999). Its amino acid sequences is highly conserved across phyla, making it easy to align sequences and making it possible to design useful universal primers (Palumbi, 1996; Zhang and Hewitt,1996; Moritz and Cicero, 2004).

According to Vilaca *et al.* (2006) and DeFilippis and Moore (2000), COI shows the evolutionary nucleotide rate three times greater than ribosomal RNA even though it has a slow rate of amino acids changes compared to other mitochondrial genes. Typically, the subsequent sequences data of COI-a (reverse) and COI-f (forward) are used to design taxon-specific primers and used to initial low-stringency amplifications (Palumbi, 1996).

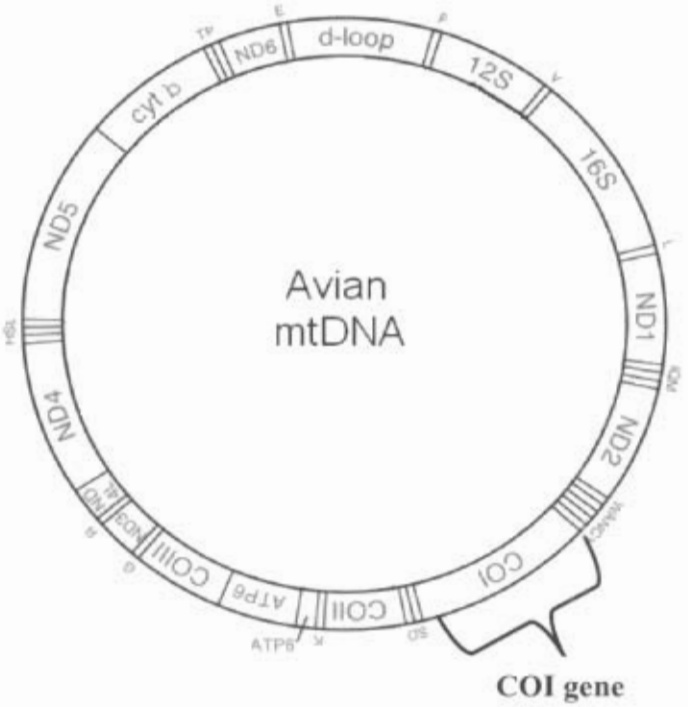

Figure 1. Gene map of the mitochondrial DNA and the location of COI (Source: Sorenson, 2003).

2.4 Previous Study

The COI gene of mtDNA had been successfully used for taxonomic studies. It had been used to identify of North America bird species and had recognized 19 new species of Thamnophilidae family since 1990. From that, nine were recently discovered species and 10 were resulted from studies before (Vilaca *et al.*, 2006). A study conducted by Vilaca *et al.* (2006) was to prove that the COI gene can be a good marker of DNA analysis for Neotropical bird family and can discriminate species within Thamnophilidae family. This study focused on 46 individuals from 16 Thamnophilidae species that were found in the Brazilian Atlantic Forest by using 418 bp of COI fragment. This study also showed that COI accurately could identify Neotropical birds even the tropical birds' show high intraspecific genetic diversity.

Seutin and Bermingham (1997) described that the systematic position of the avian species *Rhodinocichla rosea* is not clear either from the mockingbird (family Mimidae) or from a tanager (Thraupinae; Emberizidae). Many studies have been done of this species according to its morphological, anatomical, mycological and behavioral but still difficult to classify it to a particular family because *R. rosea* has a confused phenotype characteristic. The result concluded that *R. rosea* belongs to family Emberizidae (tanager) because the phenotype characteristic suggest the mimid is a convergent evolution but the relationship of tanager could not be determined.

DeFillips and Moore (2000) and Weibel and Moore (2002) study on resolution of phylogenetic relationship on avian taxa by using the COI and cytochrome b as a primer. DeFillips and Moore (2000) combines the sequences of COI and cyt. b from previous study on the same set of

specimen in order to explore the relationship between the amount of the DNA sequences and the level of phylogenetic resolution among woodpeckers (family: Picidae, subfamily Picinae). The result demonstrated those mitochondrial genomes are useful strategy for resolving phylogenetic relationships among species. Weibel and Moore (2002) focused their study on the cosmopolitan group of woodpeckers. The purpose is to reconstruct phylogenetic relationships among all New World and several Eurasian representatives of the genus Picoidae. Previous studies recommend that New World species are from a monophyletic group that develop from a single invasion by a Eurasian common ancestor but there is no clear relation has been established between the relationship of old World and New world species. All South American and North American species and six remaining species are used in this study. Although the study indicated no statistically significant differences in substitution rates, but the cyt. b genes tend to evolve at a slightly faster rate than COI genes and COI is more effective at resolving these older divergences events. The results of this study suggest that cosmopolitan genus need more study in their classification to have a clear evolutionary history.

Hussen and Rahman (2005) conducted a study by using a COI gene to construct a phylogenetic tree among selected babblers which are Grey-headed Babbler (*Stachyris poliocephala*), White-chested Babbler (*Trichastoma rostratum*) and Striped-tit Babbler (*Macronous gularis*). The result showed two clades, the first clade belongs to genus *Macronous* and *Stachyris* while another clade was genus *Trichastoma*. Hebert *et al.* (2004) found that the COI could distinguish species generally straightforward and separate individuals into the species. COI sequences differences between species are very advance than within species. Six hundred and forty eight bp region of COI was use to discriminate 260 over 667 bird's species breeding in North America.

3.0 MATERIALS AND METHODS

3.1 Samples Collection

The sampling was done twice at Kubah National Park on separates field trips between on 26th until 2nd of July and 1st until 7th of December 2006. Birds were collected by using the 2.5x 36 mm mist-net. The mist-nets were set at the suitable places such as the shelter and the main food resources at three elevations. Each captured bird was place in a cloth bag and identified using the Smythies (1981) and MacKinnon and Philips (1993) according to the body and eyes color, tail and body shape (MacKinnon and Phillipps, 1993). The birds were ringed with a serial number of UNIMAS and their morphological characters like weight, tail, total length, wing length, bill length, tarsus, bill depth, head bill and wing span were measured and recorded in bird's data book. The blood was took by piercing sterile syringe to the capillary vein and transferred to the appendorf containing the blood lysis buffer through capillary tube. The blood samples were kept at ambient temperature in the field.

The babblers also were preserved in the 70% ethanol with their body dissected. Nine samples representing five species collected in Kubah National Park and Jambusan were shown in Table 1. White-chested Babbler, Striped-tit Babbler and Grey-headed Babbler were from the previous study done by Hussen (2005).

Table 1. List of species of babblers captured.* represent the species from previous study (Hussen and Rahman, 2005).

Species	Ring Code	Individual Abbreviation	Locality
Scaly-crowned Babbler (*Malacopteran cinereum)*	B1313	SCB1	Kubah NP
Scaly-crowned Babbler (*M. cinereum)*	B1316	SCB2	Kubah NP
Scaly-crowned Babbler (*M. cinereum)*	A4787	SCB3	Kubah NP
Striped-tit Babbler (*Macronous gularis*)	A4683	STB1	Jambusan*
Striped-tit Babbler (*M. gularis*)	A4689	STB2	Jambusan*
White-chested Babbler (*Trichastoma rostratum*)	A4557	WCB2	Jambusan*
White-chested Babbler (*T. rostratum*)	A4577	WCB3	Jambusan*
Black-capped Babbler (*Pellorneum capistratum*)	B3454	BCB1	Kubah NP
Grey-headed Babbler (*Stachyris poliocephala*)	B1457	GHB1	Jambusan*

3.2 DNA Extraction

First, 700 µl of 2x CTAB (Cetyltrimethylammonium bromide) buffer was added into 1.5 microcentrifuge. The tissues were grinded and then put into microcentrifuge tube that contained a buffer. Next, 5 µl Proteinase K was added, then incubated at 50° C to 60°C until tissue dissolved completely. After that, 700 µl choloroform-isoamyl alcohol was added and vortex for two minutes. The sample was centrifuged at 130x10 rpm for 10 minutes. Then, 600 µl of upper aqueous phase was pipetted from the tube to the newly labeled tube contained 600 µl absolute ethanol and mix inversion. The sample was centrifuged again by using 130x100 rpm for 10 minutes. After 10 minutes, the pellet will be seen at the bottom of the microcentrifuge. The absolute ethanol was pipetted out carefully to make sure the pellet did not accidentally throw together. Then, 700 µl of 70% ethanol and 25 µl NaCI was added and centrifuged it again. After 10 minutes, 70 % ethanol was threw out and quick spun for five second. Tissue was used to dry

the sample from the ethanol. Lastly, 50 μl of the ddH_2O was added into the tube and shook it carefully.

3.3 Samples Visualization

All extracted, amplified and purified products were visualized by using 1% agarose gel in 1x TAE buffer. The standard marker for extracted samples used was 1 kb ladder (Fermentas) while for amplified and purified used was 100 bp ladder (GeneRular^{TM} plus, Fermentas). The gel was stained with 0.8 µL of ethidium bromide. The gel was prepared by weighed 0.5 g amount of agarose gel and heated for 1-1/2 minutes with 50 ml of 1x TAE buffer. The mixture was poured into the electrophoresis tank and the comb was inserted into the gel. When the gel was completely solidified, the 1x TAE buffer was added and the comb was removed. Then, the sample was loaded by mixed the genomic DNA with the 1 µL bromophenol loading dye (Fermentas). The electrophoresis was ran for 30 minutes at 90 volt. The visualization was done under ultraviolet (UV) radiation transiluminator.

3.4 DNA Polymerase Chain Reaction (PCR) Amplification

PCR was performed using Eppendorf Thermocycler machine. The primer used was COI, and the samples were amplified by using 700 bp section of mtDNA genome from COI. The sequence of forward (down-stream) and reverse (upper stream) of COI was shown in Table 2.

Table 2. Sequences for forward and reverse of COI primers.

Direction	COI sequences	Sequences
Forward primer	CO1-f	'5-CCTGCAGGAGGAGGAGAYCC-3'
Reverse primer	CO1-a	'5-AGTATAAGCGTCTGGGTAGTC-3'

PCR amplification was done by using 25 µL reaction consisted of 1.5 µL of 25mM $MgCl_2$ (Magnesium chloride; Promega), 2.5 µL of 10mM dNTPs (deoxynucleosides triphosphates: dATP,dCTP,dGTP and dTTP), 2.5 µL of 10x buffer (10mM Tris-HCI, pH 8.0;50 mM KCI; 0.1% [w/v] gelatin; 1%Triton x-100), 13.8 µL of ddH_2O (sterile deionized water), 1.25 µL each of 10 µM primers, 0.2 µL *Taq* Polymerase (Promega) and 2 µL of template DNA. The amplification process took 30 cycles with initial denaturation was two minutes at 94°C, one minute for denaturation at 94°C, one minute for annealing at 56°C, two minutes for extension at 72°C and five minutes for final extension at 72°C. Optimization was done started at 50°C to 57°C to determine the correct the annealing temperature. The PCR reaction mixtures and the parameters process were shown in the Table 3 and 4.

Table 3. PCR reaction mixtures (master mix).

Reagents	**Reaction Mixtures**	
	1 reaction (25 µl)	**5 reactions (µl)**
10x buffer	2.50	12.50
dNTPs (2mM)	2.50	12.50
$MgCl_2$	1.50	7.50
Primer (COI-f) (10mM)	1.25	6.25
Primer (COI-a) (10mM)	1.25	6.25
Deionized water (ddH_2O)	13.80	74.00
DNA template	2.00	10.00
Taq Polymerase (5 units/µl)	0.20	1.00

Table 4. The parameter of the PCR process for 30 cycles (Hussen, 2005).

Parameter	Temperature (° C)	Duration (mins)	Cycles
Initial denaturation	94	2	1
Denaturation	94	1	30
Annealing	56	1	
Extension	72	2	
Final Extension	72	5	1
Soaking	4	∞	

3.5 Purification and Sequencing

Twenty-four μl of the PCR amplification products were sent to a private sequencing laboratory (First BASE Laboratories Sdn. Bhd.) together with 10 μl of forward primer (COIf-L) to purify before proceed to sequencing.

3.6 Sequencing and Phylogenetic Analysis

The fluorescent based DNA sequence analysis results were flaunted using the CHROMAS program (version 2.24: McCarthy, 2004). Six hundred and thirty sites of sequences were aligned subsequently using the eyes and CLUSTAL X program (version 1.81: Thompson *et al.*, 1997) into multiple alignments together with the Barn Swallow (*Hirundo rustica*) as outgroup. For construction of phylogenetic tree the Molecular Evolutionary Genetics Analysis (MEGA) (version 3.1: Kumar *et. al.*, 2004) were used. Two types of tree were constructed. The trees were Neighbor-joining (NJ) tree and Maximum parsimony (MP). Neighbor-joining (NJ) uses discrete data while Maximum parsimony (MP) utilizes genetic distance. The level of phylogenetic confidence was estimated by bootstrap with 1000 replicates data set. Beside that, the estimation