
# PHYLOGENY OF SELECTED BABBLERS (FAMILY: TIMALIIDAE) INFERRED FROM MTDNA CYTOCHROME OXIDASE I (COI) GENE.

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# Phylogeny of selected babblers (Family: Timaliidae) inferred from mtDNA cytochrome oxidase I (COI) gene.

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## DECLARATION

I hereby declare that no portion of 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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## Table of contents

## List of Tables

## List of Figures

## List of Abbreviations

| | |
|---|---|
| A | Adenine |
| C | Cytosine |
| CI | Consistency index |
| CIA | Chloroform isoamyl alcohol |
| COI | Cytochrome oxidase subunit I |
| CTAB | Cetyltrimethyl ammonium bromide |
| dd$H_2O$ | Deionised distilled water |
| DNA | Deoxyribonucleic acid |
| EtBr | Ethidium Bromide |
| G | Guanine |
| GTR+G | General Time Reversible including Gamma |
| HI | Homoplasy index |
| K2P | Kimura-2 Parameter |
| kbp | Kilo base pair |
| MA | *Malacopteron magnirostre* |
| MC | *Malacopteron cinereum* |
| MCMC | Markov chain Monte Carlo |
| MG | *Malacopteron magnum* |
| ML | Maximum-likelihood |
| MM | *Malacocincla malaccense* |
| MP | Maximum Parsimony |
| mtDNA | Mitochondrial DNA |
| NaCl | Sodium chloride |
| NJ | Neighbor-Joining |

| | |
|---|---|
| PCR | Polymerase chain reaction |
| Pro-K | Proteinase K |
| rRNA | Ribosomal ribonucleic acid |
| SE | *Stachyris erythroptera* |
| SP | *Stachyris poliocephala* |
| T | Thymine |
| TAE | Mixture of Tris base, acetic acid and EDTA |
| UV | Ultra-violet |
| V | Volt |

# Phylogeny of Selected Babblers inferred from mtDNA Cytochrome Oxidase I (COI) Gene

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## ABSTRACT

A study on phylogeny of eight species of babblers (Family: Timaliidae) in Sarawak by using mitochondrial DNA (mtDNA) cytochrome oxidase I (COI) gene was conducted. The samples were taken from both fresh and also voucher specimens that were available in UNIMAS. Four phylogenetic trees were constructed by using four different methods namely, Neighbour-Joining (NJ), Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian inferences in this study. The ML and Bayesian trees showed that Timaliidae can be grouped into two groups that is group 1, genus *Stachyris*, and group 2, which consist of *Pellorneum*, *Trichastoma*, *Malacocincla* and *Malacopteron* genera. This showed that *Pellorneum*, *Trichastoma*, *Malacocincla* and *Malacopteron* are closely related compared to genus *Stachyris*. COI is a good genetic marker which can detect intraspecific variation within species and the relationship of the individuals by comparing the genetic divergence and the length of tree.

Key words: Phylogeny, Timaliidae, cytochrome oxidase I (COI), variation and relationship.

## *ABSTRAK*

*Satu kajian mengenai filogeni untuk lapan spesies babbler (Famili: Timaliidae) yang berada di Sarawak dengan menggunakan mitokondria DNA (mtDNA) gen sitokrom oksida I (COI) telah diadakan. Sampel diambil dari kedua-dua sampel segar dan juga dari sampel muzium yang terdapat di UNIMAS. Empat pokok filogenetik dibina dengan menggunakan empat kaedah iaitu 'Neighbor-Joining' (NJ), 'Maximum Parsimony' (MP), 'Maximum Likelihood' (ML), dan inferens 'Bayesian' di dalam kajian ini. Pokok filogenetik ML dan juga 'Bayesian' menunjukkan bahawa Timaliidae dibahagikan kepada dua kumpulan iaitu kumpulan 1, genus Stachyris dan juga kumpulan 2 yang terdiri daripada Pellorneum, Trichastoma, Malacocincla dan Malacopteron. Ini menunjukkan bahawa genus Pellorneum, Trichastoma, Malacocincla dan Malacopteron berkait lebih rapat sesama sendiri berbanding dengan genus Stachyris. COI adalah petunjuk genetik yang bagus kerana dapat mengesan variasi intraspesies dan perhubungan di antara individu dengan membandingkan kecapahan genetik dan juga kepanjangan pokok filogenetik.*

*Kata kunci: Filogeni, Timaliidae, sitokrom oksida I (COI), variasi dan perhubungan.*

## 1.0 INTRODUCTION

Each family and species of birds is adapted to a particular type of habitat for the purposes of feeding and breeding. Physically, the body shape and size, length of wings and legs, and shape, length and size of bill, birds have evolved through millions of years to fit their surroundings (Strange & Jeyarajasingam, 1993).

Timaliidae is a poorly defined family as it is a diverse group (MacKinnon and Phillips, 1993) and they occupy a range of habitats (Myers, 2009). Babblers are generally gregarious and noisy because most of them have a rather harsh, chattering call and none is migratory as they have short wings and are not strong flyers (MacKinnon & Phillips, 1994). According to Smythies (1999), babblers are poor flyers and spend much time on or near the ground in small parties. There is not much study or information on this family because they are less colourful and hard to identify between close species only by looking at their morphology. The systematics of this family is in a state of flux and many species may belong to other groups (Myers, 2009). Sibley and Ahlquist (1990) concluded that deoxyribonucleic acid (DNA) sequencing is one of the best available methods to measure degrees of similarity between the DNAs of different species.

The DNA sample were taken from mitochondrial DNA (mtDNA) because it evolves in the past 15 million years and still evolving now but the nuclear genes show very little change even after 15 million years (Simon *et al.*, 2006). Eventhough Mallet and Willmot (2003) mentioned that the gene can be uninformative due to persistant ancestral polymorphisms and the genes may introgress between closely related species, Hebert *et al.* (2003b) suggested that these complications are rare. According to Galtier *et al*. (2009), mtDNA is also maternally inherited which means that the whole genome behaves as a single, non-

recombining locus where they share a common genealogy. That is why it is more suitable to use mtDNA to do this study and to construct the phylogenetic tree while identifying the evolution and relationship among the selected species of babblers.

The Cytochrome Oxidase I (COI) gene is used in this study because it is effective for the identification of bird species (Hebert *et al.*, 2004). In another study of Hebert *et al.* (2003a), they established that the mitochondrial gene COI can serve as the core of a global bio-identification system for animals. Hebert *et al.* (2003a) also suggested that COI analysis actually provide a taxonomic system that is chasing the last digit of animal diversity. Therefore, it is more suitable to use COI gene for this study.

Previous study done by Hussen (2005) and Hasbullah (2007) still have not resolve the phylogenetic tree of babblers (Family: Timaliidae) due to small number of species and not all the genus of the family were being tested in their study.

This study is conducted by adding more species and added new genus to see the difference from the previous studies. Besides that, this study also constructed two trees, they are the Maximum Likelihood tree and the Bayesian trees, which the previous study by Hussen (2005) and Hasbullah (2007) did not produced.

Therefore, this study is conducted to construct phylogenetic trees, based on DNA sequence in order to update the previous trees. The first three individuals of each selected species of babblers were euthanized for tissue collection. The other method of collection was taking tissue samples from voucher specimens that were available in the campus.

## 2.0 OBJECTIVES

The objectives of this study are:

1. To construct phylogenetic tree of selected species of babblers in Sarawak, inferred from mitochondrial DNA (mtDNA) cytochrome oxidase I (COI) gene.

2. To identify the evolution and relationship among the selected species of babblers in Sarawak.

## 3.0 LITERATURE REVIEW

### 3.1 Techniques

From the study conducted by Sibley and Ahlquist (1990), they concluded that DNA-DNA hybridization and DNA sequencing are the best available method to measure degrees of similarity between the DNAs of different species. In their study, they have divided bablers into subfamilies Garulacinae and Sylvinae and then placed them together with warblers Megalutinae and Acropcephalinae in the family Sylviidae. By using DNA sequencing method, babblers can be grouped in their own sub families.

### 3.2 Cytochrome Oxidase I (COI) mtDNA Gene

Study done by Razak (2008) on the phylogenetic relationship of selected sunbirds and spiderhunters (Family Nectarinidae) inferred from partial mitochondrial DNA cytochrome oxidase I gene constructed the phylogenetic trees by using three different methods, which were Neighbor-Joining, Maximum Parsimony and Maximum Likelihood. Maximum Likelihood was the most preferred method as it detects slight alterations on bases on branches of sequence (Razak, 2008). This study concluded that Cytochrome Oxidase I can detect interspecific variations between the selected species and it is a good genetic marker for intra and interspecific variation studies.

### 3.3 Previous Studies

Hasbullah (2007) has investigated the phylogenetic relationship of the selected babbler species from Timaliidae family by using Cytochrome Oxidase c subunit I (COI) gene of the mitochondrial DNA (mtDNA) sequences data. The samples for this study were collected from Jambusan (Bau) and Kubah National Park (Kuching). Five blood and five tissue samples were obtained from both locations and the phylogenetic trees were constructed by using Neighbor-joining (NJ) and Maximum Parsimony (MP) methods. The phylogenetic tree showed the selected species of babblers evolved into two major clades where the first clade consist of genus *Macronus* and *Stachyris* and the second clade consist of genus *Malacopteron, Pellorneum* and *Trichastoma* (Hasbullah, 2007). It was shown by Hasbullah (2007) that the relationship of the selected species of babbler can be determined by using COI gene as genetic marker.

Another study on phylogeny of babblers using COI mtDNA gene was carried out by Hussen (2005). This study obtained samples from Jambusan (Bau) and Maludam National Park (Sri Aman). Ten blood samples from five species were collected and phylogenetic tree inferred by using Neighbor-joining (NJ) and Maximum Parsimony (MP) methods were produced. The results show that the babbler species evolved into two major clades (Hussen, 2005). The first clade consists of species from genus *Macronous* and *Stachyris* and another clade formed by the species from genus *Trichastoma* (Hussen, 2005). It is shown that COI mtDNA can detect the evolutionary and relationship of species in this family.

Previous study done by Gelang *et al.* (2009) on the phylogeny of babblers used five molecular regions to estimate the relationships among a large proportion of genera

traditionally placed in Timaliidae. The study proposed a new classification which divide the babblers into the families Sylviidae and Timaliidae and within the two, four subfamilies are recognized (Gelang *et al.*, 2009). Gelang *et al.* (2009) also proposed the family name of Pnoepygidae for the genus *Pnoepyga*.

Gilbert (2005) also did a phylogenetic study on babblers (Family: Timaliidae) by using mitochondrial DNA subunit 16s rRNA gene. She collected four samples from Maludam National Park and six samples from Jambusan, Bau. The phylogenetic tree was inferred by using Neighbor-Joining method. According to Gilbert (2005), family Timaliidae is monophyletic with genus *Macronus* being more closely related to the genus *Stachyris* and genus *Trichastoma* is split away from the other two former genera. There were still some questions after the study on the relationship between *Macronus ptilosus* and the genus *Stachyris* due to the low bootstrap value obtained (Gilbert, 2005).

Cibois (2003) did a study on the mitochondrial DNA phylogeny of babblers (Timaliidae). The study was to clarify the phylogenetic relationships using sequences of three mitochondrial genes that are cytochrome *b*, rRNA 12S and 16S. Cibois (2003) concluded that shrike babblers (*Pteruthius*) and the grey-chested thrush babbler (*Kakamega poliothorax*) are not related to Timaliidae but to other passerine groups. Through genetic analysis, we can relate the species to its family.

Another study conducted by Cibois (1999) on the molecular systematic of the Malagasy babblers (Passeriformes: Timaliidae) and Warblers (Passeriformes: Sylviidae), based on cytochrome *b* and 16S rRNA sequences. The study focus on three Malagasy genera currently assigned to the Timaliidae, *Mystacornis, Oxylabes,* and *Neomixis,* and on their relationships with other babblers and warblers using sequences of two mitochondrial genes

(cytochrome *b* and 16S rRNA). The Maximum parsimony analyses show that the Malagasy "babblers" are not related to any of the other African and Asian babblers (Cibois, 1999). Therefore the study has concluded that the genus *Mystacornis* is neither a babbler nor a warbler (Cibois, 1999). By using molecular study, we can determine where the genus *Mystacornis* belongs.

## 4.0 MATERIALS AND METHODS

### 4.1 Samples Collections

#### 4.1.1 Fresh Samples

The fresh tissue samples of babblers were collected from Gunung Gading National Park (01º44′00.00″ N 109º50′00.00″ E), Mount Jagoi, Bau (01°21.182′ N 110°02.057' E), Kubah National Park (1º35.76’ N 110º10.85’ E) and also in Universiti Malaysia Sarawak (UNIMAS) (01º27’ N 110º27’ E). All of these four sites are situated in Sarawak. The locations were indicated in Figure 1 below.

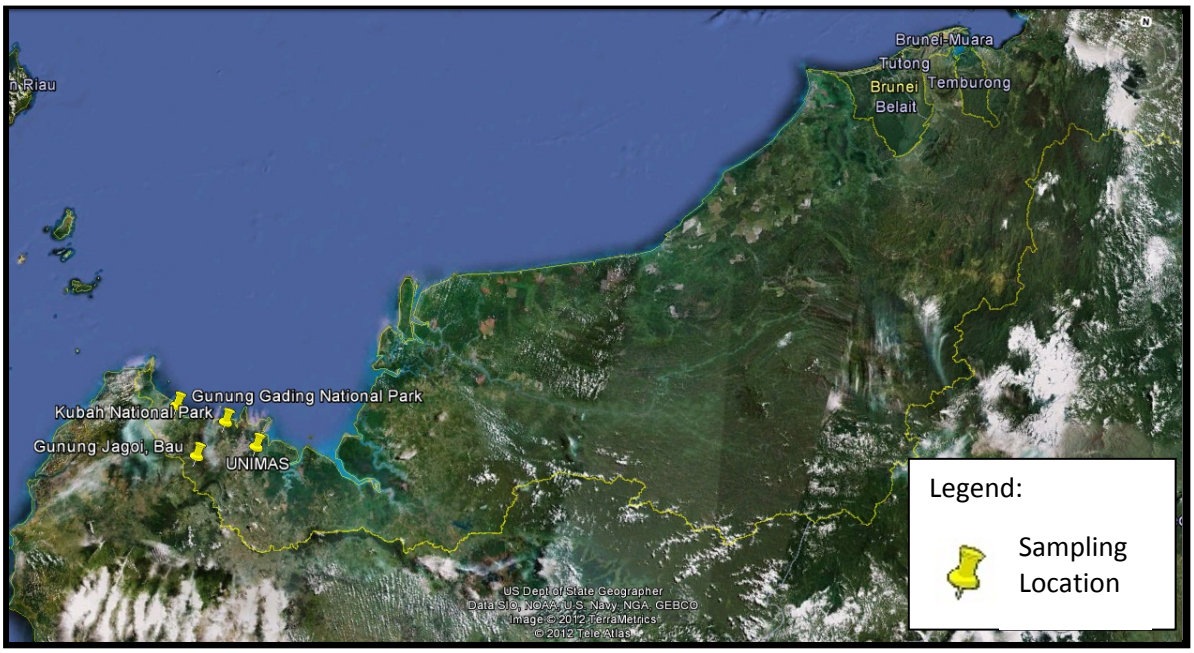


**Figure 1:** Map of sampling locations. Retrieved from Google Earth on June 5th, 2012.

The babblers were captured by using mist-netting method. The mists nets were put up in Gunung Gading National Park, Mount Jagoi, Bau, Kubah National Park and also in UNIMAS.

Mists nets were put up in various sites in each area. Number of mist nets that were put up in each area was not fixed as long as babblers can be captured. Two individuals were captured from Gunung Gading National Park, they were Moustached babbler (*Malacopteron magnirostre*) and Short tailed babbler (*Malacocincla malaccense*). Twelve individuals were then captured from Mount Jagoi, Bau which were two Moustached babblers, two Scaly-crowned babbler (*Malacopteron cinereum*), one Rufous-crowned babbler (*Malacopteron magnum*), one Black-capped babbler (*Pellorneum* capistratum), three Chestnut-winged babbler (*Stachyris erythroptera*) and three Grey headed babblers (*Stachyris poliocephala*). Four individuals were captured from UNIMAS. They were three house swallows (*Hirundo* tahitica) and one Short-tail babbler. Only one individual was captured from Kubah National Park, which was Scaly-crowned babbler.

The mist-nets were set near to the ground as babblers spend much more time on or near the ground (Smythies, 1999). Captured birds were identified based on Myers (2009).

Each babblers captured were identified and measured. The data were recorded in the data book. The first three individuals of each captured species were euthanized and their muscle and liver samples were extracted. The data were recorded in the data form as shown in Table 1.

**Table 1**: Data form for samples from fresh specimens.

| Species | Species Abbreviation | Sample number | Locality |
|---|---|---|---|
| *Malacopteron magnirostre* | MA | | |
| Sample 1 | | FMA001 | Gunung Gading National Park |
| Sample 2 | | FMA002 | Mount Jagoi, Bau |
| Sample 3 | | FMA003 | Mount Jagoi, Bau |
| *Malacocincla malaccense* | MM | | |
| Sample 1 | | FMM001 | Gunung Gading National Park |
| Sample 2 | | FMM002 | UNIMAS |
| *Malacopteron magnum* | MG | | |
| Sample 1 | | FMG001 | Mount Jagoi, Bau |
| *Malacopteron cinereum* | MC | | |
| Sample 1 | | FMC001 | Mount Jagoi, Bau |
| Sample 2 | | FMC002 | Mount Jagoi, Bau |
| Sample 10 | | FMC010 | Kubah National Park |
| *Stachyris poliocephala* | SP | | |
| Sample 1 | | FSP001 | Mount Jagoi, Bau |
| Sample 2 | | FSP002 | Mount Jagoi, Bau |
| Sample 3 | | FSP003 | Mount Jagoi, Bau |
| *Stachyris erythroptera* | SE | | |
| Sample 1 | | FSE001 | Mount Jagoi, Bau |
| Sample 7 | | FSE007 | Mount Jagoi, Bau |
| Sample 8 | | FSE008 | Mount Jagoi, Bau |
| *Pellorneum capistratum* | PC | | |
| Sample 2 | | FPC002 | Mount Jagoi, Bau |
| *Hirundo tahitica* | HT | | |
| Sample 1 | | FHT001 | UNIMAS |
| Sample 4 | | FHT004 | UNIMAS |
| Sample 5 | | FHT005 | UNIMAS |

In the field, the muscle and liver samples were extracted from each of the individuals of babblers captured and were preserved in absolute ethanol (99%) at room temperature, as recommended by BeeBee and Rowe (2006). When arrived in UNIMAS laboratory, the samples were kept in refrigerator at -20ºC.

### 4.1.2 Voucher Specimens

Tissue samples were also extracted from available sources in UNIMAS such as voucher specimens from the Institute of Biodiversity and Environmental Conservation (IBEC) and also from the Department of Zoology.

Twenty-three tissue samples were extracted from the voucher specimens of Department of Zoology and 52 tissue samples were extracted from the voucher specimens of IBEC. All of the voucher specimens were from various places within Sarawak. Their localities are Padawan, Kampung Pueh, UNIMAS, Loagan Bunut, Niah National Park, Bako National Park, Nanga Merit, Borneo highlands Resort, Maludam, Sungai Asap, Sarawak Club Golf Resort, Cermat Ceria Plantation, Betong and Mount Singai.

## 4.2 Molecular Techniques

### 4.2.1 DNA Extraction

DNA extraction was done before doing amplification by polymerase chain reaction (PCR). DNA was extracted manually by using cetyl trimethyl ammonium bromide (CTAB) method (Anon, 2012b). The chemicals or the materials needed for extraction are CTAB buffer, Proteinase-K (Pro-K), 70% ethanol, absolute ethanol (99%), Chloroform isoamyl alcohol (CIA), Sodium chloride (NaCl) and deionised distilled water ($ddH_2O$). DNA was extracted from either the muscle or liver tissues. Then all of the extracted samples were visualised using 1% agarose gel to make sure that the DNA extractions were successful and can be used for PCR in the subsequent step.

### 4.2.2 Gel electrophoresis

The extracted samples were visualised using gel electrophoresis method. TAE buffer was used to make the 1% agarose gel and also as the buffer solution during electrophoresis. 1µl of Ethidium bromide (EtBr) was mixed into the gel mixture before it hardened. The EtBr staining of agarose gels flouresence when viewed with ultra-violet (UV) light. The gels were run for an average of 30 minutes at 90V.

### 4.2.3 Polymerase Chain Reaction (PCR)

After the successful extractions, the samples were amplified by using Polymerase Chain Reaction (PCR) method. The samples were amplified so that there will be adequate amount

of specific region of DNA to be tested. The PCR reaction mixtures were prepared with the final volume of 25.0µl per tube for a single reaction including one negative control. Table 2 shows the mastermix for one reaction of PCR and the parameters used for the PCR are shown in Table 3.

**Table 2**: Mastermix for one reaction of PCR.

| **Components** | **Amount (µl)** |
|---|---|
| $ddH_2O$ | 14.20 |
| 5 x reaction buffer | 5.0 |
| $MgCl_2$ | 2.0 |
| dNTP mix (10mM) | 0.5 |
| Primer COI-f (10mM) | 1.0 |
| Primer COI-e (10mM) | 1.0 |
| Template DNA | 1.0 |
| Taq polymerase | 0.3 |

**Table 3**: Parameters for PCR.

| **Step** | **Temperature (°C)** | **Time (minutes)** | **No. of cycles** |
|---|---|---|---|
| **Pre-Denaturation** | 95 | 2 | 1 |
| **Denaturation** | 95 | 1 | |
| **Annealing** | 50.5 | 1 | } 30 |
| **Extension** | 72 | 2 | |
| **Extension** | 72 | 5 | 1 |
| **Soak** | 4 | ∞ | |