PHYLOGENY OF SELECTED FLYCATCHERS (FAMILY: MUSCICAPIDAE) INFERRED FROM MTDNA CYTOCHROME OXIDASE I GENE

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This project is submitted in partial fulfilment of the requirements of the degree of
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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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Table of contents **Pages**

List of tables **Pages**

List of Figures **Page**

List of abbreviations

DNA = Deoxyribonucleic acid

mtDNA = Mitochondrial DNA

CO I = Cytochrome oxidase I

EDTA = Ethylenetriaminetetraacetic acid

CTAB = Cetyl trimethylammonium bromide

dd$H_2$0 = deionized distilled water

NaCl = Sodium chloride

UV = Ultraviolet

Pro-K = Proteinase K

CIA = Chloroform isoamyl alcohol

TAE = Mixture of Tris base, acetic acid and EDTA

EtBr = Ethydium bromide

CC = *Cyornis caerulatus*

RU = *Rhinomyias umbratilis*

TP = *Terpsiphone paradise*

CT = *Cyornis turcosus*

FD = *Ficedula dumetoria*

HA = *Hypothymis azurea*

RP = *Rhipidura perlata*

PP = *Philentoma pyrhopterum*

RA = *Rhipidura albicollis*

CS = *Cyornis superbus*

CE = *Culicicapa ceylonensis*

RJ = *Rhipidura javanica*

CR = *Cyornis rufigastra*

AT = *Acridotheres tristis*

BPP = Bayesian posterior probability

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ABSTRACT

The phylogeny of selected flycatchers (Family: Muscicapidae) was inferred from mtDNA cytochrome oxidase I gene through DNA sequencing analysis. Polymerase chain reaction (PCR) method was incorporated in this study which resulted in approximately 498 base pairs (bp) of end product. Phylogenetic tree was constructed by using Neighbor Joining (NJ), Maximum parsimony (MP), Maximum Likelihood (ML) and Bayesian analysis method. Both NJ and MP produced 2 major clades which separated subfamily Muscicapinae from Monarchinae and Rhipidurinae which is debated to have diverged into a new family. ML and Bayesian analysis proved that genus Rhipidura and Terpsiphone are more closely related to each other than to subfamily Muscicapinae. The study indicates that using cytochrome oxidase I gene can resolve the relationship among species well.

Key words: Flycatcher, mitochondrial DNA, cytochrome oxidase I, phylogenetic tree, relationship

ABSTRAK

Kaedah penjujukan analisis DNA melalui pengaplikasian DNA mitokondria cytochrome oxidase I telah digunakan untuk mengkaji hubungan filogenetik di antara spesies flycatcher (Famili: Muscicapidae). Kaedah Polymerase Chain Reaction (PCR) telah diaplikasikan dalam kajian ini di mana produk akhir yang dihasilkan dianggar berjumlah 498 bp. Pokok filogeni dibina menggunakan kaedah 'Neighbor Joining' (NJ), 'Maximum Parsimony' (MP), 'Maximum Likelihood' (ML) dan analisis 'Bayesian'. Kedua-dua pokok NJ dan MP menghasilkan dua kelompok utama yang memisahkan sub-famili Muscicapinae daripada Monarchinae dan Rhipidurinae yang sering diklasifikasikan di bawah famili yang baru. ML dan analisis 'Bayesian' membuktikan bahawa genus Terpsiphone berkait rapat dengan genus Rhipidura daripada sub-famili Muscicapinae. Kajian ini membuktikan bahawa penggunaan 'cytochrome oxidase I' mampu menghuraikan hubungan antara spesies dengan baik

Kata kunci: Flycatcher, DNA mitokondria, cytochrome oxidase I, pokok filogeni, hubungan

1.0 Introduction

Systematic is the study of organismic diversity as that diversity is relevant to some specified kind of relationship thought to exist among populations, species or higher taxa (Wiley, 1981). According to Wiley (1981), taxonomy could be defined as theory and practice of describing and classifying organisms besides conveying information on the relationships between them. The plants and animals on earth evolved from a single origin of life and they have had a single evolutionary history, or phylogeny (Sibley and Ahlquist, 1995). Many biologists have been trying to reconstruct the phylogeny of various organisms on earth. Phylogenetic systematic is one approach to systematic that attempts to recover the phylogenetic (genealogical) relationships among groups of organisms and produce classifications that exactly reflect those genealogical relationships (Wiley, 1981). A primary objective of phylogenetic studies is to reconstruct the evolutionary history of a group of organism (Hillis, 1987). According to Page and Holmes (1998), different types of data mainly morphology, ontogeny, behavior and geographic distribution can be used for the purpose of phylogenetic inference. Page and Holmes (1998) also stated that recent advances in molecular aspects such as immunological distances, allozyme frequencies, restriction sites, DNA hybridization, and protein and nucleotide sequences have provided wide information regarding phylogenetic inference. Using DNA evolution, phylogenetic tree based on molecular perspective could be constructed to describe the evolutionary change over time of the members of a particular family. According to Page and Holmes (1998), nucleotide sequences provide wide information that is comparable across a wide taxonomic range.

Initial efforts to reconstruct phylogenetic history were based on few objective criteria, and estimates of phylogeny were little more with problems of species, speciation and geographic variation than with problems of phylogeny (Moritz and Hillis, 1996).

Flycatchers belong to the family Muscicapidae. “The true flycatchers are a cosmopolitan group, well represented in Borneo; some species are sedentary, but many are migratory to a greater or less degree” (Smythies, 1999). They build cup-shaped nests, either in fork of a tree or bush, or in a natural hole or hollow. According to Smythies (1999), the plumage of the young flycatcher is mottled or squamated. According to Phillipps and Phillipps (2009), Muscicapidae is one of the largest bird families with 328 species in the whole world and 38 species in Borneo. Bornean flycatchers include five species from the subfamily Monarchinae, 30 species from subfamily Muscicapinae and three species from subfamily Rhipidurinae.

“DNA sequences from the mitochondrially encoded genes (mtDNA) are attractive sources of characters for estimating the phylogenies of recently evolved taxa because mtDNA evolves rapidly, but its utility is limited because the mitochondrial genes are inherited as a single linkage group (haplotype) and provide only one independent estimate of the species tree”(Moore,1995). “Animal mitochondrial DNA is a small, extrachromosomal genome, typically ~16kb in size” (Boore, 1999). Boore (1999) also stated that by comparing arrangements of animal mitochondrial gene, one can determine ancient evolutionary relationships due to the uniqueness of rearrangements of the sequence. Besides that, according to Boore (1999), the arrangements of animal mitochondrial gene are unchanged over long evolutionary time although they are able to evolve rapidly.

The aim of this study is to construct the phylogeny of selected flycatchers inferred from mtDNA cytochrome oxidase I. Besides that, the evolution and relationship of selected flycatchers will also be studied in this research.

1.1 Problem statement

According to Smythies (1999), some ornithologists have included Old World insect-eaters in family Muscicapidae while treating Monarchidae separately from other members of Muscicapidae. The first ever classification of flycatchers based on molecular work were done by Sibley and Ahlquist (1990), where they classified thrushes, muscicapinae flycatchers and chats under the family Muscicapidae. Another recent study was done by Ramji (2007) where the family Muscicapidae split into two subfamilies which are Monarchinae and Rhipidurinae. According to Myers (2009), the subfamily Rhipidurinae and Monarchinae had diverged forming new family. However there are still some doubts on the phylogeny of this family. By the end of this study, researcher can provide some new findings in reconstructing and updating the phylogeny of flycatchers.

1.2 Objectives

1. To construct a phylogenetic tree of selected species of flycatchers in Sarawak.

2. To determine the evolution and relationship of selected flycatchers using mtDNA CO I gene.

2.0 Literature review

2.1 Previous study

Sibley and Ahlquist (1990), constructed the phylogeny for the family Muscicapidae by dividing them into two subfamilies which are Turdinae and Muscicapinae. Turdinae includes typical thrushes whereas Muscicapinae could be divided into two tribes mainly Muscicapini and Saxixolini. According to Sibley and Ahlquist (1990), Muscicapinae flycatchers and Saxicoline chats are more closely related to one another than either is to any other group. Sibley and Ahlquist also stated that Muscicapidae is the sister group of the Sturnidae.

Smythies (1999) stated that Muscicapidae is divided into three subfamilies which are subfamily Rhipidurinae (Fantail flycatchers), Muscicapinae (Typical flycatchers), and Monarchinae (Jungle flycatchers). According to Smythies (1999), fantail flycatchers are easily recognized by their habit of constantly cocking up and fanning out their tails. Jungle flycatchers have brownish plumage with rufescent tails, rounded head, long and heavy bills (Lekagul and Round, 1991). Typical flycatchers have an upright posture and tend to hawk after insects from a perch (MacKinnon, 1991).

The phylogenetic relationship of family Muscicapidae and the superfamily Muscicapoidea has long been an issue debated as there were many research done on the phylogeny of this family. Voelker and Spellman (2004), conducted a study on nuclear and mitochondrial DNA evidence of polyphyly in the avian superfamily Muscicapoidea. In this study, nucleotide sequences of c-mos genome and the mitochondrial cytochrome b and NB2 genes were used to assess the monophyly of superfamily Muscicapoidea as stated by Sibley and Monroe (1990). According to Voelker and Spellman (2004), Muscicapidae is polyphyletic where Turdinae (thrushes) and Muscicapidae are not the closest relatives.

Voelker and Spellman (2002) stated that,Turdinae is not a subfamily of Muscicapidae as stated by Sibley and Ahlquist (1990), but instead is sister to Sturnidae and Cinclidae clade. This clade is sister to Muscicapidae which consists of Muscicapini and Saxicolini. Voelker and Spellman (2002) also concluded that true thrushes belong to a distinct family known as Turdidae.

Cibois and Cracraft (2004) also conducted a study on assessing the passerine "Tapestry": phylogenetic relationship of the Muscicapoidea inferred from nuclear DNA sequences. Cibois and Cracraft (2004) concluded that Muscicapoidea is divided into three main groups which are the Cinclidae (dippers), the Muscicapidae which consists of thrushes and Old World flycatchers and the Sturnidae which are the starlings and mimids. This study agrees with the result from Voelker and Spellman (2004) where thrushes and Old World flycatchers form two main clades where the first clade covers all flycatchers and small thrushes. This clade consists of *Muscicapini* and *Saxicolini* tribe. The second clade consists of large thrushes known as Turdini.

Ramji (2007) constructed a phylogenetic tree of selected flycatchers using mtDNA subunit 16S rRNA and concluded that mtDNA subunit 16S is capable of constructing a phylogenetic tree of flycatcher. The tree revealed that the Monarchinae and Rhipidurinae are more closely related with each other compared to the Muscicapinae (Ramji, 2007). Ramji (2007) also suggested that the occurrence of Muscicapinae could be further expanded by having larger sample size and more individuals of the species.

The result from Cibois and Cracraft (2004) was well supported by Johansson *et.al* (2008), who conducted a study on the phylogenetic relationships within Passerida (Aves:Passeriformes): A review and a new molecular phylogeny based on three nuclear

intron markers. According to Johansson *et.al* (2004), thrushes and Old World flycatchers have a strong sister-group relationship.

Another study was conducted up to family level by Sangster *et.al* (2010). Sangster *et.al* studied the multi-locus phylogenetic analysis of Old World chats and flycatcher reveals extensive paraphyly at family, subfamily and genus level (Aves:Muscicapidae). From this study Sangster *et.al* discovered that there are mainly four major clades recognized in Muscicapidae. Those four clades are Muscicapinae, Niltavinae, Erithacinae and Saxicolinae. .

Daud (2008) who studied on the phylogentic relationship of selected species in the family Muscicapidae inferred from partial mitochondria DNA cytochrome oxidase I gene concluded that, species from genera *Rhipidura*, *Philentoma*, *Hypothymis* and *Terpsiphone* which are not included in Family Muscicapidae by Monroe and Sibley (1993), have close relationship with this family. Daud (2008) also concluded that *Philentoma* which is grouped as Monarchinae together with *Hypothymis* and *Terpsiphone*, is actually not related.

2.2 Mitochondrial DNA

The usage of mtDNA in the reconstruction of phylogeny of aves is proven to be effective. According to Wilson *et.al* (1985), mtDNA can be used in studies done above species level where it concerns genealogical tree, time scales, and the accumulation of new mutations on surviving molecular lineage. mtDNA can also be used in studies regarding below species level concerning population structure, migration concerning ancestral lineage. Wilson *et.al* (1985) also stated that mtDNA could be used in constructing trees and time scale related to molecular lineages both at and also below species level.

One of the study done was by Dumbacher *et.al* (2003) who constructed the phylogeny of the owlet-nightjars (Aves: Aegothelidae) based on mitochondrial DNA sequence. According to Dumbacher *et.al*, the molecular phylogeny constructed by using DNA extracted from museum specimen shows that most of the species in this family are monophyletic except for Aegotheles bennettii.

Another study was conducted by Whittingham *et.al* (2002) who reconstructed the phylogeny of the Tree Swallow genus, Tachycineta (Aves:Hirundidae), by Bayesian Analysis of mitochondrial DNA sequences. The phylogeny that was constructed consists of two main clades which are the South and Central American species and North American and Caribbean species.

2.3 Cytochrome oxidase I (COI)

Webb and Moore (2005) constructed a phylogenetic analysis of woodpeckers and their allies using 12S, Cyt b, and COI nucleotide sequences (class Aves; order Piciformes). The analysis which was conducted using maximum parsimony, neighbor-joining, maximum likelihood, and Bayesian algorithms shows monophyly of Indicatoridae + Picidae (infraorder Picides), Picidae, Picinae + Picumninae, and Picinae.

The usage of mtDNA cytochrome oxidase 1 is effective to determine the phylogeny of birds. Hasbullah (2007) expanded the molecular phylogenies of babblers (Family: Timalidae) using mitochondrial DNA Cytochrome Oxidase 1 (CO1) gene and concluded that it is a good genetic marker because it can detect interspecific variations between species. Phylogenetic tree shows that four genera of family Timalidae are closely related.

3.0 Materials and methods

3.1 Study site

There are five locations where sampling was conducted for this study. The first location was at Gading National Park, Lundu Sarawak (N 1º44’00.00” E109º 50’00.00”) whereas the second location is Gunung Jagoi, Bau, Sarawak (N 01º21.938’ E 110º02.395’). The other three locations are Universiti Malaysia Sarawak (N 1º27’52.05’’ E 110 º25’38.96’’), Kubah National Park (N 1º36’43.22’’ E 110º11’ 48.86’’) and Kampung Pueh, Semantan (N 1º47’ 15.93’’ E 109º45’22.25’’).

Figure 1: Study site indicated by yellow pin (Source: Google map)

3.2 Sample collection

Sampling has been conducted in various localities to obtain muscle and liver sample of flycatchers. Total of 16 individuals comprising eight species of flycatcher were captured throughout the sampling session.

Samples were collected using the standard 2.5 × 36mm mist-net. The mist nets were set up in suitable places mainly in an understory level and an open area. Other factors such as shelter and food source of flycatchers were taken into consideration while setting the mist nets. Each captured bird was placed in a cloth bag and identified using Smythies (1999), MacKinnon and Phillipps (1993). Morphological characters of the bird such as weight, total length, wing length, bill length, tarsus, bill depth, head bill and wing span were measured and recorded in bird data book.

Tissue samples were taken from muscle and liver of the bird. Tissues are collected and preserved in absolute ethanol (99%) and stored in freezer. One of the birds was made into dry specimen and the others were made into wet specimen where they are preserved in absolute ethanol (99%). These specimens were kept in UNIMAS Zoology Museum. Species of flycatchers captured are shown in Table 1. A total of 16 individuals comprising of eight species were captured from five different localities.

Besides collecting fresh samples from flycatchers which were captured, tissue samples were also collected from voucher specimen found in Department of Zoology, UNIMAS museum and IBEC museum. Sample from a total of 21 individuals comprising of nine species of flycatchers were obtained from voucher specimen. Tissue samples collected from voucher specimen are shown in Table 2. The total numbers of samples collected were from 37 individuals consisting of 13 species.

Table 1: List of species of flycatchers captured.

Individual Abbreviation	Species	Locality	Type of tissue used
PRU 001	Grey chested jungle flycatcher *(Rhinomyias umratilis)*	Gading NP	Muscle
PRU 003	Grey chested jungle flycatcher *(Rhinomyias umratilis)*	Mount Jagoi	Muscle
PFD 001	Rufous chested flycatcher *(Ficedula dumetoria)*	Mount Jagoi	Muscle
PFD 004	Rufous chested flycatcher *(Ficedula dumetoria)*	Unimas forest	Muscle
PCC 001	Large billed blue flycatcher *(Cyornis caerulatus)*	Gading NP	Muscle
PCC 002	Large billed blue flycatcher *(Cyornis caerulatus)*	Unimas forest	Muscle
PCE 001	Grey headed canary flycatcher *(Culicicapa ceylonensis)*	Mount Jagoi	Muscle
PCE 002	Grey headed canary flycatcher *(Culicicapa ceylonensis)*	Mount Jagoi	Muscle
PTP 001	Asian paradise flycatcher *(Terpsiphone paradise)*	Mount Jagoi	Muscle
PTP 002	Asian paradise flycatcher *(Terpsiphone paradise)*	Mount Jagoi	Muscle
PCT 001	Malaysian blue flycatcher *(Cyornis turcosus)*	Mount Jagoi	Muscle
PRJ 003	Pied fantail *(Rhipidura javanica)*	Unimas forest	Muscle
PRJ 004	Pied fantail *(Rhipidura javanica)*	Unimas forest	Muscle
PRJ 007	Pied fantail *(Rhipidura javanica)*	Kg Pueh	Muscle

Table 1: continued

PCR 001	Mangrove blue flycatcher *(Cyornis rufigastra)*	Unimas forest	Muscle
PCR 002	Mangrove blue flycatcher *(Cyornis rufigastra)*	Unimas forest	Muscle

Table 2: List of tissue samples collected from voucher specimen

Individual Abbreviation	Species	Collected from	Locality	Type of tissue used
PHA 001	Black naped monarch (*Hypothymis azurea)*	DZ	Samunsam WS	Muscle
PHA 002	Black naped monarch (*Hypothymis azurea)*	DZ	Kubah NP	Muscle
PHA 003	Black naped monarch (*Hypothymis azurea*)	DZ	-	Muscle
PRP 001	Spotted fantail (*Rhipidura perlata*)	DZ	-	Muscle
PRP 002	Spotted fantail (*Rhipidura perlata*)	DZ	Kubah NP	Muscle
PRP 003	Spotted fantail (*Rhipidura perlata*)	DZ	Kubah NP	Muscle
PPP 001	Rufous winged jungle flycatcher (*Philentoma pyrhopterum*)	DZ	Lambir NP	Muscle
PRU 002	Grey chested jungle flycatcher (*Rhinomyias umbratilis*)	DZ	Kubah NP	Muscle
PRA 001	White throated fantail (*Rhipidura albicollis*)	DZ	Kg Sebayor	Muscle
PRA 002	White throated fantail (*Rhipidura albicollis*)	DZ	Kg Sebayor	Muscle
PRA 003	White throated fantail (*Rhipidura albicollis*)	DZ	Kg Sebayor	Muscle
PRA 004	White throated fantail (*Rhipidura albicollis*)	DZ	-	Muscle
PCS 001	Bornean blue flycatcher (*Cyornis superbus*)	DZ	-	Muscle