PHYLOGENETICS OF TROPICAL BUTTERFLIES (FAMILY: NYMPHALIDAE) IN THE RAIN FORESTS OF SABAH, MALAYSIA

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DECLARATION

The materials in this thesis are original except for quotations, excerpts, summaries and references, which have been duly acknowledged.

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ABSTRAK

FILOGENETIK KUPU-KUPU TROPIKA (FAMILI: NYMPHALIDAE) DI HUTAN HUJAN DI SABAH, MALAYSIA

Penyelidikan ini mengkaji filogenetik kupu-kupu dari genus *Mycalesis* dan spesies kriptik dari genus *Euthalia* dan *Tanaecia*. Kawasan kajian terdiri daripada sepuluh hutan simpan tanah rendah di Sabah, Malaysia. Teknik perangkap berumpan dan penangkap tangan digunakan untuk menyampel kupu-kupu. Dalam kajian ini, gen DNA mitochondria COII digunakan untuk membentuk hubungan filogenetik bagi kumpulan yang dikaji. Dalam kajian *Mycalesis*, 396 pasangan bes jujukan DNA diperolehi daripada 42 individu mewakili 13 spesies. Analisis hubungan filogenetik mendedahkan bahawa spesiesnya membentuk empat kumpulan utama, yang juga disokong oleh persamaan yang tinggi dalam ciri-ciri morfologi berasaskan genitalia jantan dan corak warna kepak. Species yang menjadi taksa asas kepada klednya mempunyai taburan geografi yang lebih luas berbanding ahli yang lain dalam kled tersebut. Analisis perwarisan ekologi menunjukkan evolusi *Mycalesis* adalah mengikut corak evolusi berkala, iaitu evolusi spesies berkembang mengikut peredaran masa. Dalam kajian spesies kriptik genus *Euthalia* dan *Tanaecia*, 416 pasangan bes jujukan DNA diperolehi daripada 34 individu, terdiri daripada lima species dari genus *Euthalia* and lima species dari genus *Tanaecia*. Analisis filogenetik menunjukkan *Euthalia* adalah parafiletik terhadap *Tanaecia*. Tahap perbezaan genetik secara relatifnya adalah kecil bagi spesies *Tanaecia* berbanding *Euthalia*. Dua spesies *Euthalia*, *E. iapis* dan *E. godarti* muncul sebagai kumpulan monofiletik kepada spesies *Tanaecia*. Keputusan ini menunjukkan *E. iapis* dan *E. godarti* sepatutnya berada dalam kumpulan *Tanaecia* berbanding *Euthalia*. Sebaliknya, *E. iapis* dan *E. godarti* mempunyai ciri-ciri genitalia dan kepak yang sangat menyerupai spesies lain *Euthalia*. Hubungan filogenetik kedua-dua genus lebih jelas berpandukan analisis menggunakan cuma individu jantan. Keputusan ini menunjukkan identifikasi spesies tidak dapat bergantung kepada ciri-ciri morfologi betina. Informasi filogenetik *Mycalesis* serta genus *Euthalia* dan *Tanaecia* digunakan dalam kajian penggunaan diversiti filogenetik bagi pemuliharaan hutan simpan di Sabah. Keputusan menunjukkan tiada pola bagi menggambarkan hubungan di antara kedua-dua kumpulan yang dikaji. Hubungan di antara diversiti filogenetik *Mycalesis* dan beberapa parameter lain iaitu bilangan spesies *Mycalesis* yang disampel, bilangan semua spesies yang disampel, indeks diversiti Shannon-Wiener dan saiz hutan simpan dikamilkan dalam kajian ini. Keputusan menunjukkan diversiti filogenetik *Mycalesis* berhubungan positif dengan bilangan spesies *Mycalesis* yang disampel, dan bilangan spesies *Mycalesis* yang disampel berhubungan positif dengan saiz hutan simpan. Keputusan ini menunjukkan saiz habitat adalah penting dalam pemuliharaan spesies yang lemah seperti kebanyakan spesies *Mycalesis*, dan seterusnya untuk pemuliharaan diversiti filogenetik. Namun begitu, keputusan ini harus diterjemah secara berhati-hati kerana hanya melibatkan bilangan spesies yang terhad, dan tidak dapat dinafikan lebih banyak kajian diperlukan untuk menyokong kesimpulan yang dicadangkan.

ABSTRACT

PHYLOGENETICS OF TROPICAL BUTTERFLIES (FAMILY: NYMPHALIDAE) IN THE RAIN FORESTS OF SABAH, BORNEO

This study examined phylogenetics of the butterflies genus Mycalesis and cryptic species of the genera Euthalia and Tanaecia. The study sites consisted of ten lowland rain forest reserves in Sabah, Borneo. Baited traps and hand-netting techniques were used in butterfly sampling. In this study, mitochondrial DNA COII gene was used to infer phylogenetic relationships of the study groups. In the study of Mycalesis, 396 base pairs of DNA sequences were obtained from 42 individuals representing 13 species. The analyses of phylogenetic relationship revealed that the species formed four major clades, which were supported by high similarities in morphological characteristics based on male genitalia and wing colour patterns. The species that appeared as a basal taxon to its clade has wider geographical distribution compared with the other members of the clade. The analysis of ecological traits showed that species evolution of Mycalesis follows the gradual evolution patterns, of which the species evolution progressed with time. In the study of cryptic species of the genera Euthalia and Tanaecia, 416 base pairs of DNA sequences were obtained from 34 individuals, comprising of five species of the genus Euthalia and five species of the genus Tanaecia.. The phylogenetic analyses showed that the genus Euthalia is paraphyletic to Tanaecia. The level of genetic divergence is relatively low in Tanaecia species compared with Euthalia. Two of the Euthalia species, E. iapis and E. godarti appeared as a monophyletic group to the Tanaecia species. The results suggest that E. iapis and E. godarti should belong to the Tanaecia group rather than Euthalia. In contrast, E. iapis and E. godarti possessed genitalia and wing characteristics highly similar with the other species of Euthalia. By using male individuals in the phylogenetic analyses, relationships of the two genera were better resolved. The results reflected the unreliability of species identification based on female morphological characteristics. The phylogenetic information of Mycalesis and the genera Euthalia and Tanaecia were applied in the investigation of the use of phylogenetic diversity in forest reserve conservation in Sabah. Results showed no pattern on relationships between the phylogenetic diversity of the two study groups. Relationships of the phylogenetic diversity of Mycalesis and some other parameters which were species number of Mycalesis sampled, total number of species sampled, Shannon-Wiener diversity index and the size of the reserves were included in this study. The results showed that the phylogenetic diversity of Mycalesis was positively correlated with number of species of Mycalesis sampled, and the number of Mycalesis species was positively correlated with the reserve sizes. This findings suggest that habitat sizes is important in conserving vulnerable species, as many Mycalesis species and thus, is important in conserving phylogenetic diversity. However, caution must be taken when interpreting the results because this study was based on limited range of species, and undoubtedly, more studies are needed to support the proposed conclusions.

LIST OF ABBREVIATIONS

Ala	-	Alanine
Arg	-	Arginine
Asn	-	Asparagine
Asp	-	Aspartic acid
Cys	-	Cysteine
Gln	-	Glutamine
Glu	-	Glutamic acid
Gly	-	Glycine
His	-	Histidine
Ile	-	Isoleucine
Leu	-	Leucine
Lys	-	Lysine
Phe	-	Phenylalanine
Pro	-	Proline
Ser	-	Serine
Thr	-	Threonine
Trp	-	Tryptophan
Tyr	-	Tyrosine
Val	-	Valine
TER	-	Termination (stop codon)

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CHAPTER 1

INTRODUCTION

A large fraction of the terrestrial biological diversity occurs in tropical rain forests. Covering only 6% of the earth's surface, tropical rain forests contain about 50% of global species richness (Terborgh, 1992). Fossil evidence indicates that tropical forests have been present since the Cretaceous (144 to 65 million years ago) (Richards, 1996) and thus there has been a very long stable period for speciation to take place. As described by Wilson (1992), great biological diversity takes long stretches of geological time and the accumulation of large reservoirs of unique genes.

Some of the species found in tropical forests occur only in the small area where they evolved and such endemic species are especially vulnerable because of their localized distribution. The destruction of even relatively small areas of forest can therefore eliminate entire species. The pattern of decreasing species richness has been shown in moths as the forest becomes more disturbed and fragmented (Holloway *et al.,* 1992).

Over the past few decades, human activities have been the major threats to rain forest communities, mainly due to activities such as commercial logging and conversion of forest to other land uses. Therefore, a critical question in conservation biology concerns how loss to natural habitat affects the persistence of plant and animal populations and the distribution of genetic variation within them (Gibbs, 2001).

In the study of biodiversity and conservation, it is important to have a reliable system for identification of various distinct units such as species and subspecies. Undoubtedly, many groups of living organisms on earth, in particular invertebrates, remain difficult to separate into these basic units such as due to lack of differences in their morphology. In many cases, lack of information means that individuals are simply classified as morphospecies but inventories of animals and plants based on morphospecies may suffer from taxonomic validity and thus not an accurate/perfect estimators of biodiversity. This is especially true for tropical communities, which contain many cryptic species that may be highly distinct genetically yet difficult to distinguish using traditional morphological methods.

There are two important levels in the biological diversity of life; genetic variation within species and genetic differences among species. The development of molecular genetic techniques has contributed a lot of valuable information to biology and one of their most useful contributions is to enable identification of species that cannot be distinguished by morphological techniques (cryptic species). In many cases, even in well-studied groups such as butterflies, many species cannot be reliably identified through morphological differences such as wing colour patterns. Therefore, the use of molecular genetic techniques will be critically important, and their uses in invertebrate biodiversity studies need to be determined.

In recent years, molecular data have become very important in inferring phylogenies. Phylogenetic trees describe the pattern of descent among a group of species. Certain kinds of molecular data (especially DNA sequences) can provide many more characters (such as informative variable sites), and hence more information, than morphological data (especially for morphologically similar species or cryptic species). With the rapid accumulation of DNA sequence data, more phylogenies are being constructed based upon sequence comparisons.

Certain DNA sequences that evolve rapidly are useful for analysing relationships among closely related species, whereas others that evolve very slowly may be useful for assessing relationships among very old groups. A phylogenetic tree is an estimate of part of this history (a hypothesis of what the history has been) (Futuyma, 1998).

The uses of phylogenies provide new ways to measure biodiversity, to assess conservation priorities, and to quantify the evolutionary history in any set of species. Moreover, there is increasing support for the view that the most useful criterion for classifying organisms is their phylogenetic relationship (i.e common ancestry) because this relationship generally conveys the most information about the characteristics (known and yet to be described) of the members of a taxon (Mace *et al.,* 2003). All the events of biological evolution are sustained along the branches of phylogenetic trees. As a consequence, these phylogenetic trees preserve traces of the historical evolutionary processes that gave rise to the diversity of contemporary species (Pagel, 1999).

Inferring phylogenetic relationships from molecular data requires selection of methods from many techniques that have been described (Pagel, 1999). The combination of these phylogenies with powerful new statistical approaches for the analysis of biological evolution can be an accelerating tool in revealing the history and evolution of life on Earth. However, methodological problems and lack of knowledge about most species have so far restricted their use (Mace *et al.,* 2003).

Located on the northern part of Borneo, Sabah is one of the states in Malaysia. It covers a land area of 73,371 km^2, about 10% of the Borneo landmasss (Marsh & Greer, 1992). The tropical rain forest of Sabah is believed to be one of the oldest rain forests in the world (Chey *et al.,* 1997) and is rich in flora and fauna with very high diversity in many insect groups, including butterflies.

In 1986, 60.1% of Sabah's land area was lowland tropical rain forest (Marsh & Greer, 1992). Despite the fact that the region is still heavily forested by regional standards, there remains relatively little undisturbed lowland rain forest today (Marsh & Greer, 1992). Conversion of rain forest into oil palm plantation has generated most of the income to the state (Sabah Institute Development Studies, 1999). This activity has created patches of rain forest that may contribute significantly to the conservation of rain forest biodiversity. Some of the forest patches have been gazetted as Virgin Jungle Reserves (VJRs) to protect them from further disturbance. However, not much information available for forest managers to making informed recommendations as to the size and placement of forest patches to be preserved in future agricultural developments. Based on mammal study in Peninsular Malaysia, Laidlaw (2001) proposed that a positive management strategy would be to retain large, continuous areas of forest wherever possible, in preference to fragmenting forest, and to build on existing small protected-area networks, such as the VJR network, so that logged forests contain a rich mix of undisturbed protected areas.

Habitat fragmentation has the effect of creating 'islands' of remaining rain forest in a 'sea' of agricultural land. As proposed by the theory of island biogeography (Mac Arthur & Wilson, 1967), if 90% of an area is destroyed and the remaining 10% is preserved as a habitat island, this island will initially support most the original area's species, but this is more species than it can support at equilibrium. Despite the increasing threats on biodiversity from conversion of rain forest into other land uses (such as oil palm plantation) in Sabah, little is known about evolutionary histories and relationships of species that compose the complex ecosystem of rain forest in this region. Undeniably, knowledge of evolutionary history and relationships among species will contribute significantly to our understanding of species diversification, and so biodiversity. Sabah is widely known for its richness in biodiversity, but study on species evolution and diversification has receives little attention.

Due to lack of knowledge on evolutionary history and relationship of species in this region, there is a tendency that we may lose species with high conservation value following habitat fragmentation, before we are able to study them. This study attempted to use molecular genetic tools to provide some information on evolutionary relationships of butterfly species in Sabah. The study was focused on butterflies because their diversity are relatively high in Sabah (approximately 850 species, out of 910 species that have been recorded in Borneo) with about 50 species endemic to Borneo (Otsuka 1988). All of the forest patches included in this study are lowland rain forest and surrounded by oil palm plantation. The information gained from the study could be useful for creating guidelines for the conservation of butterfly biodiversity in forest patches. Even though this study focused on butterflies, the results are likely to be applicable to other groups of animal that depend on similar habitat requirements.

Butterflies are among important components of forest ecosystem for their roles as herbivores, pollinators and prey. Many butterfly species at larval stage are generally dependent on a single host-plant species, or only a small group of host-plants (Holloway *et al.*, 2001). Generally, reproductive rate of butterfly is high with short generation times, and relatively poor in dispersal ability. These characters make butterflies among the suitable groups for studying forest biodiversity and conservation.

Tropical butterfly communities consist of two major adult feeding guilds. One guild is composed of species that obtain majority of their adult nutritional requirements from flower nectar and the second guild is composed of species whose adults gain virtually all of their nutritional requirement by feeding on rotting fruits, of which many of the species belong to the family Nymphalidae (Corbet & pendlebury, 1992; Wood & Gillman, 1998). Approximately 75% of nymphalid species recorded on Borneo feed on fruit juices (Hill *et al.,* 2001).

In this study, molecular genetic techniques were used to determine evolutionary relationships of two groups of butterflies that belong to the family Nymphalidae, which are the genus *Mycalesis* and the cryptic group, consisting of the genera *Euthalia* and *Tanaecia*. The inferred phylogenetic relationships among species of the genus *Mycalesis* (subfamily Satyrinae) were used to understanding the pattern of species diversification within the genus and to identify species with high conservation value. Despite of well-studied butterfly taxonomy in Borneo, the genera *Euthalia* and *Tanaecia* are difficult to be identified at species level based on their morphological appearance. The two genera have been described into several different species but the reliability of this classification is unknown. Additionally, this assemblage of butterflies is common in rain forest and it is likely that the problems encountered with *Euthalia* or *Tanaecia* are also found in other groups of butterflies. In this study, the genetic differences in these genera were investigated in order to understand their evolutionary relationships and taxonomic classification.

Information about evolutionary relationships among species is being exploited in many ways for the purpose of optimising the conservation value of species and habitat. Gibbs (2001) proposed that conservation of genetic diversity in wild populations should be based on both habitat and population management, and this can be best achieved by maintaining large, local population management that are well-distributed among networks of breeding habitats. The use of phylogenetic diversity as a biodiversity measure has been proposed by Faith (1992). This measure is based on a phylogeny, which takes into account genetic distance among species in a given area. As a result, area that contains high value of phylogenetic diversity is considered as high priority for conservation purpose. This study applied phylogenetic diversity as a biodiversity measure in different rain forest patches in Sabah in order to assess its significance as a tool in species and habitat conservation in this region.

Aims of the study are:

1. To use molecular techniques to describe phylogenetic relationships among butterflies of the genus *Mycalesis*. Information from this study would be useful in understanding evolutionary processes that led to present biodiversity status in tropical rain forest of Sabah.
2. To use molecular techniques to investigate evolutionary relationships of the cryptic butterfly species in the genera *Euthalia* and *Tanaecia*. As a comparison to the genus *Mycalesis*, of which members of this genus are morphologically distinct, the molecular techniques applied in this study is hoped to clarify taxonomic status of the genera *Euthalia* and *Tanaecia*.
3. To assess the use of phylogenetic diversity as a biodiversity measure in prioritising conservation of forest reserves based on phylogenetic information of *Mycalesis* and the genera *Euthalia* and *Tanaecia*.

CHAPTER 2

LITERATURE REVIEW

2.1 The importance of phylogenetics in understanding biodiversity of Borneo

Biodiversity encompasses variation at all levels of organisms ranking from genes within a single population or species, to species composing all part of a local community, and finally to the communities themselves that make up the different ecosystems of the world (Wilson, 1997). The most widely recognized pattern of biodiversity is the latitudinal gradient of species richness, of which the number of species in almost all group of organisms increase towards the tropics (Wilson, 1992; Gaston, 2000).

Located between 23.5°N and 23.5°S, tropical forests are the most species rich ecosystems on earth (Rosenzweig, 1995). Tropical forests consists of two major kinds of forests: 1) rain forest which is aseasonal and 2) monsoon forest which has well defined wet and dry seasons, and the combination of these two types of forest is often referred to as tropical moist forest (Collins *et al.*, 1991). The main areas of tropical rain forest are South America, Africa and Asia (Ooi, 1993). In Asia the richest forests are in Borneo and Peninsular Malaysia (Ooi, 1993).

Unfortunately, much biodiversity of tropical forests is poorly known (Gentry, 1992). For many groups, most notably insects, the level of taxonomic knowledge is inadequate to cope with such high diversity. Most species await description, whereas other taxa have been redescribed many time.