SPATIAL DYNAMICS OF *PLASMODIUM KNOWLESI* VECTORS AND MOLECULAR CHARACTERIZATION OF ITS PARASITES IN KUDAT, SABAH, MALAYSIA

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WONG MENG LI

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SPATIAL DYNAMICS OF *PLASMODIUM KNOWLESI* VECTORS AND MOLECULAR CHARACTERIZATION OF ITS PARASITES IN KUDAT, SABAH, MALAYSIA

ABSTRACT

Plasmodium knowlesi is the fifth human malaria and poses a new threat to malaria elimination since there is a naturally existing zoonotic reservoir. In Sabah, *P. knowlesi* cases were reported in great number in 2012 and 2013 with 815 and 996 cases, respectively. Previous extensive studies had incriminated *An. balabacensis* as the vector for human malaria. However, significant environmental changes in Sabah due to land conversion from forest to plantations may have displaced the primary vector. Hence, the objective of this study was to determine the spatial dynamics of *P. knowlesi* vectors in Kudat, Sabah, and to to conduct molecular characterization of its parasites. A one year entomological survey in Kudat, Sabah was conducted using bare leg catch (BLC). A total of 2263 mosquitoes were caught consisting of 1884 anophelines (10 species) and 379 culicines in which *An. balabacensis* was the predominant species (95.1% of the total caught) while other anophelines were in low numbers. Morphologically identified *An. balabacensis, An. introlatus, An. latens, An.* cracens and *An. macarthuri* were confirmed their species status by using mtCOI, ITS2 and 18S gene markers. Restriction fragment length polymorphism analysis of ITS2 amplicons using restriction enzyme *Msp I* generated six RFLP genotype profiles whereby two of the profiles belong to *An. cracens.* Also analysis with native acrylamide gel indicated that there are variant present within the ITS2 region of *An. cracens.* Phylogenetic analysis of mtCOI, ITS2 and 18s showed that collected Leucosphyrus Group of mosquitoes clustered accordingly in their specific clade. The peak biting time of *An. balabacensis* was between 1800-2000hrs. Highest density of *An. balabacensis* was observed January in village (KP), August and November in farm (TD), and relatively low in forest (LL). Range of *An. balabacensis* caught with varied between 2-28 mosquitoes per night. In addition, the

high survival rate, parity rate (>50%), sporozoite rate (2-12.50%) and vectorial capacity (>3.0) in *An. balabacensis* was obtained in this study. There were forty five (3%) *An. balabacensis* were positive for *Plasmodium* oocysts (18), sporozoites (14) or both (13) by microscopy. Using human and simian malaria species-specific PCR primers, four simian malaria parasites were detected, namely *P. knowlesi, P. inui, P. cynomolgi* and *P. coatneyi.* There were mono-infection and mixed infections (up to quadruple infections) detected in *An. balabacensis*. Thirteen *An. balabacensis* were positive for *P. knowlesi*. The SSU rRNA gene of *Plasmodium* from 10 isolates of *An. balabacensis* were amplified, cloned and sequenced. Analysis of the SSU rRNA gene sequences confirmed the identity of the simian malaria parasites that were detected by PCR in the mosquitoes. Genetic analysis of the simian malaria parasites in *An. balabacensis* in Kudat showed high haplotype diversity indicating they were undergoing expansion. PCR of the pooled dried mosquitoes showed that *P. cynomolgi* was the predominant simian malaria identified in all study sites. Based on the current entomological findings supported by molecular data, *An. balabacensis* is a highly competent vector and will continue to pose a risk to human malaria transmission. Thus effective malaria control measures need to be devised and carried out in order for Malaysia to move towards malaria elimination.

Keywords: *Plasmodium knowlesi,* Leucosphyrus Group, identification, bionomics, phylogenetic

DINAMIK SPATIAL VEKTOR *PLASMODIUM KNOWLESI* DAN PENCIRIAN MOLEKULAR PARASIT DI KUDAT, SABAH, MALAYSIA

ABSTRAK

Plasmodium knowlesi adalah malaria manusia kelima dan satu ancaman baru kepada program eliminasi malaria memandangkan terdapatnya sumber jangkitan zoonotik semula jadi. Di Sabah, bilangan kes *P. knowlesi* yang dilaporkan pada 2012 dan 2013 adalah tinggi iaitu sebanyak 815 dan 996 kes. Kajian menyeluruh sebelum ini telah menentukan *An. balabacensis* sebagai vektor malaria manusia. Namun perubahan alam sekitar yang ketara di Sabah yang disebabkan oleh pindahguna tanah dari hutan kepada pertanian berkemungkinan menyesarkan vektor primer. Oleh itu objektif kajian ini adalah untuk menentukan dinamik spatial vektor *P. knowlesi* di Sabah dan menjalankan pencirian molekular parasit. Satu tinjau selidik entomologi selama setahun dijalankan dengan menggunakan kaedah pendedahan kaki (BLC). Sejumlah 2263 ekor nyamuk telah ditangkap yang terdiri daripada 1884 anophelines (10 species) dan 379 culicines di mana *An. balabacensis* merupakan spesies pradominan (95.1% daripada jumlah keseluruhan tangkapan) dan spesies lain dalam kuantiti yang rendah. Pengesahan status spesies *An. balabacensis, An. latens, An. introlatus, An. cracens* dan *An. macarthuri* yang dikenalpasti secara morfologi dijalankan dengan menggunakan gen mtCOI, ITS2 dan 18S. Analisis polimorfisme panjang serpihan penyekatan (RFLP) untuk amplicon ITS2 dengan enzim penyekatan, *Msp* I menghasilkan enam profil genotip RFLP di mana *An. cracens* mempunyai dua profil genotip. Tambahan lagi, analisis dengan gel akrilamid asli menunjukkan kehadiran variasi dalam bahagian ITS2 *An. cracens.* Analisis filogenetik mtCOI, ITS2 dan 18S menunjukkan nyamuk dalam kumpulan Leucosphyrus yang ditangkap dibahagikan mengikut spesies masing-masing. Dalam pada itu, puncak tertinggi masa gigitan *An. balabacensis* dikenalpasti di antara jam 1800-2000. Selain itu, kadar kegigitan tertinggi *An. balabacensis* adalah pada bulan

Januari di kampung (KP), Ogos dan November di kebun (TD) dan agak rendah di hutan (LL). Julat tangkapan *An. balabacensis* adalah antara 2-28 nyamuk setiap malam. Tambahan lagi, kadar kemandirian, kadar pariti (>50%), kadar sporozit (2-12.50%) dan kapasiti vektorial (>3.0) *An. balabacensis* yang tinggi diperoleh dalam kajian ini. Terdapat 45 (3%) ekor *An. balabacensis* yang positif untuk *Plasmodium* secara mikroskopi di mana 18 positif oosista, 14 positif sporozit dan 13 positif kedua-duanya. Dengan menggunakan primer malaria manusia dan monyet yang spesies-spesifik, empat parasit malaria monyet dikesan iaitu *P. knowlesi, P. inui, P. cynomolgi* dan *P. coatneyi.* Jangkitan mono dan bercampur didapati daripada sampel *An. balabacensis.* Tiga belas *An. balabacensis* adalah positif untuk *P. knowlesi.* Gen SSU rRNA *Plasmodium* daripada 10 *An. balabacensis* yang infektif telah diamplifikasikan, diklonkan dan dirangkaikan secara spesifik. Analisis rangkaian untuk gen SSU rRNA mengesahkan identiti parasit malaria monyet dalam nyamuk yang dikesan melalui PCR. Analisis genetik parasit malaria monyet dalam *An. balabacensis* di Kudat mendapati kadar diversiti haplotip adalah tinggi dan ini menunjukkan pengembangan populasi parasit. PCR juga dijalankan ke atas nyamuk yang kering yang dikumpulkan mengikut masa, tarikh dan lokasi unuk pengesahan identiti parasit malaria. Analisis menunjukkan *P. cynomolgi* adalah jangkitan malaria monyet yang pradominan untuk semua lokasi tangkapan. Berdasarkan kepada penemuan semasa entomologi dengan sokongan data molekular menunjukkan *An. balabacensis* adalah vektor yang sangat kompeten dan merupakan risiko bagi jangkitan malaria dalam manusia. Oleh itu, kaedah pengawalan malaria yang efektif harus dirancang dan dijalankan secara konsisten supaya Malaysia boleh bergerak ke arah eliminasi malaria.

Kata kunci: *Plasmodium knowlesi*, Leucosphyrus Group, pengenalpastian, bionomik, filogenetik

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"Let us be grateful to the people who make us happy; they are the charming gardeners who make our souls blossom." **Marcel Proust**

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%	percent
<	less than
>	more than
°C	degree Celcius
±	plus-minus sign
∞	infinity
µL	microliter
µM	micromolar
bp	base pairs
CDC	Centers for Disease Control and Prevention
CI	confidence interval
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
et al.	et alia (and others)
g	gram
hrs	hours
kb	kilobase
Kg	Kampung

m	meter
M	molar
$MgCl_2$	magnesium chloride
mL	milliliter
mM	millimolar
MOH	Ministry of Health
ng	nanogram
no	number
p	probability
PCR	polymerase chain reaction
Per	Perlis
rpm	revolutions per minute
s	seconds
SD	standard deviation
sg	sungai
SS	Sg Sendat
TL	Tawau-*latens*
TM	Tawau-*macarthuri*
UK	Ulu Kalong

V	volt
WHO	World Health Organization

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

Globally, mosquito-borne diseases have remained as one of the major problems to public health. At present, there are about 3,556 mosquito species from 113 genera reported worldwide in which a total of 540 *Anopheles* were described and classified into 8 subgenera (Harbach, 2017). In Malaysia, there are 442 species of mosquitoes representing 20 genera (Miyagi & Toma, 2000). Of these, 75 species are *Anopheles* which consists of 2 subgenera, *Anopheles* and *Cellia* (Rahman et al., 1997). *Anopheles* mosquitoes play an important role in the transmission of malaria where they act as vector. To date, about 13 species have been incriminated in Malaysia as malaria vector: *Anopheles maculatus, An. campestris, An. letifer, An. epiroticus, An. nigerrimus, An. donaldi, An. sundaicus* (Jeffery et al., 2012; Rahman et al., 1997), *An. flavirostris* (Hii et al., 1988a), *An. balabacensis* (Hii et al., 1988a), *An. hackeri (Wharton & Eyles, 1961), An. latens* (Tan et al., 2008; Vythilingam et al., 2006), *An. cracens (Jiram et al., 2012)* and *An. introlatus* (Vythilingam et al., 2014).

Malaria is a life-threatening disease caused by *Plasmodium* sp. parasites which are transmitted to human through the bites of infected female *Anopheles* mosquitoes. In 2015, about half of the world's population (~3.2 billion people) were at risk of malaria (WHO, 2015). The fifth human malaria parasite, *Plasmodium knowlesi* posed a new threat to malaria elimination as it exists naturally as a zoonotic reservoir within its primates' host. Currently, several countries in Southeast Asia (Eede et al., 2009; Figtree et al., 2010; Jiang et al., 2010; Jongwutiwes et al., 2004; Khim et al., 2011; Luchavez et al., 2008; Ng et al., 2008) were affected by *P. knowlesi*, including Malaysia (Barber et al., 2012a; Cox-Singh et al., 2008; Joveen-Neoh et al., 2011; Singh et al., 2004; Vythilingam et al., 2014; Vythilingam et al., 2008; William et al., 2013; Yusof et al.,

2014). In fact, *P. knowlesi* was responsible for the greatest number of malaria cases reported in Sabah, Malaysia with 815 and 996 cases in 2012 and 2013, respectively (William et al., 2014). In 2016, *P. knowlesi* formed about 69% of cases reported in Malaysia (MOH, 2016).

The epidemiology of *P. knowlesi* is closely related to Leucosphyrus Group of mosquitoes, as previous studies suggested that the mosquitoes belonging to Leucosphyrus Group were the vectors of simian malaria (Warren & Wharton, 1963). Known as the forest-dwelling mosquitoes, there are about 8 species occurring in Malaysia which is divided into three subgroups, namely the Leucosphyrus, Hackeri and Riparis subgroups. Within the Leucosphyrus Subgroups, there are two closely-related complexes which are morphologically similar in all the life stages, namely the Leucosphyrus and Dirus Complex (Manguin et al., 2008; Peyton, 1989; Sallum et al., 2005b). Therefore, it is of utmost importance for the development of molecular methods such as polymerase chain reaction (PCR) to precisely identify the individual species and confirm the taxonomic status of the species (Manguin et al., 2008). Correct identification of the insect vector species will have positive implications in the development of vector control strategies. Through molecular approach, species identification can be confirmed precisely, and can be applied to both the sibling species and closely-related groups with overlapping morphological characters (Garros et al., 2005; Kumar et al., 2007). In this way, phylogenetic analysis can be made and thus, assist in comprehending the species evolution over time.

The Leucosphyrus Group of mosquitoes is the main vector for the simian malaria parasite *P. knowlesi*. In Malaysia, the first study incriminated *An. hackeri* as the vector in the coastal area of Selangor (Wharton & Eyles, 1961) followed by *An. latens* in Kapit, Sarawak (Tan et al., 2008; Vythilingam et al., 2006), *An. cracens* in Kuala Lipis, Pahang (Jiram et al., 2012), and *An. introlatus* in Hulu Selangor, Selangor

(Vythilingam et al., 2014). Thus, it is of paramount importance to study the vectors in other parts of Malaysia given that the ecology of the country has changed due to deforestation and development.

There have been significant increase in the reported cases of *P. knowlesi* in Sabah from 2004 (n=59) to 2011 (n=703) (William et al., 2014). The increasing trend of knowlesi malaria cases in Sabah is also observed from 2012 onwards from 815 cases to 1064 cases in 2014 (Jelip et al., 2014; William et al., 2013). The highest proportion of the knowlesi malaria cases reported in Sabah was from Kudat Division, accounting to 87% of cases in 2009.

Therefore, the present study represents the attempt to investigate the spatial dynamics of *P. knowlesi* vectors in Kudat Division, Sabah, Malaysia, and molecular characterization of its parasites.

1.2 Significance of Study

Vital findings obtained from this study would be useful for medical entomologists, molecular biologists and public stakeholders like the Ministry of Health. Molecular identification and characterization of the mosquito's species in the Leucosphyrus Group will be helpful in aiding the precise identification of the vector species within the group, and thus allows for planning of effective control methods.

In addition, the bionomics data of the *P. knowlesi* vectors gathered from the study will enable the understanding of the seasonal abundance and biting behavior of the vectors. As a result, the findings will aid in the development of local vector programs to eliminate malaria transmission in the area. Moreover, molecular characterization of simian malaria parasites in the vector will be valuable in comprehending the relationship of the parasites from mosquitoes, humans and

macaques as there is little information available about the genetic diversity of the simian malaria parasites from mosquitoes.

1.3 Aims and Objectives

The main objective of the present study was to investigate the spatial dynamics of *P. knowlesi* vectors in Kudat division, Sabah, Malaysia and molecular characterization of its parasites. Taking this into consideration, the present study was performed according to the following specific objectives:

(1) To determine the morphological variations of adult Leucosphyrus Group of mosquitoes within Malaysia and develop a simple identification key for identification of the group from Malaysia.

(i) To develop a simple identification key of the species present in Malaysia based on the morphological characters with aid from the current available identification key.

(2) To develop molecular assay for identification of Leucosphyrus Group of mosquitoes in Malaysia.

(i) To amplify the mitochondrial cytochrome oxidase I (mtCOI), 18S small subunit ribosomal RNA (18S SSU rRNA) and internal transcribed spacer 2 (ITS2) gene of the five collected Leucosphyrus Group of mosquitoes.

(ii) To determine the genetic profiles of the mosquitoes' sample through moleculr techniques.

(3) To determine the bionomics of *P. knowlesi* vectors in Sabah

(i) To carry out mosquito sampling (longitudinal study) for a year in Kudat division, Sabah, Malaysia.

(iii) To incriminate the vectors of *P. knowlesi* by determination of entomological indicators.

(iv) To identify the malaria parasites present in the positive mosquito samples.

(4) To characterize the 18S SSU rRNA gene of simian malaria parasites found in the mosquito

(i) To characterize the 18S SSU rRNA gene of the isolates of positive mosquito samples.

(iii) To determine the genetic diversity and phylogenetic relationship of simian malaria parasites in mosquito.

A schematic flowchart of the study is as shown in Figure 1.1.

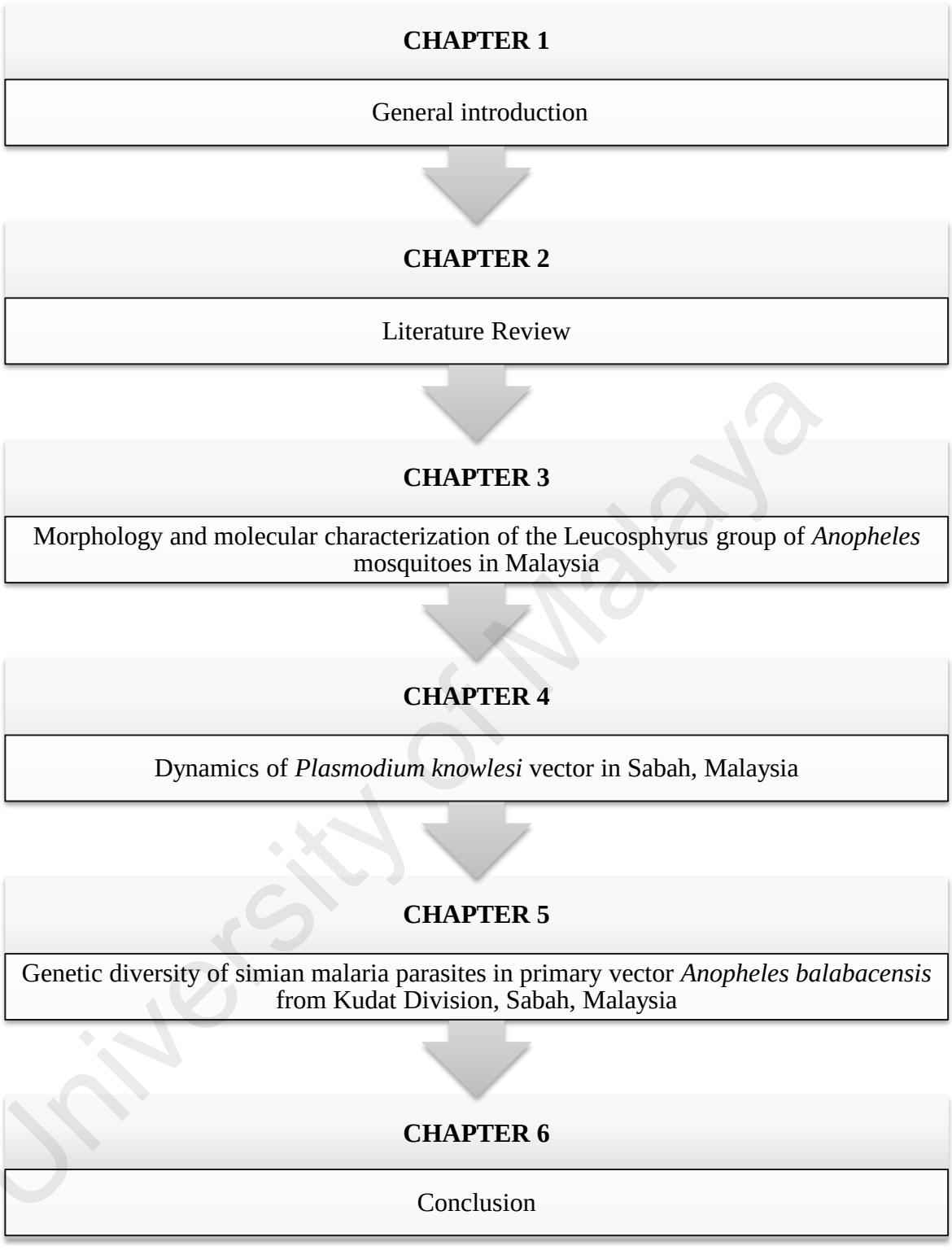

Figure 1.1: Schematic diagram of "Spatial dynamics of *Plasmodium knowlesi* vectors and molecular characterization of its parasites in Kudat, Sabah, Malaysia".

CHAPTER 2: LITERATURE REVIEW

2.1 Malaria

2.1.1 Life cycle of malaria parasites

Malaria parasites require one definitive invertebrate host and one intermediate vertebrate host to complete their life cycle. Transmission of *Plasmodium* to humans and non-human primates were from the bite of infected female anopheline mosquitoes (Coatney et al., 1971; Warrell & Gilles, 2002).

Transmission of *Plasmodium* to vertebrate hosts takes place when the sporozoites are injected into the bloodstream during blood meal from an infective mosquito (Figure 2.1). These sporozoites migrate to the liver and invade the hepatocytes. The parasites undergo intense primary schizogony to produce exoerythrocytic schizonts. However, *Plasmodium* species such as *P. vivax, P. ovale, P. cynomolgi, P. fieldi* and *P. simiovale* are known to produce hypnozoites which are capable to stay dormant in the liver for a period of time before invading the blood cells again (Coatney et al., 1971; Cogswell, 1992;2000; Collins, 2012; Collins & Contacos, 1971; Warrell & Gilles, 2002).

Each exoerythocytic schizonts contain about 30,000 to 50,000 merozoites which are released into the bloodstream when the hepatocytes rupture. The merozoites will infect the red blood cells, which is the erythrocytic stage of the *Plasmodium* life cycle. In the erythrocytes, the merozoites undergo asexual replication producing early trophozoites which are also recognized as the ring forms. These early trophozoites develop into mature trophozoites and undergo schizogony to produce schizonts. The erythrocytes eventually rupture releasing the merozoites into the bloodstream. Some merozoites will infect other erythrocytes and initiate another asexual erythrocytic cycle while some will undergo sexual erythrocytic stage (gametocytes), differentiating into

microgametocytes (male) and macrogametocytes (female). The erythocytic stage of *Plasmodium* parasites is accountable for the clinical manifestations of malaria (fever and chills) through the release of cellular contents from the lysed erythrocytes. The periodicity of malaria parasites, which is the time required to complete an erythrocytic cycle depends on the malaria parasite species. It can range from 24 hours (quotidian) to 48 hours (tertian) or 72 hours (quartan) (Coatney et al., 1971; Cogswell, 2000; Collins, 2012).

The infection cycle in mosquito begin when it bites an infected human and ingest both male and female gametocytes with the blood meal. Development of gametocytes to gametes takes place in the mosquito's gut when the infected red blood cells burst releasing the gametocytes. Both microgamete and macrogamete will fuse, forming a zygote which will further develop into an active moving ookinete. The elongated and motile ookinete will penetrate through the midgut epithelium and develops into an oocyst. Sporogony, the asexual multiplication in mosquito takes place in the oocysts and forms thousands of sporozoites which enlarge the oocysts. When the sporozoites are fully formed, the oocysts rupture and release the sporozoites into the mosquito's haemolymph. The sporozoites then migrate to the salivary glands of the mosquito. The extrinsic incubation period of *Plasmodium* parasites in mosquitoes varies with temperature and species of malaria parasites, but generally it would be between 8-15 days (Collins, 2012; Garnham, 1954). Inoculation of the sporozoites into a new vertebrate host by an infective *Anopheles* mosquito during a blood-meal continues the malaria life cycle (Collins, 2012; Ménard et al., 2013; WHO, 2013a).

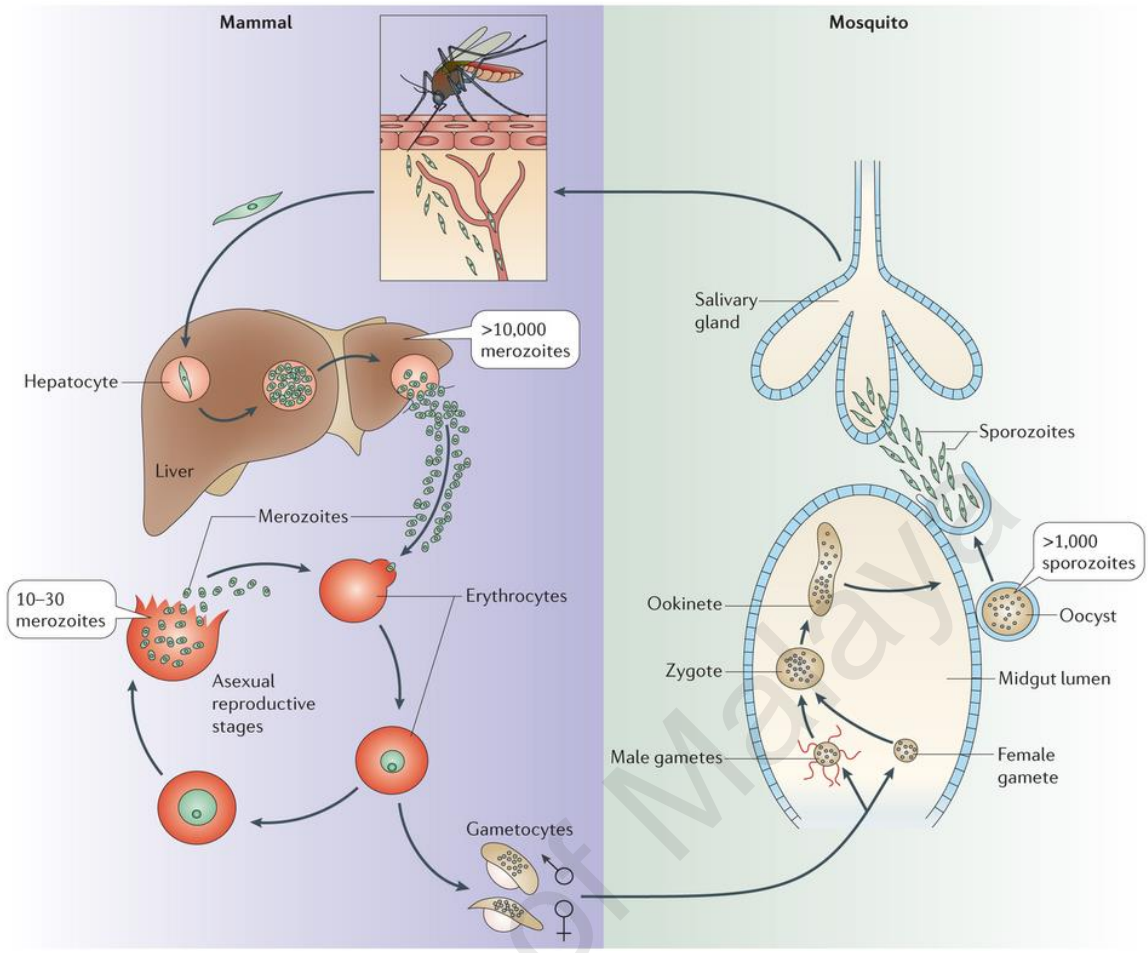

Figure 2.1: The life cycle of malaria parasite (Ménard et al., 2013).

2.1.2 Simian malaria

More than 20 species of simian malaria parasite are known to infect primates while there are 13 species found affecting non-human primates in Southeast Asia (Coatney et al., 1971; Collins, 1988) (Table 2.1). The geographical distribution of simian malaria spans across Asian, African, Central, South and North American region (Faust & Dobson, 2015; Fooden, 1994). Most simian malaria parasites can be categorized with the four known human malaria parasites based on their similar erythrocytic stage periodicity and morphology (Coatney et al., 1971). The distribution of simian malaria affecting macaques in Southeast Asia follows the geographical range of Leucosphyrus Group of mosquitoes, as previous studies have suggested that they are the vectors of simian malaria (Figure 2.2) (Fooden, 1994; Warren & Wharton, 1963).

Table 2.1: List of simian *Plasmodium* species, periodicity, distribution and natural hosts (Coatney et al., 1971; Cogswell, 2000; Deane et al., 1968; Duarte et al., 2008; Eyles, 1963; Fooden, 1994; Garnham, 1966; Lourenco-de-Oliveira & Deane, 1995).

***Plasmodium* species**	**Periodicity**	**Distribution**	**Natural Hosts**
P. inui[c]	Quartan	Southeast Asia, India, Sri Lanka, Taiwan	*Macaca fascicularis, M. nemestrina, M. cyclopis*[#], *M. irus, M. sinica*[*], *M. radiata, M. mulatta, M. nigra, M. arctoides, Presbytis melalophus*
P. cynomolgi[b]		Southeast Asia, India, Sri Lanka	
P. fieldi[d]	Tertian	Southeast Asia	
P. coatneyi[a]		Southeast Asia	
P. knowlesi[a,c]	Quotidian	Southeast Asia	
P. simiovale[d]	Tertian	Sri Lanka	*M. sinica, M. radiata*
P. fragile[a]		India, Sri Lanka	
P. shortii	Quartan	India, Sri Lanka	
P. gonderi[b]	Tertian	Africa	*Cercocebus agilis, C. atys, Lophocebus albigena, Mandrillus leucophaeus, Man. sphinx*
P. petersi	Unknown		
P. georgsi			
P. brasilianum[c]	Quartan	South American	Aotidae, Atelidae, Cebidae and Pitheciidae monkey families
P. simium[b]	Tertian	Brazil	*Alouatta* and *Brachyteles* monkeys
P. pitheci[b]	Tertian	Southeast Asia	Orang utans
P. silvaticum[b]			
P. eylesi[b]	Tertian	Southeast Asia	Gibbons
P. hylobati[b]			
P. jefferyi[b]			
P. youngi[b]			
P. schwetzi[b]	Tertian	Africa	Chimpanzees, Gorillas
P. reichenowi[a]			
P. girardi	Unknown	Madagascar	Lemurs
P. lemuris			
P. foleyi			
P. coulangesi			
P. bucki			
P. wilenbergi			
P. percygarhami			

[a,b,c,d] denotes simian malaria parasites categorized under the *falciparum*-, *vivax*-, *malarias*- and *ovale*-type family, respectively (Coatney et al., 1971; Sabbatani et al., 2010)

[#, *] denotes natural hosts restricted to Taiwan and Sri Lanka only, respectively (Fooden, 1994)

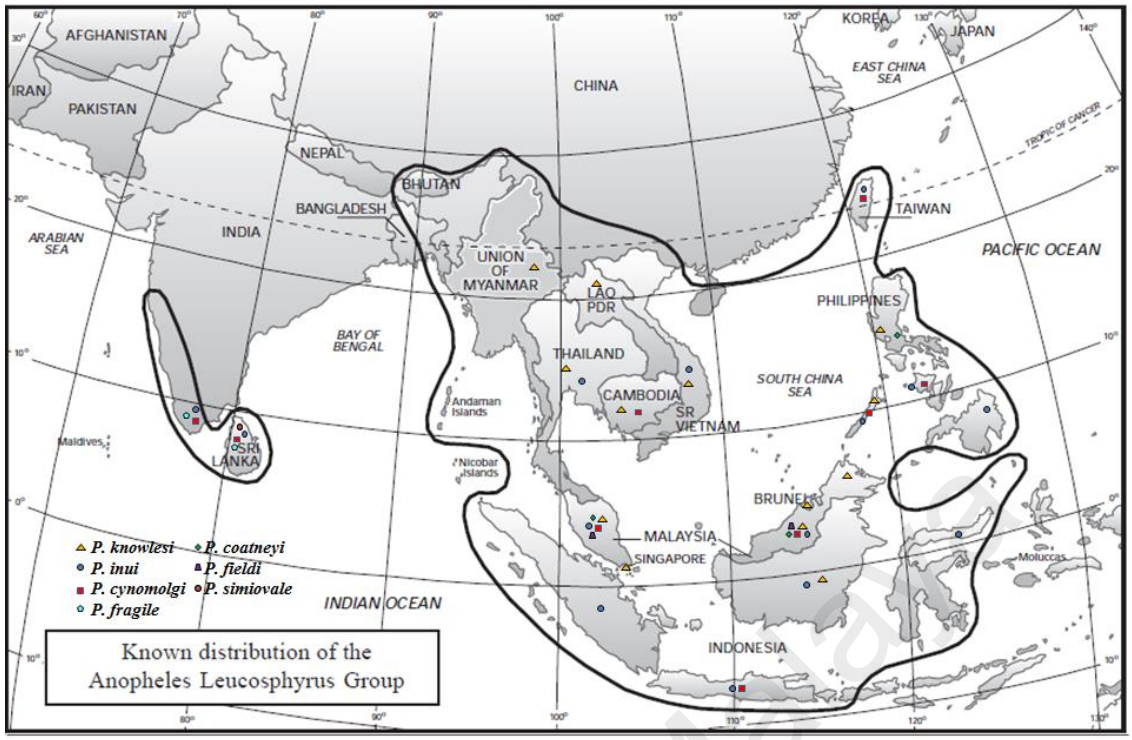

Figure 2.2: Distribution of simian malaria parasites in macaques, adopted from (Coatney et al., 1971; Eyles, 1963; Eyles et al., 1963b; Eyles et al., 1962; Fooden, 1994; Huang et al., 2010; Jeslyn et al., 2011; Jiang et al., 2010; Lee et al., 2011; Moyes et al., 2014; Sandosham et al., 1962; Seethamchai et al., 2008; Setiadi et al., 2016; Tsukamoto et al., 1978; Vythilingam et al., 2008) and the known geographical range of Leucosphyrus Group, adopted from (Sallum et al., 2005b).

2.1.3 History of simian malaria infections in human

Simian malaria parasites were first reported in Malayan monkeys by Daniels (1908). Since then, several studies have been conducted on infections of simian malaria to humans. Blacklock and Adler (1922) detected malaria parasites resembling *P. vivax, P. malariae* and *P. falciparum* in the chimpanzees, and carried out experiments to infect humans by blood passage and mosquito bites but were unsuccessful. Another experiment attempted by Clark and Dunn (1931) with *P. brasilianum* was also not successful. The first reported successful experimental transmission of simian parasites to humans was carried out by Knowles and Das Gupta (1932) by using *P. knowlesi* to infect three human volunteers through blood passage. The parasite was first discovered in *Macaca fascicularis* macaques which were brought to India from Singapore and later formally named as *Plasmodium knowlesi* by Sinton and Muliigan (1933) after Dr Knowles. The fever was observed to be of daily remittent with the clinical symptoms ranging from mild to intermittent to severe fever. In all three infections, the recovery was spontaneous. Owning to the fact that *P. knowlesi* could induce fever, it was later used by Van Rooyen and Pile (1935) as a pyretic agent to treat neuro-syphilis patients. Additionally, another successful experimental infection with *P. inui* to human volunteers was performed by Das Gupta (1938).

Then again, other attempts to infect simian malaria parasites to human via mosquito bite were unsuccessful (Coggeshall, 1941; Sinton et al., 1938), leading to the assumption that transmission of simian malaria to humans would be impossible. Nonetheless, the accidental transmission of *P. cynomolgi* to scientists in United States in 1960 (Coatney, 1963; Eyles et al., 1960) sparked the interest again on the transmissibility of simian malaria to human, and experimental mosquito infections of the simian parasites to human which were initiated showed that simian malaria transmission to human was possible under laboratory settings (Bennett & Warren, 1965;

Chin et al., 1968; Coatney et al., 1966; Coatney et al., 1961; Contacos et al., 1970; Contacos et al., 1962; Contacos et al., 1963; Schmidt et al., 1961).

In 1965, the first natural infection of *P. knowlesi* in human was reported in an American surveyor who had spent nights working in a jungle in Bukit Kertau, Pahang, Malaysia (Chin et al., 1965). Extensive surveillance surveys were performed in the area where the surveyor was infected to determine the possibility of natural simian malaria transmission in humans, in which blood from 1117 persons from 17 villages were examined for malaria parasites by microscopy using Giemsa stained slides. Of these, only 2.5% had infection where 11 were *P. falciparum*, 13 were *P. vivax* and four were not identified. Inoculation of blood into rhesus monkeys were also conducted but none of the rhesus monkeys developed malaria parasites (Warren et al., 1970). Consequently it was presumed that simian malaria infections in human were extremely rare. Few years later, in the 1970's, there was a presumptive case of *P. knowlesi* reported from Johor, Peninsular Malaysia (Fong et al., 1971).

The presumption was proven wrong when a large focus of human *P. knowlesi* cases were reported from Kapit, Sarawak, Malaysia in 2004 (Singh et al., 2004). From that study, 58% of the patients were found to be infected by *P. knowlesi* using molecular tools. These cases were previously misidentified as *P. malariae* by microscopy as the mature trophozoites, schizonts and gametocytes of *P. knowlesi* resembled those of *P. malariae* (Coatney et al., 1971). Additionally, the early trophozoites of *P. knowlesi* in the erythrocytes were indistinguishable from *P. falciparum*. Since the identification of knowlesi malaria using microscopy will be difficult, molecular tools such as nested PCR assay were used for detection of *P. knowlesi* (Singh et al., 2004).

The availability of diagnostic test for *P. knowlesi* has detected many more knowlesi malaria cases across Malaysia (Barber et al., 2011; Cox-Singh et al., 2008; Vythilingam et al., 2008; William et al., 2011) since 2004, and also from other countries except Lao PDR. Currently knowlesi malaria cases have been reported from Thailand (Jongwutiwes, 2011; Jongwutiwes et al., 2004; Putaporntip et al., 2009), Myanmar (Zhu et al., 2006), Cambodia (Khim et al., 2011), Vietnam (Eede et al., 2009), Philippines (Luchavez et al., 2008), Indonesia (Sulistyaningsih et al., 2010) and Singapore (Ng et al., 2008). Besides knowlesi malaria cases were exported from Southeast Asia by travelers from non-endemic regions such as Australia (Figtree et al., 2010), New Zealand (Hoosen & Shaw, 2011), Finland (Kantele et al., 2008), Sweden (Bronner et al., 2009), Spain (Tang et al., 2010), France (Berry et al., 2011), Netherlands (Link et al., 2012), Germany (Ehrhardt et al., 2013; Orth et al., 2013; Seilmaier et al., 2014) and Japan (Tanizaki et al., 2013) have also been reported. Notably, *P. knowlesi,* the only primate malaria with 24-hour erythrocytic cycle (Garnham, 1966) is shown to be life-threatening in which mortalities have been reported in Sabah where patients succumbed to severe knowlesi malaria infection (Cox-Singh et al., 2008; Galinski & Barnwell, 2009; William et al., 2011). The increasing incidence of *P. knowlesi* cases coupled with its fatalities alarmed the public health community to relook into the impact of *P. knowlesi* and classified it as the fifth human malaria parasite (Collins & Barnwell, 2009; Sabbatani et al., 2010; White, 2008). Nevertheless, *P. knowlesi* infection is still considered as zoonotic disease since there is no proven occurrence of human-to-human transmission. Recently, the first naturally acquired human *P. cynomolgi* was reported from east coast of state of Terengganu in Peninsular Malaysia (Ta et al., 2014).

Table 2.2: Chronology of simian malaria infections in man.

Plasmodium species	Type of infection	Year	Location of infection (case export location)	References
P. reichenowi	Experimental infection	1922	West Africa	Blacklock and Adler (1922)
P. brasilianum	Experimental infection	1931	Panama	Clark and Dunn (1931)
P. knowlesi	Experimental infection	1932	India	Knowles and Das Gupta (1932)
P. inui	Experimental infection	1938	India	Das Gupta (1938)
P. cynomolgi	Experimental infection	1938	London	Sinton et al. (1938)
P. cynomolgi	Experimental infection	1941	New York	Coggeshall (1941)
P. cynomolgi	Accidental infection	1960	Memphis	Coatney (1963); Eyles et al. (1960)
P. cynomolgi	Experimental infection	1960	Cincinnati	Schmidt et al. (1961)
P. cynomolgi	Experimental infection	1961	United States	Coatney et al. (1961)
P. cynomolgi	Experimental infection	1962	Georgia	Contacos et al. (1962)
P. cynomolgi	Experimental infection	1963	Kuala Lumpur, Malaysia	Bennett and Warren (1965)
P. brasilianum	Experimental infection	1963	United States	Contacos et al. (1963)
P. knowlesi	Natural infection	1965	Pahang, Malaysia	Chin et al. (1965)
P. inui	Experimental infection	1966	Atlanta	Coatney et al. (1966)
P. knowlesi	Experimental infection	1968	Georgia	Chin et al. (1968)
P. schwetzi	Experimental infection	1970	Georgia	Contacos et al. (1970)
P. knowlesi	Presumptive natural infection	1971	Johor, Malaysia	Fong et al. (1971)
P. knowlesi	Natural infection	2004	Kapit, Sarawak, Malaysia	Singh et al. (2004)
P. knowlesi	Natural infection	2004	Thailand	Jongwutiwes et al. (2004)
P. knowlesi	Natural infection	2006	Myanmar	Zhu et al. (2006)
P. knowlesi	Natural infection	2006	Malaysian Borneo (Sweden)	Bronner et al. (2009)

Table 2.2, continued.

Plasmodium species	Type of infection	Year	Location of infection (case export location)	References
P. knowlesi	Natural infection	Dec 2007-Nov 2009	Sabah, Malaysia	William et al. (2011)
P. knowlesi	Natural infection	2007	Peninsular Malaysia (Finland)	Kantele et al. (2008)
P. knowlesi	Natural infection	2008	Sarawak	Cox-Singh et al. (2008)
P. knowlesi	Natural infection	2008	Peninsular Malaysia	Vythilingam et al. (2008)
P. knowlesi	Natural infection	2008	Philippines	Luchavez et al. (2008)
P. knowlesi	Natural infection	2008	Singapore	Ng et al. (2008)
P. knowlesi	Natural infection	2009	Vietnam	Eede et al. (2009)
P. knowlesi	Natural infection	2009	Thailand	Putaporntip et al. (2009)
P. knowlesi	Natural infection	2009	Southeast Asia (Spain)	Tang et al. (2010)
P. knowlesi	Natural infection	2010	Indonesia	Sulistyaningsih et al. (2010)
P. knowlesi	Natural infection	2010	Thailand (France)	Berry et al. (2011)
P. knowlesi	Natural infection	2010	Indonesia (Australia)	Figtree et al. (2010)
P. knowlesi	Natural infection	2010	Malaysian Borneo (New Zealand)	Hoosen and Shaw (2011)
P. knowlesi	Natural infection	2009	Kudat, Sabah, Malaysia	Barber et al. (2011)
P. knowlesi	Natural infection	2011	Thailand	Jongwutiwes (2011)
P. knowlesi	Natural infection	2011	Cambodia	Khim et al. (2011)
P. knowlesi	Natural infection	2011	Malaysia (Netherlands)	Link et al. (2012)
P. knowlesi	Natural infection	2012	Malaysia (Japan)	Tanizaki et al. (2013)
P. knowlesi	Natural infection	2013	Thailand (Germany)	Ehrhardt et al. (2013)

Table 2.2, continued.

***Plasmodium* species**	**Type of infection**	**Year**	**Location of infection (case export location)**	**References**
P. knowlesi	Natural infection	2013	Thailand (Germany)	Orth et al. (2013)
P. knowlesi	Natural infection	2013	Thailand/ Myanmar (Germany)	Seilmaier et al. (2014)
P. cynomolgi	Natural infection	2014	Terengganu	Ta et al. (2014)

2.1.4 Simian malaria studies in macaques

Over the years, several simian malaria infections in macaques have been studied. Macaque study on *P. knowlesi* infection in Indonesia showed only one monkey (3.2%) was infected among the 31 sampled (Jones-Engel et al., 2007). Vythilingam et al. (2008) reported that monkeys trapped from three locations in peninsular Malaysia, namely Kuala Lumpur, Selangor and Kuala Lipis in Pahang showed that 13.7% (10 out of 73) macaques were positive for *P. knowlesi* using PCR in Kuala Lipis whereas none of the macaques examined from Kuala Lumpur and Selangor were positive for *P. knowlesi*. The results obtained were similar to observations reported in Thailand (Seethamchai et al., 2008) and Singapore (Li, 2011) where all the macaques caught in the urban areas were negative for malaria infection while those caught from the forest had high infection rate. The infection rate of *P. knowlesi* in wild long-tailed macaques in Singapore was as high as 68.2% (Li, 2011). However, infections in macaques from Thailand were identified as *P. inui* and *P. coatneyi* using PCR (Seethamchai et al., 2008). The low infectivity in macaques from urban areas may be due to the absence of competent vectors responsible for simian malaria transmission in the area (Li, 2011).

In a subsequent study performed in Thailand in 2010, *P. inui* accounted for the highest infection in *M. fascicularis* (38.9%) followed by *P. coatneyi* (16.7%) and *P. knowlesi* (5.6%) (Putaporntip et al., 2010). Additionally, in Selangor, out of 107 long-tailed macaques' blood samples examined, 64.5% were positive for *Plasmodium* and 23.3% from these positive samples were *P. knowlesi* infection (Ho et al.). In Sabah, Malaysian Borneo, both *M. fascicularis* (8 out of 26) and *M. nemestrina* (9 out of 15) were predominantly infected with *P. inui*. This was similar to the one carried out in Kapit Sarawak where a prevalence of *P. inui* and *P. knowlesi* infection in long-tailed macaques was 82% and 78%, respectively (Lee et al., 2011).

Screening of macaques carried out in Hulu Selangor illustrated that approximately 50% of the macaques were infected with *Plasmodium* and *P. inui* (65.7%) was the predominant infection detected with PCR followed by *P. knowlesi* (60%). Besides, other simian malaria parasites were also detected in the macaques. This indicated that there was a high intensity of transmission occurring among the long-tailed macaques in Hulu Selangor (Akter et al., 2015). In addition, an entomological survey carried out in Hulu Selangor had incriminated *An. introlatus* as the vector for *P. knowlesi* (Vythilingam et al., 2014). The results are illustrated in Table 2.3.

Table 2.3: Summary of simian malaria infections in long-tailed macaques.

Study area	Year	Pk	Pcy	Pin	Pct	Pfi	Reference
Indonesia	2007	3.2%	-	-	-	-	Jones-Engel et al. (2007)
Thailand	2008	-	-	83.3%	16.7%	-	Seethamchai et al. (2008)
Kuala Lipis, Pahang, peninsular Malaysia	2008	13.7%	NA	NA	NA	NA	Vythilingam et al. (2008)
Kuala Lumpur and Selangor, peninsular Malaysia	2008	-	NA	NA	NA	NA	
Selangor, Malaysia	2010	23.3%	NA	NA	NA	NA	Ho et al. (2010)
Thailand	2010	5.6%	NA	38.9%	16.7%	NA	Putaporntip et al. (2010)
Kapit, Sarawak, Malaysian Borneo	2011	89.0%	65.0%	86.0%	78.0%	5.0%	Lee et al. (2011)
Singapore	2011	68.2%	60.6%	1.5%	3.0%	16.7%	Li (2011)
Sabah, Malaysian Borneo	2014	15.4%	11.5%	30.8%	3.8%	3.8%	Muehlenbein et al. (2015)
Hulu Selangor, peninsular Malaysia	2015	60.0%	51.4%	65.7%	45.7%	2.9%	Akter et al. (2015)

NA denotes not available; while – denotes negative result.

2.1.5 Detection and identification of simian malaria parasites

2.1.5.1 Microscopy

In 1904, Gustav Giemsa introduced a mixture of methylene blue and eosin stains (Fleischer, 2004). Subsequently, microscopic examination of Giemsa-stained blood smears had become a universally accepted gold standard for malaria diagnosis including identification for *Plasmodium* parasites in monkeys (Ameri, 2010; Coatney et al., 1971; Cogswell, 2000; Eyles, 1963; Fooden, 1994; Rain et al., 1993; Seethamchai et al., 2008; Tsukamoto et al., 1978). Nonetheless, accurate identification of the simian malaria parasites remains challenging due to overlapping morphological characters between them (Garnham, 1966). Additionally, co-infection with two or more malaria parasite species in a single macaque combined with low parasitaemia lead to inaccurate and insensitive identification using microscopy (Coatney et al., 1971; Garnham, 1966).

Furthermore, simian malaria parasites share some similarities in morphological characters with human malaria parasites. This causes misidentification of significant proportion of *P. knowlesi* cases as *P. falciparum* and *P. malariae* with microscopy in Kapit, Sarawak (Singh et al., 2004). Likewise, the morphology of *P. cynomolgi* is similar to *P. vivax* (Sandosham et al., 1962) while *P. fieldi* resembles *P. ovale* (Eyles et al., 1960), and *P. coatneyi* and *P. inui* are alike to *P. falciparum* and *P. malariae*, respectively (Coatney et al., 1971). Consequently precise identification of malaria parasites with microscopy greatly relies on the experience and skills of the microscopists.

Anopheles mosquitoes which have *Plasmodium* in their salivary glands are potentially infectious to humans. The young oocysts in the mosquito's midgut may or may not develop into sporozoites. Therefore, it is important to differentiate between infected mosquitoes (with oocysts only) and infective mosquitoes (with sporozoites)

since sporozoite rate is epidemiologically informative as a measure of potential which a mosquito can transmit malaria (Foley et al., 2012; WHO, 2013a). Dissection of mosquitoes for examination of parity, oocysts and sporozoites is the traditional and gold standard method. Through dissection, the amount of PCR inhibitors before template preparation for PCR can be minimized (Arez et al., 2000) because the inhibitors are most likely present in the mosquito tissues (Higgins & Azad, 1995; Siridewa et al., 1996).

2.1.5.2 Polymerase Chain Reaction (PCR)

Even though microscopy detection and identification of *Plasmodium* species in Giemsa-stained thick and thin blood smears remains the gold standard for laboratory diagnosis of malaria, polymerase chain reaction (PCR) has been increasingly used to detect and identify malaria parasite infection (including mixed infections) as it is sensitive, rapid and reliable (Johnston et al., 2006; Speers et al., 2003). Consequently, it is often used when microscopic identification does not match with the epidemiological and clinical results. This is done so that effective treatment of malaria can be prescribed. Numerous PCR assays such as conventional and nested PCR with amplification of genes encoding the 18S rRNA, surface antigens and the dihydrofolate reductase-thymidylate gene (Ciceron et al., 1999) have been developed and used to detect the malaria parasite (Milne et al., 1994; Myjak et al., 2002; Ndao et al., 2004; Snounou et al., 1993; Speers et al., 2003). For instance, the nested PCR assay which is based on the 18S rRNA gene is a widely used method for malaria parasite detections (Lee et al., 2011; Singh et al., 2004; Snounou et al., 1993). The 18S SSU rRNA gene was selected as the nested PCR assay for *P. falciparum, P. vivax*, *P. malariae* and *P. ovale,* is widely utilized for molecular epidemiological studies of malaria (Snounou & Singh, 2002; Snounou et al., 1993). The nest one amplification uses the *Plasmodium* genus-specific primers to amplify the *Plasmodium* small subunit ribosomal RNA (SSU rRNA) gene.

Subsequently the PCR product of nest one was used as a template in four (human) or five (macaques) separate nest two PCR amplifications using species-specific primers to determine the *Plasmodium* species in the sample. The nested PCR assay was reported to have higher sensitivity compared to the conventional microscopy method (Lee et al., 2011; Singh et al., 1999). However, Imwong et al. (2009) reported that the species-specific primers used for detection of *P. knowlesi* infections in humans (Singh et al., 2004) cross-react stochastically with *P. vivax* genomic DNA, and developed and validated a nested primer set which targets part of the *P. knowlesi* small-subunit rRNA genes.

The extremely sensitive nested PCR assay allows the detection of malaria parasites in mosquitoes (Arez et al., 2000; Vythilingam et al., 1999) and dried blood spots on filter papers (Cox-Singh et al., 1997; Singh et al., 1999), thus is useful for detecting low parasitaemia infection in high malaria endemic areas (Bottius et al., 1996). Moreover, the overlapping morphological characteristics between humans and simian malaria parasites reduced the accuracy of microscopy identification and resulted in difficulty to ascertain the occurrence of zoonosis. Therefore, naturally-acquired human infection cases of simian malaria maybe overlooked. The designation of nested PCR using species-specific primers played a significant role in the discovery of high proportion of cases of *P. knowlesi* which was previously misdiagnosed as *P. malariae* and/ or *P. falciparum* cases (Singh et al., 2004) indicating the presence of simian malaria transmission to man. Likewise Sandosham et al. (1962) showed a slide of *P. cynomolgi bastianellii* which was similar to an aberrant form of *P. vivax* previously described from two patients from Malaysia (Sandosham & Noordin, 1967). Owing to the similarity in morphology between these parasites, there could be a possibility of *P cynomolgi* being transmitted to humans in nature without being detected. Hence, the

development of species-specific primers for PCR assay will be helpful in identification of zoonoses.

2.1.6 Malaria trends in Sabah, from 1961-1991

Since the nineteenth century, malaria is a serious disease in Malaysia and posed a challenging crisis in the Straits Settlements which is a division of British Malaya including Penang, Malacca, Labuan, a number of smaller islands and Singapore, and also Malaysian Borneo (Hodder, 1959; Mohamed, 1965; Zulueta, 1956). For instance, about 1/3 of the deaths in Penang in 1829 were caused by malaria (Hodder, 1959). Additionally, from 1940s-1950s, there were 40,000 and 250,000 malaria cases reported per year in Sarawak and Sabah (Mak et al., 1992; Rahman, 1982), respectively. The Malaria Eradication Program (MEP) was initiated in 1961 in both Sabah and Sarawak. Prior to the initiation of MEP, pilot project involving residual spraying of dichlorodiphenyltrichlorophenothane (DDT) insecticide was carried out in Keningau, Sabah (1956-1960). The project produced encouraging results in interrupting the transmission of malaria. Then, in 1971 and 1972 MEP in Sabah and Sarawak, respectively was changed to Malaria Control Program (MCP) for standardization across Malaysia (Ho, 1985).

Two main approaches were taken under the MCP in order to reduce malaria prevalence and transmission that were the application of indoor residual spraying of walls with DDT and intensive treatment of cases. Regular residual insecticide spraying with DDT 25% (emulsified concentrate) or DDT 75% (water dispersible particles) were carried out in endemic areas every 3 or 6 months when necessary, especially in the interior of aborigine settlements, logging camps and land schemes. Besides, focal spraying of houses was also carried out for malaria prone and malaria free areas when cases were reported. Larvicides were applied in drains and canals of malarious areas for larval control combined with proper water management by construction of tidal gates or

automatic siphons to control breeding of *Anopheles* vectors. In addition, rigorous entomological surveillance was carried out to investigate the vector's bionomics, the outcome of vector control and also the monitoring of the vector susceptibility against DDT. Active case detection and mass blood surveys coupled with radical and follow-up treatment were executed in problematic areas which have malaria cases increment, high influx of foreign workers, in the interior of Orang Asli settlements and where cases were reported from non-endemic malaria areas. Along with this, health education for community awareness such as exhibitions on vector-borne diseases and distribution of media like posters and videos were implemented (Mak et al., 1992; Rahman, 1982).

In 1961, the total estimated malaria cases in Sabah were approximately 100,000 cases prior to introduction of MEP. This number dropped to about 10,000 cases in 1969 (Figure 2.3). Nevertheless, the incidence of malaria rose sharply to 21,000 and more than 46,000 cases in 1971 and 1975, respectively. This was followed by a relatively high and stable number of cases for three years before a decrease of malaria cases were observed in 1979. The upsurge of malaria cases in Sabah during 1970 and 1971 maybe caused by the reduced implementation of control program in 1969. Conversely the low number of malaria cases reported in 1973 was due to prevailing drought across Sabah (Rahman, 1982).

An upsurge of malaria cases from 1975-1978 in Sabah was caused by several factors. First of all, intrinsically, Sabah's challenging terrain dampens the implementation of control measures, surveillance and supervision because of reduced accessibility to the affected areas. Secondly, the continual residual spraying with DDT resulted in resistance of vector population against the insecticide in addition to refractory nature of vectors affects the control measures. Thirdly with the presence of chloroquine-resistant *P. falciparum* (Han & Huang, 1974; Rahman, 1980), treatment for malaria was an additional factor. Moreover with the influx of migrant workers,

favorable breeding ground for vectors was created concurrently with the creation of settlements (Mak et al., 1992; Rahman, 1982). Contrastingly, the malaria control program in Sarawak yielded better results primarily due to predominance of *P. vivax* compared to *P. falciparum* in Sabah, and the principal vectors which were less efficient than those present in Sabah.

After 1980, the incidence of malaria was inconsistent with a peak of about 50,000 cases reported in 1981 followed by a decrease of cases in the following years and reaching another peak in 1985 (>35,000 cases) and 1989 (>45,000 cases). Overall, the malaria incidence in Sabah remained high as opposed to the malaria situation in Peninsular Malaysia and Sarawak (Mak et al., 1992). The unsatisfactory condition of malaria in Sabah maybe attributed to these factors, 1) the presence of drug-resistant *P. falciparum*; 2) the presence of highly effective principal human malaria vector in Sabah, *An. balabacensis* and 3) changes in ecology and socioeconomic.

The vector programs in Sabah and Sarawak were standardized with the Vector-Borne Diseases Control Program (VBDCP) of Peninsular Malaysia in 1986 under the 5th Malaysia Plan (1985-1990) which aimed to reduce malaria morbidity and mortality as well as preventing malaria from spreading to non-endemic areas in Malaysia. Consequently, for 6th Malaysia Plan (1991-1995), VBDCP aimed to bring down malaria incidence rate to <2 per 1000 population and death rate <5%.

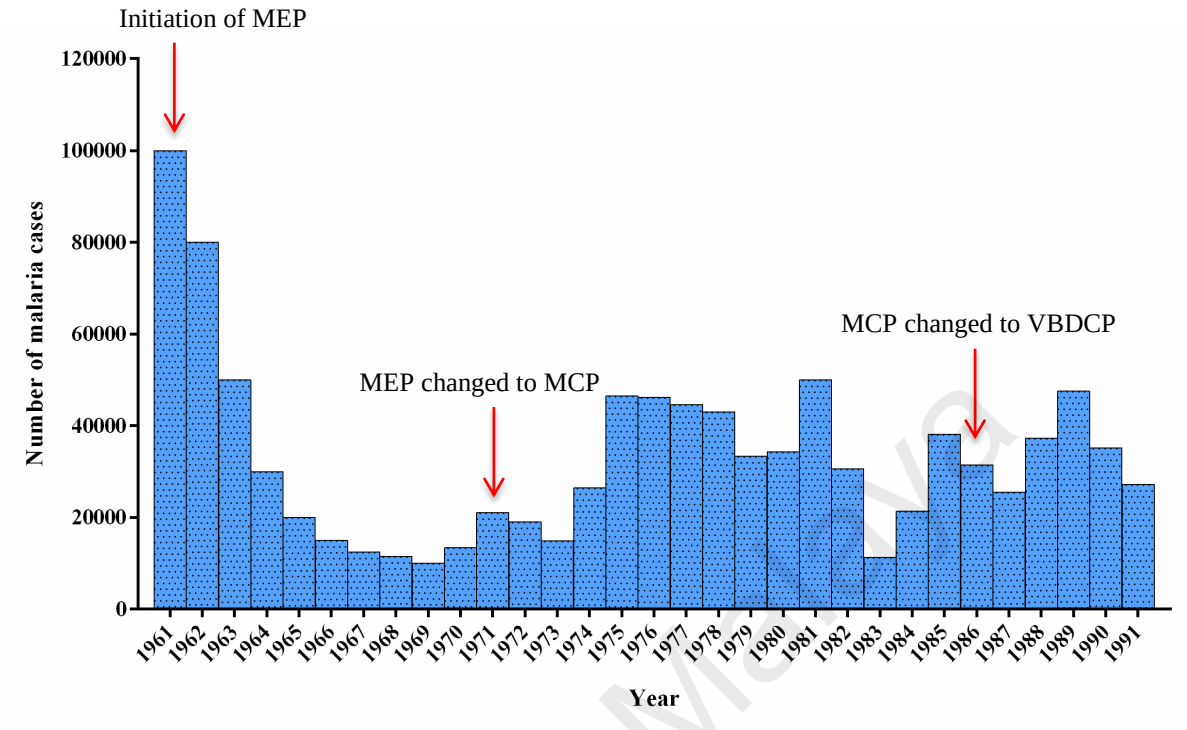

Figure 2.3: Incidence of malaria in Sabah, 1961-1991. Data are from (Mak et al., 1992; Rahman, 1982).

2.1.7 Malaria trends in Sabah, from 1992 to current

The implementation of malaria control program in Sabah starting from 1961 has reduced the malaria prevalence from ~100,000 cases to about 50,000 cases per year in 1980s (Vector Borne Diseases Control Program, 2008). Increased surveillance, vector control, and training of community volunteers coupled with early diagnosis and treatment were initiated from 1992. Moreover malaria was made mandatory notifiable disease with 100% confirmatory testing of suspected malaria cases (WHO, 2013b). Given that Malaysia had aimed to be malaria-free by 2020, the emergence of simian malaria parasite, *P. knowlesi* as the fifth human malaria parasite presents an increasing threat to malaria elimination (Barber et al., 2012b; Barber et al., 2011; Cox-Singh et al., 2008; Daneshvar et al., 2009; Joveen-Neoh et al., 2011; William et al., 2013). Currently, there has been an increasing number of knowlesi malaria cases reported in several districts throughout Sabah with the highest proportion of cases from Kudat District Hospital (KDH) where 87% of malaria patients admitted in 2009 were infected with *P. knowlesi* (Barber et al., 2011; Cox-Singh et al., 2008; Daneshvar et al., 2009; Joveen-Neoh et al., 2011).

From 1992-2011, the total number of malaria cases reported in Sabah decreased considerably, with *P. falciparum* cases reaching a peak of 33,153 cases in 1994 and dropped to as low as 605 cases in 2011. Meanwhile 15,857 cases of *P. vivax* were reported in 1995 and decreased to about 628 cases in 2011. On the other hand, the *P. malariae*/ *P. knowlesi* cases increased from 200 in 1992 to 614 in 1994 and decreased to approximately 100 cases per year in the late 1990s and early 2000s (Figure 2.4A) (William et al., 2013). The trend of *P. malariae/ P. knowlesi* cases in Sabah remained rather constant in the late 1990s to 2006, and then increased markedly from 2007 (Figure 2.4B). As mentioned previously, the notifications of *P. knowlesi* cases in Sabah continued to increase in numbers, with 815 and 996 cases in 2012 and 2013, respectively (Figure 2.5). Consequently *P. knowlesi* reported cases comprised a majority of 69% malaria cases reported in Malaysia in 2016 (MOH, 2016).

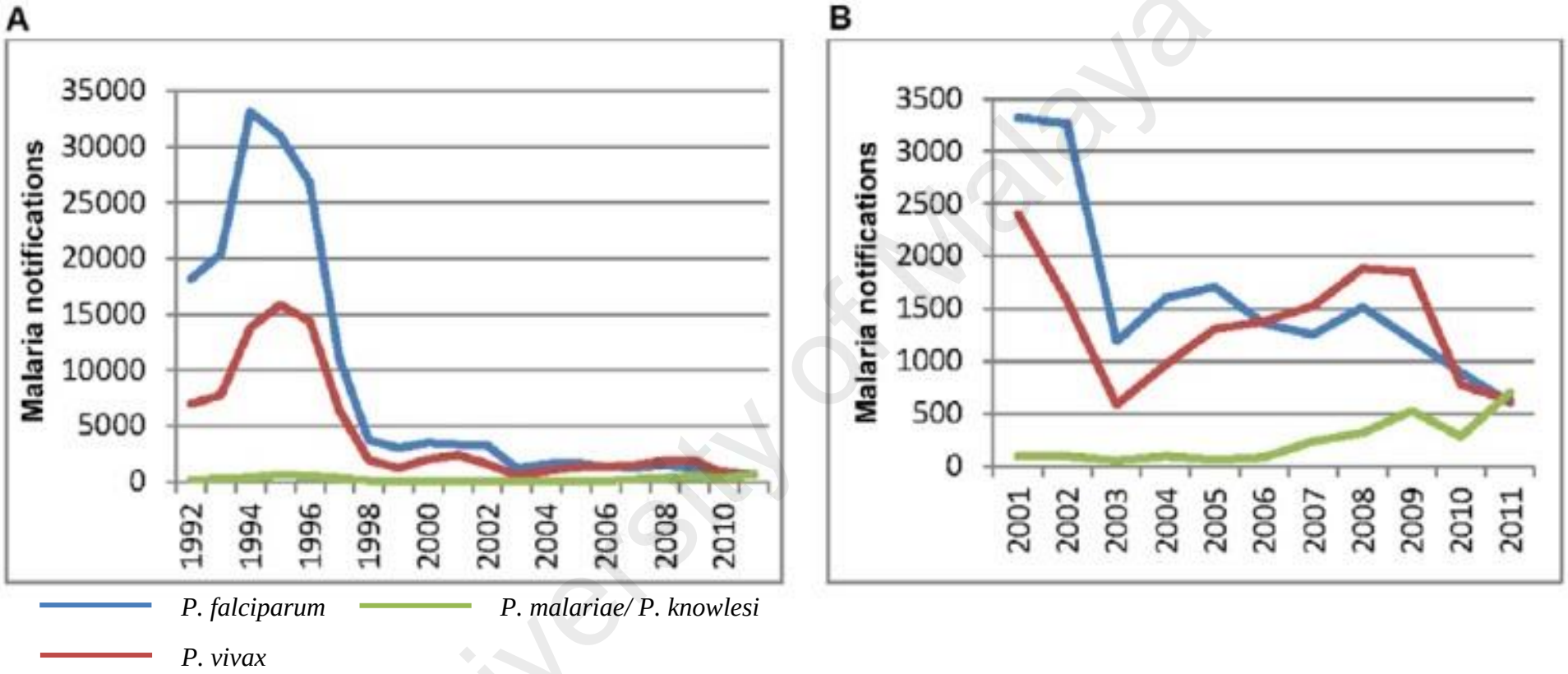

Figure 2.4: Annual malaria cases by species from 1992-2011 in Sabah, retrieved from William et al. (2013).

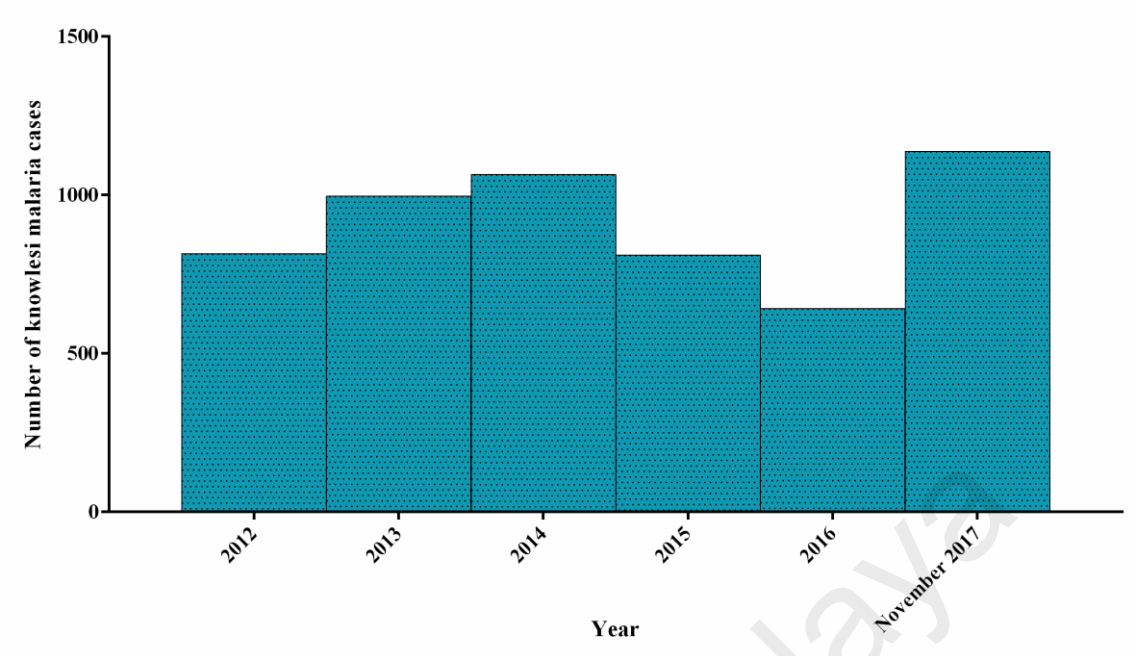

Figure 2.5: Knowlesi malaria notifications in Sabah from 2012 to November 2017. Data from (Jelip et al., 2014; Jelip et al., 2015; Jelip et al., 2016; John et al., 2017; William et al., 2014).

Certainly, the prevalence of knowlesi malaria in Sabah had increased markedly over the years, and this occurred as a result of extensive land change due to agricultural activities (Brock et al., 2016; Fornace et al., 2016) coupled with the much reduced rate of other human malaria species. The massive deforestation which has taken place across Sabah has resulted in encroachment of humans into previously forested areas, therefore, increasing human interactions with Leucosphyrus Group of mosquitoes, the vector associated with simian malaria and its simian hosts (Fornace et al., 2016). Moreover, the recent studies on epidemiology in Sabah revealed that older patients were admitted with knowlesi malaria compared to those with *P. falciparum* or *P. vivax* infections (Barber et al., 2012a; William et al., 2013). Available evidence suggested that humans get infected when they spend time in farms or forested areas with close proximity to macaques (Lee et al., 2011).

As previously described, detection and identification of simian malaria parasites by microscopy examination of Giemsa-stained thick and thin blood smears are challenging as they required skillful microscopists. However, correct identification of the malaria parasites can be achieved with PCR assays using species-specific primers for simian malaria parasites. These assays will certainly be useful in detection of zoonoses in humans, which maybe overlooked by microscopy since both human and simian malaria parasite shared similarity in their morphology.

2.2 *Anopheles* (Cellia) *leucosphyrus* group

2.2.1 Life cycle

Similar to all other mosquitoes, *Anopheles* mosquitoes go through four stages in their life cycle, namely egg, larva, pupa and adult (Figure 2.6) (CDC, 2015). The first three stages of the life cycle are aquatic and last about 5-14 days depending on the species and environment temperature. The adult *Anopheles* mosquitoes can live up to a month or longer in captivity but seldom live more than 1-2 weeks in natural environment. Since female *Anopheles* mosquitoes are the obligatory vector for *Plasmodium* transmission (Mala et al., 2016), this resulted in the parasite manipulating the life history (increased survival) and feeding behaviour (increased bloodfeeding rate) of the mosquito to increase its transmission rate (Schwartz & Koella, 2001).

During oviposition, adult female can lay about 50-200 eggs, which are laid singly on water and having lateral floats. The eggs are susceptible to drying and will hatch within 2-3 days. Nevertheless, in colder climates, eggs can take about 2-3 weeks to hatch.

Upon hatching, the larvae undergo development through four instars before they metamorphose into pupae. At the end of each instar, the larvae will molt and shed their exoskeleton to allow further growth. The larvae has the following morphology, which are well-developed head with mouth brushes for feeding, a large thorax and a segmented abdomen without legs. As compared with culicines, the anophelines' larvae lack a respiratory siphon and thus always position themselves parallel to the surface of the water and come to the water surface frequently to breathe through the spiracles located on the 8^{th} abdominal segment. The larvae feed mostly on algae, bacteria and other microorganism in the surface microlayer. They will dive below the water surface when disturbed. The larvae swim either by jerky movements of entire body or through propulsion with mouth brushes (CDC, 2015).

At the completion of four instars, the larvae will metamorphose into pupae. The pupae are comma-shaped with head and thorax merged into a cephalothorax and a curved abdomen. Simiarly with the larvae, th pupae must swim to the water surface frequently to breathe using a pair of respiratory trumpets on the cephalothorax. After a few days of pupation, the dorsal surface of the cephalothorax spilts and an adult mosquito will emerge. Depending on the species and surrounding temperatures, development from egg to adult varies considerably and in tropical climate, the life cycle will commence around 10-14 days (CDC, 2015).

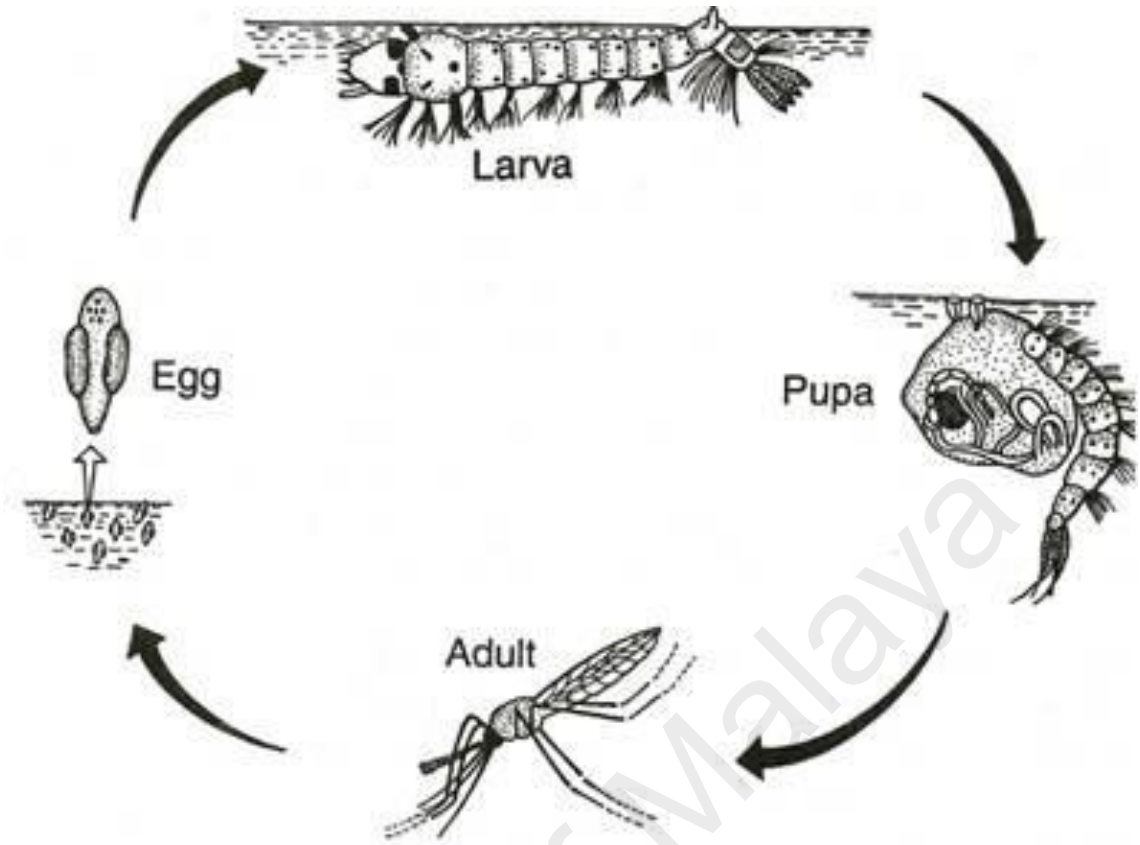

Figure 2.6: **Life cycle of *Anopheles* mosquitoes, retrieved from http://cdn.yourarticlelibrary.com/wp-content/uploads/2014/01/clip_image002158.jpg.**

2.2.2 Bionomics of *Anopheles leucosphyrus* group

The Leucosphyrus Group of mosquitoes belongs in the Neomyzomyia Series (Christophers, 1924) of the subgenus *Cellia* (Colless, 1956a; Harbach, 2004; King & Baisas, 1936; Reid, 1968; Reid & Knight, 1961) Theobald of *Anopheles* Meigen (Harbach, 2004). The range of geographical distribution of the Leucosphyrus Group extends from southwestern India eastwards to southern China, Taiwan, mainland of Southeast Asia, Indonesia and the Philippines (Figure 2.7) (Reid, 1968;1970). Colless (1956a) and Reid (1968) first proposed the classification of the Leucosphyrus Group prior to Peyton (1989) corraboration on the group. Consequently, based on morphological similarities, Peyton (1989) proposed three subgroups within the Leucosphyrus Group, namely the Elegans, Leucosphyrus and Riparis Subgroup. However, Sallum et al. (2005a) transferred *An. elegans* to the Dirus Complex and renamed the Elegans Subgroup as the Hackeri Subgroup to denote the change. This group of mosquitoes can be differentiated from the other mosquitoes in the Neomyzomyia Series by possesing a conspicuous white band on the tibio-tarsal joint of the hind leg in the adult (Colless, 1956a; Reid, 1968). Currently, there are 21 named species and two geographical forms, which are the Con Son form (under Leucosphyrus Subgroup) and Negros Form (under Riparis Subgroup) (Harbach, 2014; Peyton, 1989). Out of the 21 formally named species, 8 species are occurring in Malaysia (Table 2.4) (Colless, 1956a; Harbach, 2004; Jeffery et al., 2012; Reid, 1968; Sallum et al., 2005b). A total of 5 species occurring in Malaysia are in the Leucosphyrus Subgroup which are *An. cracens* Sallum & Peyton and *An. nemophilous* Peyton & Ramalingam (in Dirus Complex); *An. introlatus* Colless, *An latens* Sallum & Peyton and *An. balabacensis* Baisas (in Leucosphyrus Complex). *Anopheles hackeri* Edwards and *An. pujutensis* Colless are categorized in the Hackeri Subgroup. Only one species out of the total 4 species in the Riparis Subgroup is present in Malaysia, that is *An. macarthuri* Colless.

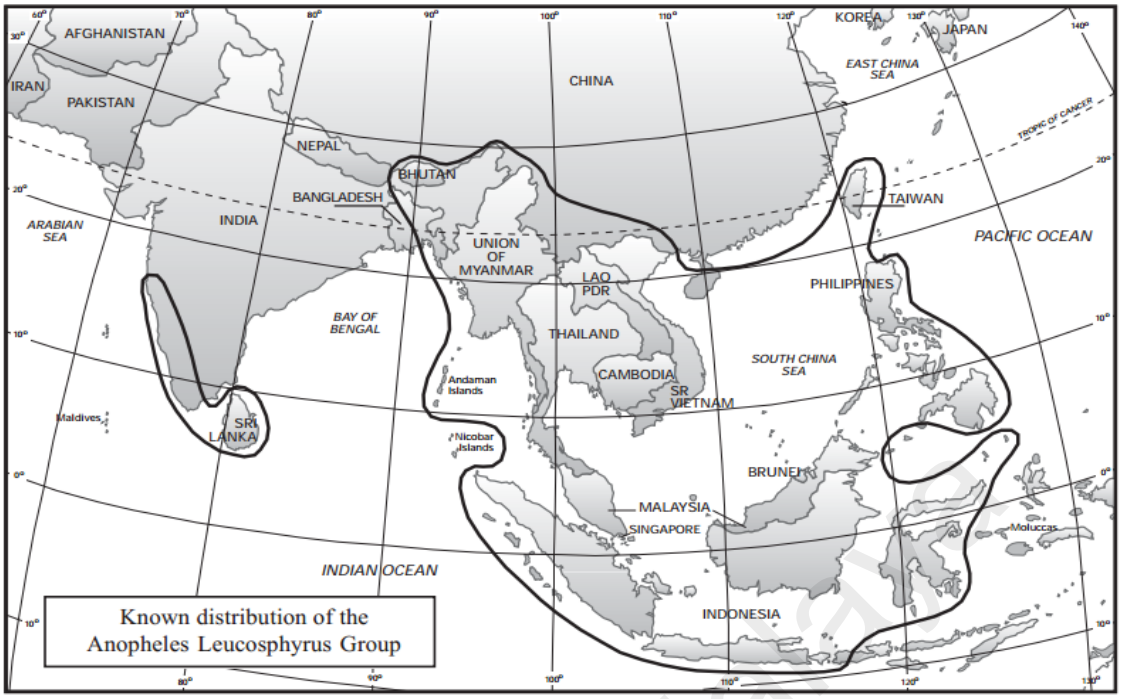

Figure 2.7: Map showing the distribution of the Leucosphyrus Group (Sallum et al., 2005b).

Table 2.4: List of *Anopheles leucosphyrus* group of mosquitoes occurring in Malaysia.

Group	Subgroup	Complex	Species
Leucosphyrus	Leucosphyrus	Dirus	*An. cracens* *An. nemophilous*
		Leucosphyrus	*An. balabacensis* *An. introlatus* *An. latens*
	Hackeri		*An. hackeri* *An. pujutensis*
	Riparis		*An. macarthuri*

2.2.2.1 Dirus Complex

The Dirus Complex is grouped under the Leucosphyrus Subgroup with Leucosphyrus Complex (Table 2.4). The taxonomy of this complex had been resolved and all the members in the complex now have their morphological descriptions and designated formal Latin names (Sallum et al., 2005a). In addition their wide distributions through all countries of Southeast Asia have been mapped (Baimai, 1998; Obsomer et al., 2007). There are seven members in the Dirus Complex (Peyton, 1989) which vary from being highly competent malaria vectors to non-vectors in tropical rainforests, cultivated forests and forest fringes of Southeast Asia (Baimai, 1998; Oo et al., 2004). *Anopheles dirus* has been incriminated as vector of human malaria in the Mekong region (Oo et al., 2003). Currently it is also a vector for *P. knowlesi* in Vietnam (Marchand et al., 2011). The members of the Dirus Complex are closely related to members in the Leucosphyrus Complex by having morphologically similar characters throughout all life stages, and there are currently no morphology characters in both larval and pupal stage to differentiate between the two complexes which lead to substantial confusion in published literature (Manguin et al., 2008; Sallum et al., 2005b). The current definition of the Dirus and Leucosphyrus Complex was based primarily on the morphological characters of the adult stages (Sallum et al., 2005b). Of the seven species in the complex, two species are occurring in Malaysia, namely *An. cracens* (= *An. dirus* B) and *An. nemophilous* (= *An. dirus* F).

2.2.2.1(a) *Anopheles cracens* Sallum & Peyton

Anopheles cracens was first differentiated from *An. balabacensis* and *An. dirus* by possesing distinctive mitotic and meiotic karyotypes, and larval salivary gland polytene chromosomes patterns of individuals from a population designated as *An. balabacensis* Perlis Form (Baimai et al., 1981). Subsequent study on the morphometry of male genitalia and frequency of clasper movements during induced mating between

An. cracens (then as *An. balabacensis* Perlis Form) and *An. dirus* (Bangkok colony) demonstrated significant differences with *An. cracens* having larger morphometrics and lesser frequency of clasper movements compared to *An. dirus* supporting that they belonged to two distinct species (Sucharit & Choochote, 1983). Further examination of polytene chromosomes and cross breeding of laboratory colonies confirmed the specific status *An. dirus* besides revealing another species nominated as *An. dirus* B (= *An. cracens*) (Baimai et al., 1984b; Hii, 1985c). Moreover, backcrosses of hybrids between *An. dirus* and *An. cracens* demonstrated both the autosomes and X chromosomes might lead to sterility in the male mosquitoes (Hii, 1985c; Hii, 1984). Also, further studies of cytogenetics and hybridization (Baimai, 1988a;1988b; Baimai et al., 1988a; Baimai et al., 1988b; Baimai & Traipakvasin, 1987; Poopittayasataporn & Baimai, 1995; Wibowo et al., 1984) coupled with morphological and morphometric characters in immature stages led to confirmation of the specific status of *An. cracens* within the Dirus Complex (Damrongphol & Baimai, 1989; Hii, 1986).

Morphologically the fourth-instar larvae of *An. cracens* has extremely light and inapparent pigmentation of sclerotized structures which ranged from light-tan to pale straw-yellow and the margins of the sclerotized tubercles of the larger seta, anterior and posterior tergal plates, and the saddle were not discernable while the head capsule including antenna were uniformly very pale tan to straw. Moreover the seta 5-C distinctively longer than antenna, seta 3-C which is short and extends to or slightly beyond anterior margin of head, seta 1-II is palmate and weakly developed with 5-17 narrow lanceolate leaflets and seta 14-P having 4-8 branches. The male pupae of *An. cracens* has genital lobe noticeably constricted at mid-length (Sallum et al., 2005a; Sallum et al., 2005b). Generally, the adult of *An. cracens* can be identified by having uniformly dark-scaled proboscis which is slightly longer than forefemur (ratio 1.09-1.17), the presector dark (PSD) spot of vein R with 1-3 small pale interruptions,

accessory sector pale (ASP) spot always absent on vein C, hindtarsomere 5 usually dark-scaled at base, rarely with an inconspicuous pale band, the apical white band of hindtibia without a dark extension into basal area and abdominal sternum VI with a small posteromedial patch of dark scales (Jeffery et al., 2012; Sallum et al., 2005a; Sallum et al., 2005b).

The larvae of *An. cracens* can be found in temporary unshaded or partially shaded fresh, stagnant water bodies such as elephant or other animals footprints, pools, streams and puddles . The habitats of the larvae are situated in both plains and mountaineous areas of secondary rain forest (Baimai et al., 1988c; Oo et al., 2002; Prakash et al., 2002; Sallum et al., 2005a; Sallum et al., 2005b).

Anopheles cracens is an anthropophilic and exophagic species exhibiting peak biting period from 2000 hours till 2100 hours (Jiram et al., 2012; Vythilingam et al., 2008) and predominantly feeding in farms (Jiram et al., 2012). The peak biting activity of *An. cracens* was similar to previous studies conducted in Thailand (1900-2100 hours) (Baimai et al., 1988c) indicating that *An. cracens* has not changed its biting activity. Moreover, *An. cracens* was known to enter shelter to feed and exit readily after a blood meal with most rarely rested on wall. Additionally, *An. cracens* has also been collected biting humans during daytime under shade (Cheong et al., 1968). Entomological surveillance carried out in Kuala Lipis, Pahang revealed that *An. cracens* was also attracted to monkey at ground level and 3m from 1900 to 0000 hours whereas from 0000 to 0500 hours, more were collected at canopy level up to 6m. The biting ratio of monkey to human of *An. cracens* was 1:2.6 (Jiram et al., 2012) and 1:5.6 (Vythilingam et al., 2008). Besides that, previous study also showed *An. cracens* fed on monkey and human on canopy and ground level, respectively (Warren et al., 1965).

The tendency of *An. cracens* to feed on both monkey and humans indicates the importance of this species in the transmission of simian malaria. The female mosquitoes

collected in areas where the houses were close to jungle was found to be infected with oocysts and sporozoites. Additionally 88% of them were parous, thus *An. cracens* was presumed to be the vector for human *Plasmodium* (Sandosham et al., 1963). Nonetheless, the involvement of *An. cracens* in malaria transmission can be secondary since they occur in areas where malaria transmission was low (Meek, 1995). Likewise, infected female mosquitoes with *P. inui* and *P. cynomolgi* were reported in the monsoon rain forest located in Perlis, Malaysia. The sporozoites which were isolated and inoculated into rhesus monkey caused malaria in the monkey (Cheong et al., 1965; Cheong et al., 1966). Moreover, 77% of *An. cracens* were infected with *P. cynomolgi* B strain in a susceptibility study when compared to other *Anopheles* mosquitoes (Klein et al., 1991). Furthermore, *An. cracens* was the predominant species collected in both bare leg catch and monkey-baited trap in Kuala Lipis, Pahang, Malaysia where three sporozoite infections in the females were identified as *P. knowlesi*. One of the infections were collected from monkey-bait trap and the other two from human-baited trap (Jiram et al., 2012). These studies showed that *An. cracens* was a competent vector in simian malaria transmission. Also, *An. cracens* was involved in the transmission of *Brugia pahangi* (filaria) since the females were orally infected (Zahedi & White, 1994).

The known distribution of *An. cracens* ranging from Indonesia to West Malaysia northwards to Thailand (Figure 2.8) (Baimai, 1988a; Baimai et al., 1988c; Sallum et al., 2005b).

2.2.2.1(b) *Anopheles nemophilous* Peyton & Ramalingam

Anopheles nemophilous was first described by Colless (1956a) as a geographical variant of *leucosphyrus* Doenitz from a number of specimens collected from Fraser's Hill, Pahang (7 females, 10 larval and pupal skins) and Central Malaya (2 females), and it was assigned to the vernacular name "Fraser's Hill form". Concurrently, "Kepong form" was also described from the same region. However, the adults of "Kepong form"

were relatively distinct from *An. nemophilous* (Fraser Hill form) though the larval stages were quite similar. In succeeding study, *An. nemophilous* was suggested as a distinct altitudinal subspecies with close affinity to *balabacensis introlatus* Colless and more specimens were needed to confirm its status (Colless, 1957). Additionally, Reid (1968) also recognized and suggested that *An. nemophilous* (= Fraser's Hill form) could be a mountain variant of *balabacensis introlatus* in which morphological differences can distinguish both the adult and pupal stages. Further cytogenetics and cross-mating studies on the new specimens lead to designation of the *An. nemophilous* (=Fraser's Hill form) as *An. dirus* species F in the Dirus Complex (Baimai et al., 1984b; Baimai et al., 1988a; Baimai et al., 1988c). *Anopheles nemophilous* was formally named and described using several morphological characters in the adult, pupal and larval stages to differentiate it from *An. dirus* and *An. introlatus* (Peyton & Ramalingam, 1988).

The larvae of *An. nemophilous* can be identified using these characters, that are seta 4-C is long and always extends well beyond base of 2-C, seta 14-P will have 6-13 branches with 8 branches on at least one side, seta 2-I and 3-I are usually single, seta 9-I usually has 5 or more branches on at least one side, seta 1-II is moderately or well-developed with distinct moderate broad, light to dark pigmented lanceolate leaflets with distinctly inflated and stout basal stem, and long seta 13-IV. Adults of *An. nemophilous* has ASP spot absent on vein C, dark-scaled proboscis which is long or slightly longer than forefemur (ratio 1.00-1.17), and a conspicuous basal pale band on the hindtarsome 4, PSD spot on vein R not extending basally beyond level of PSD wing spot on vein C, distinct whitish palpomere which strongly constrasts with light bands of palpomere 2 and 3, and all the pale scales of the wing are cream-colored to golden to yellowish (Peyton & Ramalingam, 1988; Reid, 1968; Sallum et al., 2005b).

Larvae of *An. nemophilous* can be found from rock pool, ground pool, seepage pool, stream pool or near stream margin, flood pool, wheel rut, elephant footprint and

elephant wallows in bogs located in mountaineous forest and foothills at elevations from 100m to 1500m above sea level. The habitat ranges from small to moderate in size, usually shallow and with organic matters, always freshwater bodies which either colored or turbid, and can be partially shaded or heavily shaded without grassy edges (Peyton & Ramalingam, 1988; Sallum et al., 2005b).

The adults of *An. nemophilous* were collected outdoor using human baits from 1830-2200 hours located in a village about 10m from houses and in a platform at the forest canopy. Low number were obtained and they were also found apparently to feed primarily above ground level on monkeys and other small mammals in the forest canopy. Thus, it is deliberately difficult to confirm the vector status of *An. nemophilous* though they are found to be man-biter (Peyton & Ramalingam, 1988). These low anthropophily and affinity for feeding in the forest canopy was also observed by Walton et al. (1999).

The geographical distribution of *An. nemophilous* can be found in Malaysia and Thailand (Figure 2.8) (Peyton & Ramalingam, 1988; Sallum et al., 2005b)

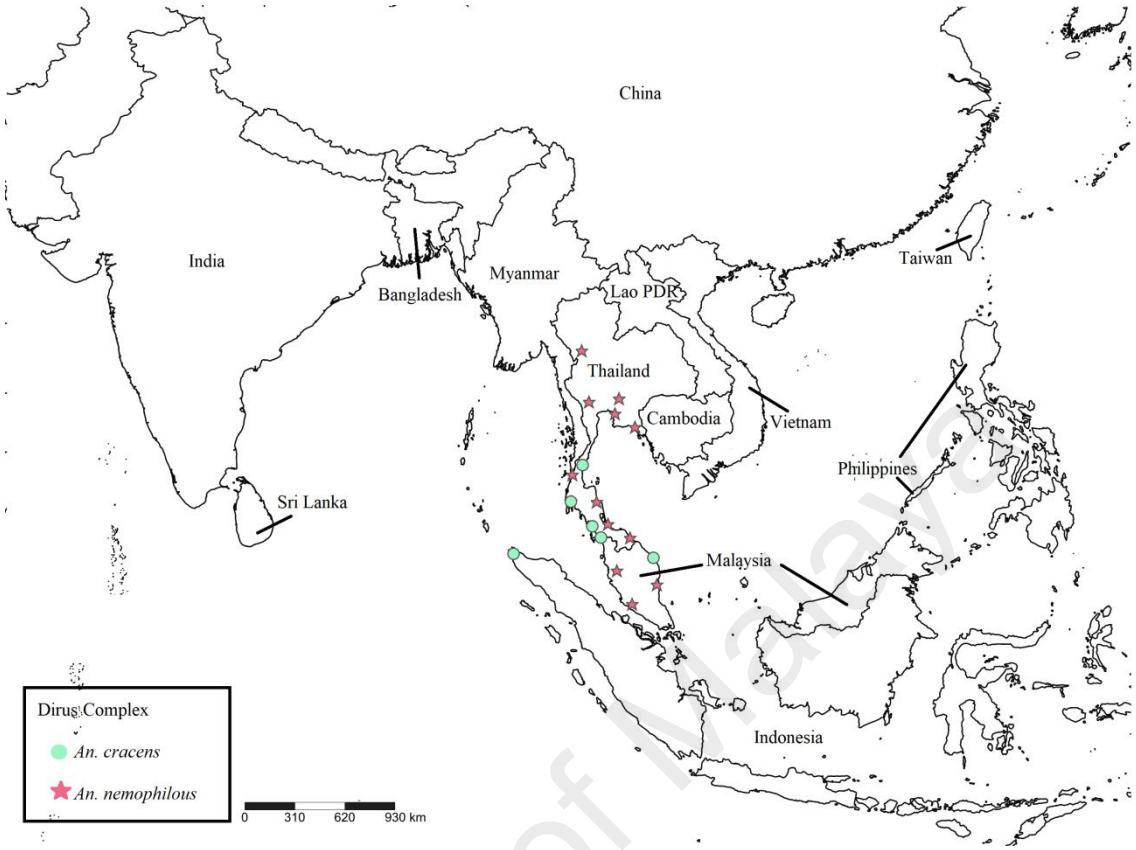

Figure 2.8: Distribution of Dirus Complex, modified from Sallum et al. (2005b).

2.2.2.2 Leucosphyrus Complex

The Leucosphyrus Complex is classified under the Leucosphyrus Subgroup and are forest mosquitoes which closely related to the members in the Dirus Complex. They also act as important vectors for human malaria parasites (Barcus et al., 2002; Hii et al., 1985; Hii et al., 1988a; Schultz, 1992; Warren & Wharton, 1963), simian malaria (Cheong et al., 1965; Eyles et al., 1963a; Tsukamoto et al., 1978; Warren & Wharton, 1963) and filaria (Atmosoedjono et al., 1993; Hii et al., 1984). Members of the Leucosphyrus Complex are distributed widely in several conutries throughout Southeast Asia but not found in India, China or Taiwan (Manguin et al., 2008; Sallum et al., 2005b). There are four species in the Leucosphyrus Complex whereby three were found in Malaysia that are *An. balabacensis, An. introlatus* and *An. latens.*

2.2.2.2(a) *Anopheles balabacensis* Baisas

Anopheles balabacensis was first described by Baisas (1936) using specimens collected from the Balabac Islands, Philippines. Based on morphology and zoogeographic distribution available then, it was proposed as a subspecies of *An. leucosphyrus* or *An. balabacensis* (Colless, 1948;1956a; Reid, 1949). Nevertheless, significant marked differences between *An. leucopshyrus balabacensis* (=*An. balabacensis*) and *An. leucosphyrus leucosphyrus* led to designation of *balabacensis* as a distinct species (Colless, 1957). Conversely, the informations obtained from the phylogenetic relationships and results of morphological confirmations revealed that *An. balabacensis* is indistinguishable from *An. dirus* at species level (Kanda et al., 1981). . However, polytene chromosome banding and cross-mating experiments by Hii (1985c) showed that *An. balabacensis* was genetically different from *An. dirus* (Thailand) and *An. cracens* (Perlis). Further study on the morphometrics of larval and pupa using the same three populations demonstrated that *An. balabacensis, An. dirus* and *An. cracens* were distinct biological species (Hii, 1986).

The larvae of *An. balabacensis* can be identified in having moderately developed seta 1-III with distinct splayed narrow lanceolate leafler and palmate seta 3-T with narrow lanceolate leaflets. The larvae also have short seta 3 and 4-C (which usually do not reach base of 2-C). Adults of *An. balabacensis* can be distinguished by having dark proboscis which is as slightly longer than forefemur (ratio 1.01-1.11) , sector pale (SP) spot always present and prominent but is reduced, presector dark (PSD) spot of vein R with 1-2 pale interruptions on one wing or can be entirely dark, distinct patch of pale scales at the base of hindtarsomere 4 on the dorsal surface and dark-scaled hindtarsomere 5 with apical pale band (Colless, 1956a;1957; Reid, 1968; Sallum et al., 2005b).

Larvae of *An. balabacensis* were found inhabiting in ground pools, temporary roadside ground pools, rocks pools along stream margings, rock pools, wheel tracks, puddles, roadside excavations and ditches, and buffalo hoofprints (Catangui, 1985; Colless, 1948;1956a;1957; Miyagi, 1973; Sallum et al., 2005b). The breeding habitat is always freshwater, stagnant, clear or colored, in full sun or partially shaded and located in hilly areas, valleys or human environments.

Females of *An. balabacensis* were highly anthrophilic and exophagic (feed outdoor) though they can bite indoors but rest outdoors (exophilic). However, they were also attracted to feed on animals (zoophilic) such as monkey and buffalo but more mosquitoes were caught frequently in monkey traps (Hii, 1985b; Miyagi, 1973). The peak biting activity period was noticed from 2200 to 0200 hours (Hii, 1985b; Hii et al., 1991; Hii et al., 1990; Hii et al., 1984; Hii & Vun, 1987). Additionally, collection of *An. balabacensis* was also obtained from CDC light traps 2-10m above ground level, with or without carbon dioxide.

Anopheles balabacensis was a highly competent vector of human malaria in Sabah and Sarawak, East Malaysia (Hii, 1980; Hii et al., 1985; Hii et al., 1988a; Hii & Vun, 1985). High frequency of feeding on humans together with increased life expectancy of *An. balabacensis* (more than 76.2% of the population lived long enough for *P. falciparum* to develop into transmission stage sporozoites) contributed to high estimates of vectorial capacity which varied from 1.44 to 19.7 in Kapitangan and from 7.44 to 9.97 in P. Darat, Banggi Island, Sabah (Hii et al., 1988a). The females of *An. balabacensis* were also found to be capable in harboring *P. knowlesi, P. cynomolgi, P. coatneyi* and *P. inui* experimentally by feeding on infective monkey (Coatney et al., 1971; Collins et al., 1967; Tsukamoto et al., 1978). In addition, *An. balabacensis* was highly competent in the transmission of *P. pitheci* and *P. silvaticum* among orang utan, *Pongo pymaeus* in the dipterocarp forest in eastern Sabah where they were attracted to feed both on orang utan and humans (Peters et al., 1976). Besides that, *An. balabacensis* was also responsible in the transmission of *Brugia* (probably *B. malayi*) (Cheong et al., 1984) and *Wuchereria bancrofti* in upper Kanibatangan and Banggi Island in Sabah, Malaysia (Hii et al., 1985; Hii et al., 1984).

Anopheles balabacensis is known from east Malaysia, Indonesia and the Philippines Islands (Figure 2.9) (Baisas, 1936; Colless, 1957; Reid, 1968; Russell et al., 1945; Sallum et al., 2005b).

2.2.2.2(b) *Anopheles introlatus* Colless

Anopheles introlatus was first described and named as *An. leucosphyrus* Kepong Form using specimens obtained from Central Malaya and Kepong, Selangor (Colless, 1956a). Further collections of specimens demonstrated morphological differences between adult, pupae and larvae of *An. introlatus* and *An. leucosphyrus* indicating that both are two are different species. Moreover, the specimens showed marked resemblance to *An. balabacensis* in the all life stages and hence *An. introlatus* was

placed as a subspecies of *balabacensis* (=*An. balabacensis introlatus*) (Colless, 1957). Based on morphological and zoogeographic data *An. balabacensis introlatus* was elevated to specific status and known as *An. introlatus* (Hii et al., 1988b).

The identification of *An. introlatus* larvae from *An. balabacensis* was based on having long seta 3-C and 4-C. Seta 3-C extends beyond anterior margin of head and seta 4-C always beyond the base of seta 2-C. Additionally, the larvae possesed seta 5-C obviously longer than antenna and individual leaflets of seta 1-VII has clear differentiation of apicolateral serrations and apical filaments (Colless, 1956a;1957; Sallum et al., 2005b). In the adults, the proboscis is dark-scaled and as long or slightly longer than forefemur (ratio 1.02-1.11), wings possessed ASP spot extending onto vein C at least on a wing and always present on subcosta vein, PSD spot of vein R on wings always with 1-2 pale interruptions, hindtarsomere 4 on hind legs without basal pale scales and hindtarsomere 5 with apical pale band but no basal white scales (Colless, 1956a;1957; Jeffery et al., 2012; Reid, 1968; Sallum et al., 2005b).

Larvae of *An. introlatus* can be collected from seepages, ground pools, rocky stream pools, aqueducts, wheel ruts and animal wallows situated in primary tropical rainforest in mountaineous area varying at elevations from 244 to 610 m above sea level (Macdonald & Traub, 1960; Sallum et al., 2005b; Scanlon et al., 1967; Warren et al., 1963).

Adults of *An. introlatus* are exophilic and attracted to feed on both humans and monkeys. They were caught using human bait at ground level and monkey trap in the forest canopy (Warren et al., 1970; Warren & Wharton, 1963). In recent study, *An. introlatus* were shown to be early biter, with peak biting period from 1900 to 2100 hours. The man-biting rate was 3.27 per man night (Vythilingam et al., 2014).

Though *An. introlatus* is not a vector for human malaria, it was incriminated as vector for simian malaria. Three females *An. introlatus* (previously known as *An.*

balabacensis introlatus) which was collected from monkey bait (1) and human bait (2) were found to harbor sporozoites in the salivary gland. When they were inoculated into rhesus monkey, a severe infection was developed on the 11th day post-infection from the mosquito caught using monkey bait. The malaria parasites were identified as *P. cynomolgi* (Eyles et al., 1963a). Additionally *An. introlatus* was also found to be involved in the transmission of *P. fieldi* in Malaysia (Wharton et al., 1964). Recently two *An. introlatus* females out of 62 caught from human landing catch were found infected with oocysts. Molecular identification using PCR confirmed the infection as *P. knowlesi* and thus, *An. introlatus* was incriminated as vector for *P. knowlesi* in Hulu Selangor (Vythilingam et al., 2014).

Anopheles introlatus is distributed in Indonesia, West Malaysia and Thailand (Figure 2.9) (Colless, 1948;1956a;1957; Reid, 1968; Sallum et al., 2005b).

2.2.2.2(c) *Anopheles latens* Sallum & Peyton

Colless (1956a) described *An. latens* based on specimens available from Malaya and Borneo. Several authors had also provided morphological descriptions on *An. latens* (Bonne-Wepster & Swellengrebel, 1953; Chow, 1961; Colless, 1957; Reid, 1968). By using morphology, hybridization and cytogenetic observations, the populations from Sabah and Kuching (SWK) and Niabet (SWN) were shown to be identical and was designated as *An. leucosphyrus sensu stricto* (Kanda et al., 1981). Confirmation of both populations as *An. leucosphyrus sensu stricto* was established using chromosomal polymorphisms (Kanda et al., 1983). By using 7 protein loci, Takai (1986) confirmed five loci are unique which could distinguish the 5 taxa within Leucosphyrus Group and verified the hypothesis by Kanda et al. (1983). Karyotypes and crossing evidence showed that there were two allopatric species of *An. leucosphyrus*, one which are inhabiting Borneo, West Malaysia and Thailand (=*An. leucosphyrus* A), and another one which is confined to Sumatra (=*An. leucosphyrus sensu stricto*) (Baimai et al.,

1988b). Based on the results of Baimai et al. (1988b), the specimens which were identified by Kanda et al. (1981); Kanda et al. (1983) and Takai (1986) as *An. leucosphyrus sensu stricto* were *An. latens* (Sallum et al., 2005a).

Generally, the larvae of *An. latens* can be differentiated from *An. balabacensis* and *An.introlatus* by possessing tubercles of seta 1-P and 2-P without a posterodorsal tooth or with a weak and subtle lip projecting forward over the base of each seta in which the tubercles usually well separated that occassionally joined basally. The seta 14-P with 5 to 8 branches, presence of 8 to 11 nearly transparent narrow lanceolate leaflets on seta 3-T and seta 14-M with 5 to 11 branches on the thorax of the larvae. The abdomen of larvae has 3 to 7 rigid branches on the minute seta 1-I, seta9-I with 3 to 5 branches and seta 1-II with 8 to 14 narrow lanceolate branches (Bonne-Wepster & Swellengrebel, 1953; Colless, 1956a;1957; Sallum et al., 2005a). The adults of *An. latens* has uniformly dark-scaled proboscis which is slightly longer or longer than forefemur (ratio 1.06-1.16), prehumeral pale (PHP) spot of vein C always present and minute, humeral pale (HP) spot always present and prominent, ASP spot usually present and can be occasionally reduced or absent, and PSD spot on vein R with 1-5 small pale interruptions while the hind tibia, femora and tarsomeres 1 are dark-scaled and speckled with pale spots (Colless, 1957; Jeffery et al., 2012; Reid, 1968; Sallum et al., 2005a; Sallum et al., 2005b).

The larvae can be found inhabiting in muddy pool in cart track running through dense jungle and in a split bamboo stem on ground (Leicester, 1903). Additionally, they can be found in ground pools along stream margins, flood pools, seepage pools, sandy pools in stream banks, small shallow running streams, elephant footprints and swamps (East Malaysia). The water in the breeding habitat of primary and secondary rain forests in mountains or valleys is always fresh, stagnant, clear and can be partially or heavily shaded (Colless, 1956a; Sallum et al., 2005b).

Like most of the members in the Leucosphyrus Complex, *An. latens* is attracted to feed on humans. Studies in the 1990s in Sarawak shown that females were more attracted to humans than to CDC light traps and found to be abundant in farm huts than in village settlements. All the mosquitoes were collected outdoors (Seng et al., 1999). The results corresponded with the previous study done in villages and forested areas where high number of mosquitoes were collected in the forested areas compared with village settlement. The females biting activity peaked around midnight from 2200-0100 hours in the forested area but soon after dusk in the village perimeter (Chang et al., 1995). The author concluded that this might be the changes in the microenvironmental conditions in both ecotypes. However, in 2008, entomological sudy carried out in Kapit, Sarawak shown that *An. latens* biting activity begins as early as 1800 hours. There were still higher number of adults collected in the farm (43.3%) compared to forest (25.4%) and village (11.7%) using human landing catch (HLC). By using monkey-baited trap, it was found that *An. latens* preferred to feed on monkeys at canopy rather than ground level also as early as 1800 hours. The ratio of *An. latens* biting monkey to human was 1:1.3 (Tan et al., 2008).

Anopheles latens was incriminated as a vector in the transmission of human and simian malaria together with filaria. In the study carried out in Baram District, Sarawak, *An. latens* (previously known as *An. leucosphyrus* A) was the predominant species caught and all of them were found positive with malaria sporozoites and filarial larvae of *W. bancrofti* (Chang et al., 1995). Moreover, it was also involved in the transmission of human malaria in the mountaineous areas in Sarawak (Zulueta, 1956) and in the Akah River region (Colless, 1956b). Sporozoites were found in females of *An. latens* in Ulu Lui and the inoculation into rhesus monkey produced *P. inui* infections (Wharton et al., 1964). Besides that, *An. latens* is also involved in transmission of *P. knowlesi, P. coatneyi,* and *P. fieldi* (Tan, 2008).

Anopheles latens is distributed from Indonesia, East Malaysia, West Malaysia and Thailand (Figure 2.9)(Colless, 1956a; Reid, 1968; Sallum et al., 2005a).

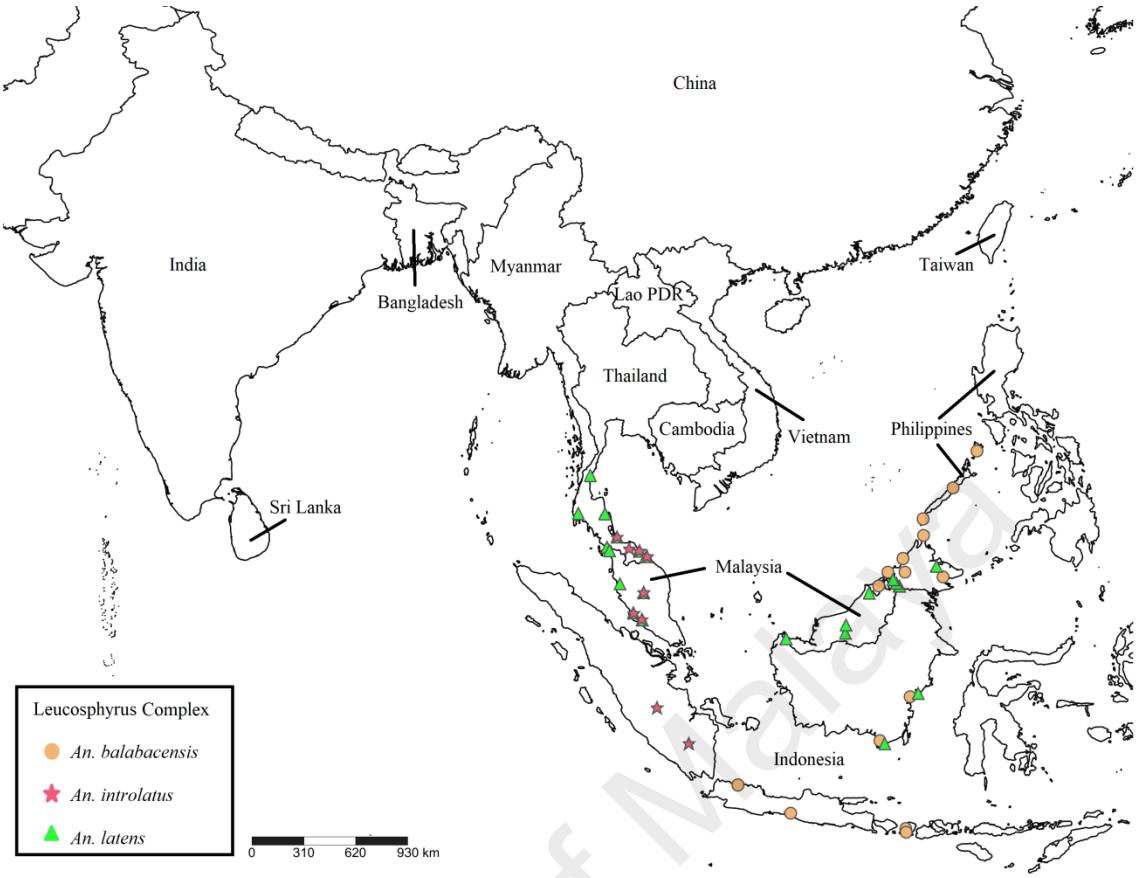

Figure 2.9: Distribution of Leucosphyrus Complex, modified from Sallum et al. (2005b).

2.2.2.3 Hackeri Subgroup

The Hackeri Subgroup was initially named as Elegans Subgroup by Colless (1956a) because *An. elegans* was considered as the nominotypical species in the subgroup. The classification of the Elegans Subgroup suggested by Colless (1956a) was confirmed by Peyton (1989) with additional morphological evidence. Nonetheless, examination on the holotype of *An. elegans* performed by Sallum et al. (2005a) showed that the species belongs to the Dirus Complex of the Leucosphyrus Subgroup (Peyton, 1989). Subsequently *An. elegans* was transferred to the Dirus Complex and since the Elegans Subgroup no longer contained its nominotypical species, the group was renamed as the Hackeri Subgroup (Sallum et al., 2005a). In addition, a new species *An. mirans* was also described and included in the Hackeri Subgroup. This subgroup can differentiated from Leucosphyrus and Riparis Subgroups by possessing proboscis which is distinctly longer than forefemur (ratio 1.16-1.45) and usually much longer than maxillary palpus (Sallum et al., 2005b). Members of the Hackeri Subgroup are medically important as they were vectors of simian or human *Plasmodium* parasites (Sallum et al., 2005b)The Hackeri Subgroup present in Malaysia consists of *An. hackeri* and *An. pujutensis*.

2.2.2.3(a) *Anopheles hackeri* Edwards

In 1921, *An. leucosphyrus* var. *hackeri* was described from Malaya by Edwards (1921) which the holotype specimen was collected by Hacker (1921) and deposited in the Natural History Museum, London. In the later years, *An. leucosphyrus* var. *hackeri* was elevated to species status, *An. hackeri* based on morphological characteristics and distinction in breeding places as compared with *An. leucosphyrus* (Colless, 1956a; Gater, 1933).

The larvae of *An. hackeri* are dark in color with conspicuous pale spots or bands on the dorsal surface (Colless, 1956a). Also the fourth instar larvae can be easily

distinguished by having fully developed seta 1-II, equal to or approximating to 1-III-VI in development, and having individual leaflets with clearly differentiated apical filaments (Colless, 1956a; Reid, 1968; Sallum et al., 2005b). In the meantime, the adults of *An. hackeri* can be distinguished from the rest in the Hackeri Subgroup in having these characters: 1) distinctly longer dark-colored proboscis than forefemur (ratio 1.22-1.38) and maxillary palpus with narrow pale apical ring basal to labella; 2) PSD spot of vein R without pale interruptions; 3) minute ASP spot and restricted to vein R which is shorter than basal dark spot of middle sark spot (MD) of vein R and sometimes reduced to 1 or 2 scales (Chow, 1961; Colless, 1956a; Gater, 1935; Jeffery et al., 2012; Reid, 1968; Sallum et al., 2005b).

In Malaysia, the larvae of *An. hackeri* can be found in breeding habitats of mangroves and rain forest in mountaineous or plain areas such as dead, hollow and split bamboos (Gater, 1933; Gater & Rajamoney, 1929; Macdonald & Traub, 1960; Reid, 1968), in muddy pools or grassy drains with partial shade and artificial containers (Gater & Rajamoney, 1929). Additionally they also can be seen in *Nipa* swamps (Gater, 1935) and rain water collections in old leaf bases cavity of *Nipa* palms (Reid, 1968; Reid & Weitz, 1961; Wharton et al., 1964; Wharton et al., 1963). The water of the breeding ground was either fresh or brackish, stagnant and colored. Breeding habitats of *An. hackeri* larvae in Thailand were in primary or secondary rain forests located in mountaineous areas ranging from 76 to 183m above sea level (Sallum et al., 2005b) whereas in Philippines, the larvae are found in heavily shaded coconut husks and artificial containers located inland or on the coast of hilly forests (Reid, 1968).

Studies and observations on the resting habits of *An. hackeri* adults shown they are exophilic. They can be found resting on the base of *Nipa* palms with close proximity to houses but apparently do not feed on humans (Reid, 1968; Wharton et al., 1964; Wharton & Eyles, 1961). Instead, the adults were found to feed predominantly on

monkeys by precipitin tests (Reid & Weitz, 1961; Wharton et al., 1964). A number adults of *An. hackeri* (13 in total) were caught biting humans on ground level using man-baited trap (Wharton & Eyles, 1961). Most adults were caught an hour after sunset (Wharton & Eyles, 1961). Subsequent entomological surveillance in the coastal area of Selangor revealed that they prefer to feed on monkeys in the canopy of mangrove forests where monkeys were frequently sighted. The forest was located few hundred yards away from their breeding ground in the *Nipa* palm area. Further experiment revealed that they moved from their breeding ground in search of blood meal in the mangrove forest (Wharton et al., 1964; Wharton et al., 1963).

Anopheles hackeri was involved in the transmission of simian malaria on the coastal mangrove of peninsular Malaysia. One of the *An. hackeri* caught was found to harbor sporozoites and when this was inoculated into rhesus monkey, *M. mulatta*, the infections built up rapidly and severely causing the monkey to die nine days post infection. The malaria parasites was identified as *P. knowlesi* and designated as *hackeri* strain of *P. knowlesi* (Wharton & Eyles, 1961). Subsequently, 12 salivary glands of *An. hackeri* were found to be infected and inoculations into rhesus monkey yielded isolation of *P. knowlesi, P. cynomolgi, P. coatneyi, P. inui* and *P. fieldi* infections (Wharton et al., 1964)

Anopheles hackeri is distributed from Malaysian Borneo to Peninsular Malaysia Malaysia, Thailand and Philippine Islands (Figure 2.10) (Gater, 1933; Jeffery et al., 2012; Reid, 1968; Sallum et al., 2005b).

2.2.2.3(b) *Anopheles pujutensis* Colless

Specimens collected by Roper (1914) presumably from Membakut, Sabah was noted to have an elongated proboscis similar to *An. hackeri* and thus was assumed the two forms are not specifically separable (Edwards, 1921). It was at that time known as *An. leucosphyrus* var *pujutensis*. Colless (1948) described it as a subspecies since there

is scarce information available on its distribution. Subsequently both the larvae and adult *An. leucosphyrus* var. *pujutensis* had been recorded from several countries, breeding in the same habitat with other members of the Leucosphyrus Group. As a result it was elevated to species status and known as *An. pujutensis* (Colless, 1956a).

The fourth instar larvae of *An. pujutensis* closely resembles to that of *An. latens* but can be distinguished by 2-4 branching of abdominal hair I.9 and having less than five branches in prothoracic hair 13 (Colless, 1956a; Rattanarithikul & Harrison, 1973; Reid, 1968). However the characters can be seen clearly only in larval pelts or cleared specimens. The integument of the head of the larvae is light brown to yellowish without pattenr of dark spots (Sallum et al., 2005b). Adults of *An. pujutensis* can be readily identified by having a distinctly long proboscis than forefemur (ratio 1.29-1.45) and maxillary palpus (ratio 0.76-0.88). The pale scale at the apex of maxillary palpus is broader than those on the maxillary palpus of *An. hackeri*. On the wings of adults, the PSD of vein R is without pale interruptions while on the hind tibia, the apical pale band is absent with longitudinal extension of dark scales (Jeffery et al., 2012; Peyton et al., 1966; Reid, 1968; Sallum et al., 2005b). Colless (1956a) noted geographical variation in several morphological characters of the adult *An. pujutensis* between Peninsular Malaysia and Borneo, namely propleural hair, apical wing fringe and maxillary index.

Larvae of *An. pujutensis* can be found in breeding habitats such as mountaineous rain forest and coconut plantations at 30-60m elevations above sea level in Peninsular Malaysia whereas in Malaysian Borneo, the habitats were situated in rain forests, rubber plantations and in hilly areas of 150-250m above sea level (Sallum et al., 2005b). The water in breeding grounds is always fresh, stagnant or slow moving, clear or colored and in partial shade or sometimes with decaying leaves. Collections of larvae have been obtained from places like deep pools, ground pools, slow moving streams, muddy pools, truck trail pools, wheel tracks, buffalo footprints, rock pools next to rivers, water

collections at the bases of *Nipa* palms (Colless, 1948; Reid, 1949; Reid & Weitz, 1961; Sallum et al., 2005b).

Adults of *An. pujutensis* can be found inhabiting the coastal mangrove and lower hills of Peninsular Malaysia and Borneo (Reid, 1968; Warren & Wharton, 1963). All the adults were exophilic and found resting in the day in the dark cavities of *Nipa* palm old leaf bases with *An. hackeri* (Reid & Weitz, 1961; Wharton et al., 1964; Wharton et al., 1963). *Anopheles pujutensis* was shown to demonstrate a clear preference for feeding on monkeys in the canopy of mangrove and lowland swamp forests (from monkey-baited trap) but rarely attracted to humans or domestic animals on ground level (Warren & Wharton, 1963; Wharton et al., 1964; Wharton et al., 1963).

Two salivary glands of *An. pujutensis* in a study in Rantau Panjang in the early 1960s were positive (Reid & Weitz, 1961) in addition to one oocysts infection found in later study (Wharton et al., 1964). This led to the assumption that *An. pujutensis* is a probable vector for simian malaria in the coastal mangrove and lowland swamp forests of Malaysia since the adults were found infected with *Plasmodium* parasites presumed to be from monkeys (Warren & Wharton, 1963).

The distribution of *An. pujutensis* ranges from Indonesia (Kalimantan and Sumatra Island) to Malaysia and northwards to Thailand (Figure 2.10) (Colless, 1948;1956a; Reid, 1968; Sallum et al., 2005b).

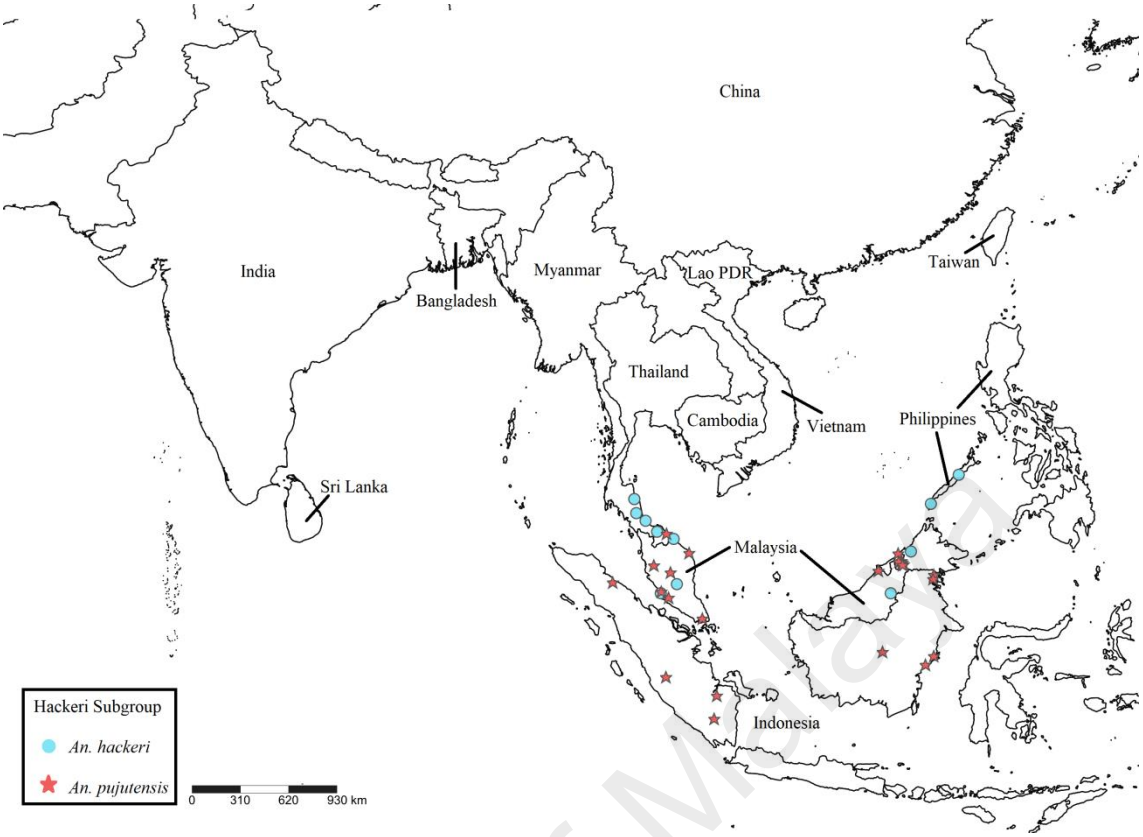

Figure 2.10: Distribution of Hackeri Subgroup, modified from Sallum et al. (2005b).

2.2.2.4 Riparis Subgroup

The Riparis Subgroup is subdivided from Leucosphyrus Group together with Leucosphyrus Subgroup and Hackeri Subgroup by comparison of differences in the length ratio of the adult female proboscis/ forefemur and maxillary palpus/ proboscis (Colless, 1956a; Peyton, 1989; Reid, 1949; Sallum et al., 2005a). The proboscis of the adults in the Riparis Subgroup is shorter than forefemur (ratio 0.90-0.99) (Peyton, 1989). Only one species in the Riparis Subgroup is present in Malaysia which is *An. macarthuri*. Based on morphological characters and geographical distributions comparison with other species in the Leucosphyrus Group, *An. macarthuri* was designated as separate and distinct species in the Riparis Subgroup (Hii et al., 1988b).

2.2.2.4(a) *Anopheles macarthuri* Colless

Anopheles macarthuri was first described as Crawford's "Spine IV short" pupal type (Crawford, 1938) of *An. leucosphyrus*. This is followed by a description as a form similar to *An. leucosphyrus* var *riparis* (Reid, 1949) before Colless (1956a) described and elevated it to subspecies of *An. riparis.*

The larvae of *An. macarthuri* are generally indistiguishable from larvae of *An. riparis* and *An. cristatus* except that there are dark spots present on the dosal apoteme laterally located between setae 7-C and 8-C on head integument. Additionally, the seta 4-C of *An. macarthuri* larvae is not well developed, short (not extending to the base of 2-C) and usually single besides possesing a prominent, broad and apically rounded posterodorsal process or lip arising from the tubercle of seta 1-P as compared with *An. riparis* larvae. Few other morphological characters of larvae used to differentiate *An. macarthuri* includes seta 1-I (somewhat palmate), seta 1-VII (with minute non-evident apicolateral serrations) and seta 13-IV (shorter than 0.5 length of seta 10-IV) (Colless, 1956a; Rattanarithikul & Harrison, 1973; Sallum et al., 2005b). Adult *An. macarthuri* can be identified by having proboscis shorter than forefemur (ratio 0.90-0.99) without

apical pale ring, presector dark spot (PSD) of vein R without pale interruptions on both wings, accessory sector pale (ASP) spot on vein C usually absent, and if present short on both vein C and R, and hind tarsus 4 is absent with basal pale band while the apical pale band of hind tibia had a linear dark stripe on the ventral (Jeffery et al., 2012; Reid, 1968; Sallum et al., 2005b).

The larvae of *An. macarthuri* can be found particularly in clear rock pools, sandy pools at the edges of small streams with decaying leaves, ground pools beside rivers, seepage pools, rock pools in bamboo, marshy depression beside streams, elephant footprints, in water collection of fallen tree trunks and natural container habitats. The habitats ranges from jungle to primary and secondary rainforests situated either at valleys or in hill or mountaineous areas with elevation up to 884m above sea level (Reid, 1968; Sallum et al., 2005b).

Anopheles macarthuri demonstrates exophilic behaviour. A study in Pos Lenjang, Pahang, Malaysia showed that *An. macarthuri* mosquitoes fly as far as 800m post-feeding in search of breeding ground (Ahmad et al., 2011). Only two adults of *An. macarthuri* were caught from monkey-baited trap in the forest canopy in hill forest and none were caught feeding human on ground (Wharton et al., 1964).

The distribution of *An. macarthuri* is known from Malaysian Borneo, West Malaysia and Thailand (Figure 2.11) (Chow, 1961; Harrison et al., 1990; Reid, 1949; Scanlon & Sandhinand, 1965).

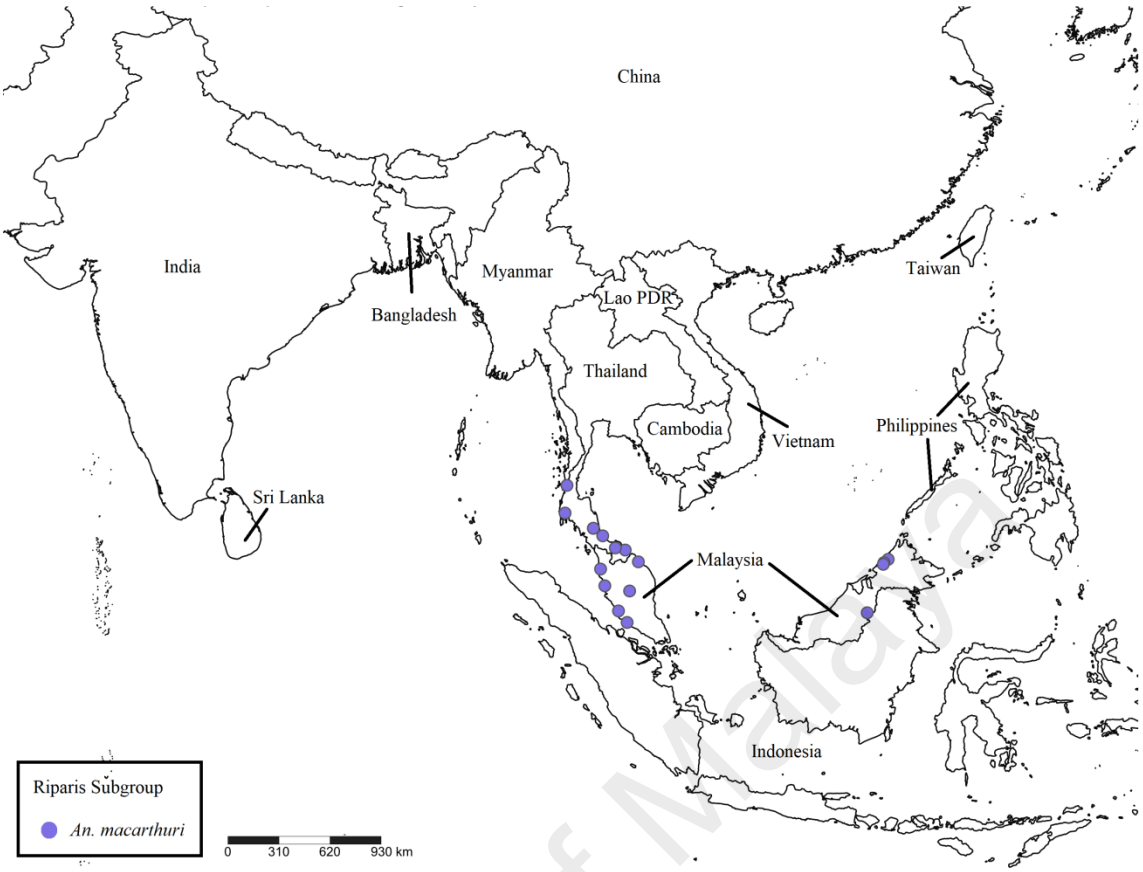

Figure 2.11: Distribution of Riparis Subgroup, modified from Sallum et al. (2005b).

2.2.3 Identification of *Anopheles leucosphyrus* group

2.2.3.1 Morphological characteristics

Both the epidemiology of vector-borne diseases and biodiversity of known or potential vectors are firmly interconnected (Manguin et al., 2008).The *Anopheles* diversity in Asia is much richer compared to the other regions (Foley et al., 2007) and since the main malaria vectors in Southeast Asia belong to species complexes and groups comprising of closely-related species (Harbach, 2004) which are hard to distinguish morphologically and often differ in their bionomics, this poses a challenge to comprehend malaria transmission and epidemiology (Manguin et al., 2008).

Both the Leucosphyrus and Dirus Complex in the Leucosphyrus Group of mosquitoes consists of species complexes which are closely related. The females of *Anopheles introlatus* can be mistakenly identified as *An. nemophilous* of Dirus Complex since some of the latter species hindtarsome 4 are absent with pale scales at the base (Sallum et al., 2005b). Additionally *An. balabacensis* is polymorphic in the morphology characters used for distinguishing the species between Leucosphyrus and Dirus Complex, that are absence of basal pale scales on hindtarsomere 4 and presence of accessory sector pale (ASP) spot on vein C, subcosta and R (Sallum et al., 2007). Consequently precise identification of *Anopheles* vectors using morphological characters greatly relies on the experience and skills of the entomologists.

2.2.3.2 Polymerase chain reaction (PCR)

Using deoxyribonucleic acid (DNA) analyses, there is an increase of discovery in new species, mainly sibling or cryptic species (Wilkerson et al., 2004). This is due to molecular tools such as PCR are extremely sensitive, rapid, reliable and relatively tolerant to degraded DNA from poorly preserved specimens as they require small amount of DNA such as one mosquito leg and thus, allows the rest of the specimens to be used for other analyses (Walton et al., 1999). Several studies using genetic and

molecular tools were carried out to investigate the members of Leucosphyrus Group for species recognition, gene flow and genetic population structure (Sallum et al., 2005b).

Four members of *An. dirus* complex (species A, B, C and D) have been distinguished using isozyme electrophoresis (Green et al., 1992), DNA probes (Panyim et al., 1988), non-radioactive DNA hybridization (Audtho et al., 1995), rDNA PCR of ITS 2 (Xu et al., 1998) and PCR-RFLP (Yasothornsrikul et al., 1988). By using allele-specific polymerase chain reaction, *An. dirus* species A, B, C, D or F were able to be identified precisely (Walton et al., 1999). Additionally development of six sequence characterized amplified regions (SCAR) resulted in the precise identification of three important malaria vectors among the *An. dirus* species A, B, C and D in Thailand (Manguin et al., 2002).

Identification of the Leucosphyrus Group mosquitoes using the mitochondrial cytochrome c subunit I (mtCOI) and NADH dehydrogenase subunit six genes showed low intraspecific variation among the members but higher interspecific variation was observed between *An. dirus* and *An. baimaii*. However, phylogenetic relationships between the members are moderately to weakly supported though it is clearly supporting the monophyly of the Dirus Complex (Sallum et al., 2007). Extensive sampling of species in the Leucosphyrus Group and further development of molecular tools will critically aid in establishing the precise identification of the members and resolution of phylogenetic relationship between them. Moreover, it will assist in comprehending future biogeographical studies and co-evolutionary studies of *Anopheles* species in relation to simian and human malaria (Sallum et al., 2007).

CHAPTER 3: MORPHOLOGY AND MOLECULAR CHARACTERIZATION OF THE LEUCOSPHYRUS GROUP OF *ANOPHELES* MOSQUITOESIN MALAYSIA

3.1 Introduction

Globally approximately 424 anopheline species have been described and are divided among six subgenera (Harbach & Kitching, 2005; Krzywinski & Besansky, 2003; WHO, 2008). These subgenera are further divided into groups, subgroups, complexes and species. Groups, subgroups and complexes are informal taxonomic classifications which become important with the increase of cryptic species within a number of anopheline taxa. Most of the cryptic species were reported as important malaria vectors worldwide. Thus, precise identification of the species plays a major role in both understanding the disease transmission and targeting the accurate species for vector control. However, cryptic species become problematic for systematic and phylogenetic studies since both studies are based on morphological characteristics which are widely used for the classification of *Anopheles* (Harbach, 1994).

The Leucosphyrus Group of mosquitoes (Reid, 1949;1968) are classified in Neomyzomyia Series (Christophers, 1924) of subgenus *Cellia* (Colless, 1956a; Harbach, 2004; King & Baisas, 1936; Reid, 1968; Reid & Knight, 1961) Theobald of *Anopheles* Meigen (Harbach, 2004) and ranges from southwestern India eastwards to southern China, Taiwan, mainland of Southeast Asia, Indonesia and the Philippines (Reid, 1968;1970). The taxonomy of Leucosphyrus Group was initially proposed by Colless (1956a) and Reid (1968), and corroborated later by Peyton (1989) which Sallum et al. (2005a) then confirmed the Hackeri, Leucosphyrus and Riparis Subgroups based on morphological similarities . Currently, there are 20 formally named species and two geographical forms

within the Leucosphyrus Group (Peyton, 1989) in which eight species can be found in Malaysia. There are five species classified under Leucosphyrus Subgroup, namely *An. cracens* and *An. nemophilous* (Dirus Complex), and *An. balabacensis, An. latens* and *An. introlatus* (Leucosphyrus Complex). The Hackeri Subgroup consists of both *An. hackeri* and *An. pujutensis* while *An. macarthuri* is classified in the Riparis Subgroup.

Within the Leucosphyrus Group, five species are of medical importance as they are highly competent disease vectors of malaria, namely *An. hackeri* (Wharton & Eyles, 1961), *An. balabacensis* (Hii, 1985a; Hii et al., 1985; Hii et al., 1988a), *An. latens* (Tan et al., 2008; Zulueta, 1956), *An. cracens* (Jiram et al., 2012) and *An. introlatus* (Vythilingam et al., 2014). In addition, *An. balabacensis* is also vector for Bancroftian filariasis (Hii et al., 1985). However, it is difficult to distinguish the vector involved in transmission using morphological characters due to the presence of closely related species within the Leucosphyrus Group. Though non-morphological features such as polytene chromosomes, karytotyping, crossing studies (Baimai et al., 1984a; Baimai et al., 1987; Baimai & Green, 1985; Baimai et al., 1984b; Baimai et al., 1988a; Baimai et al., 1988b; Baimai et al., 1988d; Baimai & Traipakvasin, 1987; Poopittayasataporn & Baimai, 1995; Sawadipanich et al., 1990) and allozymes (Green et al., 1992) had been used to distinguish the mosquitoes, they have major disadvantages which preclude their large-scale application (Walton et al., 1999). Inherently they require considerable technical skills (cytotaxonomy), fresh or frozen materials (allozymes) or time consuming (cross-mating experiments) (Manguin et al., 2002; Walton et al., 1999).

Through molecular approach such as PCR, species identification can be confirmed precisely since it is fast, reliable and extremely sensitive (Manguin et al., 2008; Walton et al., 1999). Both degraded DNA from poorly preserved specimens or DNA from a single leg

of a mosquito can be used for PCR for species identification. Thus, this allows the rest of the specimens to be used for other analyses, for instance parasite detection. Few molecular markers have been used for species identification and phylogenetic studies, namely mitochondrial cytochrome c oxidase subunit I (COI), internal transcribed spacer (ITS2) and small subunit ribosomal RNA (18S SSU rRNA).

The mitochondrial gene encoding the cytochrome c oxidase subunit I (COI) which is one of the largest protein-coding gene and the largest compared to COII and COI is present in high copy number (Harrison, 1989; Moritz et al., 1987; Simon, 1991; Wolstenholme, 1992) with a mixture of highly conserved and variable regions within its gene (Lunt et al., 1996), easily isolated, and lack of recombination. The eukaryotic ribosomal DNA (rDNA) genes which encode the rRNA are an extremely well-studied gene family and have been used to distinguish mosquito species such as *An. gambiae* complex (Scott et al., 1993) and *An. punctulatus* complex (Beebe & Saul, 1995). They are arranged in tandem repeats with each unit comprising of 18S, 5.8S and 28S ribosomal RNA separated by spacers, namely the intergenic spacer (IGS), external transcribed spacer (ETS) and internal transcribed spacer (ITS 1 and ITS 2) (Gerbi, 1985). The IGS separates the repeat units while ETS lies between the promoter and 18S gene. The ITS 1 is located between 18S and 5.8S whereas the ITS 2 separates both 5.8S and 28S genes (Gerbi, 1985; Schlötterer et al., 1994). Since the coding regions and spacers evolve at different rate, they aid in comprehending phylogenetic relationships between species (Hillis & Dixon, 1991; Honda et al., 1998; Kuperus & Chapco, 1994; Van de Peer et al., 1993).

Since the Leucosphyrus Group of *Anopheles* mosquitoes seem to play a major role in simian malaria, it is pertinent to have a simple morphological key for the species present in Malaysia. Thus, this study aims to build a simple identification key for the adult of the

Leucosphyrus Group of *Anopheles* present in Malaysia based on previous references (Jeffery et al., 2012; Reid, 1968; Sallum et al., 2005a; Sallum et al., 2005b) besides carrying out identification based on molecular techniques. Unlike Dirus Complex which has been extensively studied using molecular techniques such as allele-specific polymerase chain reaction (ASPCR) (Walton et al., 1999) and development of sequence characterized amplified regions (SCAR) markers (Manguin et al., 2002), there is limited information on Leucosphyrus Complex and other species in the Leucosphyrus Group. Therefore, this study also aims to determine the mtCOI, ITS 2 and 18S SSU rRNA sequences from the five species of *An. leucosphyrus* group collected in Malaysia (Leucosphyrus Complex: *An. balabacensis, An. latens, An. introlatus*; Dirus Complex: *An. cracens*; Riparis Subgroup: *An. macarthuri*) to investigate the evolutionary relationships and for species discrimination.

3.2 Materials and methods

3.2.1 Mosquito isolates

Five species of the *An. leucosphyrus* group were examined for this study. Ten *Anopheles balabacensis* specimens were from field material collected in Kudat, Sabah. Specimens of *An. balabacensis* (6), *An. latens* (7) and *An. macarthuri* (2) from Tawau, Sabah were collected by Hayley Brant of Imperial College, London. Specimens of *An. cracens* was from a colony maintained at the Department of Parasitology, Faculty of Medicine, University Malaya which were established from mosquitoes collected in Kampung Sungai Ular, Kuala Lipis, Pahang (courtesy of Dr Amirah Amir, University of Malaya, Malaysia). Another colony of *An. cracens* was obtained from a colony maintained at Chiang Mai University which was originally obtained in the wild from Perlis, Malaysia. Specimens of *An.* introlatus were collected from Sungai Sendat and Ulu Kalong, Selangor,

Malaysia. The list of the isolates used is summarized in Table 3.1. Available pinned adult mosquitoes' specimens were examined under stereomicroscope for species identification using morphological characters. A simple identification key for the female adult of the Leucosphyrus Group of *Anopheles* in Malaysia was built based on Reid (1968), Sallum et al. (2005b) and Jeffery et al. (2012). The mosquito legs were removed and kept in labelled microcentrifuge tubes at -20°C freezer for molecular identification.

Table 3.1: List of mosquito isolates used in molecular identification.

Mosquito species	Taxon	Isolates ID	Location of collection
An. balabacensis	Leucosphyrus Complex	Banggi1 Banggi2 Banggi3 Banggi4 Banggi5 Banggi6 Banggi7 Banggi8	Banggi Island, Kudat, Sabah
		KP1 KP2	Kg Paradason, Kudat, Sabah
		TL8 TM3 TM4 TM5 TM6 TM7	Tawau, Sabah
An. introlatus	Leucosphyrus Complex	SS1 SS2 SS3 SS4 SS5	Sg Sendat, Selangor
		UK1 UK3 UK4 UK5	Ulu Kalong, Selangor
An. latens	Leucosphyrus Complex	TL1 TL2 TL3 TL4 TL5 TL6 TL7	Tawau, Sabah

Table 3.1, continued.

Mosquito species	Taxon	Isolates ID	Location of collection
An. cracens	Dirus Complex	Per1 Per2 Per3 Per4 Per5 Per6 Per7 Per8	Perlis
		SU1 SU2	Kampung Sg Ular, Kuala Lipis, Pahang
An. macarthuri	Riparis Subgroup	TM1 TM2	Tawau, Sabah

3.2.2 Mosquito DNA extraction

Genomic DNA was extracted from the mosquito's legs using DNeasy® Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. Mosquito's legs were homogenized in their respective labelled micro-centrifuge tubes using a hand held homogenizer (Kontes Thompson Scientific) with plastic pestle in 180µL ATL. After brief homogenizing for 30 seconds, 20µL of Proteinase K was added into the tubes to aid protein digestion and to aid removal of any present nucleases. The mixture was then vortexed briefly before incubating overnight at 56°C at 300rpm in a thermomixer.

The next day, each tube was centrifuged briefly to bring down the condensation. Two hundred microliter of molecular grade ethanol and 200 µL of buffer AL were added into the sample and vortexed to yield a homogenous solution. The entire mixture was pipetted into DNeasy Mini spin column placed in a collection tube. The column was centrifuged at 8000 rpm for a minute. Both the flow-through and collection tube were discarded. The spin column was placed into a new collection tube and 500 µL of buffer AW1 was added into the column and centrifuged for 1 minute at 8000 rpm. The flow-through and collection tube were discarded. Five hundred microliter of buffer AW2 was added to the spin column which was placed into a new collection tube. The spin column was then centrifuged for 3 minutes at 14000 rpm. The flow-through and collection tube were discarded. The spin column was placed into a new collection tub and centrifuged for 1 minute at 14000 rpm to remove any remaining wash buffer. For DNA elution, the column was placed into a sterile 1.5mL micro-centrifuge tube and 100 µL of buffer AE was added directly onto the column membrane. The column was incubated for 1 minute at room temperature and centrifuged at 8000 rpm for 1 minute to elute the DNA.

All mosquitoes' legs were extracted individually and stored in a sterile 1.5mL micro-centrifuge tube in -20°C freezer until required.

3.2.3 PCR primers, amplification and sequencing

Three individual sets of primers were chosen to amplify the mtCOI, ITS 2 and 18S SSU rRNA genes of the mosquitoes as shown in Table 3.2.

Amplification of the mtCOI (Folmer et al., 1994), ITS 2 (Alquezar et al., 2010) and 18S SSU rRNA (Beebe et al., 2000b) were performed in a 20 µL reaction mixture as shown in Table 3.3. The cycling parameters for the amplification of mtCOI, ITS 2 and 18S SSU rRNA were illustrated in Table 3.4.

The PCR products were run on 1% agarose gel stained with Midori Green Direct (Nippon Genetics, Japan) at 90V for 30 minutes and visualized using an ultraviolet transilluminator. Amplified PCR products were clean-up using ExoSap (Antarctic Phosphatase and Exonuclease I-New England Biolabs, USA) (Nordström et al., 2000) prior to sequencing. The Antarctic Phosphatase removes dNTPs and pyrophosphates from PCR reactions while the Exonuclease I removes leftover primers. A master mix of 10µL were prepared immediately before use and kept on ice. The constituents of the master mix are as shown in Table 3.5. The master mix was added to the PCR samples. The samples were incubated at 37°C for 30 minutes followed by 80°C for 5 minutes (to deactivate the enzyme). The products were sent for sequencing (Macrogen, Geumchun-gu, Seoul, Korea).

Table 3.2: Oligonucleotide sequences of PCR primers used for molecular identification of mosquitoes.

Primers	Sequence (5'-3')	Annealing temperature (°C)	Expected product size (bp)
LCO1490	GGTCAACAAATCATAAAGATATTGG	50	710
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA		
ITS 2FH	GGATCGATGAAGACCGCAGCTA	55	650; 950
ITS2 RH	CCGTTTGCGCTCGCAGCTACTCAGG		
18SAP1	GAGGGAGCCTGAGAAATG	51	1800
18SAP2	CGGAAACCTTGTTACGACT		

Table 3.3: Components of master-mix for PCR identification of mosquitoes.

Components	Final concentration	Volume (µL)
RNase and DNase-free molecular grade water	-	12.5
5X MyFi Reaction Buffer (Bioline)	1x	4.0
Forward primer, 10µM	0.5µM	1.0
Reverse primer, 10µM	0.5µM	1.0
MyFi DNA Polymerase, 2U/µL (Bioline)	1.0U	0.5
DNA template	-	1.0
Total volume per reaction	-	20.0

Table 3.4: Cycling parameters for amplification of mtCOI, ITS 2 and 18S SSU rRNA in mosquitoes.

Steps	Temperature (°C)	Time (s)	Number of cycles
Initial denaturation	95	180	1
Denaturation	95	60	
Annealing	X (refer to Table 3.1)	60	35
Extension	72	60	
Final extension	72	300	1
	4	∞	-

Table 3.5: Components of master-mix for ExoSap.

Components	Final concentration	Final volume (µL)
RNase & DNase-free molecular grade water	-	8.75
10x Antarctic Phosphatase reaction buffer (New England Biolabs)	1x	1.0
Antarctic Phosphatase, 5U/µL (New England Biolabs)	1.0U	0.2
Exonuclease I, 20U/µL (New England Biolabs)	1.0U	0.05
Total volume per reaction	-	10.0

3.2.4 Restriction fragment length polymorphism (RFLP)

Restriction digestion analysis of the ITS 2 product was performed using the restriction enzyme *Msp* I (New England Biolabs, USA) which recognizes the motif "CCGG". A total volume of 10 µL reaction mixture was prepared as shown in Table 3.6. The samples were incubated at 37°C for 2 hours. Then, 5 µL of the digested product was run on a 3% agarose gel stained with Midori Green Direct (Nippon Genetics, Japan) and visualized using ultraviolet transilluminator. Genotype profiles were scored.

Table 3.6: Components of RFLP analysis.

Components	Final concentration	Final volume (µL)
RNase & DNase-free molecular grade water	-	3.0
10x NE4 buffer (New England Biolabs)	2x	1.0
Msp I restriction enzyme, 20U/µL (New England Biolabs)	20U	1.0
ITS2 PCR product	-	5.0
Total volume per reaction	-	10.0

3.2.5 ITS 2 copy variant analysis and DNA sequencing

A 10% non-denaturing acrylamide gel (BioRad, USA) was prepared in a 50mL Falcon tube prior to analysis of ITS 2 copy variant by electrophoresis. Casting plates were first set up. In a 50mL Falcon tube, 10 mL of 40% acrylamide/bis solution (BioRad, USA) was added in with 8mL of 5x Tris-Borate-EDTA buffer (TBE), 400µL of 10% ammonium persulfate (BioRad, USA) and double distilled water up to final volume of 40 mL. The solution was mixed homogenously by inverting the tubes 6-8 times. Then, 32µL of tetramethylethylenediamine (TEMED) (BioRad, USA) was added into the solution and mixed gently. Immediately after, the gel was poured carefully into the casting plates and allowed to set. After the gel was set, 2.5µL of the ITS 2 PCR product was loaded into the wells and electrophoresed for 2.5 hours at 200 V. The gel tank was placed in a plastic container containing ice and water to prevent the gel from heating and subsequent denaturing of the DNA duplex. After the electrophoresis, the gel was stained with Midori Green Direct (Nippon Genetics, Japan) for 1 minute, rinsed twice with TBE buffer and visualized under ultraviolet transilluminator. As native acrylamide gels are sensitive to double stranded duplex formation, heteroduplexes (duplex mispairing) will migrate across the gel slower than homoduplexes (no mispairing). Thus, this permits a qualitative assessment of the major ITS 2 copy variants amplified in the ITS 2 PCR products. Hence, individuals which produced only homoduplexes (a single band corresponding to the size of the PCR product) could be directly sequenced whereas heteroduplexes will produce multiple band profiles in the acrylamide gel signifying the presence of ITS 2 copy variants in the PCR product. As a result, cloning will be required prior to DNA sequencing.

3.2.6 Isolates used for ITS 2 gene characterization and amplification of gene

The internal transcribed spacer 2 gene of mosquitoes from *An. balabacensis* (3), *An. cracens* (3), *An. introlatus* (3), *An. latens* (3) and *An. macarthuri* (2) were characterized and denoted in Table 3.7.

Characterization of the isolates were performed using primers based on ITS 2 gene as shown in Table 3.2 using primers ITS 2FH and ITS 2RH (Alquezar et al., 2010). Amplification was prepared in 50µL reaction mixture as illustrated in Table 3.7. A total of 5µL DNA template was used. Both positive and negative controls were included for each batch of PCR. The PCR parameters for the amplification are as shown in Table 3.3. All PCR products were analyzed by electrophoresis in 1% agarose gel, stained with Midori Green Direct (Nippon Genetics, Japan) and observed under ultraviolet illumination. PCR product from this amplification was excised for gel purification, and subsequently cloned into a pGEM®-T Easy Vector.

Table 3.7: List of isolates used for characterization of ITS2.

Mosquito species	Isolates ID
An. balabacensis	Banggi2 Banggi3 Banggi5
An. introlatus	SS1 SS2 UK1
An. latens	TL2 TL5 TL7
An. cracens	Per7 SU1 SU2
An. macarthuri	TM1 TM2

3.2.7 Gel purification of PCR product

The DNA fragment was excised from the agarose gel with a sterile scalpel, transferred to a sterile labelled microcentrifuge tube and weighed. The gel slice was purified using NucleoSpin® Gel and PCR Clean-up (Machery-Nagel, Germany) according to manufacturer's protocol. For each 0.1g of agarose gel which was <1%, 200µL of buffer NTI was added into the microcentrifuge tube and incubated for 5-10 minutes at 50°C with occasional shaking to ensure the gel slice was completely dissolved. A NucleoSpin® Gel and PCR Clean-up Column was placed into a collection tube (2mL) and up to 700µL of sample was loaded into the column. The column was centrifuged for 30s at 10,700rpm. After centrifugation, the flow-through was discarded and the column was placed back into the collection tube. Any remaining sample was loaded into the column and centrifugation step was repeated. The silica membrane of the column was washed by adding 700µL of buffer NT3 into the column and centrifuged for 30s at 10,700rpm. The flow-through was discarded after centrifugation and the column was placed back into the collection tube. The column was then centrifuged for 1 minute at 10,700rpm to remove buffer NT3 completely. Then, the spin column was removed carefully from the centrifuge and flow-through was discarded. The spin column was then incubated for 5 minutes at 70°C for total removal of any residual ethanol from buffer NT3 prior to elution. Next the spin column was placed into a new sterile 1.5mL microcentrifuge tube. For elution, 20µL of buffer NE was added directly onto the spin column membrane. The spin column was then incubated at room temperature for 1 minute and centrifuged for 1 minute at 10,700rpm to elute the DNA. All purified DNA was stored in -20°C freezer until required.

3.2.8 Cloning of purified PCR amplicon

The pGEM®-T Easy Vector System I kit (Promega, Madison, WI, USA) was used and performed according to the manufacturer's protocol. Briefly the ligation reaction was carried out in a 10µL reaction volume containing 3µL of the purified PCR amplicon, 5µL of 2x Rapid Ligation Buffer, 1µL of pGEM®-T Easy Vector (50ng) and 1µL of T4 DNA Ligase (3 Weiss units/µL). A positive control using 2µL of Control Insert DNA instead of purified PCR product and a negative control were prepared. Both the pGEM®-T Easy Vector and Control Insert DNA tubes were centrifuged prior to ligation preparation. Additionally, the 2x Rapid Ligation buffer was vortexed vigorously before each use. The ligation reactions mixture was mixed thoroughly by pipetting. The reaction mixture was incubated overnight at 4°C for the maximum number of transformants.

For transformation, One Shot® TOP10 *Escherichia coli* competent cells (Invitrogen, Carlsbad CA, USA) were used. The vial of One Shot® TOP10 competent cells were allowed to thaw on ice prior to transformation. Then, 3µL of the ligation reaction mix was added into the vial and mixed gently before incubating on ice for 30 minutes. For pUC19 control, 1µL of DNA was added into a separate vial of competent cells, mixed gently and incubated on ice for the same duration. For the heat-shock procedure, the reaction mixture was placed in a 42°C waterbath for 50 seconds without shaking, and thereafter immediately placed on ice for 5 minutes. Then, 950µL of pre-warmed S.O.C medium was aseptically added into each vial. The vial was capped tightly and incubated at 37°C with horizontal shaking in a shaking incubator for 2 hours at 225rpm. After incubation, the vial was centrifuged at 5000rpm for 5 minutes and the supernatant was discarded carefully leaving the pellet. The pellet was resuspended in 200µL Luria Bertani (LB) broth. Two volumes of 100µL transformant culture were then

spread on pre-warmed labelled LB agar plate supplemented with ampicillin. The plates were inverted and incubated overnight at 37°C for bacterial growth.

Colony PCR was conducted using M13F (-40) and M13R (-48) to screen the *E. coli* transformants for the gene insert. A single colony of the *E. coli* transformant was picked using a sterile 10µL pipette tip and dipped into 10µL PCR reaction mixture containing 1x green GoTaq® reaction buffer (Promega, Madison, WI, USA), 3mM $MgCl_2$ (Promega, Madison, WI, USA), 0.2mM dNTP mix (Promega, Madison, WI, USA), 0.5µM of each primer, 0.5 units of GoTaq® DNA polymerase and sterile deionized water up to 10µL volume. The colony PCR amplification was carried out with an initial denaturation at 95°C for 4 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds. The final extension was 72°C for 2 minutes. At the end of the amplification cycle, the temperature was reduced to 4°C. The PCR products were analyzed by electrophoresis in 1% agarose gel, stained with Midori Green Direct (Nippon Genetics, Japan), and observed under ultraviolet illumination.

3.2.9 Extraction of plasmid DNA

Plasmid DNA was extracted using NucleoSpin® Plasmid (Machery-Nagel, Germany) according to manufacturer's protocol. An overnight Luria Bertani (LB) broth culture of transformants was centrifuged at 8300rpm for 6 minutes to pellet the cells. The supernatant was removed and cell pellet was resuspended completely by adding 250µL of Buffer A1 with RNase A, vortexed and transferred into a new sterile 1.5mL microcentrifuge tube. To lyze the cells, 250µL Buffer A2 was added and mixed gently by inverting the tubes 6-8 times, followed by incubation at room temperature for 5 minutes until the lysate becomes clear. Then, 300µL Buffer A3 was added and mixed thoroughly by inverting the tubes 6-8 times and followed by centrifugation at

10,700rpm for 5 minutes. For DNA binding, a NucleoSpin® Plasmid Column was placed in a 2mL collection tube and 750µL of supernatant was pipetted onto the column. The flow-through was discarded and the column was placed back into the collection tube. The step was repeated for any remaining lysate. To wash the column membrane, 500µL Buffer AW was added onto the column, centrifuged for 1 minute at 10,700rpm and the flow-through was discarded. This was followed by addition of 600µL Buffer A4 onto the column and centrifugation for 1 minute at 10,700rpm. The flow-through was discarded and the column was placed back into the collection tube. For drying of the column membrane, the column was centrifuged for 2 minutes at 10,700rpm. The collection tube was discarded and the column was placed into a new sterile 1.5mL microcentrifuge tube. For DNA elution, 50µL of Buffer AE was added directly onto the column membrane, incubated at room temperature for 1 min and centrifuged for 1 minute at 10,700rpm. Twenty microliters of eluted plasmid DNA was sent for sequencing to a commercial laboratory (Macrogen, Geumchun-gu, Seoul, Korea). The remaining plasmid DNA was stored at -20°C until required.

3.2.10 Sequence alignment and phylogenetic analyses

All sequences obtained from sequencing of mtCOI, 18S and ITS 2 were analyzed and edited using Geneious software (Kearse et al., 2012), and aligned with ClustalW using the default parameters. By using Basic Local Alignment Search Tool (BLAST), sequence identity comparison and confirmation were carried out. As the ITS2 regions were comprised of many short repeats and gaps, the final alignment required some manual fine-scale modifications. Subsequently, multiple sequence alignment of mtCOI, 18S and ITS2 were conducted.

By using MEGA 6.06, the best model for Maximum Composite-Likelihood (bootstrap= 1000) were chosen by analyzing using best DNA/ Protein model menu and

employed to study the evolutionary relationship among the Leucosphyrus Group of *Anopheles* mosquitoes obtained.

3.3 Results

3.3.1 Identification key of the species in Leucosphyrus Group of *Anopheles* mosquitoes in Malaysia

An identification key for the adults of Leucosphyrus Group of *Anopheles* mosquitoes occurring in Malaysia was built using morphological characters such as proboscis, wing spots and legs (Appendix A-*An. pujutensis*; Appendix B-*An. nemophilous*)

1. Proboscis always much longer than maxillary palpus, longer than forefemur (Figure3.1)…….....2

Figure 3.1

Proboscis at most slightly longer than maxillary palpus, as long as or slightly longer than forefemur (Figure 3.2)…….....3

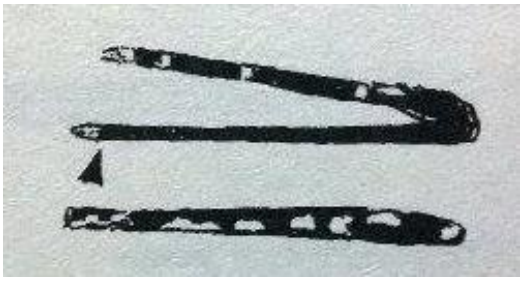

Figure 3.2

2(1). Apical pale band of palpomere 5 very narrow, proboscis is dark with narrow apical ring basal to labella (Figure 3.3)…...……...*hackeri*

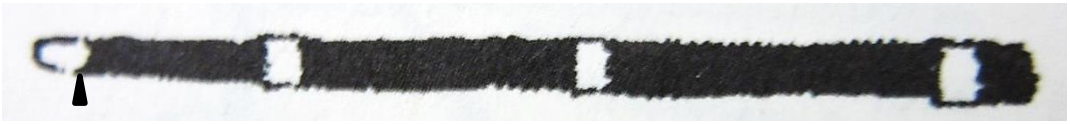

Figure 3.3

Apical pale band of palpomere 5 very broad, proboscis has pale scales at apex basal to labella (Figure 3.4)……..……*pujutensis*

Figure 3.4

3(1). Accessory sector pale (ASP) spot usually extending onto costa (C) and subcostal (SC) at least on one wing (Figure 3...…….4

Figure 3.5

ASP spot absent on costa and usually absent on subcostal (Figure 3.6)

……..…….5

Figure 3.6

4(3). Hindtarsomere 4 without obvious basal pale band on dorsal surface (Figure 3.6)

…….6

Figure 3.6

Hindtarsomere 4 with patch of pale scales on dorsal surface basally (Figure 3.7)

……..*balabacensis*

Figure 3.7

5(3). Presector dark (PSD) spot on vein R without pale interruptions (Figure 3.8)

…….*macarthuri*

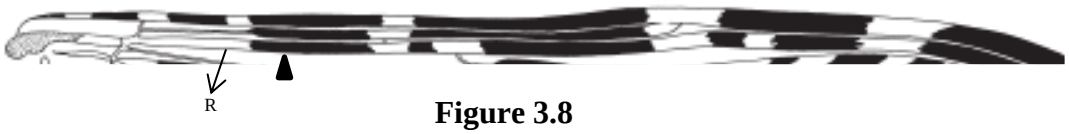

Figure 3.8

PSD spot on vein R with or without pale interruptions (Figure 3.9)

…….7

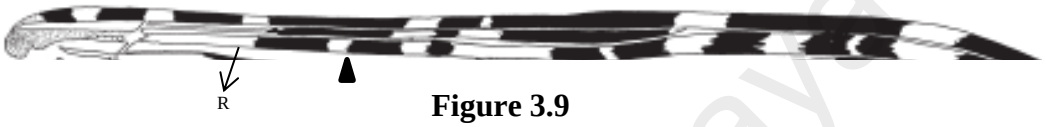

Figure 3.9

6(4). PSD spot of vein R often not extending basally beyond PSD spot on costa and rarely extending onto apical ½ of humeral dark (HD) spot on costa, apical pale band of palpomere 5 light cream-colored, not strongly contrasting with pale bands on palpomere 2 and 3 (Figure 3.10)……..……...*introlatus*

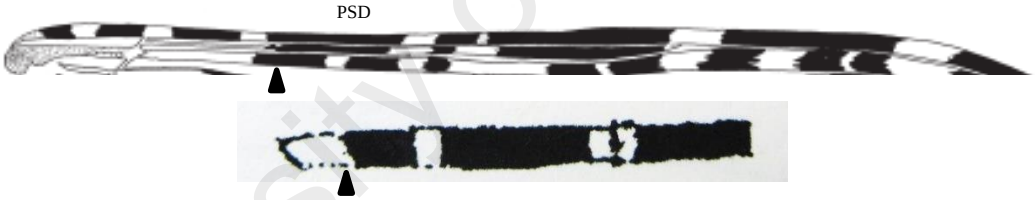

Figure 3.10

PSD spot of vein R often not extending basally onto level of HD on costa or beyond middle of HD, apical pale band of palpomere 5 distinctly cream-colored, strongly contrasting with silvery white band on palpomere 2 and 3 (Figure 3.11)

…….*latens*

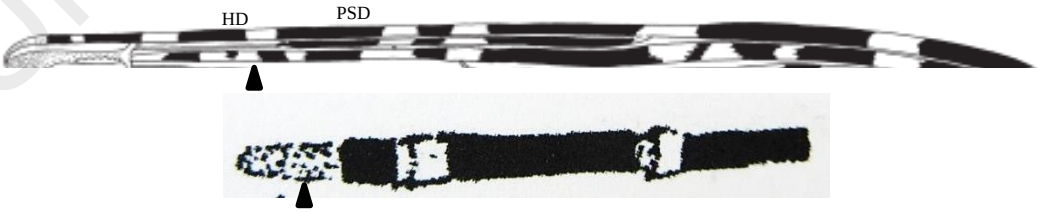

Figure 3.11

7(5). All pale scales of wing cream-colored to golden to yellowish, including presector pale (PSP) and sector pale (SP) spots, PSD spot of vein R often not extending basally beyond level of PSD wing spot on vein C (Figure 3.12)

……...……..*nemophilous*

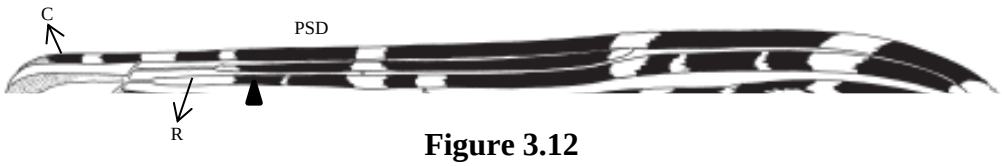

Figure 3.12

All pale scales on anterior veins of wings (including PSP and SP on costa) white, contrasting with pale spots on posterior, PSD spot of vein R frequently extending basally beyond ½ of PSP but not beyond HD, sternum VI with a small posteromedial patch of dark scales (Figure 3.13)

……..*cracens*

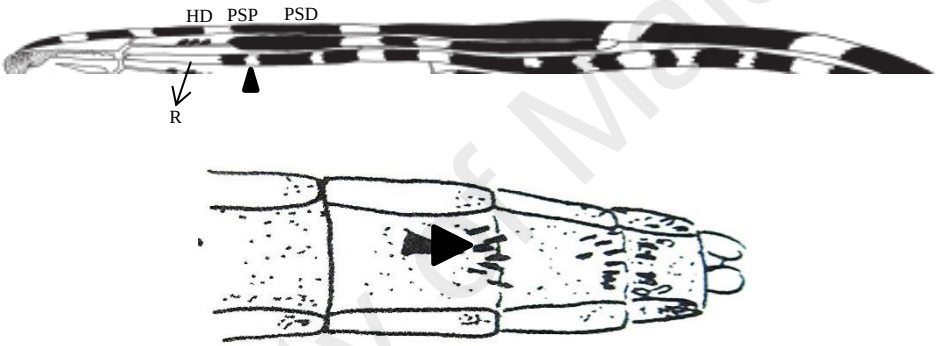

Figure 3.13

3.3.2 Molecular identification of mosquito isolates

In total, 44 Leucosphyrus Group of *Anopheles* mosquitoes consisting of five different species, namely *An. balabacensis* (16), *An. introlatus* (9), *An. latens* (7), *An. cracens* (10) and *An. macarthuri* (2) were collected from 6 different locations across Malaysia (Table 3.1). These mosquitoes were collected from forested areas, village and farming areas. From these mosquitoes, 44 sequences of both mtCOI and 18S, and 64 ITS2 sequences were obtained. The amplification using mtCOI gene marker yielded a size of about 710bp (Figure 3.14) while with 18S gene marker amplified a region of approximately 1800bp (Figure 3.15). On the other hand, amplicons with two consistently distinct sizes were yielded from each species, with *An. cracens, An. balabacensis* and *An. introlatus* being approximately 950bp, and *An. latens* and *An. macarthuri* yielding about 650bp (Figure 3.16). The ITS2 region was subsequently assessed using three different approaches: 1) primary assessment of sequence variation by RFLP analysis; 2) presence of intragenomic copy variants using heteroduplex analysis and 3) detailed DNA sequence variation by cloning and sequencing of the ITS2.

Extraction of DNA and PCR amplification were also carried out for pinned old specimens of *An. nemophilous* and *An. pujutensis* available in the Department's collection. However, none of the specimens were successfully amplified from the PCR.

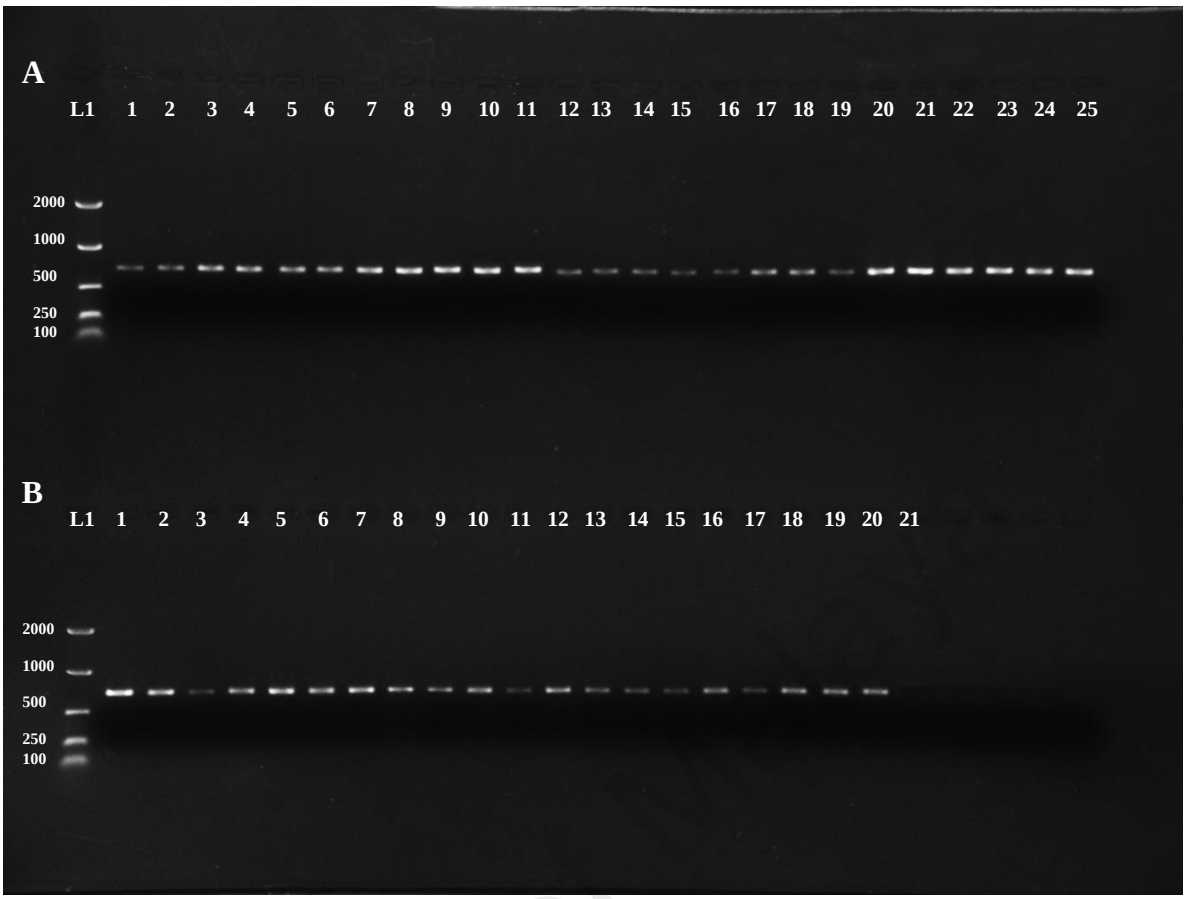

Figure 3.14: Gel picture showing PCR amplicons generated from the five species of the Leucosphyrus Group of *Anopheles* mosquitoes using LCO1490 and HCO2198 primers. Agarose gel of 1% was used. L1 denotes lane with DNA ladders of 100bp. (A) Specimens of *An. balabacensis* are from lane 1-8 and lane 24, lane 9-16 indicates specimens of *An. introlatus,* specimens of *An. latens* are from lane 17-23 lane 25 indicates specimen of *An. cracens*.(B) Specimens of *An. cracens* are denoted from lane 1-7, lane 8-9 indicates specimens of *An. macarthuri,* specimens of *An. balabacensis* from lane 10-16, lane 17 is *An. introlatus,* lane 18-19 denotes specimens of *An. cracens,* lane 20 is positive control used (*An. farauti*) and lane 22 is negative control (no DNA template). The amplicons generated were about 710bp.

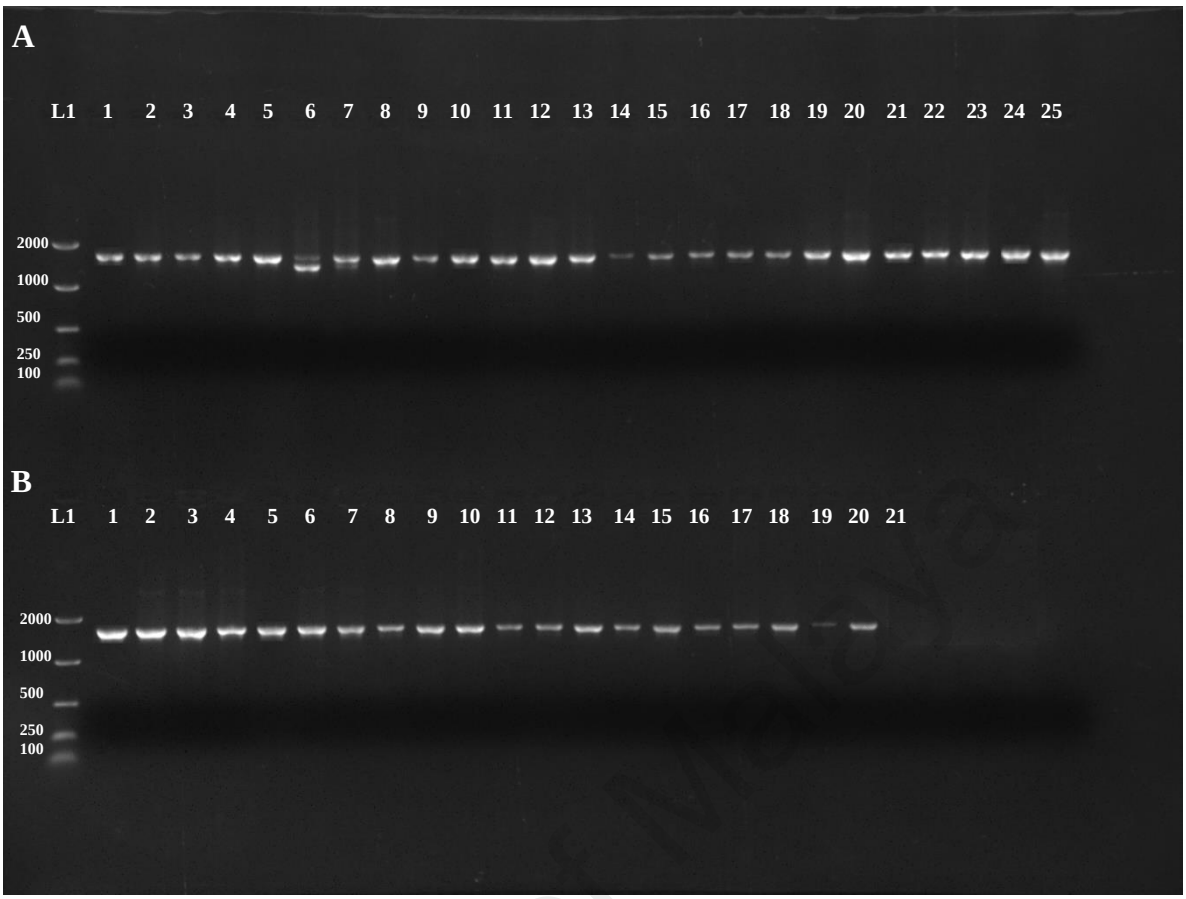

Figure 3.15: Gel picture showing PCR amplicons generated from the five species of the Leucosphyrus Group of *Anopheles* mosquitoes using 18SAP1 and 18SAP2 primers. Agarose gel of 1% was used. L1 denotes lane with DNA ladders of 100bp. (A) Specimens of *An. balabacensis* are from lane 1-8 and lane 24, lane 9-16 indicates specimens of *An. introlatus,* specimens of *An. latens* are from lane 17-23, lane 25 indicates specimen of *An. cracens*. (B) Specimens of *An. cracens* are denoted from lane 1-7, lane 8-9 indicates specimens of *An. macarthuri,* specimens of *An. balabacensis* from lane 10-16, lane 17 as *An. introlatus*, lane 18-19 denotes specimens of *An. cracens*, lane 20 is positive control used (*An. farauti*) and lane 21 is negative control (no DNA template). The amplicons generated were approximately 1800bp.

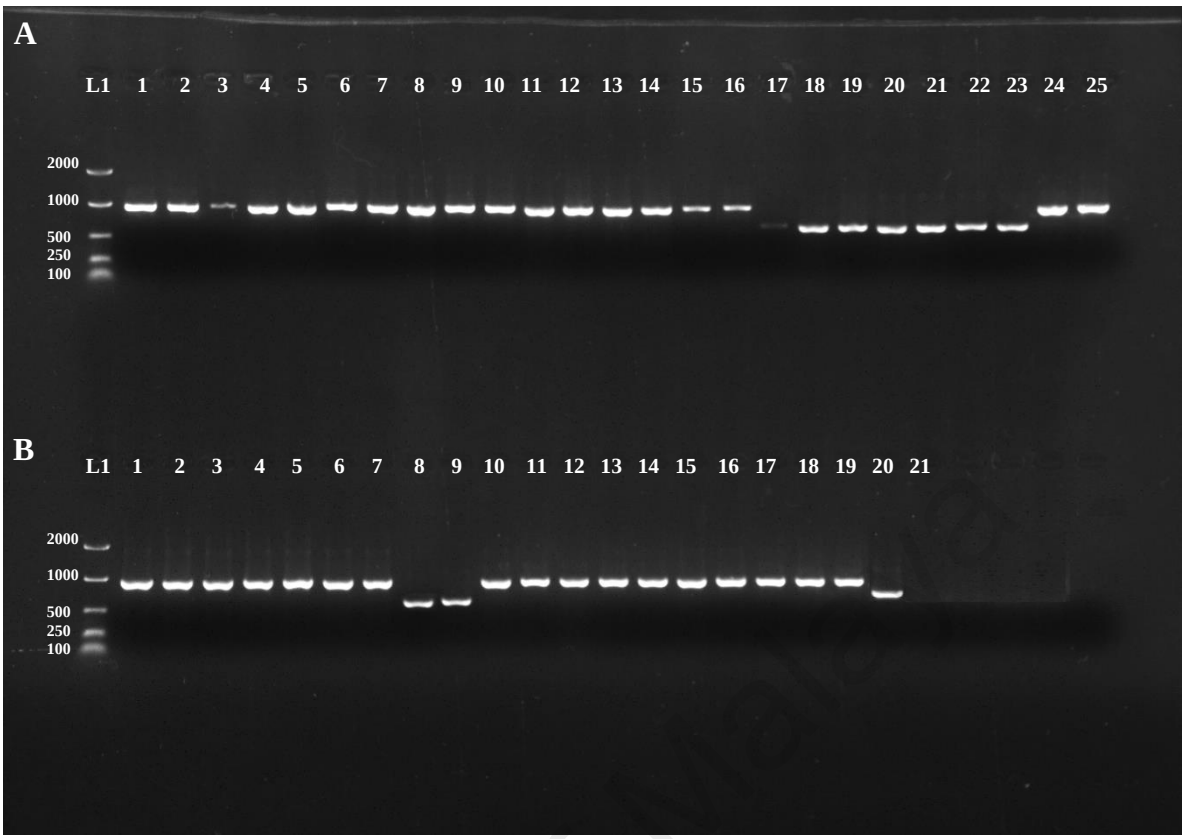

Figure 3.16: Gel picture showing PCR amplicons generated for five species of the Leucosphyrus Group of *Anopheles* mosquitoes using ITS2FH and ITS2RH primers. Agarose gel of 1% was used. L1 denotes lane with DNA ladders of 100bp. (A) The amplicons of *An. balabacensis* (lane 1-8; lane 24) and *An. introlatus* (lane 9-16) were shown to be approximately 950bp, isolates of *An. latens* (lane 17-23) were of about 650bp, lane 25 denotes amplicons of *An. cracens* approximately 950bp. (B) Isolates of *An. cracens* (lane 1-7) were shown to be approximately 950bp, lane 8-9 denotes amplicons of *An. macarthuri* with about 650bp, isolates of *An. balabacensis* (lane 10-16), *An. introlatus* (lane 17) and *An. cracens* (lane 18-19) with about 950bp, lane 20 is positive control used (*An. farauti*) and lane 21 is negative control (no DNA template).

3.3.3 Analysis of the ITS2 RFLP data

Species-specific banding patterns were observed from restriction digestion of the ITS2 product with enzyme *Msp* I (recognition motif CCGG). Size separation through a 3% agarose gel revealed six RFLP profiles with bands ranging from 200bp to 600bp. These six profiles have been designated as I-VI (Figure 3.17). The digestion of ITS2 sequences with *Msp* I produced two fragments in *An. balabacensis* (200 and 300bp), *An. introlatus* (300 and 400bp) and in *An. cracens* from Pahang (200 and 510bp), and three fragments in *An. cracens* from Perlis (200, 300 and 600bp) and in *An. macarthuri* (100, 200 and 250bp). Meanwhile, in specimens of *An. latens*, the digestion of ITS2 PCR products with *Msp* I produced uncut fragment at 400bp.

3.3.4 Identification of ITS2 copy variants from within each genome

For assessment of ITS2 homogenization status that is to determine the presence of copy variants within each genome and to confirm on the potential for direct DNA sequencing, all the PCR products were run through a 10.0% native acrylamide gel. Distinct homogenization profiles were consistent for all of the six RFLP genotypes obtained in Section 3.3.3 (Figure 3.18).

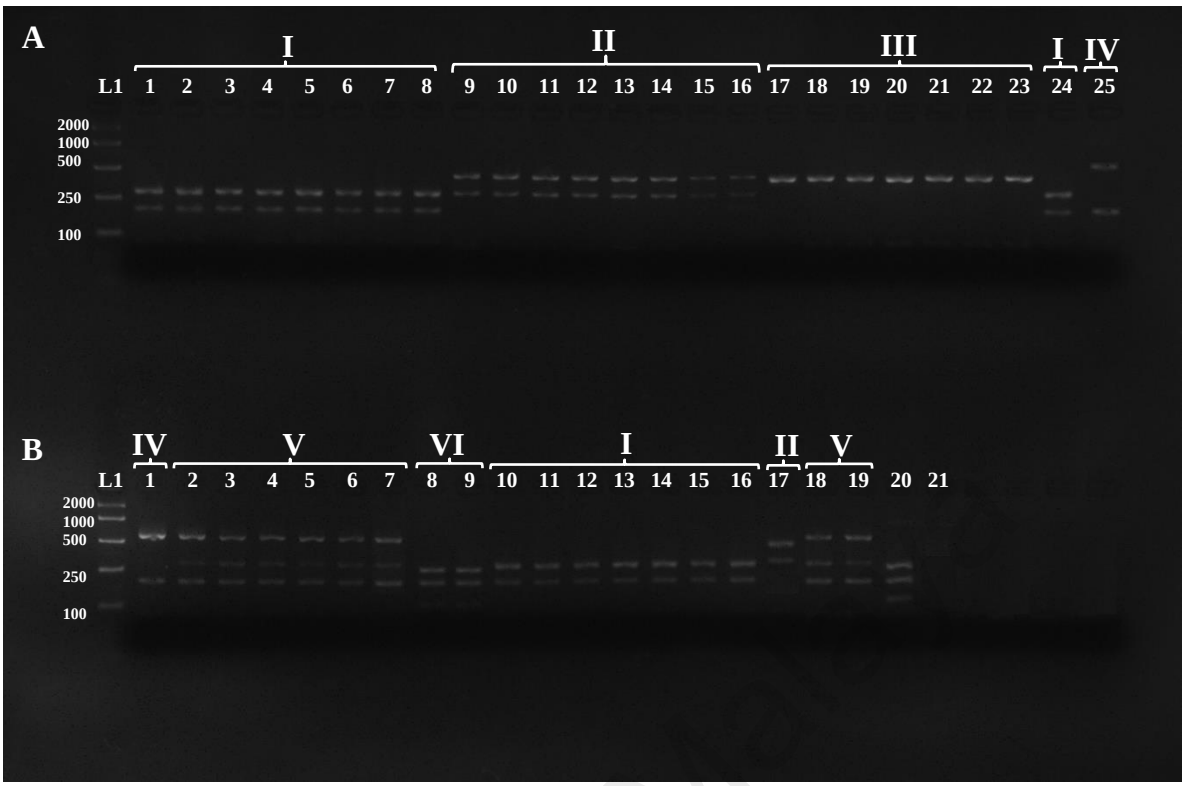

Figure 3.17: Gel picture showing the ITS2 genotypes of the five species of the Leucosphyrus Group of *Anopheles* mosquitoes. The same ITS2 products were cut with *Msp* I and run through a 3.0% agarose gel reveals six distinct RFLP profiles. L1 denotes lane with DNA ladders of 100bp. (A) The amplicons of *An. balabacensis* from Kudat, Sabah (lane 1-8; lane 24) as profile I and *An. introlatus* (lane 9-16) were shown as profile II, isolates of *An. latens* (lane 17-23) were of profile III, lane 25 denotes amplicons of *An. cracens* from Pahang as profile IV. (B) Isolates of *An. cracens* (lane 1) were from Pahang shown profile IV, isolates of *An. cracens* (lane 2-7) were from Perlis and depicted as profile V, lane 8-9 denotes amplicons of *An. macarthuri* with genotype VI, isolates of *An. balabacensis* from Tawau, Sabah (lane 10-16) as profile I, lane 17 denotes *An. introlatus* and *An. cracens* (lane 18-19) which from Perlis are of profile V, lane 20 is positive control used (*An. farauti*) and lane 21 is negative control (no DNA template).

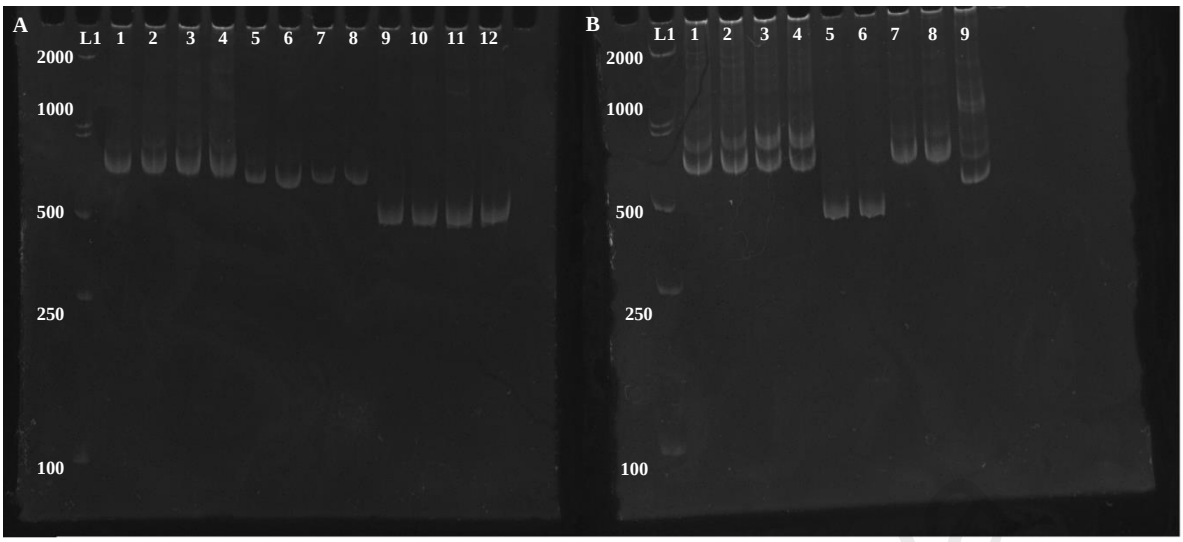

Figure 3.18: Gel picture showing the heteroduplex analysis of ITS2 PCR products of the five species of the Leucosphyrus Group of *Anopheles* mosquitoes. L1 denotes lane with DNA ladders of 100bp. (A) Lane 1-4 depicts genotype I of *An. balabacensis* from Kudat, Sabah, genotype II of *An. introlatus* from lane 5-8, and genotype III *An. latens* from lane 9-12. (B) Lane 1-4 shows homoduplex (bottom band) and heteroduplex products of *An. cracens* (genotype IV: lane 1-2 and V: lane 3-4), *An. macarthuri* (genotype VI) from lane 5-6 and lane 7-8 depicts specimens of *An. balabacensis* (genotype I) from Tawau, Sabah. Lane 9 denotes positive control, *An. farauti*. The ITS2-RFLP genotypes shown the same heteroduplex profiles when the PCR products were run through a 10.0% acrylamide gel which suggested that the copy variants are fixed within individuals and within interbreeding populations.

3.3.5 DNA sequence analysis

Mitochondrial cytochrome oxidase subunit I (mtCOI) and nuclear SSU regions (18S) from single specimens of the collected species in the Leucosphyrus Group of *Anopheles* mosquitoes were sequenced directly from PCR products. By analyzing and aligning the sequences of mtCOI and 18S PCR products, a final 658bp and 1496bp in length nucleotide sequences alignment, respectively were obtained. All obtained sequences from each of the amplified mtCOI, and 18S of mosquitoes were uploaded to GenBank and the accession numbers are as listed in Table 3.8. The ITS2 alignment length was found to vary between species ranging from 600bp to 910bp. Within the same species, sequence variation of ITS2 was found. The clones of *An. balabacensis* (N=12) and *An. introlatus* (N=10) were all unique sequences. Meanwhile, the clones of *An. cracens* (N=16) were comprised of 15 unique sequences, the *An. latens* clones (N=17) consists of 13 unique sequences and the *An. macarthuri* clones (N=9) were observed to have 7 unique sequences. All obtained sequences from each of the amplified mtCOI, 18S, and ITS2 clones of mosquitoes were deposited in GenBank and the accession numbers are as listed in Table 3.9.

Table 3.8: List of mtCOI and 18S sequences with their respective accession number.

Mosquito species and ID	Accession number	
	mtCOI	18S
An. cracens		
Per1	MG002540	MG002584
Per2	MG002541	MG002585
Per3	MG002542	MG002586
Per4	MG002543	MG002587
Per5	MG002544	MG002588
Per6	MG002545	MG002589
Per7	MG002546	MG002590
Per8	MG002547	MG002591
SU1	MG002548	MG002592
SU2	MG002549	MG002593
An. balabacensis		
Banggi1	MG002524	MG002568
Banggi2	MG002525	MG002569
Banggi3	MG002526	MG002570
Banggi4	MG002527	MG002571
Banggi5	MG002528	MG002572
Banggi6	MG002529	MG002573
Banggi7	MG002530	MG002574
Banggi8	MG002531	MG002575
KP1	MG002532	MG002576
KP2	MG002533	MG002577
TM3	MG002534	MG002578
TM4	MG002535	MG002579
TM5	MG002536	MG002580
TM6	MG002537	MG002581
TM7	MG002538	MG002582
TL8	MG002539	MG002583
An. introlatus		
SS1	MG002550	MG002594
SS2	MG002551	MG002595
SS3	MG002552	MG002596
SS4	MG002553	MG002597
SS5	MG002554	MG002598
UK1	MG002555	MG002599
UK3	MG002556	MG002600
UK4	MG002557	MG002601
UK5	MG002558	MG002602

Table 3.8, continued.

Mosquito species and ID	Accession number	
	mtCOI	18S
An. latens		
TL1	MG002559	MG002603
TL2	MG002560	MG002604
TL3	MG002561	MG002605
TL4	MG002562	MG002606
TL5	MG002563	MG002607
TL6	MG002564	MG002608
TL7	MG002565	MG002609
An. macarthuri		
TM1	MG002566	MG002610
TM2	MG002567	MG002611

Table 3.9: List of ITS2 clone sequences with their respective accession number.

Mosquito species and ID	ITS2 clone sequences	Accession number
An. cracens		
Per7	Per7C12	MG008561
	Per7C13	MG008562
	Per7C15	MG008563
	Per7C18	MG008564
	Per7C19	MG008565
SU1	SU1C1	MG008566
	SU1C3	MG008567
	SU1C4	MG008568
	SU1C5	MG008569
	SU1C7	MG008570
	SU1C8	MG008571
	SU1C9	MG008572
SU2	SU2C1	MG008573
	SU2C5	MG008574
	SU2C9	MG008575
	SU2C10	MG008576
An. balabacensis		
Banggi2	Banggi2C4	MG008613
	Banggi2C6	MG008614
	Banggi2C8	MG008615
	Banggi2C12	MG008616
	Banggi2C13	MG008617
Banggi3	Banggi3C3	MG008618
	Banggi3C4	MG008619
	Banggi3C10	MG008620
	Banggi3C12	MG008621
Banggi5	Banggi5C1	MG008622
	Banggi5C22	MG008623
	Banggi5C24	MG008624
An. introlatus		
SS1	SS1C3	MG008577
	SS1C4	MG008578
	SS1C5	MG008579
	SS1C9	MG008580
SS2	SS2C3	MG008581
	SS2C7	MG008582
UK1	UK1C1	MG008583
	UK1C2	MG008584
	UK1C7	MG008585
	UKC9	MG008586

Table 3.9, continued.

Mosquito species and ID	ITS2 clone sequences	Accession number
An. latens		
TL1	TL1C1	MG008596
	TL1C4	MG008597
	TL1C5	MG008598
	TL1C6	MG008599
	TL1C7	MG008600
	TL1C8	MG008601
TL5	TL5C1	MG008602
	TL5C3	MG008603
	TL5C4	MG008604
	TL5C5	MG008605
	TL5C6	MG008606
	TL5C8	MG008607
TL7	TL7C2	MG008608
	TL7C3	MG008609
	TL7C4	MG008610
	TL7C5	MG008611
	TL7C7	MG008612
An. macarthuri		
TM1	TM1C1	MG008587
	TM1C3	MG008588
	TM1C5	MG008589
	TM1C6	MG008590
TM2	TM2C1	MG008591
	TM2C4	MG008592
	TM2C6	MG008593
	TM2C11	MG008594
	TM2C12	MG009595

3.3.6 Phylogenetic analysis

Based on sequence alignment and comparison, all maximum composite likelihood phylogeny trees for mtCOI and 18S SSU sequences, and ITS2 clone sequences produced nearly identical topologies. The maximum composite likelihood topology of mtCOI, 18S and ITS2 sequences revealed two major clades supported by strong bootstrap value (>90%) whereby the first clade comprised of Leucosphyrus Subgroup (A: *An. cracens,* B: *An. balabacensis*, C: *An. introlatus* and D: *An. latens*) which can be differentiated from Riparis Subgroup (second clade denoted as E: *An. macarthuri*) by having proboscis as long as or slightly longer than forefemur and presector dark spot of vein R with or without pale interruptions.

The first clade is further divided into four subclades for both mtCOI and ITS2 maximum composite likelihood topology whereas for 18S, three subclades were recovered. In the mtCOI topology, *An. cracens* (denoted as A) collected from Pahang and Perlis were separated into two clusters, with the first cluster originated from Perlis shares similarity with the sequence obtained from GenBank (Thailand: JX219733) (Logue et al., 2013) whereas both SU1 and SU2 in the second cluster (from Pahang). The mtCOI sequences of related species (Logue et al., 2013), *An. dirus* A from GenBank were also included for phylogenetic analysis. As predicted, *An. cracens* is closely related but was distinct from *An. dirus.* However, no distinct clustering was seen for 18S SSU and ITS2 maximum composite likelihood topology for *An. cracens* specimens from Pahang and Perlis. Due to lack of sequences of the Leucosphyrus Complex of *Anopheles* mosquitoes from other countries, comparisons were only made for specimens collected in this study.

Anopheles balabacensis (denoted as B) of Leucosphyrus Complex formed the second subclade clustered together with subclade of *An. cracens* for both mtCOI and

ITS2 ML topology as compared to the ambiguous clustering in the 18S maximum composite likelihood topology. The phylogenetic position of *An. balabacensis* needs to be confirmed as it is seen more closely related to *An. cracens* (Dirus Complex) rather than to the other members from Leucosphyrus Complex.

Both *An. introlatus* (denoted as C) and *An. latens* (denoted as D) formed the third and fourth clade with strong bootstrap value (>95%), and were clustered together. Both of the species are grouped in Leucosphyrus Complex along with *An. balabacensis.* There are two clusters of *An. introlatus* observed for mtCOI maximum composite likelihood topology which were collected from two different locations in Hulu Selangor, namely Sg Sendat (SS) and Ulu Kalong (UK).

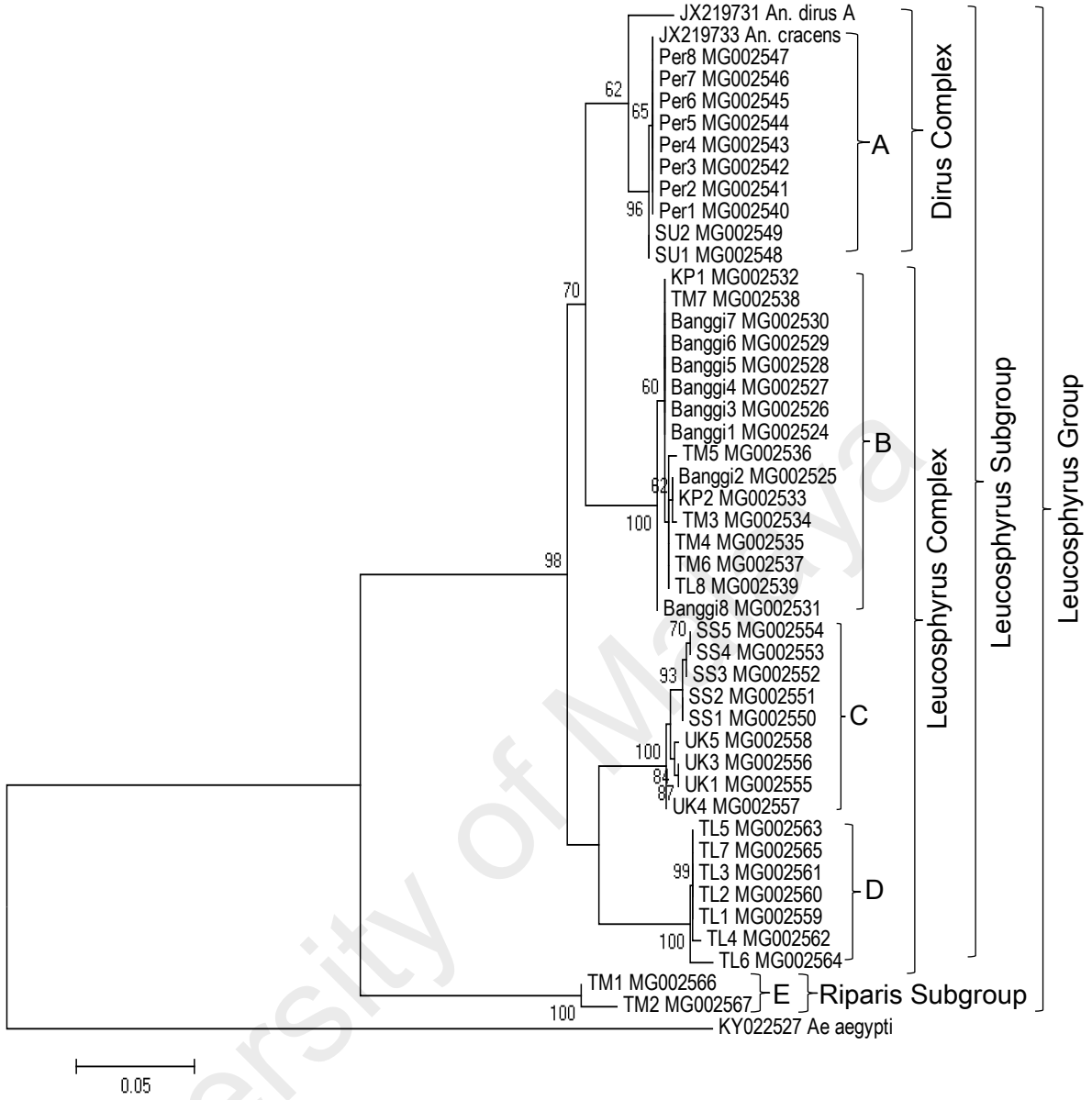

Figure 3.19: Phylogeny tree of mtCOI using Maximum Composite Likelihood (bootstrap=1000) of the five species of the Leucosphyrus Group of *Anopheles* mosquitoes collected for this study and sequences obtained from GenBank. Letter denotes the species (A: *An. cracens;* B: *An. balabacensis,* C: *An. introlatus*, D: *An. latens*; E: *An. macarthuri*). Both JX219731 and JX219733 were obtained from GenBank. *Aedes aegypti* (KY022527) is used as outgroup.

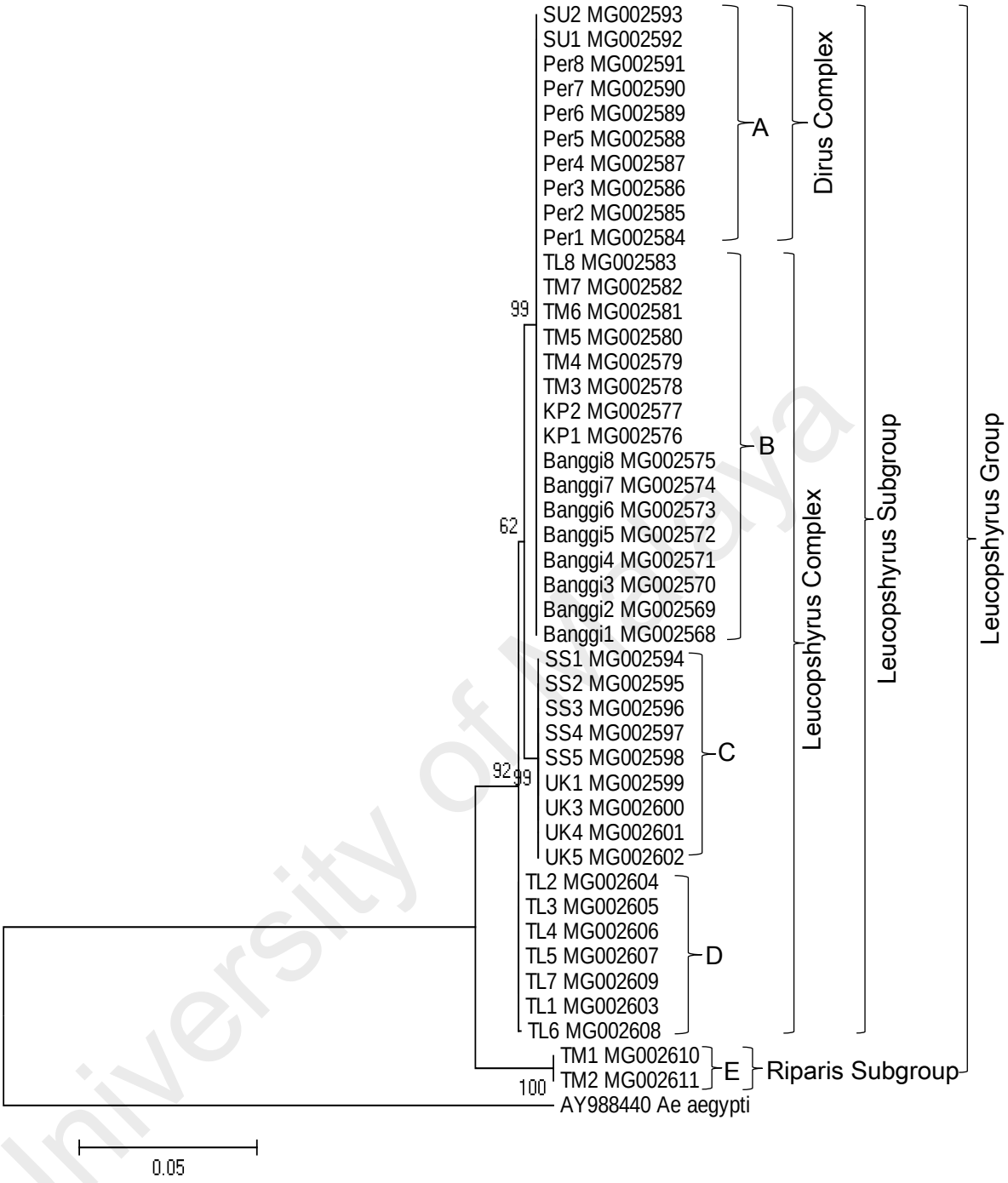

Figure 3.20: Phylogeny tree of 18S using Maximum Composite Likelihood (bootstrap=1000) of the five species of the Leucosphyrus Group of *Anopheles* mosquitoes collected for this study and sequence obtained from GenBank. Letter denotes the species (A: *An. cracens;* B: *An. balabacensis,* C: *An. introlatus*, D: *An. latens*; E: *An. macarthuri*). *Aedes aegypti* (AY988440) is used as outgroup.

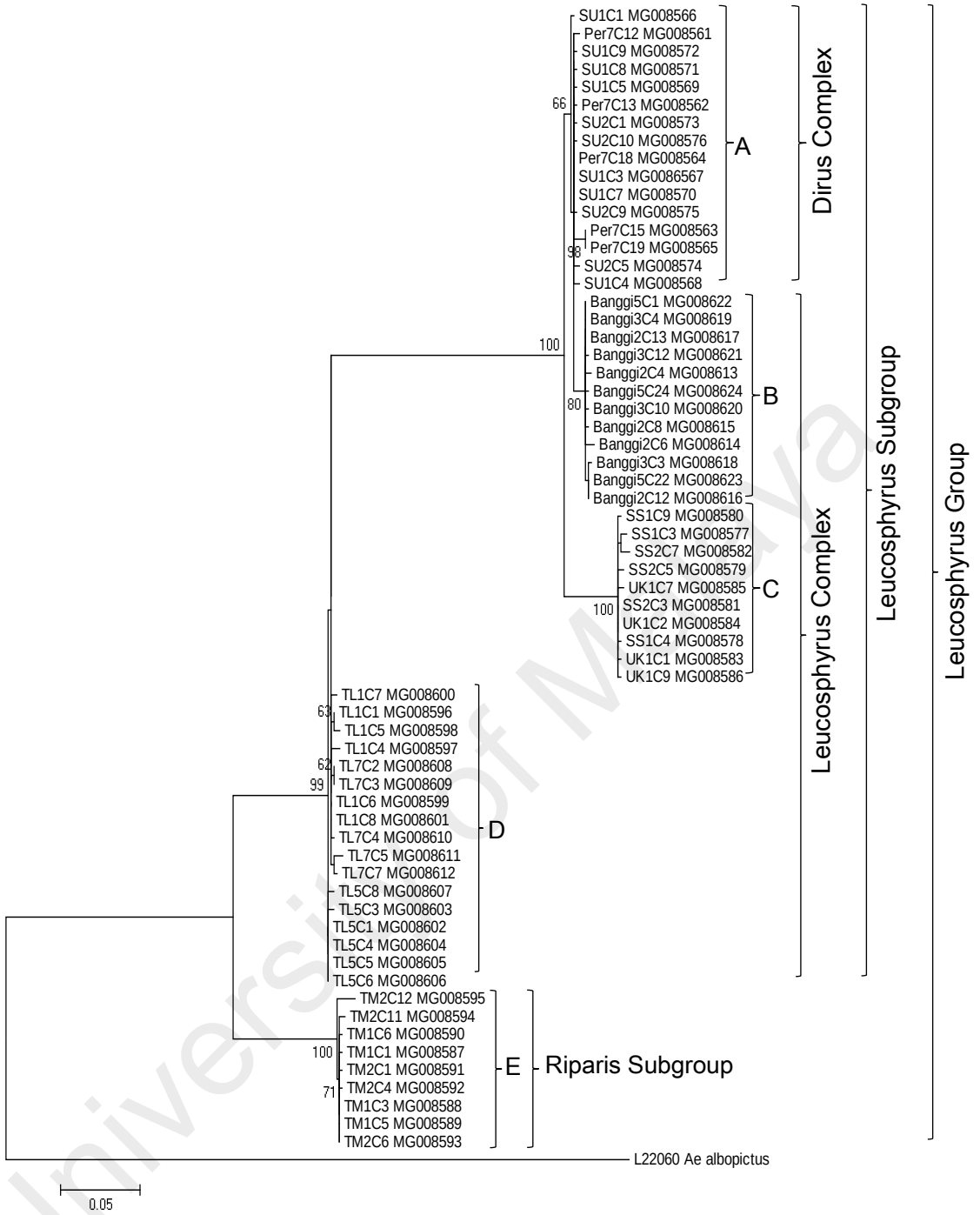

Figure 3.21: Phylogeny tree of ITS2 using Maximum Composite Likelihood (bootstrap=1000) of the five species of the Leucosphyrus Group of *Anopheles* mosquitoes collected for this study and sequence obtained from GenBank. Letter denotes the species (A: *An. cracens;* B: *An. balabacensis,* C: *An. introlatus*, D: *An. latens*; E: *An. macarthuri*). *Aedes albopictus* (L220060) is used as outgroup.

3.4 Discussion

Knowledge of vector species and their precise identification is an important criteria for a proper understanding of the epidemiology and transmission dynamics of malaria in the region. Therefore a simple identification key to identify the species of the Leucosphyrus Group of *Anopheles* mosquitoes in Malaysia was built based on morphological characters so that it could be used to identify the specimens in the field. However, morphological distinction among species of the Leucosphyrus Complex as well as the members in the Dirus Complex is challenging since these two complexes exhibit polymorphism for some of the characters used for identification (Sallum et al., 2005b). Thus, molecular technique was also conducted for correct identification of the species.

The five species in the Leucosphyrus Group collected in this study could be resolved by utilizing these three molecular markers- the mtCOI, 18S SSU and rDNA ITS2. The mitochondrial DNA is fast evolving and able to provide information on the presence of divergent lineages since it is maternally inherited. However, mtCOI cannot corroborate the existence of reproductive isolation between lineages (Alquezar et al., 2010). The maternally inherited COI which is widely used as a DNA barcode was able to show and support the clustering of the species of Leucosphyrus Group collected in this study. Nonetheless, all the ML topology of the three genes places *An. balabacensis* as sister to the Dirus Complex instead of clustering within the Leucosphyrus Complex clade. Morphologically, members of the Leucosphyrus Complex can be differentiated from the species in Dirus Complex by having accessory sector pale (ASP) wing spot on vein C, subcostal and R, and the absence of basal pale scales at the hindtarsomere 4 but *An. balabacensis* is polymorphic for these two characters and hence can overlap with members from both the Dirus Complex and Leucosphyrus Complex. Furthermore, a phylogenetic and chromosomal study on the seven populations of members in the

Leucosphyrus Group showed that *An. balabacensis* was not distinct from *An. dirus* (Kanda et al., 1983). Besides, it also demonstrated that *An. dirus, An. cracens* and *An. balabacensis* were monomorphic in all the 15 loci of gene-enzyme systems tested in a study by (Yong et al., 1983). Consequently it was observed that *An. balabacensis* was genetically closely related to the species in Dirus Complex and probably is a widespread species complex in Southeast Asia (Sallum et al., 2007). Nevertheless, polytene chromosomes and cross-mating studies done by Hii (1985c) demonstrated there are 3 distinct X chromosomes, namely X_A, X_B and X_C which represents *An. dirus* A (Bangkok), *An. cracens* (Perlis) and *An. balabacensis* (Sabah) specimens, respectively. Additionally, though these three forms hybridized successfully, sterile male offsprings were produced in at least one of the reciprocal cross. By applying the 'isolation species concept' (Dobzhansky, 1951;1980), , this indicated that they were true biological species. Further study by (Hii, 1986) on the morphometrics of the immature stages of these three species showed that all were distinguishable by using multivariate analysis on the setal branching of both larvae and pupae. The robust studies by (Hii, 1985c;1986) concluded that *An. balabacensis* was distinct from *An. dirus* A (Bangkok) and *An. cracens* (Perlis). .

The ITS2 is a transcribed spacer which is a part of the nuclear rDNA multicopy gene family and it evolves through non-Mendelian inheritance processes (Liao, 1999). Through evolution of ITS2, development of simple species-diagnostic molecular tools to distinguish cryptic species can be accomplished and also in providing evidence of reproductive isolation at the rDNA locus (Alquezar et al., 2010). The PCR-RFLP analysis based on the ITS2 region of the rDNA was able to distinguish all the species collected in this study by revealing six different profiles denoted from I-VI. The discrimination required only 4-bp restriction enzyme (*Msp* I which recognizes the motif CCGG) which on average, should cut every 256 bases (4^4) by assuming random basepair arrangements. In the study, only *An. latens* showed a clear single band which contains no restriction sites for *Msp* I whereas *An. cracens* samples with restriction enzyme produced two profiles. Samples from Pahang had showed two clear bands (200 and 510bp) whereas those from Perlis n to had three bands (200, 300 and 600bp). Subsequently, analysis of heteroduplex generated a single heteroduplex profile for both of the samples of *An.*

cracens from Pahang and Perlis suggesting there are only a copy variant present within these two geographical locations. Phylogenetic analysis of ITS2 sequences from *An. cracens* individuals indicated no distinct clustering as compared with maximum composite likelihood topology of mtCOI. Further confirmation on *An. cracens* from Pahang needs to be established since only two specimens were tested.

The PCR-RFLP analysis is advantageous to be applied as one of the molecular tool besides SCAR markers (Manguin et al., 2002) and allele-specific polymerase chain reaction (ASPCR) (Walton et al., 1999) as it is accurate, reliable, has minimal storage prerequisites and can act as another potential tool for rapid discrimination of species type.

The 18S SSU rRNA gene which is also part of the nuclear rDNA multicopy gene family are commonly used and proven to be informative for analyses of distantly related organisms since it is relatively long (usually about 1800-1900 nucleotides) and highly conserved (Bargues & Mas-Coma, 1997). However, it may lack sufficient resolution to distinguish cryptic or sibling species (Beebe et al., 2000a) when compared with ITS2 (Collins & Paskewitz, 1996; Wilkerson et al., 2004). This was observed in the topology of the maximum composite likelihood produced for 18S SSU gene for all the species included in this study.

The results of the current study do not fully resolve the relationships within the Leucosphyrus Subgroup. The phylogenetic position of *An. balabacensis* is ambiguous as it is placed closer to Dirus Complex rather than with the clusters of Leucosphyrus Complex. However, this study corroborates the monophyly of the Riparis Subgroup. Future studies should encompass on sampling of species within the Leucosphyrus Complex so that a multiplex PCR can be designed as has been done for the Dirus Complex.

3.5 Conclusion

Morphological identification of the species within Leucosphyrus Group is challenging due to the polymorphism present in the characters used to differentiate between members of the Leucosphyrus Complex and Dirus Complex. By utilizing molecular markers such as mtCOI, rDNA ITS2 and 18S SSU rRNA, species identification can be carried out precisely. Additionally PCR-RFLP method presented here can aid in resolving the identity of the *An. leucosphyrus* group of mosquitoes with distinct banding pattern besides other routine identification method used such isoenzyme analysis since it does not rely on skillful interpretation and thus, no subjective bias is introduced in the identification process.

CHAPTER 4: DYNAMICS OF *PLASMODIUM KNOWLESI* VECTOR IN SABAH, MALAYSIA

4.1 Introduction

Over the last decade, significant progress was made in malaria control, thus reducing the incidence of cases and mortality by 30% and 47%, respectively on a global scale (WHO, 2013b) while countries in the Pacific region had a 76% reduction in cases (Cotter et al., 2013). These successes were aided by the development and use of better tools for malaria diagnostic and treatments (Wongsrichanalai et al., 2007) as well as increased coverage of vector control methods such as Long Lasting Treated bed nets (LLINs) and Indoor Residual Spraying (IRS) (Yukich et al., 2008).

The presence of a zoonotic reservoir poses a challenge for malaria elimination as recently, *Plasmodium knowlesi*, the primate malaria has been documented causing human infections in several countries in Southeast Asia (Eede et al., 2009; Figtree et al., 2010; Jiang et al., 2010; Jongwutiwes et al., 2004; Khim et al., 2011; Luchavez et al., 2008; Ng et al., 2008) and creating a serious public health problem within Malaysia (Barber et al., 2012a; Cox-Singh et al., 2008; Joveen-Neoh et al., 2011; Singh et al., 2004; Vythilingam et al., 2014; Vythilingam et al., 2008; Yusof et al., 2014). In Sabah, *P. knowlesi* contributes to a large number of reported cases with 815 and 995 cases, respectively in 2012 and 2013 (William et al., 2014).

The growing burden of *P. knowlesi* poses threat to malaria elimination in Malaysia given that in the past, malaria transmission were mostly human-specific malaria parasite species (William et al., 2013). Since 2011, significant progress has been achieved for human malaria elimination in Malaysia, advancing towards complete elimination by 2020 (Rundi, 2011). However, in 2016, *P. knowlesi* cases comprised 69% of the cases in Malaysia and this is of great public health concern (MOH, 2016).

There are two facets influencing the effective control of *P. knowlesi* through conventional methods. The first is the large zoonotic reservoir within the macaques, which may in future spillover to humans on a larger scale due to loss of immunity because human infections were removed completely. Secondly, evidences had shown that the incriminated vectors of *P. knowlesi* in Malaysia were both exophagic and exophilic, thus conventional methods such as LLINs and IRS may not be effective against them (Jiram et al., 2012; Tan et al., 2008; Vythilingam et al., 2014).

Incriminating vector species responsible for *P. knowlesi* is crucial for vector control yet little data is available on the vectors of simian malaria in this region. Mosquitoes belonging to Leucosphyrus Group of *Anopheles* are considered to play a role in the transmission of *P. knowlesi*. Few species have been incriminated as vectors with the first being *Anopheles hackeri* in the coastal area of Selangor (Wharton & Eyles, 1961), followed by *An. latens* in Kapit, Sarawak (Tan et al., 2008; Vythilingam et al., 2006). *An. cracens* in Kuala Lipis, Pahang (Jiram et al., 2012) and *An. introlatus* in Hulu Selangor (Vythilingam et al., 2014). *Anopheles dirus* was incriminated as vector for *P. knowlesi* transmission in Vietnam (Marchand et al., 2011; Nakazawa et al., 2009). The considerable spatial variation of *P. knowlesi* vector species within and beyond Malaysia emphasizes the crucial need for localized comprehensive study of vector ecology to facilitate the planning of control strategy.

Anopheles balabacensis is postulated to play the role as primary vector of *P. knowlesi* in the current extensive transmission in Sabah. Through the numerous studies carried out in the 1980s in the region (Hii et al., 1991; Hii et al., 1985; Hii et al., 1988a; Hii & Vun, 1985), *An. balabacensis* was incriminated as the vector for human malaria. Additionally it is shown that *An. balabacensis* can be experimentally infected with *P. knowlesi* in the laboratory (Collins et al., 1967). Throughout the years since the early studies, there has been substantial amount of ecological changes in Sabah resulting from

conversion of forest to oil palm plantations (Bryan et al., 2013; Fornace et al., 2014; Hansen et al., 2013). There is a dire need to investigate how the land change affects the abundance, diversity and transmission potential of *P. knowlesi* vectors. Hence it is of utmost importance to verify and incriminate the vector species in addition to characterization of abundance and biting behavior of the vector. Consequently these findings will aid in the development of local vector programs to eliminate malaria transmission. Thus, a longitudinal study was carried out to determine the behavioural characteristics of the vectors.

4.2 Materials and methods

4.2.1 Description of study area

The study was carried out in three selected sites in the northern part of Sabah.The sites were Timbang Dayang (TD) (117°102’92”E, 7°155’85”N) and Limbuak Laut (LL) (117°065’75”E, 7°215’84”N) on Banggi Island, and Kampung Paradason (KP) (116°786’35”E, 6°768’37”N) on mainland Kudat (Figure). These sites were selected to represent the ecotypes mostly existing in the study area in Northern Sabah, comprising of small scale farming (TD), secondary forest (LL) and village settlement (KP). There were reports of recent human *P. knowlesi* cases and macaques’ sightings nearby all the sites.

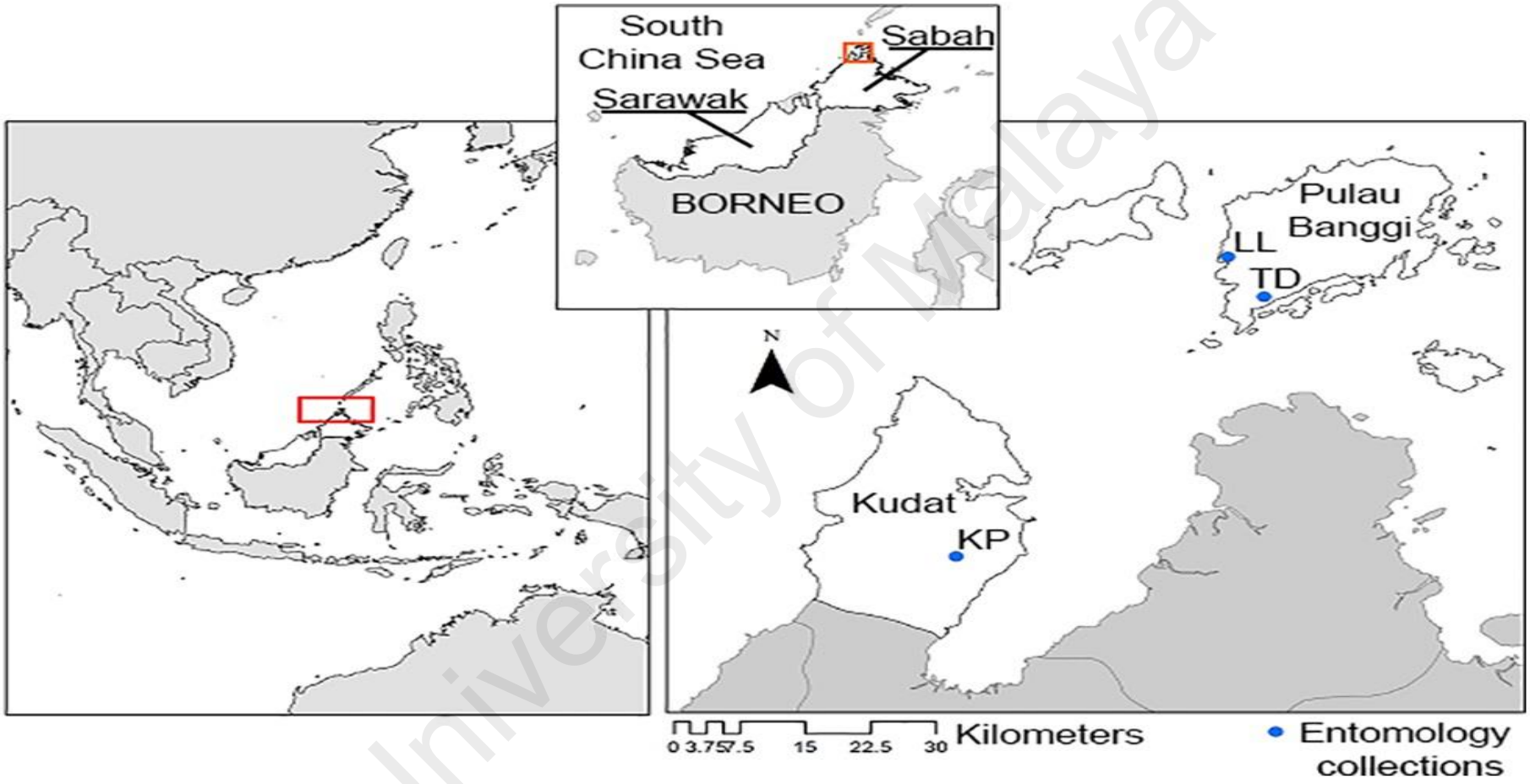

Figure 4.1: Map showing study sites in Banggi Island and mainland Kudat.

Timbang Dayang (TD) is a village comprising of 180 people. It is located on a hilly landscape whereby the houses were surrounded by small scale farming areas situated approximately 200 meters from the edge of secondary forest. These small farms are less than 1 hectare and consist of maize, banana and fruit trees cultivated primarily for house consumption. Mosquito sampling was carried out near the edge of the farm located approximately 150 meters from the surrounding houses.

Limbuak Laut (LL) is a village with a population of 144 people. The houses were located on a road bordering the closed canopy secondary forest. Mosquito collection site was situated inside the forest at a point approximately 500 meters from the edge of the forest.

Kampung Paradason (KP) in mainland Kudat is a village accommodating 160 people. The village is established in a heavily cultivated area which is characterized by swidden farming and small rubber and palm oil plantations. The area is experiencing high rate of environmental change such as frequent burning and land clearing. There is little remaining of secondary forest in the area. The local community lives in both individual houses and a traditional communal longhouse shared between six households. Mosquito collections were carried out in two points: one located about 100 meters from the longhouse and another in an associated garden area situated 75 meters from the first collection point.

4.2.2 Mosquito collection

Mosquitoes were collected using human landing collections (HLC). Mosquitoes sampling were carried out monthly at all sampling sites for a year from August 2013 to July 2014. Three nights per month of collections were performed at both TD and LL while two nights per month in KP. Two men per team were assigned for mosquitoes collections at each site from 1800-0600 hours. Mosquitoes landing on the legs of

catchers were captured individually in vials plugged with cotton wool and placed into bags labelled accordingly to the hour and collection sites. Prior to sampling, the bottom of the glass vial is stuffed with a piece of tissue paper (as shown in Figure 4.2B) and wetted with water to create humid micro-environment. A simple cardboard box was used for storing and transporting the mosquitoes from the field to the laboratory. On the interior, a thin plastic sheet was lined and fixed with adhesive bands. The collected mosquitoes were arranged inside and covered with a thin plastic sheet. A wet towel was then placed on top to ensure humidity. A supervisor was appointed to visit the team hourly to ensure collections were being carried out. In both TD and LL collections were conducted by one team each night while two teams were allocated for collections each night in KP. Therefore, a total of six individual human landing catches were carried out each month in TD and LL, and eight per month in KP.

Figure 4.2: A) Field workers performing human landing catch (HLC). B) glass vial with collected mosquitoes.

4.2.3 Mosquito identification

In the laboratory, *Anopheles* mosquitoes were identified using the morphological keys built in Chapter 3 with a dissecting microscope. Specimens morphologically identified as *An. balabacensis* were further confirmed by polymerase chain reaction (PCR) using mtCOI (Folmer et al., 1994), ITS2 (Alquezar et al., 2010) and 18S genes (Beebe et al., 2000b). Amplified products were sent to sequencing to a commercial laboratory (MyTACG Bioscience, Malaysia). Sequence identity comparison and confirmation were performed using Basic Local Alignment Search Tool (BLAST).

4.2.4 Mosquito dissection and examination of ovary, midgut and salivary glands

Anopheles mosquitoes were dissected using a dissecting microscope (made by inserting a minute entomological pin into a wood applicator stick) to extract their ovaries, midguts and salivary glands. Live *Anopheles* mosquitoes were knocked down by placing them in -20°C for 30 seconds. After immobilization, the legs and wings of the mosquito were removed one at a time. The mosquito was placed on a slide afterwards and arranged in a suitable position for dissection of the ovaries, midgut and salivary glands. All the wings and legs were removed prior to dissection to avoid contamination by scales from the mosquitoes' wings or legs.

Extraction of ovaries and midgut by dissection was carried out to determine the parity and oocysts rate, respectively. The mosquito was laid on a dry slide with the apex of the abdomen to the right and a drop of saline was added near the extremity of the abdomen. The abdomen was separated from the thorax by a cut using left dissecting needle, leaving part of the metanotum attached to the abdomen. The left dissecting needle was then fixed in the attached part of the thorax. By using the right needle, a small cut in the integument was made carefully on each side on the seventh abdominal segment without tearing the internal organs. The left needle was used to hold the abdomen while the right

needle was gently placed on the apex of the abdomen to draw out the ovaries, Malphigian tubules and stomach. The ovaries were detached from the hindgut. While the stomach was partially extracted, the Malphigian tubules were slowly cut away from around the stomach and as close as their insertion without tearing the gut wall. The midgut was drawn out completely from the abdomen and the rectum was cut out from the midgut just below the pyloric ampulla using the dissection needle. The extracted ovaries were transferred to a new labelled slide and were allowed to dry. Drying was important as the tracheolar skeins can be seen more clearly when filled with air. Once they were dried, the slides were examined under a microscope with magnification of 40x. Identification of parity in female adult mosquitoes was determined by the presence of coiled tracheolar skeins and uncoiled tracheolar skeins in the ovary of nulliparous and parous mosquitoes, respectively. Meanwhile, the dissected stomach was transferred to another clean labelled slide with a drop of 2% mercurochrome and the specimen was covered with a round coverslip. If there was excess mercurochrome, it was absorbed with a small piece of filter paper. The examination of midgut was carried out by viewing the slide 10x magnifications using a light microscope, starting from the posterior end of the midgut to the anterior part. Both sides of the stomach was examined. The other side of the stomach was examined by gentle pushing of the coverslip along the slide so that the stomach was rolled over, revealing the cysts along the edge of the stomach. The oocysts can be recognized as corpuscles (stained in red pigment under mercurochrome) on the stomach walls of an infected female adult mosquito.

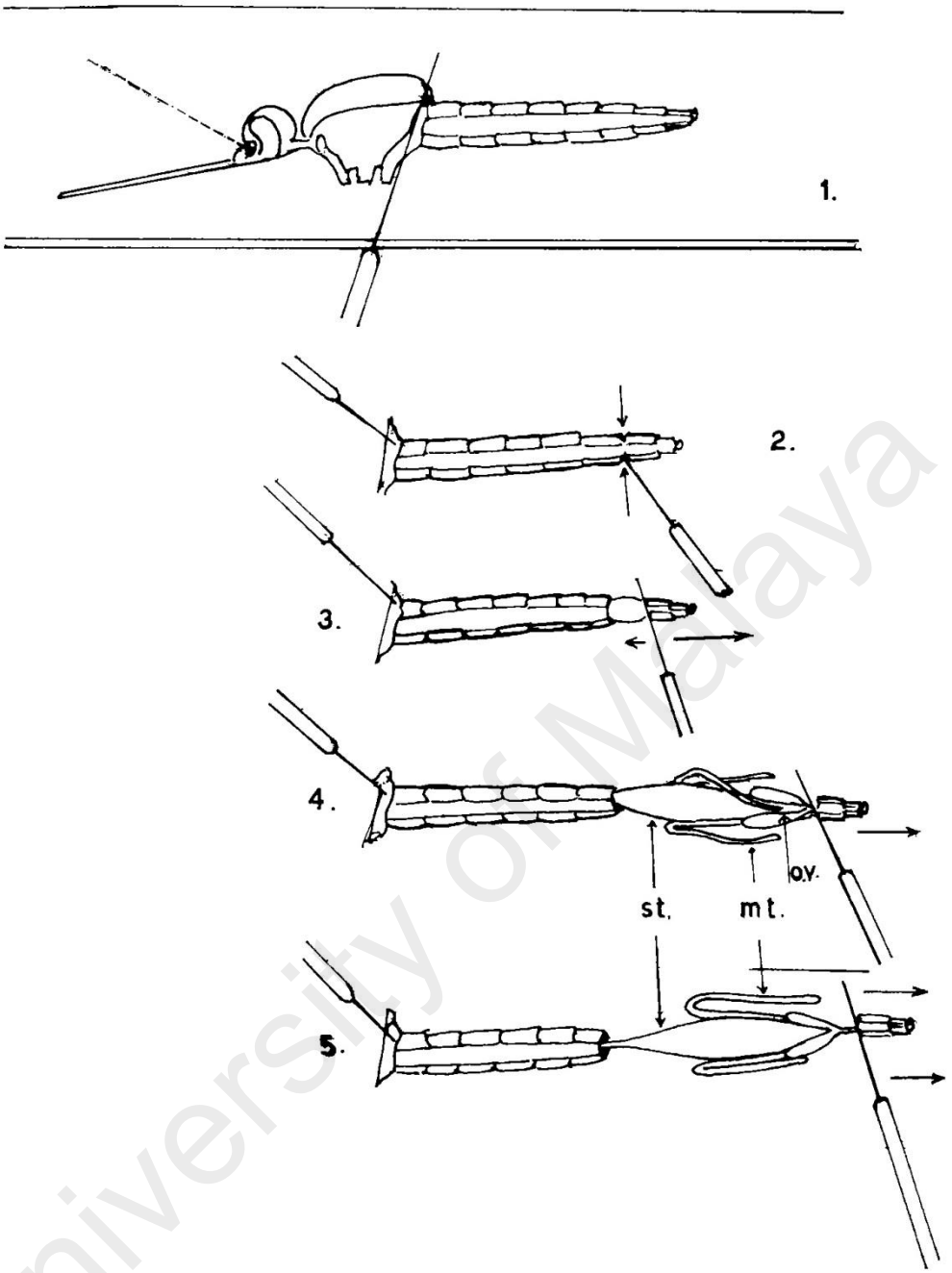

Figure 4.3: Dissection of ovary and midgut (adapted from (WHO, 1975).

mt= Malphigian tubules; st= stomach; ov= ovary

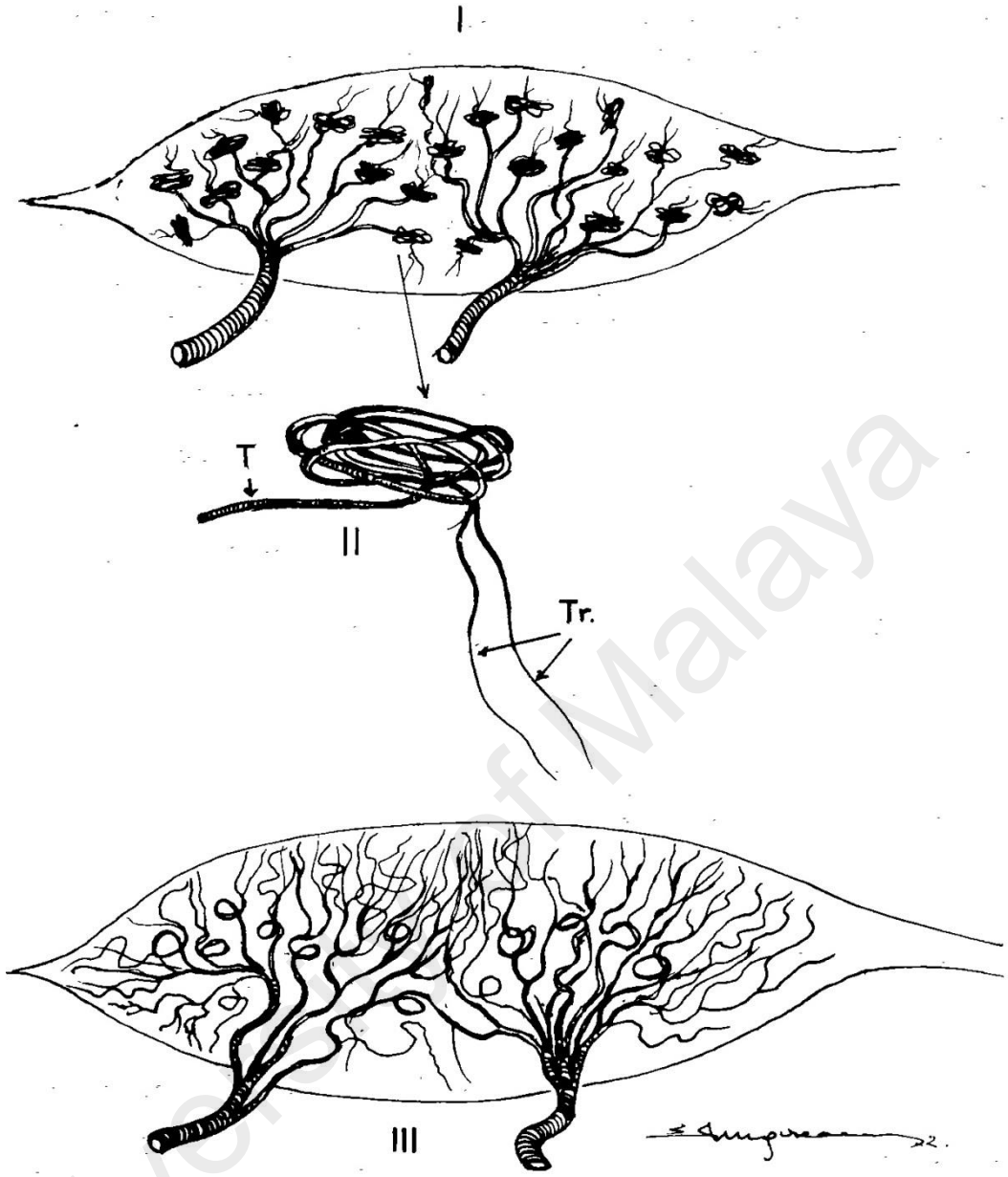

Figure 4.4: Ovary of a nulliparous and parous female adult mosquitoes (adapted from (WHO, 1975).

T= trachea; Tr= tracheoles

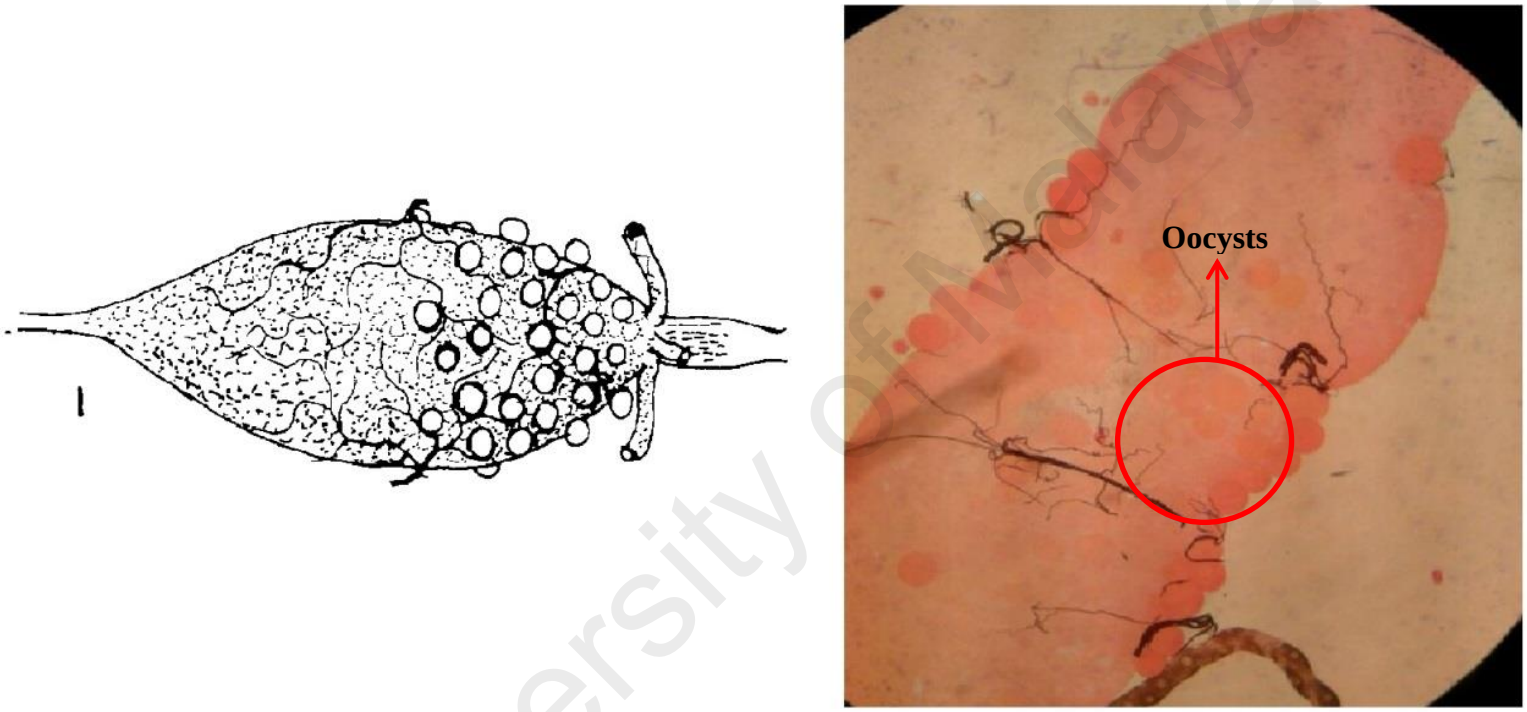

Figure 4.5: Oocysts in midgut.

Dissection of salivary glands of adult mosquitoes was carried out mainly for establishment of sporozoite rate. Preparation of mosquitoes for salivary gland dissection was carried out in the same way as above. The mosquito was arranged on the slide with the head pointing to the right. The left needle was then be placed gently on the thorax just below the region where the salivary glands lie. By using the right needle, the neck was cut. Then, a drop of saline was placed close to the neck section. The gland was squeezed out from the thorax by pressing gently on the thorax using the left needle a little above the first coxae of the mosquito. Immediately, the point of the right needle was dipped into the saline to bring it into contact with the extracted salivary glands. A pair of salivary glands was placed onto a drop of saline (size of a pinhead) on a new slide and covered with a coverslip for examination of sporozoites under a light microscope set at 40x magnifications. Salivary glands cells were ruptured gently using dissection needle to free the sporozoites (if present). If the mosquito was positive, the sporozoites could be clearly seen as minute spindle-like forms either isolated or clustered as they come out from the glands and dispersed once they were in saline solution.

All positive midguts and salivary glands, and the respective head and thorax of the positive specimens were transferred into individual labelled 1.5 mL micro-centrifuge tubes (Axygen, USA) containing 95% ethanol for subsequent molecular analysis, namely, identification of *Plasmodium* parasites.

Dried mosquitoes which could not be dissected were kept in labelled individual microcentrifuge tubes. They were pooled according to time, site and month for malaria parasite detection and characterization. Details regarding the protocol are provided in Chapter 5.

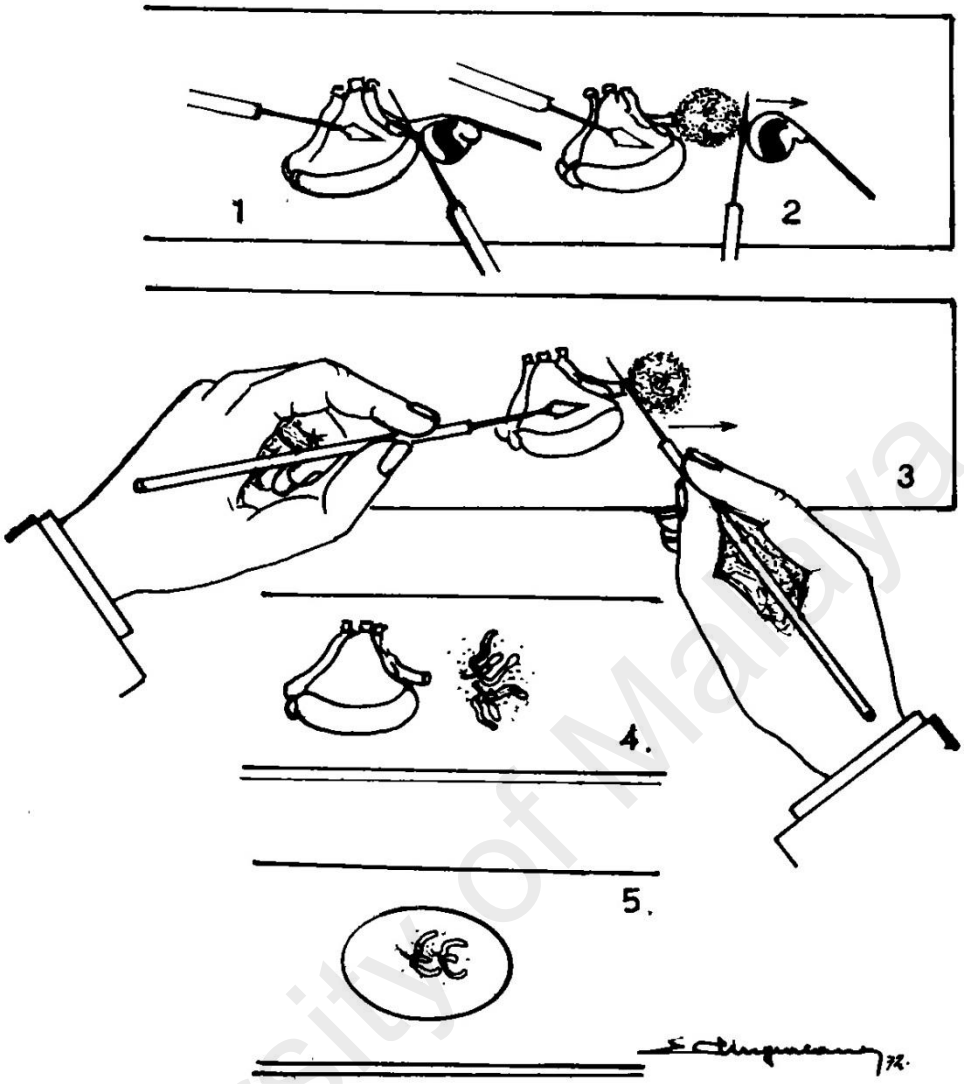

Figure 4.6: Dissection of salivary glands (adapted from (WHO, 1975).

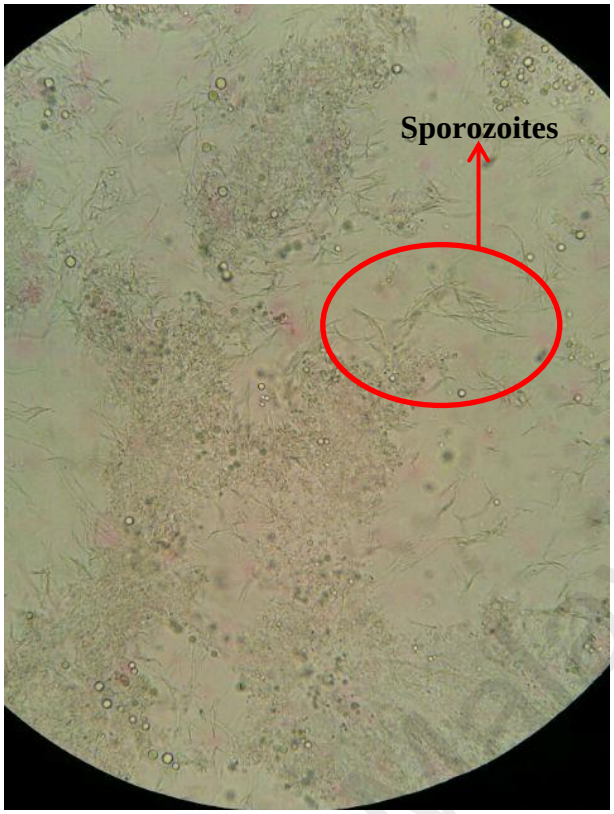

Figure 4.7: Sporozoites released from the ruptured salivary glands.

4.2.5 DNA extraction of infected mosquito samples

In the laboratory, the specimens were centrifuged before the ethanol was allowed to evaporate completely from the specimen tubes by placing them in a thermomixer (Eppendorf, Germany) at 70°C. Genomic DNA was extracted from the midguts and salivary glands or pooled mosquitoes using DNeasy® Blood and Tissue kit (Qiagen, Germany) according to the manufacturer's protocol. Midguts and salivary glands were homogenized in their respective labelled micro-centrifuge tubes using a hand held homogenizer (Kontes Thompson Scientific) with plastic pestle in 180 µL buffer ATL. After brief homogenizing for 30 seconds, 20 µL of Proteinase K was added to aid protein digestion and removal of possible nuclease contamination. The mixture was then vortexed before incubating at 56°C overnight at 300 rpm in a thermomixer.

The following day, each tube was centrifuged briefly to bring down the condensation in the tube. 200 µL of molecular grade ethanol and 200 µL of buffer AL were added into the sample and vortexed to yield a homogenous solution. The entire mixture was pipetted into DNeasy Mini spin column placed in a collection tube. The column was centrifuged at 8000 rpm for 1 minute. The flow-through and collection tube were discarded. The spin column was placed into a new collection tube and 500 µL of buffer AW1 was added into the column and centrifuged at 8000 rpm for a minute. The flow-through and collection tube were discarded and 500 µL of buffer AW2 was added into the spin column, with a new collection tube. The spin column was centrifuged at 14000 rpm for 3 minutes. The flow-through and collection tube were discarded. The spin column was placed into a new collection tube and centrifuged for a minute at 14000 rpm to remove any remaining wash buffer. For DNA elution, the column was transferred into a sterile 1.5 mL micro-centrifuge tube and 100µL of buffer AE was added directly onto the column membrane. The column was incubated for a minute at room temperature and centrifuged at 8000 rpm for a minute to elute the DNA.

All positive midguts and salivary glands were extracted individually and stored in sterile 1.5 mL microcentrifuge tube in -20°C freezer until required.

4.2.6 Detection of *Plasmodium* in infected midgut and salivary glands

Detection and identification of *Plasmodium* parasites found in infected mosquitoes were carried out in nested PCR using primers based on the *Plasmodium* small subunit ribosomal RNA (ssurRNA) as shown in Table 4.1 (Lee et al., 2011; Singh et al., 1999; Singh et al., 2004). Amplification was performed in a 50µL reaction mixture for nest 1 (Table 4.2) and PCR parameters for nest 1 is shown in Table 4.3. For nest 2 PCR, 20µL reaction mixture was prepared (Table 4.4) according to (Lee et al., 2011; Singh et al., 1999; Singh et al., 2004). The PCR parameters for nest 2 is as listed in Table 4.5. In nest 1, 5µL of DNA template was used whilst for nest 2, 2µL of nest 1 product was used as template. Both positive and negative controls were included for each batch of PCR.

Table 4.1: **Oligonucleotide sequences of PCR primers used for detection and identification of *Plasmodium* parasites in mosquitoes.**

Plasmodium	**Primers**	**Sequence (5'-3')**	**Nested PCR**	**Annealing temperature (°C)**	**Expected product size (bp)**
Genus-specific	rPLU 1	TCAAAGATTAAGCCATGCAAGTGA	1	55	1640
	rPLU 5	CCTGTTGTTGCCTTAAACTCC			
falciparum	rFAL1	TTAAACTGGTTTGGGAAAACCAAATATATT	2-human *Plasmodium*	58	205
	rFAL2	ACACAATGAACTCAATCATGACTACCCGTC			
malariae	rMAL1	ATAACATAGTTGTACGTTAAGAATAACCGC		58	144
	rMAL2	AAAATTCCCATGCATAAAAAATTATACAAA			
vivax	rVIV1	CGCTTCTAGCTTAATCCACATAACTGATAC		58	117
	rVIV2	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA			
ovale	rOVA1	ATCTCTTTTGCTATTTTTTAGTATTGGAGA		58	787
	rOVA2	GGAAAAGGACACATTAATTGTATCCTAGTG			
knowlesi	Pmk8	GTTAGCGAGAGCCACAAAAAAGCGAAT		60	153
	Pmkr9	ACTCAAAGTAACAAAATCTTCCGTA			
coatneyi	PctF1	CGCTTTTAGCTTAAATCCACATAACAGAC		60	503
	PctR1	GAGTCCTAACCCCGAAGGGAAAGG			
cynomolgi	CY2F	GATTTGCTAAATTGCGGTCG	2-simian *Plasmodium*	60	137
	CY4R	CGGTATGATAAGCCAGGGAAGT			
inui	PinF2	CGTATCGACTTTGTGGCATTTTTCTAC		58	479
	INAR3	GCAATCTAAGAGTTTTAACTCCTC			
fieldi	PfldF1	GGTCTTTTTTTTGCTTCGGTAATTA		63	421
	PfldR2	AGGCACTGAAGGAAGCAATCTAAGAGTTTC			

Table 4.2: **Components of master-mix for nest 1 PCR.**

Components	**Final concentration**	**Volume (µL)**
RNase & DNase-free molecular grade water	-	25.25
5x green Gotaq reaction buffer (Promega)	1x	10.0
$MgCl_2$ 25mM (Promega)	3mM	6.0
dNTP mix, 10mM each (Promega)	0.2mM	1.0
Forward primer (10µM	0.25µM	1.25
Reverse primer (10µM)	0.25µM	1.25
GoTaq DNA polymerase, 5U/µL (Promega)	1.25U	0.25
DNA Template	-	5.0
Total volume per reaction	**-**	**50.0**

Table 4.3: **Cycling parameters for nest 1 PCR.**

Steps	Temperature/ °C	Time/ s	Number of cycles
Initial denaturation	94	240	1
Denaturation	94	30	
Annealing	55	60	35
Extension	72	60	
Final extension	72	240	1
	4	∞	-

Table 4.4: **Components of master-mix for nest 2 PCR.**

Components	**Final concentration**	**Volume (µL)**
RNase & DNase-free molecular grade water	-	10.1
5x green GoTaq reaction buffer (Promega)	1x	4.0
$MgCl_2$ 25mM (Promega)	3.0mM	2.4
dNTP mix, 10mM each (Promega)	0.2mM	0.4
Forward primer (10µM)	0.25µM	0.5
Reverse primer (10µM)	0.25µM	0.5
GoTaq DNA polymerase, 5U/µL (Promega)	0.5U	0.1
DNA template (from nest 1)	-	2
Total volume per reaction	**-**	**20.0**

Table 4.5: **Cycling parameters for nest 2 PCR.**

Steps	Temperature/ °C	Time/ s	Number of cycles
Initial denaturation	94	240	1
Denaturation	94	30	
Annealing	X	60	35
Extension	72	60	
Final extension	72	240	1
	4	∞	-

4.2.7 Determination of entomological indicators

Malaria transmission in a geographical area can be determined through the incrimination of vector based on several parameters on its ecology and behavior.

4.2.7.1 Man-biting rate (Ma)

Man-biting rate (Ma) refers to the average number of bites a person would receive per night by a vector species (Silver, 2007; WHO, 2013a). However, according to WHO (2013a) guidelines, it is needless to permit mosquito to feed and should be collected as soon as they land on the skin since it is assumable that biting will follow after. Ma is obtained as follows:

$$\text{Ma} = \frac{\text{Total number of bites from a mosquito species}}{\text{Total number of voluteers} \times \text{Total number of collection nights per month}}$$

4.2.7.2 Parity rate (PR)

Parity rate (PR) refers to the proportion of parous mosquitoes in a vector population and is an entomological indicator used for determination of rate of malaria transmission. It is also used to establish longevity and infectivity of a mosquito (WHO, 2013a). PR is obtained as follow:

$$\text{PR} = \frac{\text{Total number of parous mosquitoes}}{\text{Total number of dissected mosquitoes}} \times 100$$

4.2.7.3 Sporozoite rate (S)

Sporozoite rate (S) is the proportion of female mosquitoes of a particular species which is infective. By determining S, confirmation of vector species, intensity of malaria transmission and impact of malaria control interventions can be resolved (WHO, 2013a). Calculation of sporozoite rate is as follows:

$$\mathrm{S} = \frac{\text{Total number of mosquitoes with sporozoites}}{\text{Total number of dissected mosquitoes}} \times 100$$

4.2.7.4 Oocysts rate

Oocysts rate refers to the proportion of female mosquitoes infected with oocysts (WHO, 2013a). Oocysts rate is calculated as follows:

$$\text{Oocycts rate} = \frac{\text{Total number of mosquitoes with oocysts}}{\text{Total number of dissected mosquitoes}} \times 100$$

4.2.7.5 Entomological inoculation rate (EIR)

Entomological inoculation rate (EIR) refers to the number of infectious bites per person per unit time (Shaukat et al., 2010). EIR is used as a direct index to reflect the human exposure to pathogenic malaria parasites (Burkot et al., 1987; Burkot et al., 1990; Killeen et al., 2000) and for estimation of malaria transmission intensity in field conditions (Beier et al., 1999). EIR (per night) is calculated as:

$$\mathrm{EIR} = \frac{Ma \times S\ (\%)}{100}$$

4.2.7.6 Longevity and infectivity

The survival of a female mosquito after a blood meal can be denoted as *p* (the probability of surviving one day after blood-meal) (Davidson, 1954; WHO, 2013a). Calculation of *p* can be computed by using parity rate and gonotrophic cycle (gc) of the mosquito species. Value of *p* can be estimated as:

$$\mathrm{p} = \sqrt[gc]{proportion\ parous}$$

The probability that a population of female mosquitoes survive and becomes infective can be expressed as p^n, where n is the extrinsic incubation period for

Plasmodium to complete the sporogonic cycle in the vector (Garrett-Jones & Shidrawi, 1969). The vector's life expectancy is described as the mean number of days of life in the infective condition per mosquito receiving the infection (Garrett-Jones & Shidrawi, 1969). Life expectancy of a vector can be estimated as follows:

$$\text{Life expectancy for each mosquito species} = \frac{1}{-\ln p}$$

4.2.7.7 Vectorial capacity (VC)

Vectorial capacity (VC) is an index which is defined as the maximum average daily number of possible infective bites between a vector population and human assuming that all the mosquito biting the infective person becomes infected (Garrett-Jones & Shidrawi, 1969; WHO, 2013a). The formula of VC is given as follows:

$$\text{VC} = \frac{Ma \times a \times p^n}{-\ln p}$$

Where,

Ma= man-biting rate

a= the daily rate of blood feeding on man (0.33 was chosen, assuming a blood meal every third day on man

p= probability of daily survival

n= length of sporogonic cycle (10 days based on *P. knowlesi* extrinsic incubation period)

4.2.8 Statistical analysis

All statistical analysis was performed using PASW Statistics 18 and R programming language for statistical analysis (version 3.2.0). Generalized linear mixed models (GLMM) were built to analyze these parameters, namely, the abundance of *An. balabacensis*, their time of biting, and the proportion of vectors which were infected with oocysts, sporozoites and were parous. In all analyses, the localities, namely Timbang Dayang, Limbuak Laut and Kampung Paradason were fitted as a fixed effect. Months of sampling were fitted alternatively either as fixed (for monthly values' prediction) or random effect (to test the differences between localities while controlling for seasonal variation).

Poisson and negative binomial distribution were used for the analysis of mosquito abundance while binomial distribution was assumed in all the analysis of parity and infection rates. Zero inflation in count data, namely mosquito abundance was assessed. Models testing associations between response variables (vector abundance, parity and infection rates), descriptive variables (month and locality) and random effects of sampling nights were assessed through comparison on the basis of having higher log-likelihood and lower *Akaike information criterion* (AIC) values, along with the result of analysis of variance (ANOVA) of nested models. Tukey post hoc contrasts were used to differentiate the statistical difference between localities. The confidence level (CI) for man-biting rate, percentage biting, sporozoite rate, oocysts rate and parity rate was 95% CI and calculated using the Fleiss quadratic (Fleiss et al., 2013) with OpenEpi (http://www.openepi.com/Proportion/Proportion.htm). All graphs were constructed using GraphPad Prism 6.0.

4.2.9 Ethical clearance

This project was approved by the NMRR Ministry of Health Malaysia (NMRR-12-786-13048). All volunteers who conducted the mosquito collections had signed the informed consent forms and were given antimalarial prophylaxis throughout the period of participation.

4.3 Results

4.3.1 Species composition of *Anopheles* in all study sites

A total of 1884 *Anopheles* consisting of 10 different species was collected. From the total collection, *An. balabacensis* was the predominant mosquito (95.1% of the total) present in all localities. All the other species of *Anopheles* were found in low numbers and present only in one or two localities. The lowest number of *Anopheles* caught were *An. tesselatus* (0.1), followed by *An. watsonii* (0.2%), *An. flavirostris* (0.3%), *An. maculatus* (0.4%), *An. umbrosus* group (0.4%), *An.* aconitus (0.6%), *An. barbirostris* group (0.7%), *An. vagus* (1.0%) and *An. donaldi* (1.3%). Although there are five species of the Leucosphyrus Group of *Anopheles* mosquitoes in Sabah (Sallum et al., 2005b), only *An. balabacensis* was obtained. A total of 379 Culicines were collected but only identified to genus.

Table 4.6: *Anopheles* species collected in study sites in Kudat Division, Sabah from August 2013 to July 2014.

Mosquito species	Banggi Island		Kudat Kg Paradason	Total (%)
	Limbuak Laut	Timbang Dayang		
An. balabacensis	479	464	848	1791 (95.1)
An. donaldi	10	3	12	25 (1.3)
An. vagus	1	18	0	19 (1.0)
An. barbirostris group	2	0	11	13 (0.7)
An. aconitus	0	11	0	11 (0.6)
An. maculatus	0	0	8	8 (0.4)
An. umbrosus group	7	0	0	7 (0.4)
An. flavirostris	0	5	0	5 (0.3)
An. watsonii	1	0	2	3 (0.2)
An. tessellatus	0	0	2	2 (0.1)
Total	**500**	**501**	**883**	**1884 (100)**

4.3.2 Abundance of *Anopheles balabacensis* over time

The number of *An. balabacensis* collected ranged from about 2-28 per man night. However, there was no clear consistent seasonal trend observed. The pattern of seasonal fluctuation differed between sites (Figure 4.8). In Limbuak Laut which represents the forest ecotype, *An. balabacensis* abundance was relatively low (less than 15 per night) and constant across months. The abundance of *An. balabacensis* in the small farming site however varied more than 10-fold over a year with a high peak in August (~25 per night) and November (~18 per night) 2013, and low from February to May (less than 4 per night) and July (2 per night) 2014. Nevertheless, the abundance of *Anopheles balabacensis* was fluctuated in village settlement. The highest monthly density of *An. balabacensis* was seen in January 2014 (27 per night) whereas the lowest (less than 1 per night) in both October and November 2013.

By using GLMM models, the results indicated that the Poisson distribution was generally better in representing *An. balabacensis* abundance data than with negative binomial. From analysis of statistical models assuming a Poisson distribution, Tukey post hoc test revealed that *An.* balabacensis abundance was significantly higher in village settlement (KP) than in forest (KP and LL, p=0.04) and small farming site (KP and TD, p= 0.02) on the basis of statistical models assuming a Poisson distribution. Controlling for variation across months, *An. balabacensis* abundance in the village site was estimated to be about 15-20% higher than in both forest and small farming site.

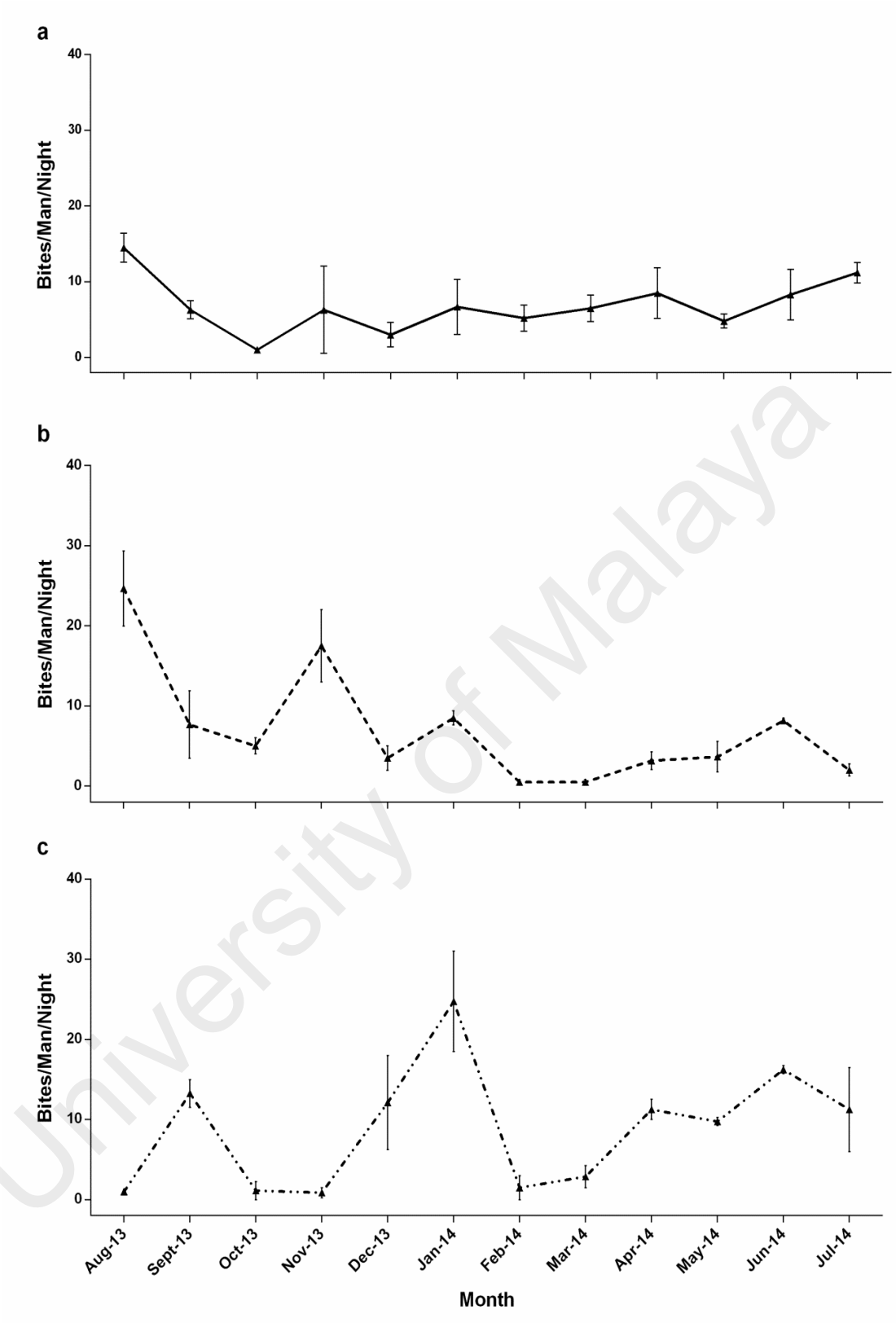

Figure 4.8: Bites/ Man/ Night of *An.balabacensis* measured monthly in three study sites.

a. Limbuak Laut, b. Timbang Dayang, c. Kg Paradason. Error bars are 95% CI.

4.3.3 Biting cycles of *Anopheles balabacensis*

Figure 4.9 demonstrates the biting cycle of *An. balabacensis.* It shows that they bite as early as 1800 hours and continued to bite throughout the night until early hours of the morning. The peak biting time was observed to be between 1800 to 2200 hours in both forest site and village site, accounting for 38% of the total night catch. In small farming site (TD), biting rates were relatively similar between 1800-2400 hours, and then began to fall with a second small peak in the early part of the morning (0300-0400 hours).

4.3.4 Transmission efficiency of *Anopheles balabacensis*

The parity rates of *An. balabacensis* were more than 50% on most collections for all study sites as shown in Figure 4.10. The mean parous rate varied from 58-65% with minor fluctuation (Figure 4.10, Table 4.7). Tukey's test shown that there is no significant differences observed in parity rates between all study sites ($p>0.05$, Table 4.8). Estimation of daily survival rate, life expectancy and vectorial capacity values based on parity rates were computed for *An. balabacensis* at each site (Table 4.7). Estimates of *An. balabacensis* survival and vectorial capacity were higher in LL and TD compared to KP. In both LL and TD respectively, 24% and 22% of *An. balabacensis* would be assumed to live the 10 days necessary for *P. knowlesi* to develop into transmission stage sporozoites, contrasting with only 16% in KP. Moreover, those surviving the 10 days would have a further life expectancy of 7 and 6.7 days in LL and TD, respectively, compared to 5.4 days in KP. Vectorial capacity was highest in LL with a calculated value of 3.85 followed by TD (3.36) and KP (2.50).

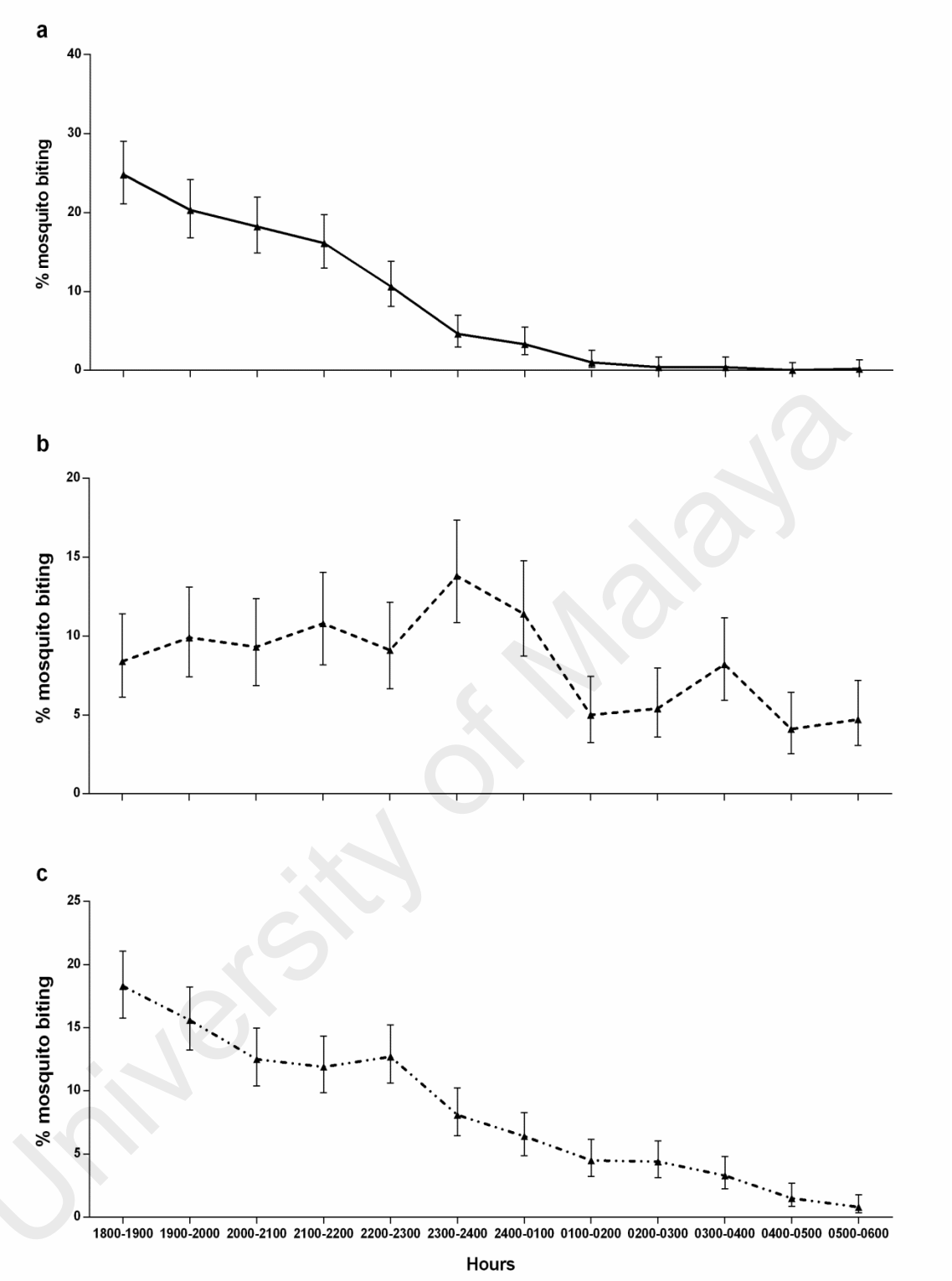

Figure 4.9: Percentage of *An. balabacensis* (from the total collection) which were captured biting hourly during night sampling period (1800-0600hrs) in each study sites (pooled across the whole year).

a. Limbuak Laut, b. Timbang Dayang, c. Kg Paradason. Error bars are 95% CI.

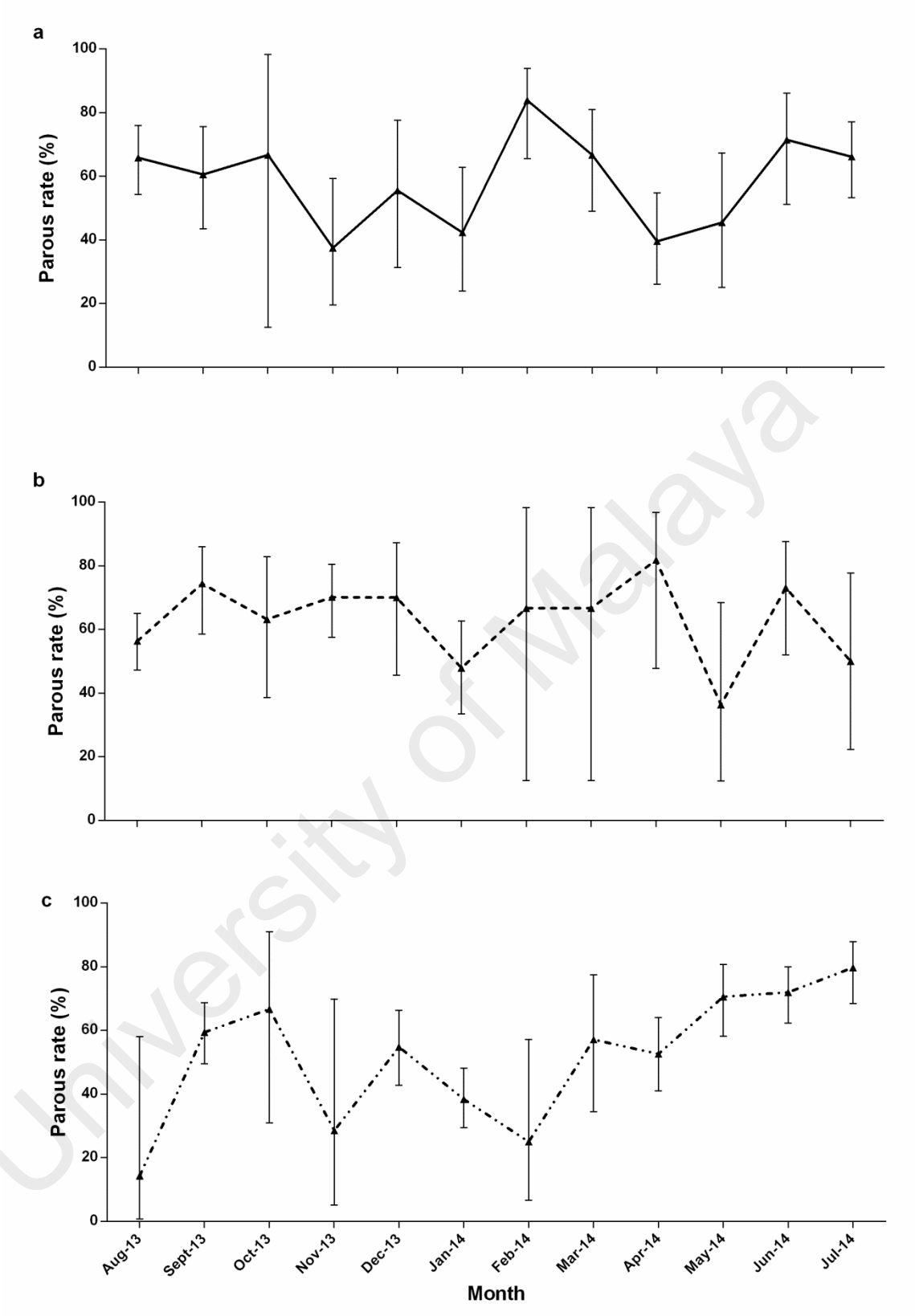

Figure 4.10: Parity rates in three study sites.

a. Limbuak Laut, b. Timbang Dayang, c. Kg Paradason. Errors bars are 95% CI.

Table 4.7: Annual infection rates, man-biting rate, entomological inoculation rate, parous rate, probability of daily survival, life expectancy and vectorial capacity of *An. balabacensis* in study sites.

Parameters	Banggi Island		Mainland Kudat
	Limbuak Laut	Timbang Dayang	Kg Paradason
Sporozoite rate (S) (95% CI)	3.42 (1.91-5.93)	1.93 (0.85-4.12)	1.03 (0.45-2.20)
Oocysts rate (95% CI)	3.16 (1.72-5.60)	3.23 (1.76-5.74)	1.03 (0.45-2.21)
Man-biting rate (ma)	7.0	6.8	8.8
Entomological inoculation rate (EIR)	0.24	0.13	0.09
Parity rate (95% CI)	65.00 (59.94-69.75)	63.78 (58.63-68.65)	57.53 (53.68-61.29)
Probability of daily survival (p)[1]	0.87	0.86	0.83
p^{10} (%)	24	22	16
Life expectancy[3]	7.0	6.7	5.4
Vectorial capacity (VC)[4]	3.85	3.36	2.50

[1] The probability of daily survival (p) was taken as $\sqrt[3]{P}$ where P= percentage parous.
[2] p^{10} is the percentage of population expected to survive to become infective with an extrinsic cycle of 10 days based on *P. knowlesi* extrinsic incubation period.
[3] Refer to section 4.2.7.5
[4] Refer to section 4.2.7.6

Table 4.8: Generalised linear mixed model fitting of the data. The model used is of the form "glmm<-glmmadmb (parameter ~ locality+(1 | month), zero Inflation = T, data = data, family = "pdf")"/ KP= Kampung Paradason, LL= Limbuak Laut, TD= Timbang Dayang. AIC= Akaike information criterion. Means with different superscript letters indicate they are significantly different.

Parameter	n	Zero-inflation	Log-lik-hood	AIC	Mean predicted values			Tukey's test between means
					KP	LL	TD	
Bites per man/ night	92	False	-357.36	722.72	7.84^{a}	6.26^{b}	6.13^{b}	KP-LL; p= 0.04, KP-TD; p=0.02
Sporozoite rate	83	True	-57.93	125.86	0.01^{a}	0.04^{b}	0.02^{ab}	KP-LL; p= 0.04
Oocyst rate	83	True	-59.298	128.59	0.01^{a}	0.03^{ab}	0.03^{b}	KP-TD; p= 0.035
Parity	83	False	-158.127	324.25	0.58	0.59	0.63	No difference between means (p> 0.05)

4.3.5 Infection rates and entomological inoculation rates by months and sites

Forty five (3%) *An. balabacensis* out of the 1482 dissected were found to be positive for *Plasmodium* infection in terms of sporozoite (14), oocysts (18) or both (13) by microscopy. Of these only 10 salivary glands and 3 midguts were positive for *P. knowlesi* by PCR. Besides *P. knowlesi*, other simian malaria parasites were also present as shown in Table 4.9. This shows that in addition to *P. knowlesi*, *An. balabacensis* is also a vector to other simian *Plasmodium* species as well.

There was no consistent seasonal pattern of mosquito infection rates across sites (Figure 4.11). In LL, sporozoite rates were highest from December to February (4-16.67%). In TD, sporozoite rates were high in December (5.00%) and from June to July (7.69-12.50%). In March, only three mosquitoes at TD were dissected, of which two were found to be positive where one for sporozoite and one for oocyst. Thus, sporozoite rates appeared to be extremely high at this time, but it is likely an artifact of low sample size. In KP, the highest sporozoite rate was obtained in May 2014 (2.86%). The highest entomological inoculation rate (EIR) was 0.6 in TD in June. Tukey post hoc tests performed on the results of statistical models of *An. balabacensis* infection rates indicated there was variation between sites. Specifically, sporozoite rates were lower in KP compared to LL ($p= 0.04$), and oocyst rates were lower in KP than in TD ($p= 0.035$) (Table 4.8). Sporozoite rates were estimated to be approximately 2 to 3 times higher in LL and TD respectively than in KP (Table 4.7).

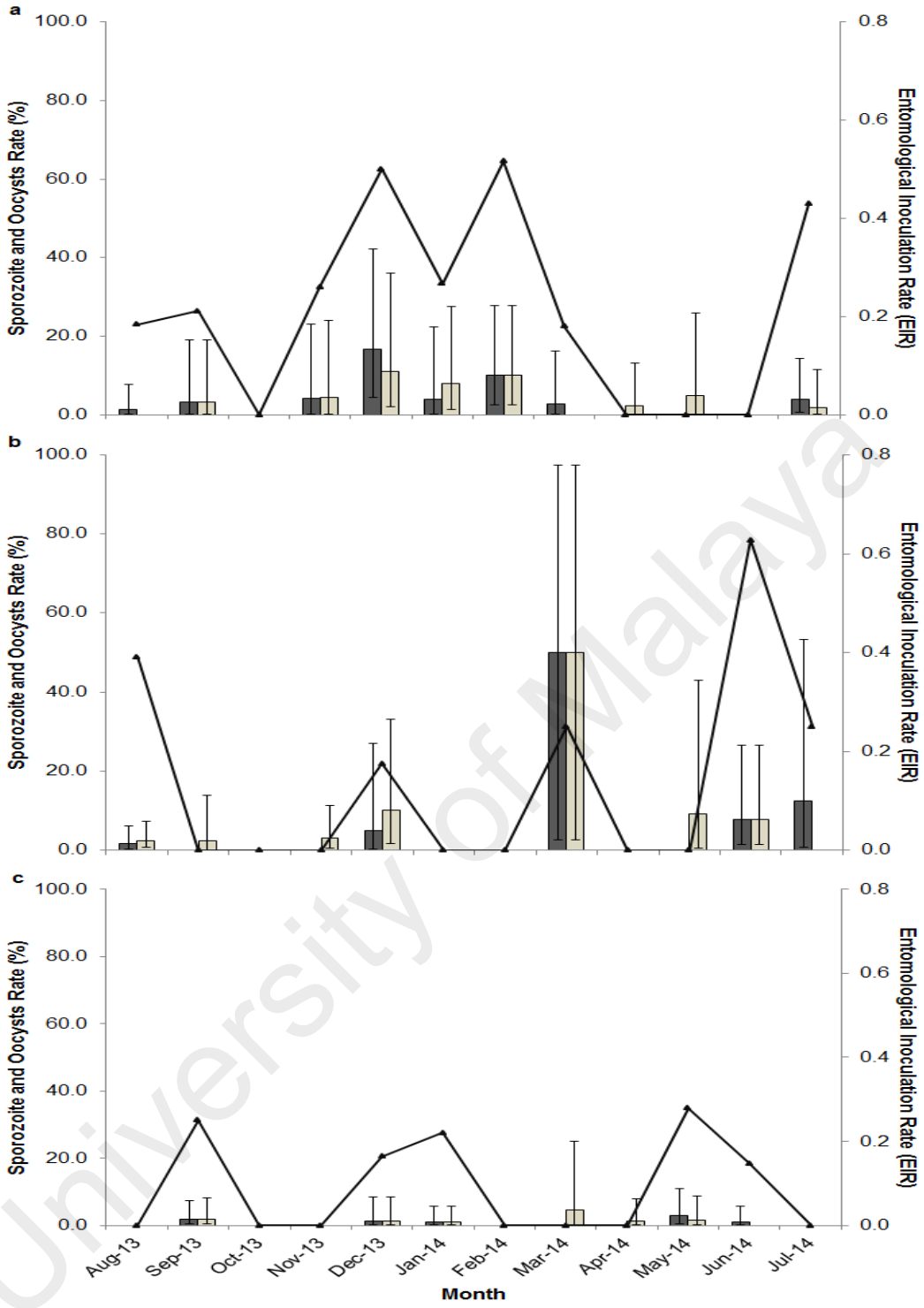

Figure 4.11: Infection rates and entomological inoculation rate (EIR) of *Anopheles balabacensis* in the study sites. The line connects the points of EIR for *An. balabacensis*. Bars indicate the infection rates, which are sporozoite rates (dark grey with 95% CI) and oocyst rates (light grey with 95% CI).

a. Limbuak Laut, b. Timbang Dayang, c. Kg Paradason.

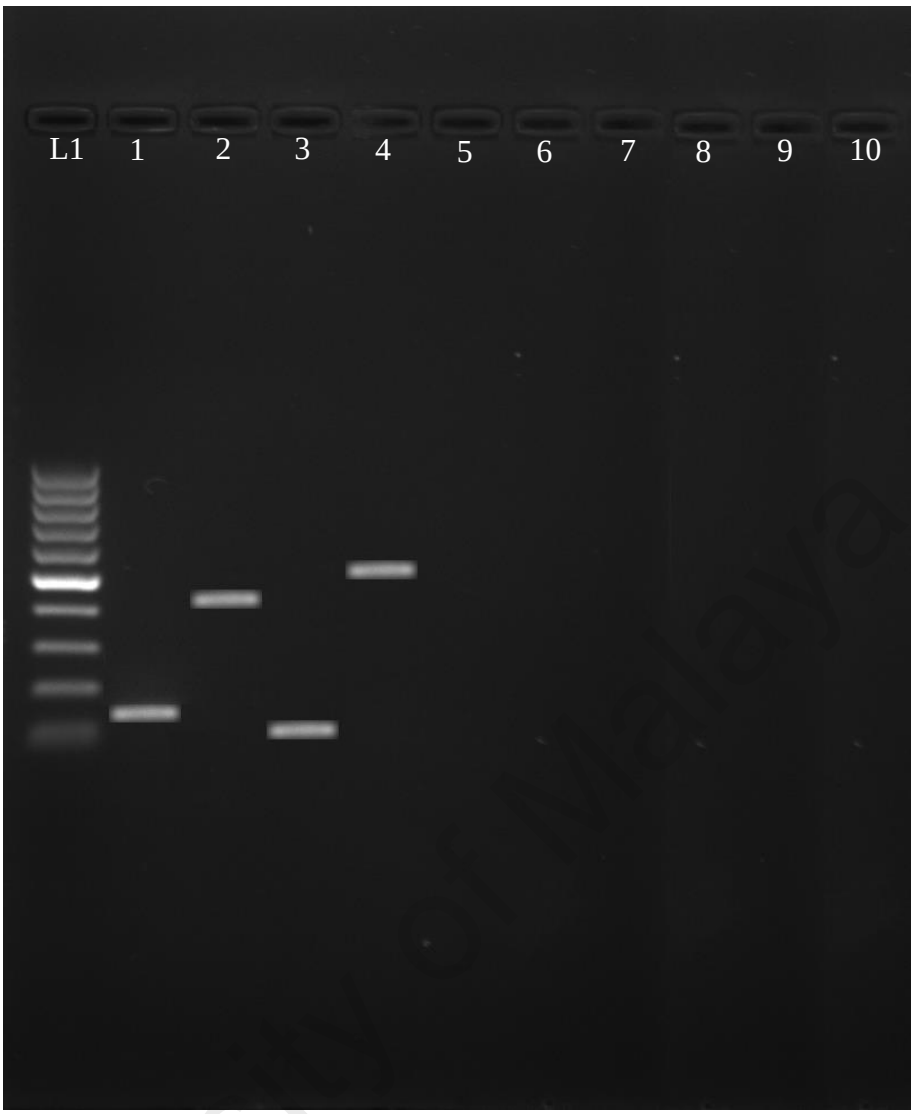

Figure 4.12: Gel picture showing molecular identification of simian malaria parasites in salivary glands of *Anopheles balabacensis* (sample LL477sg) by nested PCR. Agarose gel of 1.5% was used. L1 denotes 100bp ladder. Lane 1 is *P. knowlesi* (153bp), lane 2 is *P. inui* (479bp), lane 3 is *P. cynomolgi* (137bp), lane 4 is *P. coatneyi* (503bp), lane 5 is *P. fieldi* (421bp) but the sample is negative for the species and lane 6-10 is negative control for each of the species (*P. knowlesi, P. inui, P. cynomolgi, P. coatneyi* and *P. fieldi*, respectively).

Table 4.9: Species of *Plasmodium* identified from midgut and salivary glands of the *An. balabacensis* using nested PCR.

***Plasmodium* species**	**Mosquito organs**	
	Midgut	**Salivary glands**
Pk	0	1
Pcy	7	6
Pin	5	5
Pk+Pin	2	2
Pk+Pcy	1	2
Pcy+Pin	8	4
Pk+Pcy+Pin	0	4
Pk+Pct+Pcy+Pin	0	1
Not identified	8	2
Total	**31**	**27**

Key: Pk= *P. knowlesi*; Pcy= *P. cynomologi*; Pct= *P. coatneyi*; Pin= *P. inui*
14 *An. balabacensis* had salivary glands positive; 18 *An. balabacensis* had midgut positive and 13 had both midgut and salivary glands positive. Thus the total 58.

4.4 Discussion

Our study provides the first evidence to confirm that *An. balabacensis* is the vector of the zoonotic malaria *P. knowlesi* within the substantial foci of human infection in Sabah (Wong et al., 2015). It was the predominant species found in all sites with mean biting rates ranging between 6.8 and 8.8. A substantial proportion of *An. balabacensis* (32.8%) were captured biting outdoors in the early part of the evening (1800-2000hrs), a time when humans would not be expected to be using LLINs, which is the current front line malaria control strategy in Malaysia. This also corroborates well with a study conducted in the east coast of Sabah and case control study in Kudat where *An. balabacensis* was found biting in the early part of the night (Brant et al., 2016; Manin et al., 2016). In this study all collections were made outdoors, as previous studies have found that this is where the majority of *An. balabacensis* (~76%) seek their hosts (Hii et al., 1988a; Vythilingam et al., 2005). A recent study also showed that *An. balabacensis* was about 5.5 folds higher biting outdoors compared to indoors (Manin et al., 2016). However, total amount of human exposure to infectious bites from *An. balabacensis* may be even higher than indicated here if the additional contribution of limited indoor exposure were to be incorporated. In comparing the density and bionomics of *An. balabacensis* populations between three sites, there was geographical variation in both their abundance and sporozoite infection rates. Vector abundance was highest in the village site (KP), whereas sporozoite rates were higher in the forest (LL) and small farming site (TD) compared to the village site. However, it is unknown whether these differences are truly the result of habitat-dependent transmission efficiencies, as only one site from each ecotype was sampled. However these findings reinforce the hypothesis that spatial heterogeneity in *P. knowlesi* exposure risk may be driven by variation in mosquito vector demography in addition to the presence of the reservoir macaque host.

Although it has been postulated that *P. knowlesi* was present in macaques before the arrival of humans in Southeast Asia (Lee et al., 2011), and a large number of *P. knowlesi* malaria cases has been reported in Sabah (William et al., 2014), the identity of the vector remained elusive. Whilst it has been demonstrated by Chin et al. (1968) that *An. balabacensis* can transmit *P.knowlesi* from monkey to man, man to monkey and man to man under experimental conditions, this study is the first to confirm that it acts as a vector under natural conditions. *Anopheles balabacensis* was also incriminated as the primary vector of human malaria in Sabah in the 1950s (Colless, 1952; McArthur, 1950) which was further supported with extensive studies in the 1980s confirming its role as the main vector for human malaria infections (Hii, 1985a; Hii et al., 1988a). Given *An. balabacensis* is the likely vector of other primate malaria species in this area, it could also be the conduit for other zoonotic malaria spillovers to humans. This indicates that these *Plasmodia* species are not partitioned amongst different vector species, and emphasizes that *An. balabacensis* should be the primary target for all malaria control efforts in the area.

There was a significant difference in the *Anopheles* species composition found here relative to previous studies in Sabah (Hii et al., 1985; Vythilingam et al., 2005). Currently *An. balabacensis* and *An. donaldi* constituted >95% and 1.3% of all *Anopheles* recorded on Banggi Island respectively, while studies in this area in the 1980s estimated the relative proportion of these species to 13.6% and 39% of *Anopheles* respectively (Hii et al., 1985). In the central region of Sabah, *An. donaldi* was incriminated as the dominant vector for human malaria parasites in studies carried out in 2001-2002 (Vythilingam et al., 2005). There was no infection in *An. donaldi* within this study, but this may be because too few were collected (n=25) for reliable detection. Thus, it cannot be dismissed that *An. donaldi* remains in other areas of Sabah where it is most abundant. The cause of this apparent shift in malaria vector species composition

over the past 40 years in Banggi Island is uncertain although it coincides with a period of extensive deforestation in Sabah (Bryan et al., 2013; Fornace et al., 2014). One possibility is that this is just an artefact of sampling, as here sampling was not conducted in the exact same locations as historical studies, but instead targeted sites of known human *P. knowlesi* infection. These sites may have inherently higher densities of *An. balabacensis* (thus triggering *P. knowlesi* infection) than other locations within the area. However, there is grounds to hypothesize this could be evidence of long-term shift in species composition in response to the rapid deforestation or prolonged use of interventions such as LLINs or IRS as has been documented elsewhere (Yasuoka & Levins, 2007). Previous work within the Kinabatangan area of Sabah has also documented a shift from a high proportion of *An. balabacensis* to dominance of *An. donaldi* within the same sites over the period 1980s to 2000 (Hii et al., 1985; Vythilingam et al., 2005). Regardless of the explanation for the dominance of *An. balabacensis* within this study, the relatively high survival and sporozoite rates in this vector coupled with the potentially increased contact of human-vector-macaques have likely made major contributions to the increase in *P. knowlesi* cases in the area. Recent studies in the east coast of Sabah in Tawau division also showed that *An. balabacensis* was the predominant species found in greater abundance in the logged forest compared to virgin jungle or primary forest (Brant et al., 2016)

Although Sabah has reported a large number of *P. knowlesi* cases in the past few years especially in Kudat division, it is hypothesized that people are only getting infected when they visit forested areas. Within our current study sites, the number of malaria cases occurring over the sampling period ranged from 1.9 to 2.5 cases per 100 people (Grigg et al., 2014). As positive *An. balabacensis* were present in most months of the year and most of the infective mosquitoes (40%) were captured biting in the early part of the evening between 1800 to 2000hrs, people could be exposed when they return

from work in or around forested areas. Case control studies have also shown that more *An. balabacensis* were caught in case houses compared to control houses (Manin et al., 2016). Studies now and previously have demonstrated that the *Anopheles* mosquitoes start biting only after 1800hrs. The average biting rate reported for *An. balabacensis* here is much higher than in previous studies conducted in the 1980s (e.g 6.8 to 8.8/ night compared to 0.75 to 4.44) (Hii et al., 1988a). These biting rates are also considerably higher than has been reported for *An. latens* (0.95 to 4.71 bites per night) in Sarawak (Tan et al., 2008). The high density of *An. balabacensis* in this area combined with its relatively high sporozoite rates with all simian malaria (1.82%) and *P. knowlesi* in particular (0.67%) indicate it is most likely responsible for the majority of transmission in this area.

In this study all mosquitoes were collected using human bait, thus results are only directly informative for estimating potential human exposure and not transmission between macaques. Ideally parallel collections of mosquitoes attracted towards macaques would have been conducted but this was not possible due to logistical constraints and ethics regulations for working with macaques. Previous work (Jiram et al., 2012; Tan et al., 2008) showed that the *P. knowlesi* vectors in other areas, namely *An. latens* and *An. cracens* were attracted to both humans and macaques. Furthermore in Palawan Island, Philippines, *An. balabacensis* was more attracted to a monkey-baited trap than traps baited with water buffalo or humans and individuals host seeking on macaques had oocysts and sporozoites (although malaria species unconfirmed) (Tsukamoto et al., 1978). Thus, although data for mosquitoes biting macaques are not available here, it could be expected that transmission between macaques to be at least as high as or much greater than predicted for humans here.

It is also unfortunate here that comparison of the mosquito density against rainfall was not conducted due to unforeseen circumstances. The meteorological

department was only able to provide rainfall data for one locality in Kudat. It is known that rainfall for the three locations were not the same. At the start of the project, another team was responsible for the collection of the environmental data in all the study sites but that failed.

To further resolve the transmission dynamics of *P. knowlesi* in primates, these studies should be expanded to incorporate assessment of the host preference and choice of *An. balabacensis* and other potential vectors most directly through analysis of the blood meals in randomly sampled resting females (Valinsky et al., 2014). However, collection of recently blood fed mosquitoes resting outdoors has proved challenging. To overcome this limitation ongoing work is also investigating the use of new sampling methods to increase feasibility of such data collection in the future.

4.5 Conclusion

The high rate of parity, survival and sporozoite infections in this mosquito indicates that *An. balabacensis* is a highly competent vector. With a very high vectorial capacity and life expectancy, *An. balabacensis* will continue to pose a risk of human infection. As Malaysia moves towards malaria elimination, breaking transmission under these conditions will be extremely challenging, further complicated by the presence of sizeable macaque reservoir.

Current frontline malaria control measures in this area are insecticide treated bednets and indoor residual spraying but more innovative control methods that specifically target outdoor biting mosquitoes such as the use of repellents or attractive toxic sugar baits will be essential.

CHAPTER 5: GENETIC DIVERSITY OF SIMIAN MALARIA PARASITES IN PRIMARY VECTOR *ANOPHELES BALABACENSIS* FROM KUDAT DIVISION, SABAH, MALAYSIA

5.1 Introduction

Currently only the *Anopheles* mosquitoes belonging to the Leucosphyrus Group have been incriminated as vectors. In the early 1960s when the first case of *P. knowlesi* was reported in human (Chin et al., 1965), *An. hackeri* was incriminated as the vector for *P. knowlesi* and all the other simian malaria parasites (Warren & Wharton, 1963; Wharton & Eyles, 1961), while *An. cracens* (at that time reported as *An. balabacensis*) was reported to be positive for *An. inui* in the northern region of peninsular Malaysia (Cheong et al., 1965). Since the report of a large focus of *P. knowlesi* cases in 2004 in Kapit, Sarawk, Malaysian Borneo (Singh et al., 2004), studies were undertaken in various parts of Malaysia to determine the vectors involved in the transmission of *P. knowlesi*. In Kapit, *An. latens* was incriminated as the vector (Tan et al., 2008; Vythilingam et al., 2006); *An. cracnes* in Kuala Lipis (Jiram et al., 2012), *An. introlatus* in Hulu Selangor (Vythilingam et al., 2014) and *An. balabacensis* in Sabah (Wong et al., 2015). Besides Malaysia, in Vietnam *An. dirus* has been incriminated as the vector (Marchand et al., 2011; Nakazawa et al., 2009).

In Sabah and in Sarawak, *An. balabacensis* and *An. latens* were the predominant vectors of human malaria respectively (Chang et al., 1995; Hii et al., 1988a). However, in peninsular Malaya, *An. maculatus* was the predominant vector (Reid, 1968). It is unfortunate that in the 1960s molecular tools were not available and thus to determine the species of sporozoites, it had to be inoculated into macaques. When the first case of *P. knowlesi* was reported from Bukit Kertau, Pahang, peninsular Malaysia, they

obtained infected gut and glands from *An. introlatus* and *An. latens* which were then inoculated into rhesus monkeys but no infection developed (Warren et al., 1970).

Dissection of mosquitoes to obtain midgut and salivary glands to determine the presence of oocysts and sporozoites respectively is gold standard. On examination, if found to be positive, the midgut and salivary glands can be transferred to 90% alcohol for molecular detection of the parasites. In the study carried out in Kudat, Sabah, *An. balabacensis* was the predominant mosquito obtained and 45 of them were positive for either oocysts, salivary glands or both (Wong et al., 2015).

In order to identify the oocysts and sporozoites to species level, the SSU rRNA gene was selected as more data of that gene is available in the GenBank and will help in the comparison of sequence data from humans and monkeys. The transmission of simian malaria to humans is complex and many questions remain unanswered. One needs to understand the link between hosts, parasites vectors and the environment. This will perhaps help us to understand transmission dynamics of simian malaria parasites infection in macaques and humans. In this study, we determined the genetic diversity, circulating haplotypes, haplotype diversity and the population structure of the malaria parasites obtained from *An. balabacensis* based on the SSU rRNA genes. Little information is available about the genetic diversity and nature of gene flow of the simian malaria parasites from mosquitoes. Thus, published sequences of the parasites from humans and macaques were used to determine their inter-population genetic differentiation based on sample origin.

5.2 Materials and methods

5.2.1 Identification of malaria parasites from field collected *Anopheles balabacensis*

Entomological investigations were carried out in Limbuak Laut (LL) (117°065'75"E, 7°215'84"N) and Timbang Dayang (TD) (117°102'92"E, 7°155'85"N) on Banggi Island, and Kampung Paradason (KP) (116°786'35"E, 6°768'37"N) on mainland Kudat from August 2013 to July 2014 as described in the previous chapter. *Anopheles* mosquitoes were dissected to extract ovaries for determination of parity, and midguts and salivary glands for examination of oocysts and sporozoites, respectively. All positive midguts and salivary glands were transferred to individual microcentrifuge tubes for molecular identification of malaria parasites. Details regarding identification and spatial dynamics of the vector have been reported in chapter 4.

5.2.2 Extraction of DNA

DNA extracted from individual positive midguts and salivary glands described in the previous chapter were used for studies mentioned here. However, dried mosquitoes that could not be dissected were pooled according to time, site and month, and each pool contained from 1-5 mosquitoes. Any blood-fed mosquitoes that died were discarded. Ethanol was allowed to evaporate from the specimen tubes using Thermomixer (Eppendorf, Germany) at 70°C after brief centrifugation. Genomic DNA was extracted from dried mosquitoes using DNeasy Blood and Tissue Kit (Qiagen, Germany) according to manufacturer's recommendation and details as mentioned in the previous chapter. The eluted DNA was kept at -20°C until required.

5.2.3 PCR amplification for detection of *Plasmodium* genus in pooled mosquitoes

Pooled mosquitoes were screened for *Plasmodium* using 18S ribosomal RNA genus primer (Plas F: 5'-AGTGTGTATCAATCGAGTTTCT-3'; Plas R: 5'-CTTGTCACTACCTCTCTTCTTTAGA-3') as previously described (Li, 2011). Amplification of PCR was performed in 50µL reaction as illustrated in Table 5.1. The PCR parameters are as listed in Table 5.2. To save cost, only DNA from pooled mosquitoes was subjected to this single PCR so that all the samples which were positive for genus can be tested by nested PCR. Positive and negative controls were included in each batch of PCR assay. The PCR products were analyzed on 1.5% agarose gel electrophoresis, stained with SYBR Safe DNA gel stain (Invitrogen, Carlsbad CA, USA) and observed under ultraviolet transillumination.

Table 5.1: Components of master-mix for PCR assay with Plas F and Plas R primers.

Components	Final concentration	Volume (µL)
RNase and DNase-free molecular grade water	-	27.25
5x green GoTaq® reaction buffer (Promega)	1x	10.0
$MgCl_2$, 25mM (Promega)	2.0mM	4.0
dNTP mix, 10mM each (Promega)	0.2mM	1.0
Forward primer, 10µM	0.25µM	1.25
Reverse primer, 10µM	0.25µM	1.25
GoTaq® DNA polymerase, 5U/µL (Promega)	1.25U	0.25
DNA template	-	5.0
Total volume per reaction	**-**	**50.0**

Table 5.2: **Cycling parameters for PCR assay with Plas F and Plas R primers.**

Steps	Temperature/ °C	Time/ s	Number of cycles
Initial denaturation	95	240	1
Denaturation	95	30	
Annealing	52.8	30	44
Extension	72	30	
Final extension	72	120	1
	4	∞	-

5.2.4 Nested PCR amplification for detection of *Plasmodium* species and sequencing

All positive midguts, salivary glands and positive pools of mosquito samples were subjected to nested PCR assay based on small subunit ribosomal RNA genes and PCR cycling parameters as previously described (Lee et al., 2011; Singh et al., 2004) (Table 5.3). Positive and negative controls were included in all PCR assays. The PCR amplifications for nest 1 and 2 were performed as described in Section 4.2.6. Nest 2 amplicons were analyzed with 1.5% agarose gel electrophoresis, stained with SYBR Safe DNA gel stain (Invitrogen, Carlsbad CA, USA) and observed under an ultraviolet transilluminator. Positive samples were excised from gel and sent for sequencing to a commercial laboratory (MyTACG Bioscience, Malaysia).

5.2.5 Isolates used for SSU rRNA gene characterization and amplification of gene

Thirteen isolates were further processed as denoted in Table 5.3. Of these, three isolates were infected macaque blood collected from Hulu Selangor by Akter et al. (2015). Nest 1 PCR amplifications of SSU rRNA gene was performed with *Plasmodium*-specific primers, rPLU 1 and rPLU5 as described (Singh et al., 1999). PCR assay were carried out in a 50µL reaction mixture as shown in Table 5.4. PCR amplification parameters were as listed in Table 5.5

The nest 1 PCR product was subjected to nest 2 PCR amplification using modified forward *Plasmodium*-genus primer and reverse *Plasmodium*-species primers (Chua et al., 2017; Imwong et al., 2009; Lee et al., 2011) listed in Table 5.11 with their respective annealing temperatures. Two microliters of nest 1 PCR product was used for nest 2 PCR amplifications. The concentration of each constituent for PCR assay and cycling parameters were as listed in Table 5.7 and Table 5.8, respectively.

Table 5.3: Isolates used in characterization of simian malaria parasites based on SSU rRNA gene.

Isolates	Locality	Host
LL237sg LL442sg LL477sg	Limbuak Laut, Banggi Island, Kudat, Sabah	*An. balabacensis*
TD92sg TD397sg TD456sg TD470sg	Timbang Dayang, Banggi Island, Kudat, Sabah	*An. balabacensis*
KP82sg KP646sg KP730sg	Kg Paradason, Kudat, Sabah	*An. balabacensis*
B21 M28 M1	Hulu Selangor, Selangor	Macaque

Table 5.4: Components of master-mix for nest 1 PCR.

Components	Final concentration	Volume (µL)
RNase and DNase-free molecular grade water	-	13.25
2x Accura HF reaction buffer (Lucigen)	1x	25.0
dNTP mix, 10mM each (Promega)	0.2mM	1.0
Forward primer, 10µM	0.5µM	2.5
Reverse primer, 10µM	0.5µM	2.5
High-fidelity DNA polymerase, 2U/µL (Accura)	1.5U	0.75
DNA template	-	5.0
Total volume per reaction	-	**50**

Table 5.5: Cycling parameters for nest 1 PCR.

Steps	Temperature/ °C	Time/ s	Number of cycles
Initial denaturation	94	300	1
Denaturation	94	60	
Annealing	55	60	35
Extension	72	60	
Final extension	72	600	1
	4	∞	-

Table 5.6: Oligonucleotide sequences of PCR primers used for characterization of simian malaria parasites in the isolates.

Plasmodium	Primers	Sequence (5'-3')	Nested PCR	Annealing temperature (°C)	Expected product size (bp)
Genus-specific	rPLU 1	TCAAAGATTAAGCCATGCAAGTGA	1	55	1640
	rPLU 5	CCTGTTGTTGCCTTAAACTCC			
Forward genus-specific	UMSF[1]	GGATAACTACGGAAAAGCTGT		-	-
knowlesi	Pkr1550[2]	GAGTTCTAATCTCCGGAGAGAAAAGA		50	1050
cynomolgi	CYN1R[3]	GATTAACTCCGAAGAGAAAATC	2-simian	55	1015
inui	INAR3[4]	GCAATCTAAGAGTTTTAACTCCTC	*Plasmodium*	60	1039
coatneyi	PctR1[4]	GAGTCCTAACCCCGAAGGGAAAGG		60	1029
fieldi	PfldR2[4]	AGGCACTGAAGGAAGCAATCTAAGAGTTTC		63	1039

[1] modified forward *Plasmodium*-genus primer based on Singh et al. (1999)

[2] reverse *P. knowlesi* primer based on Imwong et al. (2011)

[3] reverse *P. cynomolgi* primer based on Chua et al. (2017)

[4] reverse *Plasmodium*-species primers based on Lee et al. (2011)

Table 5.7: Components of master-mix for nest 2 PCR.

Components	Final concentration	Volume (µL)
RNase and DNase-free molecular grade water	-	16.25
2x Accura HF reaction buffer (Lucigen)	1x	25.0
dNTP mix, 10mM each (Promega)	0.2mM	1.0
Forward primer, 10µM	0.5µM	2.5
Reverse primer, 10µM	0.5µM	2.5
Accura High-fidelity DNA polymerase, 2U/µL (Lucigen)	1.5U	0.75
DNA template (from nest 1)	-	2.0
Total volume per reaction	**-**	**50**

Table 5.8: Cycling parameters for nest 2 PCR.

Steps	Temperature/ °C	Time/ s	Number of cycles
Initial denaturation	94	300	1
Denaturation	94	60	
Annealing	X	90	35
Extension	72	60	
Final extension	72	600	1
	4	∞	-

5.2.6 Gel purification of PCR product and A-tailing preparation for cloning

The amplified PCR fragment was excised from the agarose gel with a sterile scalpel, transferred to a sterile labelled microcentrifuge tube and weighed. The gel slice was purified using NucleoSpin® Gel and PCR Clean-up (Machery-Nagel, Germany) according to manufacturer's recommendations. For each 0.1g of agarose gel which is <1.0%, 200µL of buffer NT1 was added into the microcentrifuge tube and incubated for 5-10 minutes at 50°C with occasional shaking to ensure the gel slice was completely dissolved. A NucleoSpin® Gel and PCR Clean-up column was placed into a 2mL collection tube and up to 700µL of sample was loaded into the column. The column was centrifuged for 30s at 10,700rpm. After centrifugation, the flow-through was discarded and the column was placed back into the collection tube. Any remaining sample was loaded into the column and centrifugation step was repeated. The silica membrane of the column was washed by adding 700µL of buffer NT3 into the column and centrifuged for 30s at 10,700rpm. The flow-through was discarded after centrifugation and column was placed back into the collection tube. The column was then centrifuged for 1 minute at 10,700rpm to remove buffer NT3 completely. Then, the spin column was removed carefully from the centrifuge and flow-through was discarded. The spin column was then incubated for 5 minutes at 70°C for total removal of any residual ethanol from buffer NT3 prior to elution. Next the spin column was placed into a new sterile 1.5mL microcentrifuge tube. For elution, 20µL of buffer NE was added directly onto the spin column membrane. The spin column was incubated at room temperature for 1 minute and centrifuged for 1 minute at 10,700rpm to elute the DNA. All purified DNA was stored in -20°C freezer until required.

Accure High-fidelity polymerase has proof-reading activity for robust amplification of DNA templates. Therefore, the PCR amplicons generated will be blunt-ended. The PCR amplicons generated which were purified can be modified using the A-

tailing procedure and ligated into pGEM®-T Easy Vector system I. Briefly, A-tailing of purified PCR amplicon was prepared in a 10µL reaction mixture as listed in Table 5.9 and incubated in a Thermomixer (Eppendorf, Germany) for 30 minutes at 70°C. The A-tailed purified PCR amplicon was then ligated into pGEM®T Easy Vector system I as mentioned in Section 5.2.7.

Table 5.9: Components of master-mix for A-tailing procedure.

Components	Final concentration	Volume (µL)
RNase and DNase-free molecular grade water	-	2.8
5x Colorless GoTaq® reaction buffer (Promega)	1x	2.0
$MgCl_2$, 25mM (Promega)	2.5mM	1.0
dNTP mix, 10mM each (Promega)	0.2mM	0.2
GoTaq® DNA polymerase, 5U/µL (Promega)	5U	1.0
Purified DNA amplicon	-	3.0
Total volume per reaction	**-**	**10.0**

5.2.7 Cloning of *Plasmodium* SSU rRNA gene

The pGEM®-T Easy Vector System I kit (Promega, Madison, WI, USA) was used according to the manufacturer's protocol. Briefly, the ligation reaction was carried out in a 10µL reaction volume containing 3µL of the A-tailed purified PCR amplicon, 5µL of 2x Rapid Ligation buffer, 1µL of pGEM®-T Easy Vector (50ng) and 1µL of T4 DNA Ligase (3 Weiss units/µL). A positive control using 2µL of Control Insert DNA (provided with the kit) and negative control was prepared. Both the pGEM®-T Easy Vector and Control Insert DNA tubes were centrifuged prior to ligation preparation. Additionally, the 2x Rapid Ligation buffer was vortexed vigorously before each use. The ligation reactions mixture was mixed thoroughly by pipetting. The reaction mixture was then incubated overnight at 4°C for the maximum number of transformants.

For transformation, the chemically competent cells used were One Shot® TOP10 *Escherichia coli* competent cells (Invitrogen, Carlsbad CA, USA). The vial of One Shot® TOP10 competent cells were allowed to thaw on ice prior to transformation. Then, 3µL of the ligation reaction mix was added into the vial and mixed gently before incubation on ice for 30 minutes. For pUC19 control, 1µL of DNA was added into a separate vial of competent cells, mixed gently and incubated on ice for the same duration. For the heat-shock procedure, the reaction mixture was placed in a 42°C waterbath for 50s without shaking, and thereafter immediately placed on ice for 5 minutes. Then, 950µL of pre-warmed S.O.C medium was aseptically added into each vial. The vial was capped tightly and incubated at 37°C with horizontal shaking in a shaking incubator for 2 hours at 225rpm. After incubation, the vial was centrifuged at 5000rpm for 5 minutes and the supernatant was discarded carefully leaving the pellet in the tube.

The pellet was resuspended in 200µL of Luria-Bertani (LB) broth supplemented with ampicillin. Two volumes of 100µL transformant culture were then spread on pre-warmed labelled LB agar plate supplemented with ampicillin aseptically. The plates were inverted and incubated overnight at 37°C for bacterial growth.

Colony PCR was conducted using M13F (-40) forward primer and M13R (-48) reverse primer to screen the *E. coli* transformants for the gene insert. A single colony of the *E. coli* transformant was picked using a sterile 10µL pipette tip and dipped into 10µL PCR reaction mixture as listed in Table 5.10. The colony PCR amplification parameters are as shown in Table 5.11. The PCR products were analyzed by 1% agarose gel electrophoresis, stained with SYBR Safe DNA gel stain (Invitrogen, Carlsbad CA, USA) and observed under ultraviolet illumination.

Table 5.10: Components of master-mix for colony PCR.

Components	Final concentration	Volume (µL)
RNase and DNase-free molecular grade water	-	5.5
5x green GoTaq® reaction buffer (Promega)	1x	2.0
25mM $MgCl_2$ (Promega)	3.0mM	1.2
dNTP mix, 10mM each (Promega)	0.2mM	0.2
Forward primer, 10µM	0.5µM	0.5
Reverse primer, 10µM	0.5µM	0.5
GoTaq® DNA polymerase, 5U/µL (Promega)	0.5U	0.1
DNA template	-	-
Total volume per reaction	**-**	**10.0**

Table 5.11: Cycling parameters for colony PCR.

Steps	Temperature/ °C	Time/ s	Number of cycles
Initial denaturation	95	240	1
Denaturation	95	30	
Annealing	55	30	35
Extension	72	30	
Final extension	72	120	1
	4	∞	-

5.2.8 Preparation of glycerol stock

Escherichia coli colonies containing the plasmid DNA of targeted fragment were each inoculated into 5mL of LB broth supplemented with ampicillin. The culture was grown overnight at 37°C in a shaking incubator at 225rom for subsequent preparation of glycerol stock and plasmid extraction.

Glycerol stocks were prepared for long term storage of the individual bacterial cultures with the targeted DNA fragment at -80°C. They were prepared by adding 500µL of culture to 500µL of sterile glycerol, followed by storage at -80°C freezer. The sterile glycerol is prepared by adding 25mL of sterile deionized water to 25mL of glycerol (Biotechnology Grade, Amresco, Ohio, USA) and filter-sterilized.

5.2.9 Extraction of plasmid DNA

Plasmid DNA was extracted using NucleoSpin® Plasmid (Machery-Nagel, Germany) according to manufacturer's protocol. An overnight LB broth culture of tranformants was centrifuged at 8300rpm for 6 minutes to pellet the cells. The supernatant was removed and cell pellet was resuspended completely by adding 250µL of Buffer A1 with RNase A, vortexed and transferred into a new sterile 1.5mL microcentrifuge tube. To lyze the cells, 250µL Buffer A2 was added and gently mixed by inverting the tubes 6-8 times, followed by incubation at room temperature for 5 minutes until the lysate becomes clear. Then, 300µL buffer A3 was added and mixed thoroughly by inverting the tubes 6-8 times and followed by centrifugation at 10,700rpm for 5 minutes. For DNA binding, a NucleoSpin® Plasmid column was placed in a 2mL collection tube and 750µL of supernatant was pipetted onto the column followed by centrifugation at 10,700rpm for 1 minute. The flow-through was discarded and the column was placed back into the collection tube. The step was repeated for any remaining lysate. To wash the column membrane, 500µL buffer AW was added onto

the column, centrifuged for 1 minute at 10,700rpm and the flow-through was discarded. This is followed by addition of 600µL buffer A4 onto the column and centrifuged for 1 minute at 10,700rpm. The flow-through was discarded and the column was placed back into the collection tube. For drying the column membrane, the column was centrifuged for 2 minutes at 10,700rpm. The collection tube was discarded and the column was placed into a sterile 1.5mL microcentrifuge tube. For elution, 50µL buffer AE was added directly onto the column membrane, incubated at room temperature for 1 minute and centrifuged for 1 minute at 10,700rpm. Twenty microliters of eluted plasmid DNA was sent for sequencing to a commercial laboratory (MyTACG Bioscience, Malaysia). The remaining plasmid DNA was stored at -20°C freezer until required.

5.2.10 Sequence editing and alignment

Sequences from midgut, salivary glands and pooled mosquitoes were aligned with ClustalW using Geneious 9.1.6 software (http://www.geneious.com) (Kearse et al., 2012). Similarity searches using Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were performed. Nucleotide sequences of the SSU rRNA obtained from section 5.2.4 were phylogenetically compared with those obtained from GenBank for a number of *Plasmodium* species with MEGA 7.0 software (Kumar et al., 2016). The phylogenetic tree was constructed using neighbor-joining method based on Maximum Composite Likelihood distance matrix including transitions and transversions.

Nucleotide sequences obtained from cloned samples in section 5.2.9 were aligned with ClustalW using Geneious 9.1.6 software (http://www.geneious.com) (Kearse et al., 2012). Sequence analysis and comparison at the nucleotide level were performed against the reference sequence for *P. knowlesi* H-strain (GenBank accession number AM910985), *P. inui* San Antonio I strain (GenBank accession number

XR606809 and *P. cynomolgi* Berok strain (PlasmoDB Gene ID PCYB_032923). Results were exported to MEGA 7.0 software (Kumar et al., 2016) for further analysis. An additional of 39 *PkA-type 18S rRNA* sequences derived from *P. knowlesi* infected samples (2 from *An. balabacensis*; 25 from macaques; 12 from humans), 38 *PinA-type 18S rRNA* sequences of *P. inui* infected macaque samples and 8 *PcyA-type 18S rRNA* sequences from *P. cynomolgi* infected samples (7 from macaques; 1 from human) were obtained from GenBank and PlasmoDB, and included in the analysis. The sequences accession numbers are as listed in Table 5.12.

Table 5.12: Accession numbers of sequences retrieved from GenBank database included in the analyses.

Plasmodium species	Region/ Country	Accession no.		
		Mosquito	Macaque	Human
P. knowlesi	Peninsular Malaysia	NA	AY327557 (Nuri strain)	AM910985 (H strain)
			L07560	
	Hulu Selangor (B21)	NA	**MF370091-MF370100**	
	Malaysian Borneo			
	Sarawak	NA	DQ350264-DQ350269	AY327549-AY327556
		NA	DQ641518-DQ641525	EU807923
		NA	FJ619069; FJ619087-FJ619090; FJ619097-FJ619098	FJ804768
	Sabah	MF582564-MF582565	MF582566	MF582562-MF582563
	Sabah (LL477sg)	**MF370081-MF370090**	NA	NA
	Sabah (KP730sg)	**MF370101-MF370107**	NA	NA
	Sabah (TD397sg)	**MF370108-MF370109**	NA	NA
P. inui	Peninsular Malaysia			NA
	Hulu Selangor (R1)	NA	**MF370156-MF370160**	NA
	Malaysian Borneo			
	Sarawak	NA	FJ619065, FJ619067; FJ619073-FJ69074, FJ619076; FJ619078-FJ619079, FJ619081-FJ619082; FJ619085; FJ619093, FJ619095-FJ619096; FJ619104	NA
	Sabah (KP82sg)	**MF370110-MF370119**	NA	NA
	Sabah (KP646sg)	**MF370120-MF370124**	NA	NA

[1]Accession numbers in bold are sequences generated in this study and deposited in GenBank

Table 5.12, continued.

Plasmodium species	Region/ Country	Accession no.		
		Mosquito	Macaque	Human
P. inui	Malaysian Borneo			
	Sabah (TD92sg)	**MF370125-MF370130**	NA	NA
	Sabah (TD470sg)	**MF370131-MF370140**	NA	NA
	Sabah (LL237sg)	**MF370141-MF370148**	NA	NA
	Sabah (LL477sg)	**MF370149-MF370155**	NA	NA
	Thailand	NA	EU400384-EU400392; EU400395-EU400397	NA
	Celebes	NA	AB287276-AB287277	NA
	Taiwan	NA	FN256224-FN256230; FN430724-FN430725	NA
P. cynomolgi	Hulu Selangor (M28)	NA	**MF370174-MF370183**	NA
	Terengganu			JQ794445
	Malaysian Borneo	NA	AB287289-AB287290 (Mulligan strain)	NA
	Sabah (LL442sg)	**MF370161-MF37063**	NA	NA
	Sabah (LL477sg)	**MF370164-MF370166**	NA	NA
	Sabah (TD397sg)	**MF370167-MF370170**	NA	NA
	Sabah (TD456sg)	**MF370171-MF370173**	NA	NA
	Sarawak	NA	FJ619084	NA
	Ceylon	NA	L07559; L08241	NA
	Kalimantan	NA	DQ660816	NA

[1]Accession numbers in bold are sequences generated in this study and deposited in GenBank

5.2.11 Genetic diversity, haplotype network and spatial structuring

The polymorphism of the A-type 18S SSU rRNA genes for *P. knowlesi, P. inui* and *P. cynomolgi* was estimated by computing haplotype diversity (Hd), number of haplotypes (h), nucleotide diversity (π), number of polymorphic sites and the average number of pairwise nucleotide differences using DnaSP version 5.10.01 (Librado & Rozas, 2009). Haplotype networks for *P. knowlesi, P. inui* and *P. cynomolgi* A-type 18S SSU rRNA based on their polymorphic sites were constructed by using median-joining method in NETWORK version 5.0.0.1 software (Fluxus Technology LTD Suffolk, UK). The genealogical haplotype network for *P. knowlesi, P. inui* and *P. cynomolgi* was inferred using the sequences of human and macaque isolates from other geographical locations. Where available, sequences from reference strains of *P. knowlesi, P. inui* and *P. cynomolgi* were included as references.

STRUCTURE version 2.3.4 software (The Pritchard Lab, Stanford University, Stanford, CA, USA) was used to define the genetic structure of the *P. knowlesi, P. inui* and *P. cynomolgi* parasite population in LL, TD and KP. This software used the Bayesian model-based clustering approach. Admixture model was used to estimate the most probable number of populations (K). All sample data was run for values K=1-8, each with a total of 15 iterations. For each run, 100,000 Markov Chan Monte Carlo generations was used after burn-in of 50,000 steps. The most likely number K in the data was estimated by calculating ΔK values and the K value which maximizes the log probability data, 1nP (D) was identified. The most probable K value was calculated according to Evanno's method by using STRUCTURE Harvester. ARLEQUIN version 3.5.1.3 software (University of Berne, Berne, Switzerland) was also used to compute pairwise differences (F_{ST}) between each populations of mosquitoes (*An. balabacensis*), macaques and humans from haplotypes with 10,100 permutations. F_{ST} is defined as comparison of the sum of genetic variability within and between populations on the

basis of the differences in allelic frequencies. F_{ST} values were interpreted as no (0), low (>0-0.05), moderate (0.05-0.15) and high (>0.15) genetic differentiation.

5.2.12 Neutrality and demographic analysis

Departure and demographic expansions were examined from a strict neutral model, on the basis of pairwise mismatch distribution, Tajima D test (Tajima, 1989), Fu and Li D* (Fu, 1997), and Fu and Li F* (Fu & Li, 1993) statistics using DnaSP version 5.10.01 (Librado & Rozas, 2009).

5.2.13 Ethics

This project was approved by the NMRR Ministry of Health Malaysia (NMRR-12-786-13048).

5.3 Results

5.3.1 Identification of *Plasmodium* species in pooled mosquitoes

Identification of the malaria parasites in the dried pooled mosquito's samples by nested PCR (Figure 5.1) showed that *P. cynomolgi* was the predominant simian malaria species detected in all three study sites followed by *P. inui*, *P. fieldi*, *P. knowlesi* and *P. coatneyi* (Table 5.13). Similarity searches and comparison of all the sequences obtained in this study with the available sequences in GenBank using BLAST revealed 97-99% similarity. Phylogenetic analysis of 18S SSU rRNA gene was constructed using the sequences obtained from this study as well as other previous published sequences along with available references sequences (Figure 5.2). The phylogenetic tree revealed five major clades corresponding to the five simian *Plasmodium* species, namely *P. knowlesi*, *P. inui*, *P. cynomolgi*, *P. coatneyi* and *P. fieldi* with high bootstrap values. Every pooled mosquito samples were confirmed as *P. knowlesi*, *P. inui*, *P. cynomolgi*, *P. coatneyi* and *P. fieldi* respectively by performing BLAST in GenBank. There are no samples

branching out from the major clade for each simian *Plasmodium* species except the outgroup, *P. berghei* (GenBank accession number: AJ243513).

Table 5.13: Summary of identified simian *Plasmodium* in pooled dried *An. balabacensis* from three study sites.

Infection type	*Plasmodium* species	Limbuak Laut (LL)	Timbang Dayang (TD)	Kg Paradason (KP)
Single	Pk	0	0	0
	Pin	1	2	3
	Pcy	3	11	5
	Pct	1	0	2
	Pfi	0	1	0
Double	Pk+Pcy	0	0	3
	Pk+Pin	1	0	0
	Pin+Pcy	1	0	6
	Pin+Pct	1	0	0
	Pin+Pfi	2	2	0
	Pcy+Pfi	0	2	0
	Pcy+Pct	0	0	0
Triple	Pk+Pcy+Pct	0	0	1
	Pk+Pin+Pfi	0	1	0
	Pk+Pin+Pcy	0	0	0
	Pin+Pcy+Pct	0	0	1
	Pin+Pcy+Pfi	1	0	0
Quadruple	Pk+Pin+Pcy+Pct	0	0	1
	Pk+Pin+Pcy+Pfi	1	0	0
TOTAL		**12**	**19**	**22**

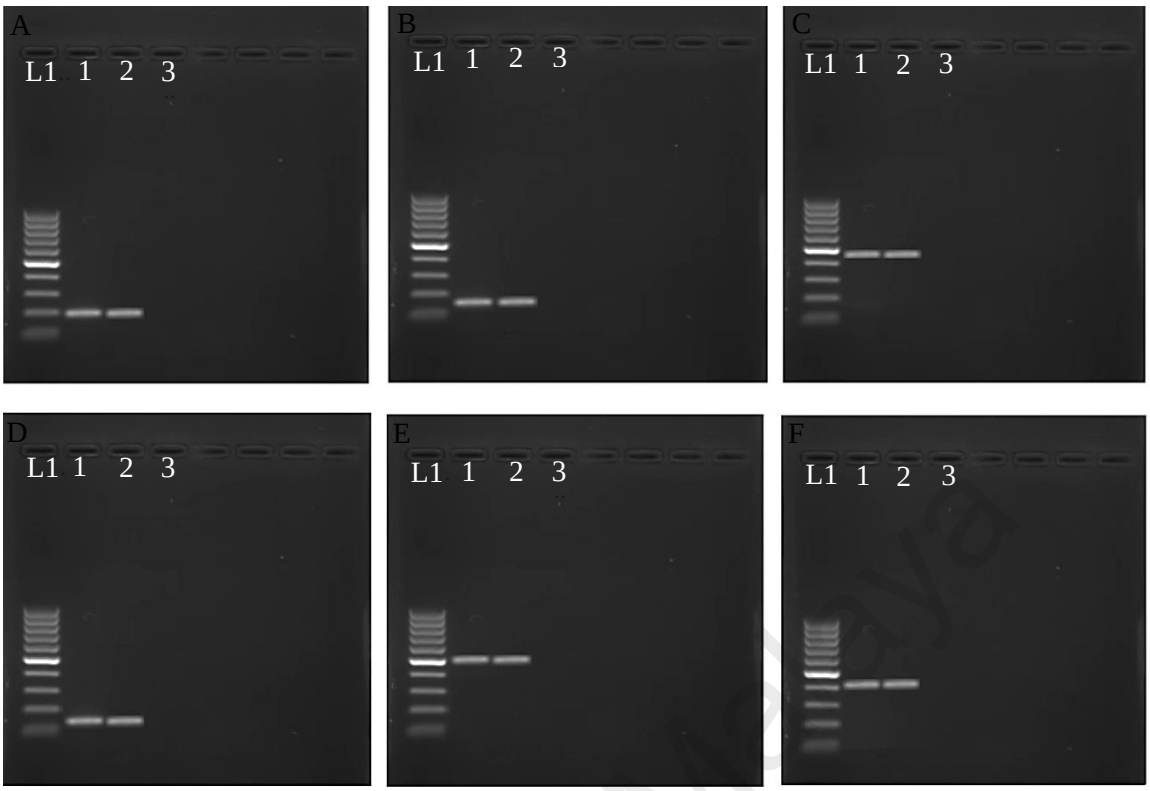

Figure 5.1: Gel electrophoresis result image for detection of *Plasmodium* in pooled mosquitoes. L1 denotes 100bp ladder. Lane 1 is the sample while Lan 2 is positive control. Lane 3 is negative control. A) Plas F and Plas R detection for positive *Plasmodium* in pooled mosquitoes (188bp). B) *P.knowlesi* positive sample (153bp). C) *P. inui* positive sample (479bp). D) *P. cynomolgi* positive sample (137bp). E) *P. coatneyi* positive sample (503bp). F) *P. fieldi* positive sample (421bp).

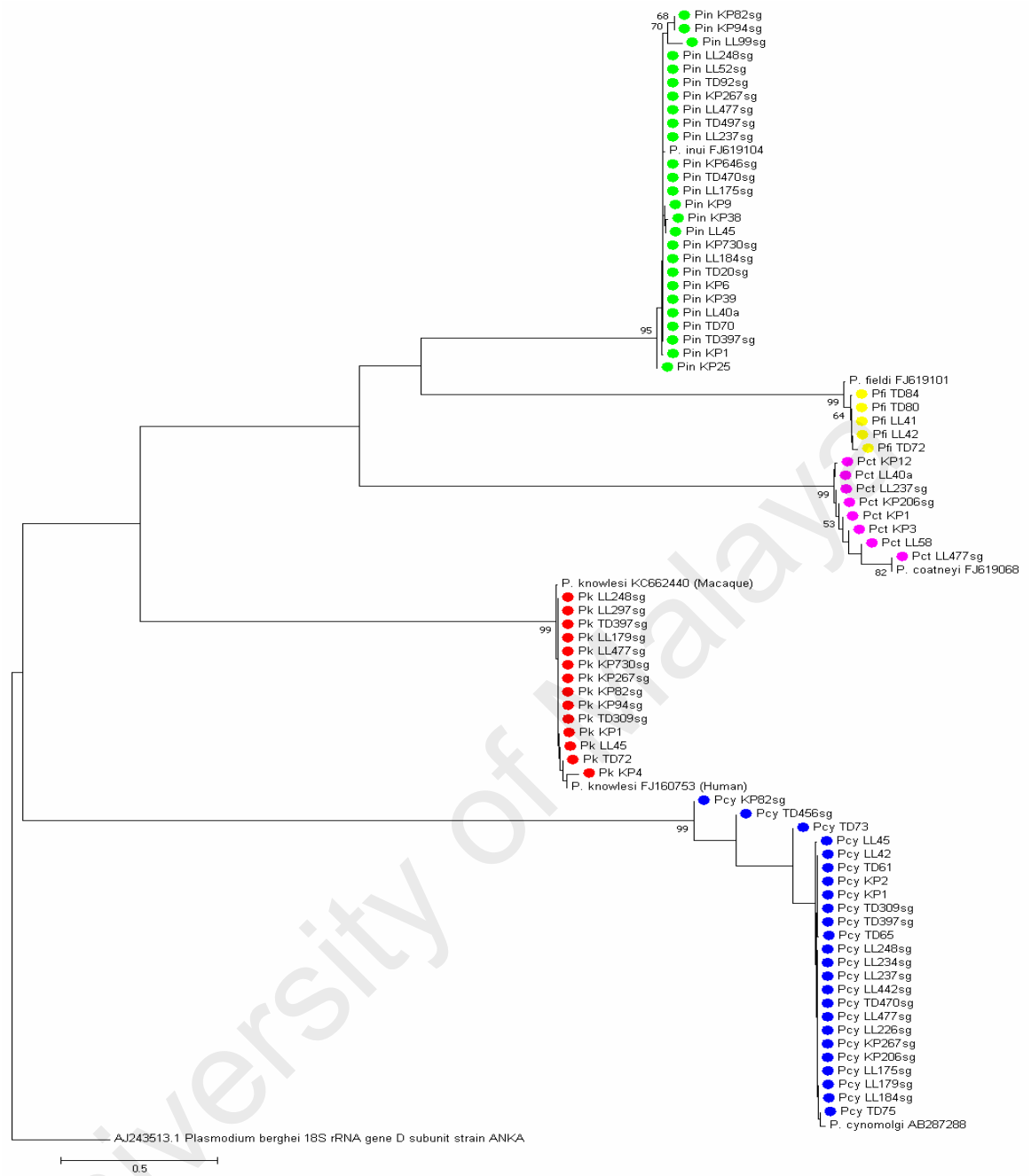

Figure 5.2: Phylogenetic tree of 18S SSU rRNA gene of the positive infected samples and pooled dried mosquitoes samples of *An. balabacensis.* Neighbor-joining method was used to construct the phylogeny tree. Number at nodes indicate percentage support of 1000 bootstrap replicates with only bootstrap values above 50% are displayed on the tee. Sequences obtained from this study were marked with colored circle.

5.3.2 DNA sequence analysis

5.3.2.1 *P. knowlesi*

A total of 68 sequences were aligned and analyzed for *PkA-type 18S rRNA* (1004bp) after multiple sequence alignment. Of these, 19 sequences were generated from 3 infected *An. balabacensis* samples and 10 sequences were from 1 infected macaque sample of *P. knowlesi*. The sequences were deposited in GenBank and their associated accession numbers listed in Table 5.12. The remaining 39 sequences were obtained from GenBank.

5.3.2.2 *P. inui*

Eighty nine sequences of *PinA-type 18S rRNA* (994bp) were used for analysis. A total of 51 sequences were generated from 6 infected *An. balabacensis* samples (number of sequences=46) and one infected macaque sample (number of sequences=5). The sequences were deposited in GenBank and their accession numbers are shown in Table 5.12.

5.3.2.3 *P. cynomolgi*

A total of 23 sequences (972bp) were obtained for analysis and comparison from Timbang Dayang, Limbuak Laut and peninsular Malaysia. Those from peninsular Malaysia were of macaque infected sample whereas those from Timbang Dayang and Limbuak Laut were from infected *An. balabacensis*. The sequences were deposited in the GenBank and their accession numbers are shown in Table 5.12.

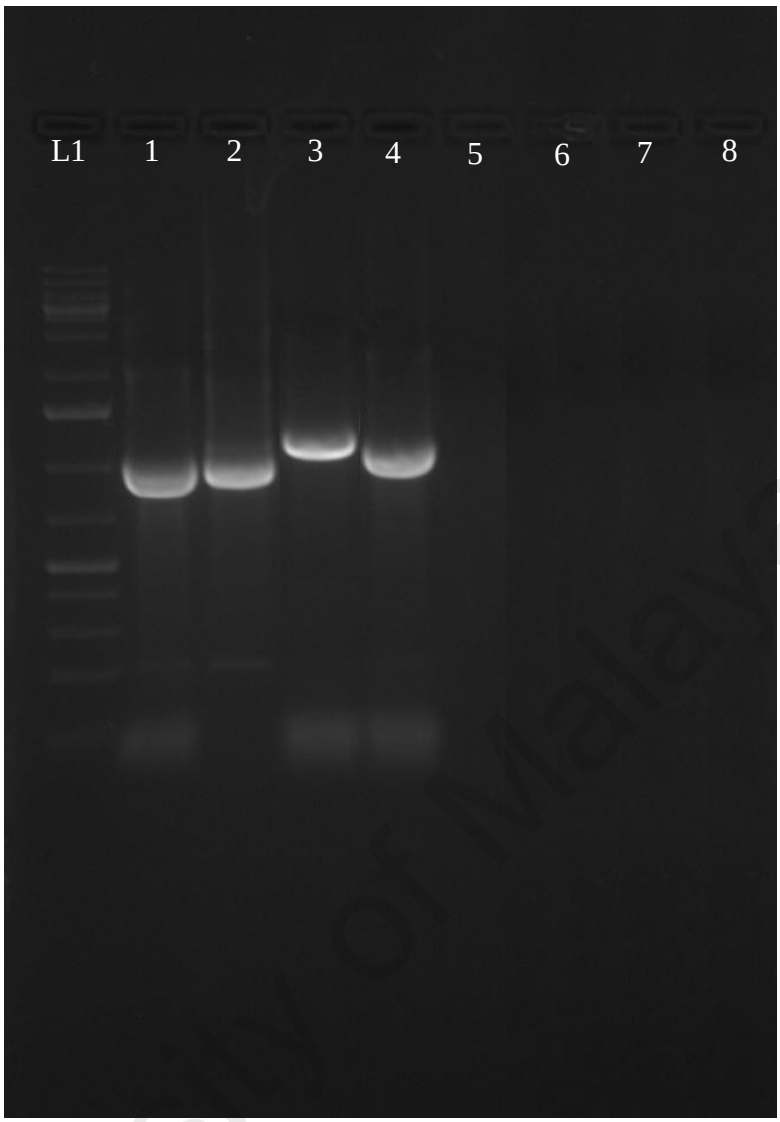

Figure 5.3: Gel picture showing amplified amplicons from infected salivary glands of *An. balabacensis* using oligonucleotides listed in Table 5.6. L1 denotes 1kb ladder. Lane 1-4 denotes *P. cynomolgi, P. coatneyi, P. knowlesi* and *P. inui,* respectively. Lane 5-8 denotes negative control for each of the species (*P. cynomolgi, P. coatneyi, P. knowlesi* and *P. inui*).

5.3.3 Gene diversity indices

5.3.3.1 *P. knowlesi*

Analysis of the molecular polymorphism within the 65 partial *PkA-type 18S rRNA* (1004bp) showed low polymorphic sequences (π=0.00515±0.00062) (Table 5.14) compared to *P. knowlesi*. Overall, there were 107 polymorphic sites yielding 49 haplotypes. High haplotype diversity with low nucleotide diversity was observed in the *P. knowlesi* populations from LL (Hd=1.0±0.045, π=0.00609±0.00069), KP (Hd=1.0±0.052, π=0.00433±0.00050), TD (Hd=1.0±0.500, π=0.01±0.00502) and Peninsular Malaysia (Hd=1.0±0.045, π=0.0068±0.00067). However, for TD, only two sequences were analyzed and compared. Statistical analysis of Tajima D, Fu and Li D*, and Fu and Li F* showed significant negative values for LL (p=0.05) (Table 5.15) which indicates that the *P. knowlesi* populations in LL is undergoing expansion. Overall, the *P. knowlesi* population in Malaysia were expanding as the statistical analysis using Tajima D, Fu and Li D*, and Fu and Li F* yielded significant negative values (p=0.0001-0.02). Population expansion was also evident from the unimodal shape of the pairwise mismatch distribution for *P. knowlesi* A-type 18S rRNA obtained (Figure 5.4A).

5.3.3.2 *P. inui*

Molecular polymorphism analysis of the 66 sequences from Malaysia of *PinA-type 18S rRNA* showed relatively low polymorphism (π=0.00170±0.00035) (Table 5.14). There were 31 polymorphic sites which led to 18 haplotypes within Malaysian samples of mosquitoes (*An. balabacensis*) and macaques. Number of haplotypes was low yielding to low haplotype diversity. Among the mosquito populations, highest haplotype diversity was observed in the populations from KP (Hd=0.838±0.049), followed by LL Hd=0.714±0.081), TD (Hd=0.425±0.133) and Peninsular Malaysia

(Hd=0.400±0.23). Nevertheless, relatively low nucleotide diversity was observed for all locations. Overall, the populations of *PinA-type 18S rRNA* in Malaysia were expanding as the calculations using Tajima D, Fu and Li D*, and Fu and Li F* statistics yielded significant negative values (p=0.001-0.02) (Table 5.15). Pairwise mismatch distribution analysis revealed unimodal distribution suggesting that the *P. inui* population was expanding (Figure 5.4B).

5.3.3.3 *P. cynomolgi*

The analysis of sequence polymorphism in 23 sequences of *PcyA-type 18S rRNA* showed moderate polymorphism (π=0.0080±0.00114) (Table 5.14) compared to *P. knowlesi* and *P. inui*. Overall, there are 52 polymorphic sites which led to 15 haplotypes within the samples of infected mosquitoes and macaques in Malaysia. High haplotype diversity and low nucleotide diversity were observed in LL (Hd=1.0±0.096, π=0.01±0.0017), TD (Hd=0.810±0.130, π=0.0032±0.00071) and peninsular Malaysia (Hd=0.889±0.075, π=0.00581±0.00134) populations. Statistical analysis using Tajima D Fu and Li D*, and Fu and Li F* indicated population of *P. cynomolgi* in Malaysia were expanding though it is not significant (Table 5.15). Analysis on pairwise mismatch distribution revealed a unimodal shape distribution suggesting an expanding population of *P. cynomolgi* (Figure 5.4C).

Table 5.14: Genetic characteristics of the simian *Plasmodium* populations.

Plasmodium species	Location	No. sequences	No. haplotypes	No. polymorphic sites	Haplotype diversity ±SD	Nucleotide diversity ±SD	Average number of nucleotide differences, k
P. knowlesi	Peninsular Malaysia						
	MaPen	10	10	29	1.0±0.045	0.00680±0.00067	6.82222
	Malaysian Borneo						
	BalLL	10	10	29	1.0±0.045	0.00609±0.00069	6.11111
	BalTD	2	2	10	1.0±0.500	0.0100±0.00502	10.00000
	BalKP	9	9	16	1.0±0.052	0.00433±0.00050	4.33333
	MaBor	22	12	30	0.935±0.029	0.00492±0.00163	5.35931
	HuBor	12	10	8	0.970±0.044	0.00309±0.00047	2.37879
	Overall	65	49	107	0.975±0.011	0.00528±0.00067	5.24519
P. inui	Peninsular Malaysia						
	MaPen	5	2	1	0.400±0.237	0.00040±0.00024	0.40000
	Malaysian Borneo						
	BalLL	15	5	5	0.714±0.081	0.00107±0.00027	1.06667
	BalTD	16	3	2	0.425±0.133	0.00045±0.00016	0.45000
	BalKP	15	5	6	0.838±0.049	0.00211±0.00024	2.09524
	MaBor	15	7	18	0.657±0.0138	0.00288±0.00117	2.85714
	Overall	66	18	31	0.738±0.056	0.00170±0.00035	1.67879
	Others						
	MaThai	12	11	47	0.985±0.04	0.011±0.003	10.95455
	MaWan	9	9	22	1.000±0.052	0.0049±0.001	4.88889

[1]BalLL, *An. balabacensis* haplotypes from Limbuak Laut; BalTD, *An. balabacensis* haplotypes from Timbang Dayang; BalKP, *An. balabacensis* haplotypes from Kg Paradason; MaBor, macaque haplotypes from Malaysian Borneo; HuBor, human haplotypes fromMalaysian Borneo; MaPen, human haplotypes from Peninsular Malaysia; MaThai, macaque haplotypes from Thailand; MaWan, macaque haplotypes from Taiwan.

Table 5.14, continued.

Plasmodium species	Location	No. sequences	No. haplotypes	No. polymorphic sites	Haplotype diversity ±SD	Nucleotide diversity ±SD	Average number of nucleotide differences, k
P. cynomolgi	Peninsular Malaysia						
	MaPen	10	6	23	0.889±0.075	0.00581±0.00134	5.64444
	Malaysian Borneo						
	BalLL	6	6	22	1.00±0.096	0.010±0.0017	10.53333
	BalTD	7	4	8	0.810±0.130	0.0032±0.00071	3.14286
	Overall	23	15	52	0.953±0.027	0.0080±0.00114	8.08696

[1]BalLL, *An. balabacensis* haplotypes from Limbuak Laut; BalTD, *An. balabacensis* haplotypes from Timbang Dayang; BalKP, *An. balabacensis* haplotypes from Kg Paradason; MaBor, macaque haplotypes from Malaysian Borneo; HuBor, human haplotypes fromMalaysian Borneo; MaPen, human haplotypes from Peninsular Malaysia; MaThai, macaque haplotypes from Thailand; MaWan, macaque haplotypes from Taiwan.

Table 5.15: Results of statistical testing for neutrality of simian *Plasmodium* populations.

***Plasmodium* species**	**Location**	**Tajima D**	**Fu and Li D***	**Fu and Li F***
P. knowlesi	Peninsular Malaysia			
	MaPen	-1.60	-2.04[5]	-2.18
	Malaysian Borneo			
	BalLL	-1.936[5]	-2.18[5]	-2.39[5]
	BalTD	NA	NA	NA
	BalKP	-1.27	-1.48	-1.60
	MaBor	-1.76	-2.22	-2.24
	HuBor	-0.42	-0.24	-0.08
	Overall (Malaysia)	-2.62[2]	-6.23[4]	-5.78[4]
P. inui	Peninsular Malaysia			
	MaPen	-0.81	-0.81	-0.77
	Malaysian Borneo			
	BalLL	-1.03	-1.74	-1.78
	BalTD	-0.64	-0.50	-0.61
	BalKP	0.47	1.21	1.21
	MaBor	-1.96[4]	-2.09	-2.36
	Overall (Malaysia)	-2.38[3]	-3.26[5]	-3.50[4]
	MaThai	-1.48	-0.95	-1.24
	MaWan	-1.95[3]	-2.23[4]	-2.42[4]
P. cynomolgi	Peninsular Malaysia			
	MaPen	-1.44	-1.38	-1.58
	Malaysian Borneo			
	BalLL	0.194	-0.064	-0.010
	BalTD	-0.197	-0.47	-0.44
	Overall (Malaysia)	-1.73	-2.30	-2.49

[1]BalLL, *An. balabacensis* haplotypes from Limbuak Laut; BalTD, *An. balabacensis* haplotypes from Timbang Dayang; BalKP, *An. balabacensis* haplotypes from Kg Paradason; MaBor, macaque haplotypes from Malaysian Borneo; HuBor, human haplotypes from Malaysian Borneo; MaPen, macaque haplotypes from Peninsular Malaysia; MaThai, macaque haplotypes from Thailand; MaWan, macaque haplotypes from Taiwan.

[2]$p<0.001$

[3]$p<0.01$

[4]$p<0.02$

[5]$p<0.05$

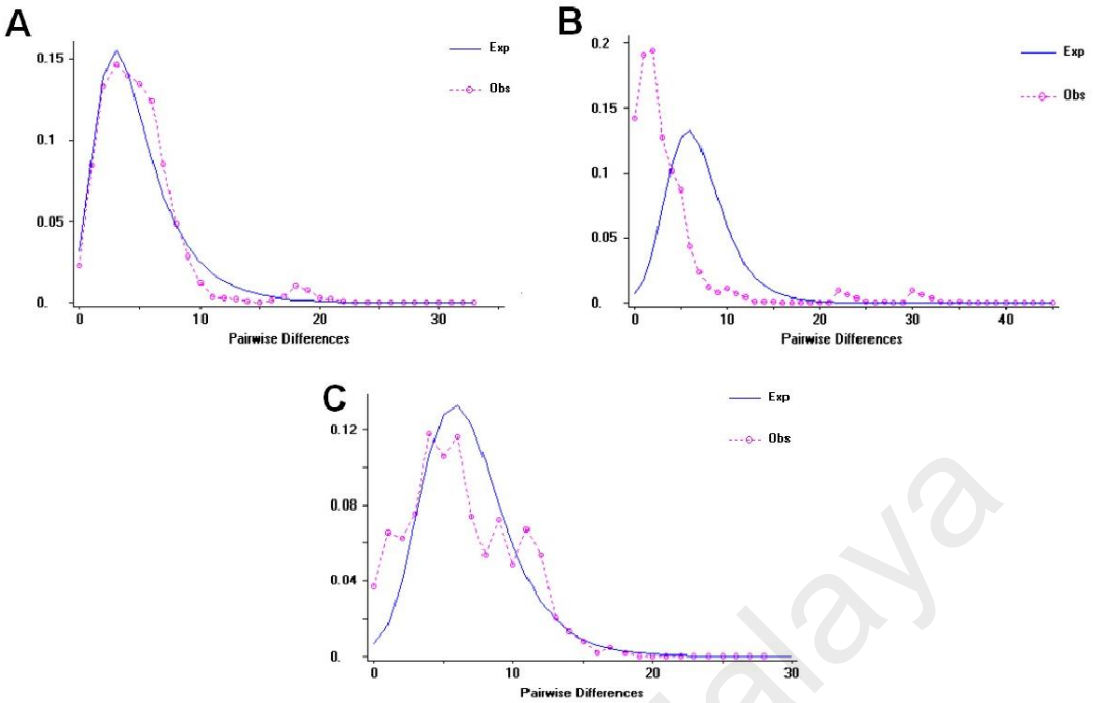

Figure 5.4: Pairwise mismatch distribution of *P. knowlesi, P. inui* and *P. cynomolgi* parasite populations, Malaysia. A) *P. knowlesi* A-type 18S rRNA B) *P. inui* A-type 18S rRNA C) *P. cynomolgi* A-type 18S rRNA. Red dotted lines represent the observed frequencies of the pairwise differences; blue lines represent the expected curve for a population that has undergone a demographic expansion.

5.3.4 Haplotype network

5.3.4.1 *P. knowlesi*

The network tree for the *PkA-type 18S rRNA* haplotypes also showed "star-like" networks suggesting a population expansion (Figure 5.5). There are three haplotypes shared by humans and macaques within the Malaysian Borneo clusters (in Malaysian Borneo, haplotype 27: n=3, human=1, macaque=2; haplotype 28: n=5, human=1, macaque=4; haplotype 29: n=9, human=4, macaque=5). The haplotypes derived from *An. balabacensis* sample (LL and KP) were closer to the shared haplotypes from macaque and human samples from Sarawak (H28 and H29). The two haplotypes obtained from *An. balabacensis* sample from TD (H8 and H9) were clustered along with the human haplotypes from Sarawak. *Plasmodium knowlesi* populations of the macaque originating from Peninsular Malaysia were clustered distantly from the Malaysian Borneo clusters but seem closely related to a shared haplotype (H28) from Malaysian Borneo. The polymorphic sites corresponding to each of the haplotypes were illustrated in Figure 5.6.

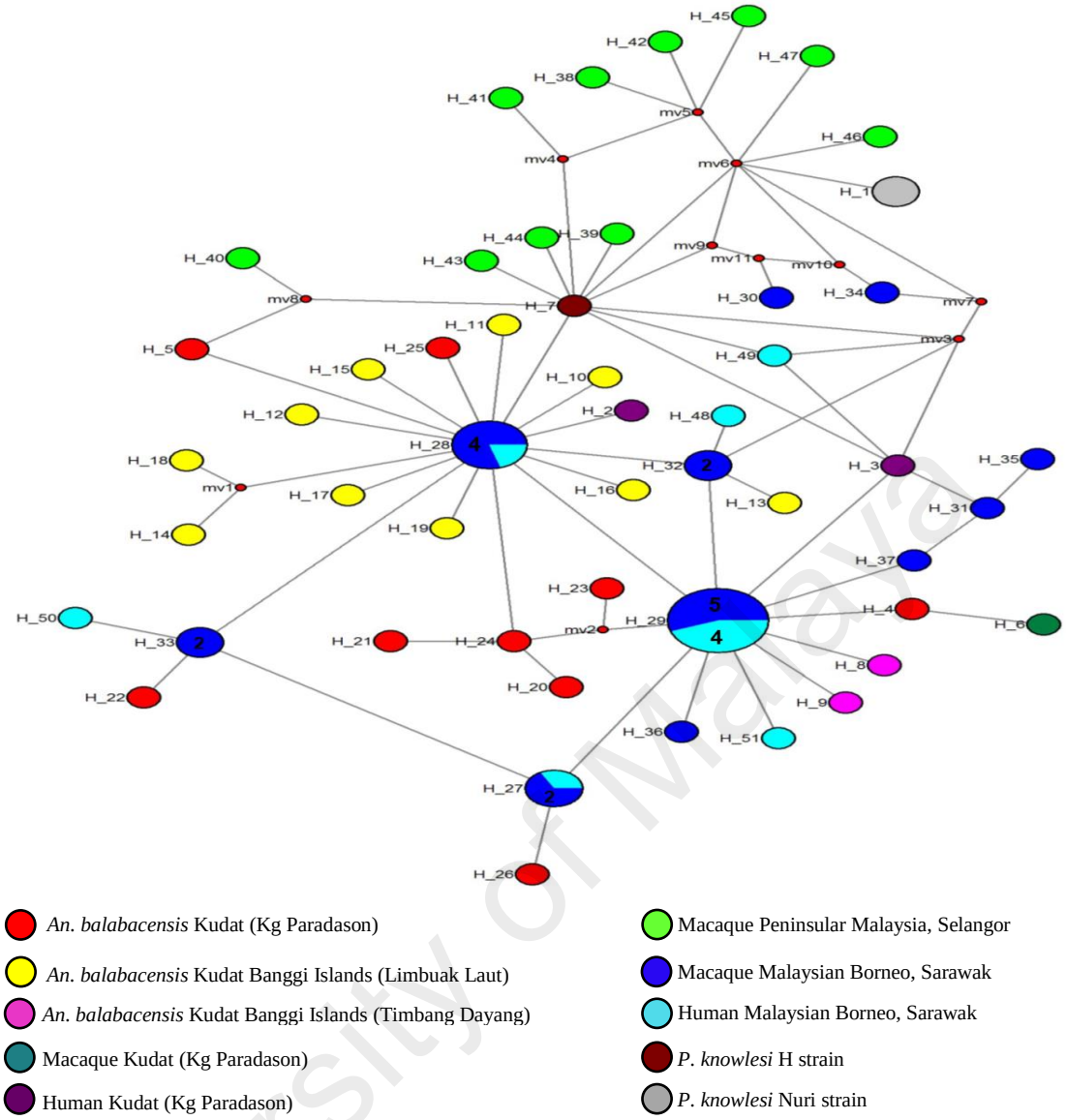

Figure 5.5: Median-joining networks of *P. knowlesi* type A small subunit ribosomal 18S RNA haplotypes from Malaysia. The genealogical haplotype network shows the relationships among the 51 haplotypes present in the 68 sequences obtained from mosquito (*An. balabacensis*), macaques and humans samples from peninsular Malaysia and Malaysian Borneo. Each distinct haplotype has been designated a number (H_n). Circle sizes represent the frequencies of the corresponding haplotype (the number is indicated for those that were observed >1x). Small red nodes are hypothetical median vectors created by the program to connect sampled haplotypes into a parsimonious network. Distances between nodes are arbitrary.

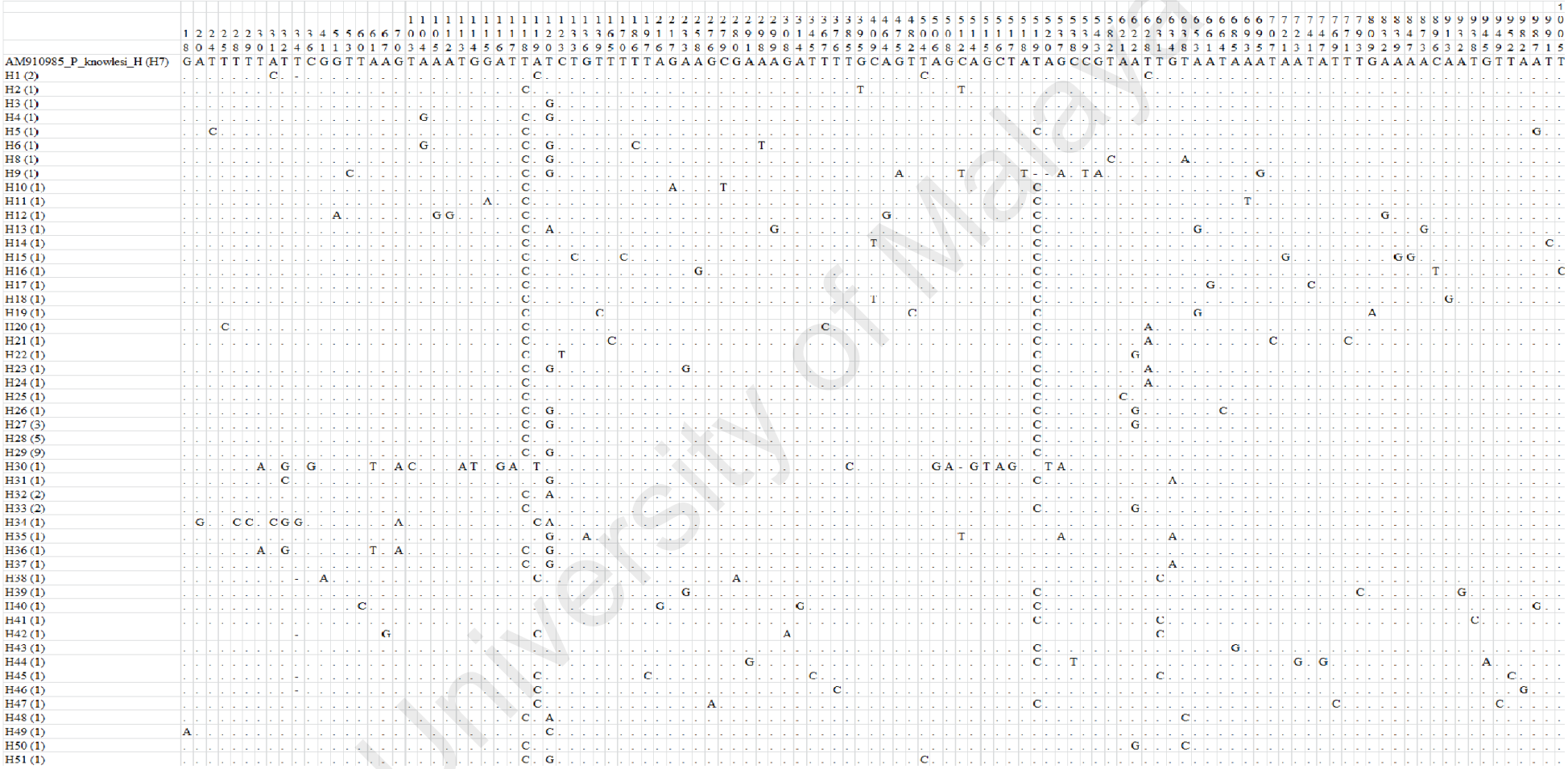

Figure 5.6: Polymorphic regions of *PkA-type 18S rRNA* haplotype.

5.3.4.2 *P. inui*

The network tree for *PinA-type 18S rRNA* genes showed the macaques from Malaysia and infected *An. balabacensis* samples from Kudat shared the most dominant haplotype (H3) (n=33: macaque=13; *An. balabacensis*=20) (Figure 5.7). There are four haplotypes of *P. inui* infected *An. balabacensis* samples from LL (H11, H6, H12 and H7) which are distinctly different from those in H3. In addition, 2 haplotypes from TD (H8 and H9) and 4 haplotypes from KP (H10, H1, H2 and H4) are clustered with H3 displaying the "star-like" network. In the network, the Malaysian samples are clustered separately from the Thailand (H30-H38) and Taiwan (H21-H29) clusters revealing they are distinctly different populations. The polymorphic sites corresponding to each of the haplotypes were illustrated in Figure 5.8.

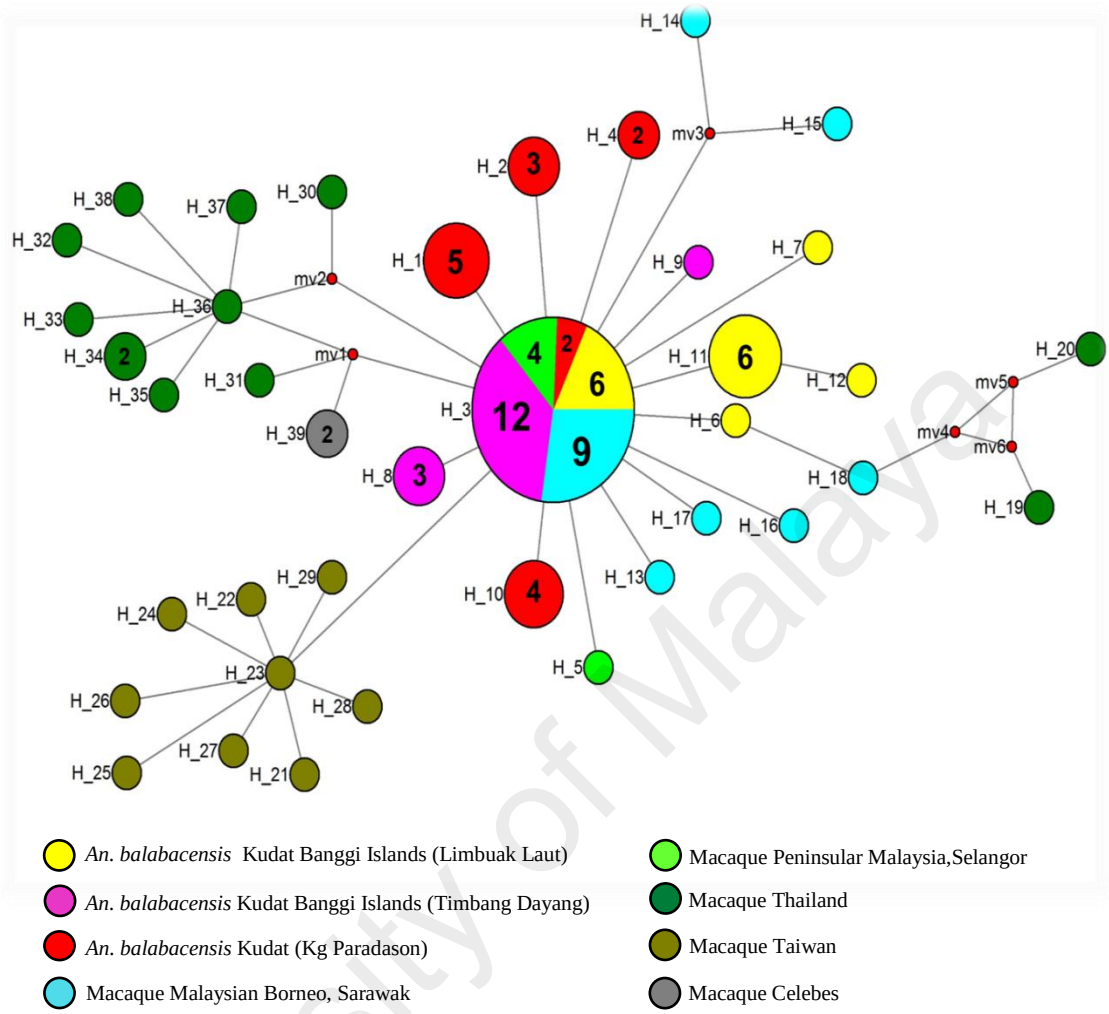

Figure 5.7: Median-joining networks of *P. inui* type A small subunit ribosomal 18S RNA haplotypes from Malaysia. The genealogical haplotype network shows the relationships among the 39 haplotypes present in the 89 sequences obtained from mosquito (*An. balabacensis*), macaques and humans samples from peninsular Malaysia and Malaysian Borneo. Each distinct haplotype has been designated a number (H_n). Circle sizes represent the frequencies of the corresponding haplotype (the number is indicated for those that were observed >1x). Small red nodes are hypothetical median vectors created by the program to connect sampled haplotypes into a parsimonious network. Distances between nodes are arbitrary.

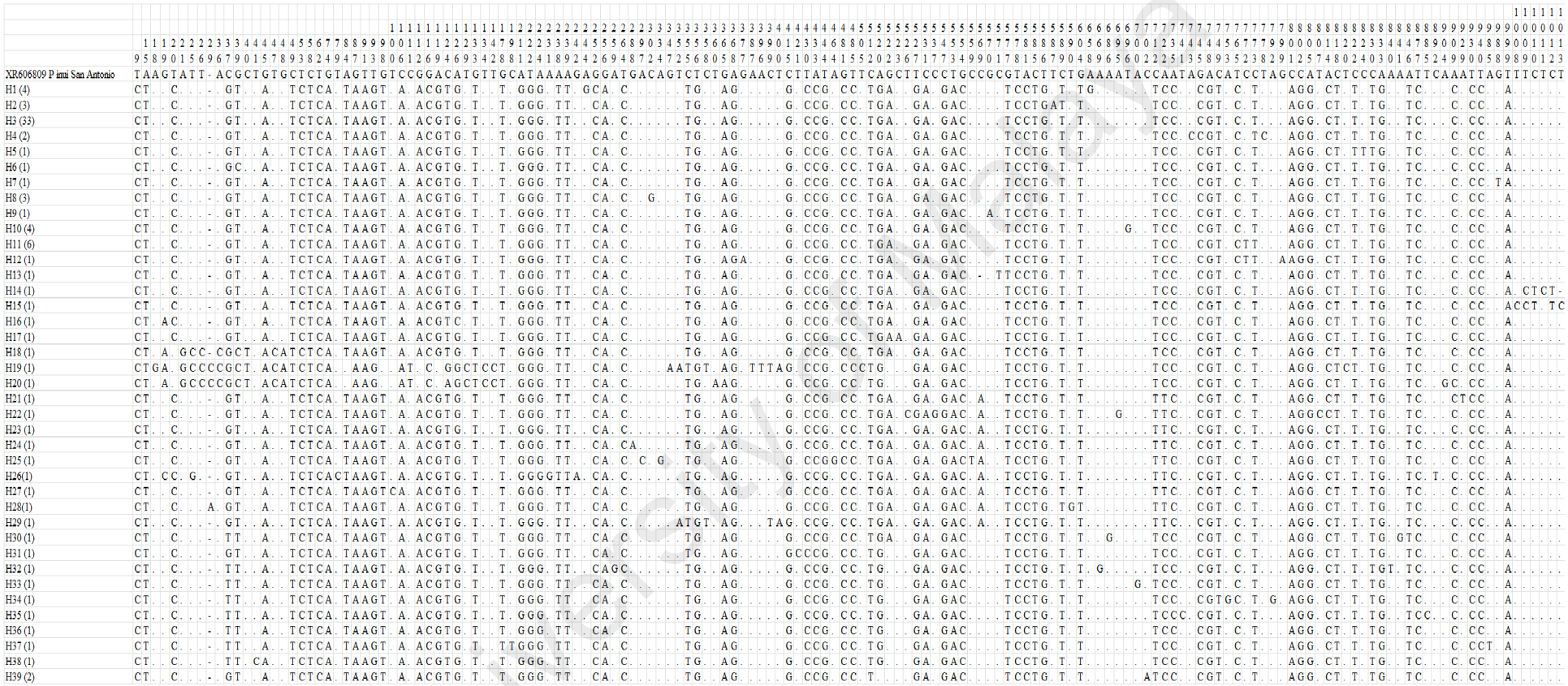

Figure 5.8: Polymorphic regions of *PinA-type 18S rRNA* haplotypes.

5.3.4.3 *P. cynomolgi*

The network of *PcyA-type 18S rRNA* showed geographical clustering of the macaque and mosquito haplotypes (Figure 5.9). The macaque haplotypes from Peninsular Malaysia formed a separate cluster along with the Berok strain while mosquito haplotypes formed a distant and diverse haplotype cluster. *Anopheles balabacensis* derived samples from LL and TD shared the same haplotype (H6). The Mulligan strain shared haplotypes with LL. The macaque parasite population from peninsular Malaysia is distinctly different from the *An. balabacensis* parasite population from Kudat, Sabah.

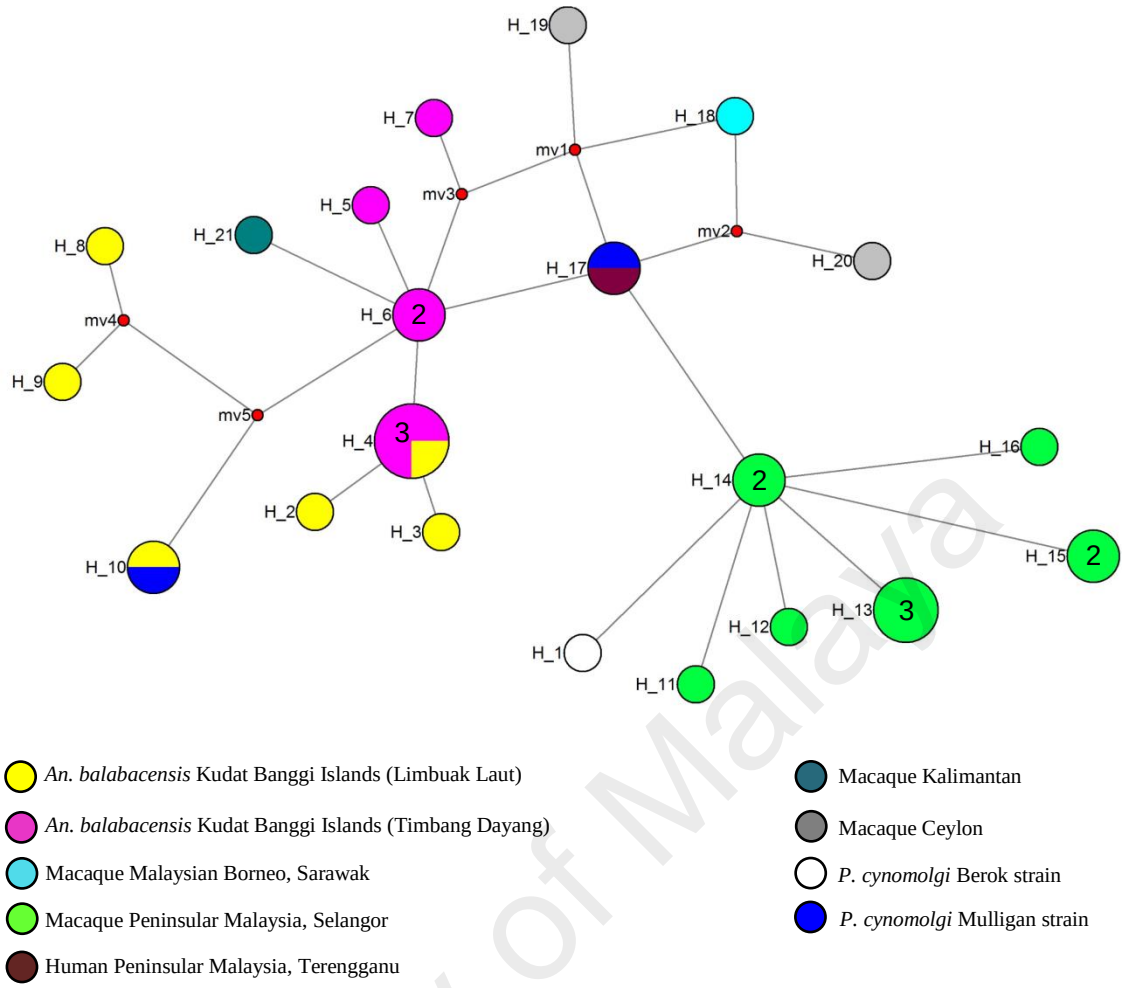

Figure 5.9: Median-joining networks of *P. cynomolgi* type A small subunit ribosomal 18S RNA haplotypes from Malaysia. The genealogical haplotype network shows the relationships among the 21 haplotypes present in the 34 sequences obtained from mosquito (*An. balabacensis*), macaques and humans samples from peninsular Malaysia and Malaysian Borneo. Each distinct haplotype has been designated a number (H_n). Circle sizes represent the frequencies of the corresponding haplotype (the number is indicated for those that were observed >1x). Small red nodes are hypothetical median vectors created by the program to connect sampled haplotypes into a parsimonious network. Distances between nodes are arbitrary.

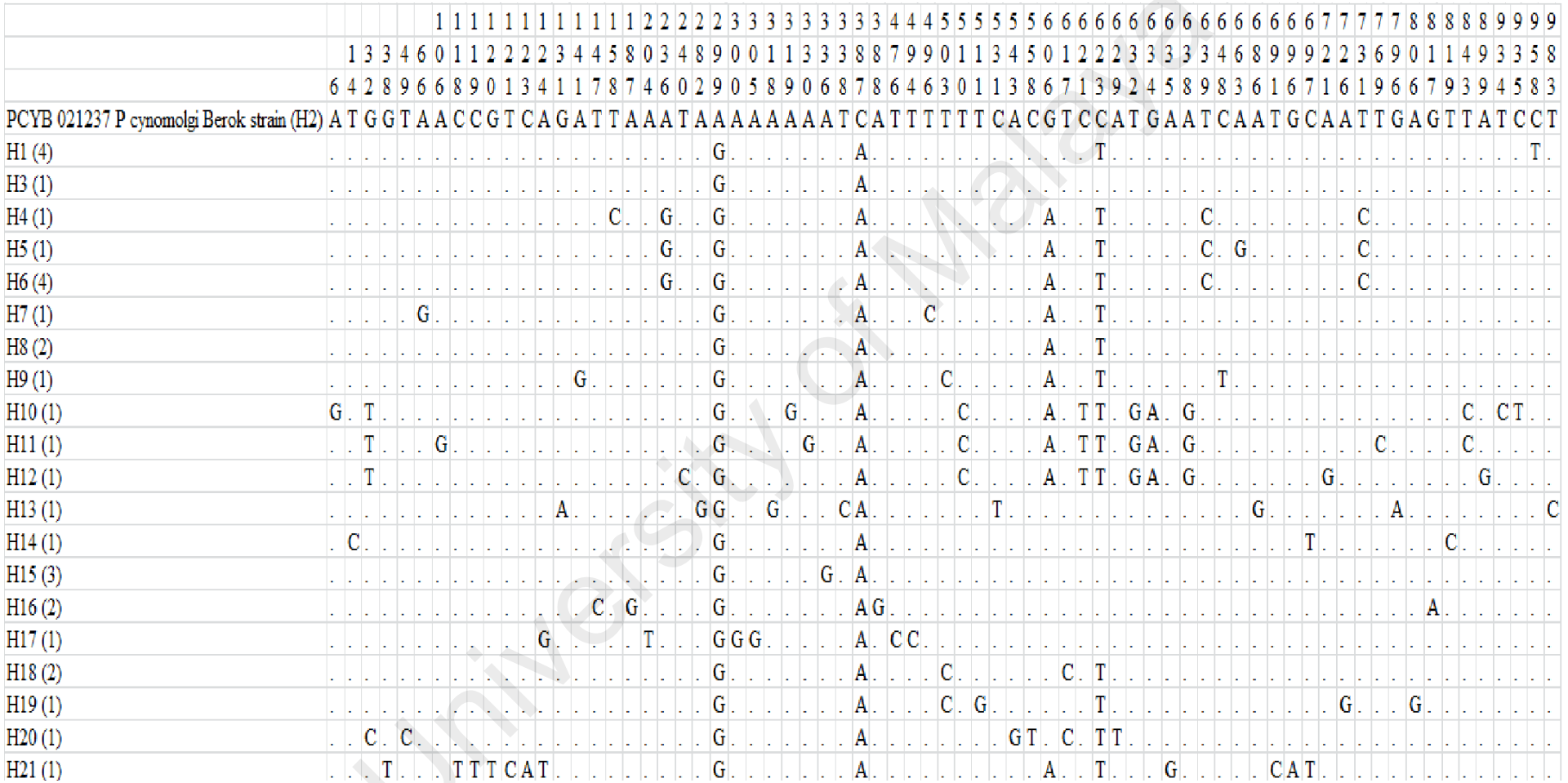

Figure 5.10: Polymorphic regions of *PcyA-type 18S rRNA* haplotypes.

5.3.5 Population structure

A Bayesian admixture model in STRUCTURE was utilized to calculate the potential number of *P. knowlesi, P. inui* and *P. cynomolgi* parasite populations in infected *An. balabacensis* within Kudat, Sabah, Malaysia. K values from 1 to 8 were used for the analysis. The estimation of pairwise F_{ST} values was also performed using ARLEQUIN software to determine to what extent population differentiation exists within *P. knowlesi, P. inui* and *P. cynomolgi* in Malaysia on the basis of its host , *An. balabacensis*.

5.3.5.1 *P. knowlesi*

Significant genetic structure was found when K=2 (ΔK=115.673) (Figure 5.11) indicating two sub-populations of *P. knowlesi* within the mosquito samples from 3 different regions of Sabah. The analysis of pair-wise F_{ST} values showed moderate population differentiation where F_{ST} value was 0.062 (between KP and LL populations) (Table 5.16). High genetic differentiation was observed between LL and TD populations (F_{ST}=0.198) and between KP and TD populations (F_{ST}=0.291). These values indicated that there were at least two sub-populations of *P. knowlesi* in the mosquitoes.

Inter population differentiation between mosquitoes and macaques showed high population differentiation F_{ST} values (>0.18, p=0.001-0.05) for infected macaque with *P. knowlesi* in Peninsular Malaysia and *P. knowlesi* infected *An. balabacensis* from Kudat. Similar results were obtained for comparisons of Peninsular Malaysia populations (F_{ST}=0.2-0.3, p<0.001) indicating geographical distance is limiting gene flow.

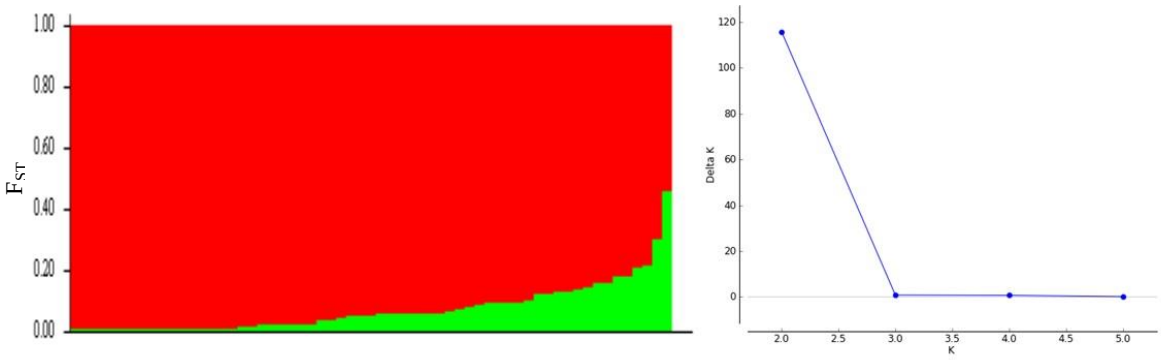

Figure 5.11: Most likely number of *P. knowlesi* parasite subpopulations haplotype clusters (K) from *An. balabacensis* (K=2, ΔK=115.673). Relationships were determined by using Bayesian model-based STRUCTURE version 2.3.4 software (The Pritchard Laboratory, Stanford University, Stanford, CA, USA) including comparison of K and ΔK (=mean (| L”(K) |)/sd(L(K)).

Table 5.16: F_{ST} results for pairwise population comparisons of *Plasmodium knowlesi* parasite populations and associated significance, Malaysia[‡and§].

Gene and location	Haplotype	F_{ST} values					
		BalKP	**BalLL**	**BalTD**	**MaPen**	**MaBor**	**HuBor**
PkA-type 18S rRNA							
Mainland Kudat, Sabah	BalKP	NA	§	§	‡	§	§
Banggi Island, Kudat	BalLL	0.062	NA	§	‡	‡	‡
	BalTD	0.291	0.198	NA	§	NS	§
Peninsular Malaysia	MaPen	0.242	0.187	0.319	NA	‡	‡
Sarawak	MaBor	0.093	0.094	0.265	0.236	NA	NS
	HuBor	0.163	0.124	0.419	0.319	-0.008	NA

[1]BalKP, *An. balabacensis* haplotypes from Kg Paradason; BalLL, *An. balabacensis* haplotypes from Limbuak Laut; BalTD, *An. balabacensis* haplotypes from Timbang Dayang; MaPen, macaque haplotypes from Peninsular Malaysia; MaBor, macaque haplotypes from Malaysian Borneo; HuBor, human haplotypes from Malaysian Borneo, NA, not applicable; NS, not significant; *PkA-type 18S rRNA*, *P. knowlesi* type A small subunit ribosomal 18S RNA.

[2]ARLEQUIN (University of Berne, Berne, Switzerland) software package version 3.5.1.3 was used to compute pairwise differences between populations (i.e., mosquitoes, macaques and humans from Peninsular Malaysia, Malaysian Borneo and Kudat division Sabah).

[‡]$p<0.001$; p values computed with 10,100 permutations.

[§]$p<0.05$.

5.3.5.2 *P. inui*

Population structure analysis of *P. inui* also showed two sub-populations when K=2 (ΔK=49.76) (Figure 5.12). The analysis of pair-wise F_{ST} values showed high population differentiation values for populations of infected *An. balabacensis* in Kudat, ranging from 0.165 (between KP and TD populations) to 0.209 (between KP and LL populations) and 0.232 (between LL and TD populations) (Table 5.17). Comparisons of populations from peninsular Malaysia with those in Kudat revealed moderate population differentiation F_{ST} values (0.04-0.142) but not significant. High F_{ST} values were obtained for comparisons between Malaysian populations and Thailand (>0.2), and between Malaysia and Taiwan populations (>0.19) which indicated distance playing a role.

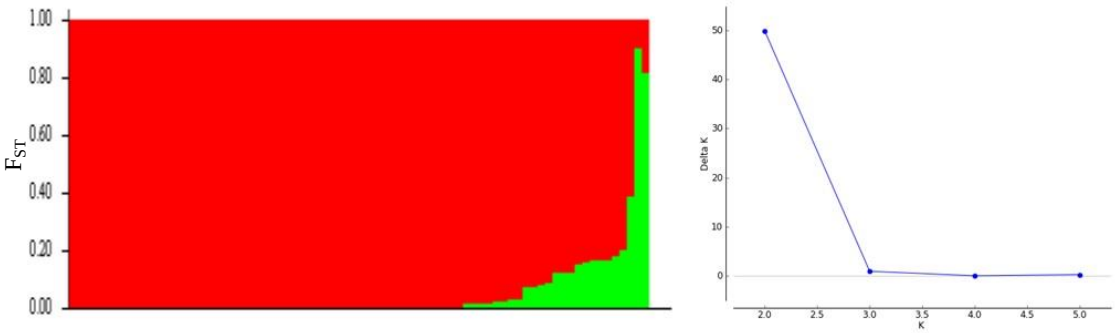

Figure 5.12: Most likely number of *P. inui* parasite subpopulations haplotype clusters (K) from *An. balabacensis* (K=2, ΔK=49.76). Relationships were determined by using Bayesian model-based STRUCTURE version 2.3.4 software (The Pritchard Laboratory, Stanford University, Stanford, CA, USA) including comparison of K and ΔK (=mean (| L"(K) |)/sd(L(K)).

Table 5.17: F_{ST} results for pairwise population comparisons of *Plasmodium inui* parasite populations and associated significance[‡and§].

Gene and location	Haplotype	F_{ST} values						
		BalKP	**BalLL**	**BalTD**	**MaPen**	**MaBor**	**MaThai**	**MaWan**
PinA-type 18S rRNA								
Mainland Kudat	BalKP	NA	‡	‡	NS	‡	‡	‡
Banggi Island, Kudat	BalLL	0.209	NA	‡	NS	§	‡	‡
	BalTD	0.165	0.232	NA	NS	§	‡	‡
Peninsular Malaysia	MaPen	0.054	0.142	0.046	NA	NS	NS	‡
Malaysian Borneo	MaBor	0.094	0.104	0.040	-0.061	NA	‡	‡
Thailand	MaThai	0.248	0.264	0.271	0.112	0.194	NA	‡
Taiwan	MaWan	0.292	0.344	0.363	0.187	0.228	0.228	NA

[1]BalKP, *An. balabacensis* haplotypes from Kg Paradason; BalLL, *An. balabacensis* haplotypes from Limbuak Laut; BalTD, *An. balabacensis* haplotypes from Timbang Dayang; MaPen, macaque haplotypes from Peninsular Malaysia; MaBor, macaque haplotypes from Malaysian Borneo; MaThai, macaque haplotypes from Thailand; MaWan, macaque haplotypes from Taiwan; NA, not applicable; NS, not significant; *PinA-type 18S rRNA*, *P. inui* type A small subunit ribosomal 18S RNA.

[2]ARLEQUIN (University of Berne, Berne, Switzerland) software package version 3.5.1.3 was used to compute pairwise differences between populations (i.e., mosquitoes, macaques and humans from Peninsular Malaysia, Malaysian Borneo and Kudat division Sabah).

[‡]$p<0.001$; p values computed with 10,100 permutations.

[§]$p<0.05$.

5.3.5.3 *P. cynomolgi*

Population structure analysis of *P. cynomolgi* populations in mosquitoes using STRUCTURE revealed significant genetic structure when K=2 (ΔK=13.58) (Figure 5.13) indicating two sub-populations within the mosquitoes. Comparisons of populations between LL and TD revealed moderate population differentiation though it is not significant (F_{ST}=0.149). The F_{ST} values were ranged from 0.35 (between LL and peninsular Malaysia populations) to 0.36 (between TD and peninsular Malaysia populations) (Table 5.18) suggesting high population differentiation.

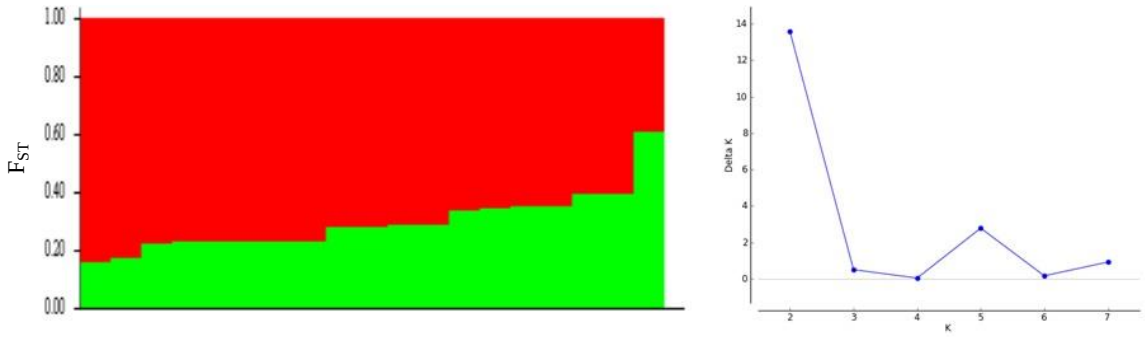

Figure 5.13: Most likely number of *P. cynomolgi* parasite subpopulation haplotype clusters (K) from *An. balabacensis* (K=2, ΔK=13.58). Relationships were determined by using Bayesian model-based STRUCTURE version 2.3.4 software (The Pritchard Laboratory, Stanford University, Stanford, CA, USA) including comparison of K and ΔK (=mean (│L"(K)│)/sd(L(K)).

Table 5.18: F_{ST} results for pairwise population comparisons of *Plasmodium cynomolgi* parasite populations and associated significance[‡and§].

Gene and location	Haplotype	F_{ST} values		
		BalLL	BalTD	MaPen
PcyA-type 18S rRNA				
Banggi Island, Kudat	BalLL	NA	NS	‡
	BalTD	0.149	NA	‡
Peninsular Malaysia	MaPen	0.369	0.354	NA

[1]BalLL, *An. balabacensis* haplotypes from Limbuak Laut; BalTD, *An. balabacensis* haplotypes from Timbang Dayang; MaPen, macaque haplotypes from Peninsular Malaysia; NA, not applicable; NS, not significant; *PinA-type 18S rRNA*, *P. inui* type A small subunit ribosomal 18S RNA.

[2]ARLEQUIN (University of Berne, Berne, Switzerland) software package version 3.5.1.3 was used to compute pairwise differences between populations (i.e., mosquitoes, macaques and humans from Peninsular Malaysia, Malaysian Borneo and Kudat division Sabah).

[‡]$p<0.001$; p values computed with 10,100 permutations.

[§]$p<0.05$.

5.4 Discussion

The results of the various analyses conducted on the *P. knowlesi, P. inui* and *P. cynomolgi* parasites collected from Kudat, Sabah (*An. balabacensis*) suggest that all the three parasites population were genetically distinct from each other. Phylogenetic analysis of all the sequences from the three species formed distinct groups with strong bootstrap values. Population wise analysis indicated that nucleotide diversity low for all the three species. However, for *P. knowlesi,* haplotype diversity also known as gene diversity is the probability that two random sequences are different (Rozas, 2009) and a measure of species evenness was high for infected *An. balabacensis* isolates from LL (Hd=1.0±0.045), KP (Hd=1.0±0.052), TD (Hd=1.0±0.50) and infected macaque isolate from peninsular Malaysia (Hd=1.0±0.045) for *P. knowlesi.* Similar results were also observed for isolates infected with *P. inui* and *P. cynomolgi* from Kudat and peninsular Malaysia. The high haplotype diversity among the infected *An. balabacensis* isolates of *P. knowlesi* and *P. cynomolgi* may indicate high parasite transmission in these regions over long periods. Similar high haplotype diversity values have been reported for *P. knowlesi* csp gene from Sarawak (Lee et al., 2011) and *P. cynomolgi* reticulocyte binding protein (RBP) and Duffy-binding protein (DBP) gene (Sutton et al., 2016). The haplotype diversity was highest for *P. knowlesi* followed by *P. cynomolgi* and lowest in *P. inui.* Similar high haplotype diversity was observed in a recent study of *P. knowlesi* from macaque as well as humans from Malaysian Borneo and Peninsular Malaysia (Yusof et al., 2016). This may be due to the fact that these two parasites, *P. knowlesi* and *P. cynomolgi* are able to invade humans and there is always transmission going on from monkeys to humans via the *Anopheles* mosquito. It is obvious that large number of knowlesi malaria has been reported in humans but currently only a single case of *P. cynomolgi* has been reported (Ta et al., 2014). Perhaps people are asymptomatic and have not been screened. Nevertheless, the nucleotide diversity (π) which are defined as

average number of nucleotide differences per site between two sequences (Rozas, 2009) were low for *P. knowlesi, P. inui* and *P. cynomolgi* isolates, regardless of the isolates' geographic or host origins.

In order to observe clearly the DNA sequence variation in *P. knowlesi, P. inui* and *P. cynomolgi* populations, the sequences of each respective simian parasite species were subjected to a median-joining network analysis of haplotypes. The three haplotype networks generated individually for *P. knowlesi, P. inui* and *P. cynomolgi* showed an excess of unique infected *An. balabacensis* 18S rRNA haplotypes around the dominant haplotypes resulting in a "star-like"structure (Figure 5.5, 5.7 and 5.9). The shared dominant haplotype, H3 between mosquito (*An. balabacensis*) and macaques in the *P. inui* network suggested evidence of parasite transmission from one species to another. Meanwhile the clustering of haplotypes of infected *An. balabacensis* along with the haplotypes shared between humans and macaques coupled with their high haplotype diversity in *P. knowlesi* may indicate that there is possibility of transmission between mosquitoes, humans and macaques given that Sabah has undergone extensive ecological changes resulting from conversion of forest to oil palm plantations over the years (Bryan et al., 2013; Fornace et al., 2014; Hansen et al., 2013). Population genetic analysis using Tajima's D, Fu and Li's D*, and Fu and Li's F* values also indicated that each parasite population is undergoing expansion and this can beattributed to the high haplotype diversity. Significant negative values for each of these tested parameters showed that same trend (*P. knowlesi*: -2.62 to -6.23, p= 0.001-0.02). From this study and others (Brant et al., 2016; Manin et al., 2016), it is obvious *An. balabacensis* has become the predominant species comprising more than 90% of the population due to environmental changes. It is known that malaria parasite infectivity and *Anopheles* susceptibility are environment dependent (Tripet et al., 2008). Certain parasite genotypes are more successful than others and these could also change when host and

parasites are exposed to different environment (Wolinska & King, 2009). A recent study has shown that vector related genes points towards a strong evolutionary pressure on the parasite driven by the *Anopheles* species (Benavente et al., 2017). This is also shown in this study where there is higher haplotype number as well as haplotype diversity values in mosquitoes from different sites compared to macaques and humans.

Significant genetic differentiations among all three populations (LL, TD and KP) of *P. knowlesi* (F_{ST}=0062-0.291) and *P. inui* (F_{ST}=0.165-0.232) from Kudat suggested that they are distinct genetic populations infecting *An. balabacensis*. Both LL and TD were located on Banggi Island with geographical distance more than 10km where the former is nearer to the coastal and the latter is nearer to inland, and they are distinguishable from each other. This might be possible if one experienced a more preceding immigration to the region and with enough relapse of time caused the both of them to be different. Additionally, the high population differentiation between these two population of parasites maybe attributed to the ecological differences as well as the narrow range of dispersion of its vector (Hii & Vun, 1985). Perhaps the macaques close to the coastal area of LL do not migrate inland to TD which is a farming site. Collaring of maaques followin their movement will shed more light on this issue. Recent genomic study from Malaysian Borneo using clinical samples also identified two *P. knowlesi* sub-populations and co-existence, and independent transmission in the same geographical region (Assefa et al., 2015)

Coadaptation of parasites and vectors (Cohuet et al., 2006) affects a mosquito's susceptibility to malaria infection and capacity to transmit the disease (Alavi et al., 2003; Billingsley & Sinden, 1997). For a successful transmission to human, the malaria parasite will have to overcome the defense mechanisms within the mosquito vector and completes its developmental stage. Hence, the mechansim in which a parasite is co-adapted with its host is attributed to the selection pressure and evolutionary potential it

endured (Brandt et al., 2007; Gandon & Michalakis, 2002). In this study, we proposed that distinct *P. knowlesi, P. inui* and *P. cynomolgi* genetic populations in Kudat had been infecting the local occurring predominant mosquito vector, *An. balabacensis*. It is important to analyze more of these parasites from humans and macaques in Sabah. It will gave a bigger picture of what is actually ongoing. This is just a start with the parasites from the mosquito and further work may unveil new evidences.

5.5 Conclusion

In conclusion, the present novel study reveals a high level of haplotype diversity and low nucleotide diversity in the A-type 18S rRNA gene of *P. knowlesi, P. inui* and *P. cynomolgi* infected isolates of *An. balabacensis.* It can be attributed that there are distinct genetic populations of these three simian parasites in Kudat in which evolution and distribution has been shaped by past and recent events. It has been shown that there is a close relatedness between simian malaria parasites from *An. balabacensis* and the host be it humans or macaques from Kudat, Sabah although only few samples were available (Chua et al., 2017). Further analyses using larger number of samples from disparate geographical locations as well as from different Leucosphyrus Group of *Anopheles* should be performed in order to clearly understand the phylogeographic distribution and population structure of the parasites which will provide a more comprehensive structure. These simian parasites pose a threat to the malaria elimination program as they can be transmitted to human. Thus more investigations on the epidemiologic and biology of the parasite will aid in devising an effective control program.

CHAPTER 6 CONCLUSION

Plasmodium knowlesi, a simian malaria parasite is the current major cause of human malaria posing as a new public health problem due to its natural existence as a zoonotic reservoir within its primates' host. Of late, there have been reported cases of *P. knowlesi* in Aceh and Northern Sumatera (Indonesia) which were asymptomatic (Herdiana et al., 2016; Lubis et al., 2017). The numbers are alarming and thus it is timely that concerted effort should be established towards recognizing that *P. knowlesi* is a public health threat.

Notably, in Sabah, increasing incidences of knowlesi malaria have been reported. From 2004 to 2011, the reported cases of *P. knowlesi* increased significantly from 59 to 703 (William et al., 2014). Since then, there were increased notifications of knowlesi malaria cases throughout Sabah with the highest proportion of cases reported from Kudat Division (Barber et al., 2011; Cox-Singh et al., 2008; Daneshvar et al., 2009; Joveen-Neoh et al., 2011). Consequently, in Malaysia, the reported cases of knowlesi malaria comprised a majority of 69% of the total malaria cases (Ministry of Health personal communication). Additionally, *P. knowlesi* is life threatening since it is the only simian malaria parasite with 24-hour erythrocytic cycle (Garnham, 1966), and there have been reported mortalities in Sabah where patients succumbed to severe knowlesi malaria infection (Cox-Singh et al., 2008; Galinski & Barnwell, 2009; Rajahram et al., 2012; William et al., 2011). Without doubt, the extensive land change due to agricultural activities has resulted in encroachment of humans into previously forested areas thus increasing the contact between human, the mosquitoes and macaque (simian host) (Bryan et al., 2013; Fornace et al., 2014). The information regarding the vector responsible for the transmission of simian malaria in Kudat, Sabah is scanty though *An. balabacensis* have been incriminated as vector for human malaria (Hii et al.,

1988a) in Banggi Island and *An. donaldi* in the Kinabatangan area (Vythilingam et al., 2005). There is always a misconception that everything is known about *An. balabacensis* as it was the primary vector of human malaria.

This study was designed to determine the spatial dynamics of the *P. knowlesi* vectors in Kudat, Sabah and to conduct molecular characterization of its parasites in order to comprehend the phylogenetic relationship between the parasites in mosquitoes, macaques and humans.

The Leucosphyrus Group of *Anopheles* mosquitoes, also known as the forest-dwelling mosquitoes is the main vector for simian malaria parasite. There are 20 named species and two geographical forms in which 8 species are occurring in Malaysia and divided into three subgroups. Since the Leucosphyrus Group of *Anopheles* mosquitoes is closely related to the epidemiology of simian malaria, a simple morphological key for adults were built based on Jeffery et al. (2012) and Sallum et al. (2005b) to identify the Leucosphyrus Group of mosquitoes present in Malaysia. These keys were developed mainly to help the vector control personnel in Malaysia to identify the mosquitoes in the field as a first step. Molecular studies should then be carried out in established laboratories to determine the exact species. In this study, molecular identification using three different gene markers (mtCOI, 18S and ITS2) were used to precisely identify the mosquitoes caught from the sampling site.

From the restriction digestion of amplified ITS2 product, the five species of mosquitoes in the Leucosphyrus Group obtained could be differentiated as they have specific banding patterns using restriction enzyme, *Msp* I which recognized the motif CCGG. . By utilizing mtCOI, ITS2 and 18S gene markers, the five species of the Leucopshyrus Group of *Anopheles* collected in the study was grouped accordingly in their specific clade though *An. balabacensis* clade were placed as sister to the Dirus

Complex instead of within the Leucosphyrus Complex clade. Out of the three markers used to resolve the phylogenetic relationships, mtCOI ML topology was able to show and support the clustering of the species in the Leucosphyrus Group of *Anopheles* collected in this research since mtCOI is maternally inherited, it is fast evolving and able to provide information on the presence of divergent lineages.

Nonetheless, the results obtained from this study do not fully resolve the relationships within the members in the Leucosphyrus Group of *Anopheles*. In future, there should be molecular studies such as designation of a multiplex PCR for Leucosphyrus Complex like what has been done for Dirus Complex since the members of Leucosphyrus Complex play important role as vectors in simian malaria transmission (Tan et al., 2008; Vythilingam et al., 2014; Vythilingam et al., 2006).

In this study, *An. balabacensis* was confirmed as vector of *P. knowlesi* within the foci of human infection in Sabah (Wong et al., 2015) and was the predominant occurring species (95%) found in all sampling sites followed by *An. donaldi* (1.3%). This is in contrast with the study in the 1980s where lower proportion of *An. balabacensis* were caught than *An. donaldi* (Hii & Vun, 1985). Previous work done in the Kinabatangan area also revealed a shift of high proportion of *An. balabacensis* to dominance by *An. donaldi* within the same site from 1980s to 2000 (Hii & Vun, 1985; Vythilingam et al., 2005). This may be caused by extensive deforestation for agricultural activities in Sabah during that period (Bryan et al., 2013; Fornace et al., 2014). Regardless of the dominance of mosquitoes, the high survival and sporozoite rates in *An. balabacensis* obtained in this study combined with the increased contact between human, vector and macaques could probably increase the *P. knowlesi* cases in the area. In addition, since *An. balabacensis* was caught biting between 1800-2000 hours, workers could be exposed when they return from work in or around forested areas. The high survival rate, parity rate and sporozoite rate in *An. balabacensis* coupled

with high vectorial capacity proved that it is a highly competent vector and will continue to be a risk in malaria transmission to human.

In this research, mosquitoes were dissected to extract ovaries, midgut and salivary glands for examination of parity, oocysts and sporozoites. Dissection of mosquitoes to distinguish between infected mosquitoes (oocysts only) and infective mosquitoes (with sporozoites) is the traditional and gold standard method. It is important to distinguish between infected and infective mosquitoes because oocysts rates are considered to be less epidemiologically informative as a measure to determine the potential of a mosquito species in malaria transmission (Foley et al., 2012). Moreover, dissection of mosquitoes to detect oocysts and salivary glands before template preparation for PCR minimizes the amount of PCR inhibitors present (Arez et al., 2000). In this study, 45 mosquitoes were found to be infected with either oocysts (18) or sporozoites (14) or both (13) by microscopy. Dissection requires fresh specimens and some of the specimens which died were too dry and could not be dissected. They were pooled together according to time, month and location for *Plasmodium* detection since it was too expensive to process mosquitoes individually using molecular techniques. A number of pooled mosquitoes were positive but these were not taken into account for calculation of sporozoite rate as the exact number was not known. If all the mosquitoes could be dissected, the sporozoites rates would have been higher.

In this study, all the mosquitoes were collected using human bait and thus reflecting only on the estimation of potential human exposure and not transmission between macaques. However, macaque-baited trap was not possible due to logistical constraints and ethics regulations for working with macaques. In future studies, collections on resting mosquitoes and analysis of blood meal should be performed as

this will aid in comprehending the host preference and choice of the vector and other potential vectors.

This research provides information on the genetic diversity of simian malaria parasites found in the vector, *An. balabacensis* and comparison was made with those found in humans and macaques. From the analysis, there is high level of haplotype diversity but low nucleotide diversity found in the A-type 18S rRNA gene of *P. knowlesi*, *P. inui* and *P. cynomolgi* parasite populations in *An. balabacensis*. Median-joining network analysis of the haplotypes in each respective simian malaria parasites revealed a "star-like" haplotype network suggesting that the parasite population is undergoing expansion. In reference to the *P. inui* network, the shared dominant network between *An. balabacensis* and macaque may signify that there is transmission ongoing between the vector and macaques. The high haplotype diversity in *P. knowlesi* parasites in the vector combined with the clustering of the haplotypes found in them might imply that transmission between human, *An. balabacensis* and macaques is possible. A clear geographical clustering of the parasite populations from Peninsular Malaysia and Malaysian Borneo was observed in the haplotype network of *P. cynomolgi*. These results were supported by population genetic analysis using Tajima's D, Li and Fu's D* and Li and Fu's F*revealing significant negative values which indicated that each of the parasite's population in Malaysia is undergoing expansion. A recent study by Benavente et al. (2017) revealed that vector related genes which are driven by *Anopheles* species placed strong selection pressure on the parasite population. Significant genetic differentiations among the three parasites populations (LL, TD and KP) of *P. knowlesi* and *P. inui* indicated they are distinct genetic populations infecting *An. balabacensis.* The high population differentiation especially between LL and TD maybe resulted from ecological differences which could possibly cause the two parasites populations to evolve separately or due to the narrow range of dispersion of the vector (Hii & Vun,

1985) or lack of migration of macaques from one area to another area and vice versa. Additionally, the evolutionary mechanism in which the co-adaptation of the parasite with its host is affected by selection pressure and evolutionary potential the parasite has to endure (Brandt et al., 2007; Gandon & Michalakis, 2002). Hence, the distinct parasite populations of *P. knowlesi, P. inui* and *P. cynomolgi* found in the three sampling sites in Kudat infecting *An. balabacensis* may play a bigger role in the simian malaria transmission and thus, will probably affect malaria elimination program in Malaysia. There are very few sequences of the parasites from the mosquitoes in the GenBank. In actual fact besides Malaysia, only in Vietnam, the vector for simian malaria has been established (Marchand et al., 2011). Therefore it is important for more studies to be conducted on these parasites from the vectors, humans and macaques in order to comprehend what is actually ongoing. This should be extended to the Southeast Asia region.

Malaria vector control programs in Southeast Asia including Malaysia are largely dependent on the use of insecticides for indoor residual spraying (IRS) and insecticide-treated nets (ITNs). However, the vectors involved in simian malaria transmission in Malaysia are early biters, as early as 1800 hours which means people can be exposed to them when they are returning from work. This indicates that in order for a control program to succeed, one must have thorough knowledge on the behavior and bionomics of the vector (precisely identified) so that appropriate control measures can be taken (Trung et al., 2005). Therefore, the reliability on IRS and ITNs should be reduced. Instead, control methods such as environmental management for larval control can be used if feasible. Both *An. epiroticus* and *An. maculatus* populations in Malaysia werecontrolled by building bunds and digging drains for excluding brackish water (for *An. epiroticus*) (Moorhouse & Wharton, 1965), and periodic flushing of streams using small dams controlled by siphons (for *An. maculatus*) (Williamson & Scharff, 1936).

The presence of mixed simian malaria parasites infections in *An. balabacensis* along with its high sporozoite rate and high haplotype diversity (found in *P. cynomolgi* population) may lead to the possibility of this simian malaria being transmitted to human. Currently, only a one single case of *P. cynomolgi* infection in human has been reported (Ta et al., 2014).

It is known that the two human malaria species (*P. falciparum* and *P. vivax*) which infects millions of people is actually of zoonotic origin (from the African apes) and happened thousands of years ago (Liu et al., 2010; Liu et al., 2014). Thus, there is also a possibility that perhaps in future *P. knowlesi* and other simian malarias may become established in humans, especially when human malaria is eliminated. Currently human to human transmission of knowlesi malaria has not been established. In order to explore the possibility, feeding of mosquitoes on infected humans' blood and allowing it to develop into infective stages before feeding the mosquitoes with clean blood to check its transmission status should be given more thought.

Macaques have shown to be infected with multiple parasites (Akter et al., 2015; Lee et al., 2011) and studies have confirmed that mosquitoes can establish multiple infection over successive bloodmeals (Pollitt et al., 2015). This has also been observed in field collected mosquitoes (Manin et al., 2016; Tan, 2008; Wong et al., 2015). Thus, in the light of malaria elimination, it is timely for countries in Southeast Asia to take a concerted effort to study the vectors and to design a new vector control strategies as current control methods are not appropriate.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications related to the study

1) Wong, M. L., Chua, T. H., Leong, C. S., Khaw, L. T., Fornace, K., Wan-Sulaiman, W.-Y., William, T., Drakeley, C., Ferguson, H. M. & Vythilingam, I. (2015). Seasonal and spatial dynamics of the primary vector of *Plasmodium knowlesi* within a major transmission focus in Sabah, Malaysia. *PLoS Neglected Tropical Diseases, 9*(10), e0004135.

2) Vythilingam, I., Wong, M. L., Wan Yussof, W. S. (2016). Current status of *Plasmodium knowlesi* vectors: a public health concern? *Parasitology.*

Papers presented

1) Oral presentation at 6th ASEAN Congress of Tropical Medicine and Parasitology 2014, Kuala Lumpur, Malaysia.

Meng Li WONG, Indra VYTHILINGAM, Cherng Shii LEONG, Tock Hing CHUA, Benny OBRAIN, Heather FERGUSON, Loke Tim KHAW and Chris DRAKELY. (2014). Preliminary study of entomologic parameters in relation to simian malaria in Kudat Division, Sabah, Malaysia

2) Oral presentation at 7th International Congress of the Asia Pacific Society of Infection Control 2015, Taipei, Taiwan.

Meng Li WONG, Indra VYTHILINGAM, Cherng Shii LEONG, Loke Tim KHAW, Tock Hing CHUA, Benny OBRAIN, Heather FERGUSON and Chris DRAKELY. (2015). Incrimination of *Anopheles balabacensis* as the vector for simian malaria in Kudat Division, Sabah, Malaysia.