TAXONOMIC AND ECOLOGICAL STUDIES OF THE GENUS *SIMULIUM* (DIPTERA: SIMULIIDAE) IN PENINSULAR MALAYSIA

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ABSTRACT

A thorough black fly investigation was carried out in Peninsular Malaysia (1) to report the detailed identification keys and taxonomic descriptions of new species, (2) to elucidate their nationwide distribution pattern and (3) to investigate their assemblage pattern along an elevational gradient. Five new species, *Simulium vanluni*, *Simulium ledangense*, *Simulium pairoti* and *Simulium azhari* and *Simulium johorense* were discovered and described based on mature larva, pupa, male and female. A total of 47 *Simulium* species were revealed through the nationwide surveys in Peninsular Malaysia. The most frequently collected species were *Simulium trangense*, followed by *Simulium angulistylum*, *Simulium cheongi*, *Simulium tani*, *Simulium vanluni*, *Simulium sheilae* and *Simulium bishopi*. *Simulium vanluni*, *Simulium trangense* and *Simulium angulistylum* were the three most abundant species. High species richness was associated with larger, deeper, faster and higher discharge streams with larger streambed particles, more riparian vegetation and low pH. *Simulium vanluni* and *Simulium tani* prefer large, fast flowing streams with higher pH, large streambed particles and riparian trees. *Simulium bishopi* was commonly found at high elevation with cooler stream, low conductivity and more riparian trees. In contrast, *Simulium sheilae* was positively related with streams at low elevation, warmer stream with low conductivity and less riparian trees. On the other hand, a total of 35 black fly species were recorded along an elevational gradient. The most frequently collected species were *Simulium tani* and *Simulium whartoni*, while the relatively common species were *Simulium* sp. (nr. *feuerborni*), *Simulium decuplum*, *Simulium angulistylum*, *Simulium bishopi* and *Simulium izuae*. Six simuliid species were distributed below 500 m, whereas eight species were distributed above 1,400 m. *Simulium* sp. (nr. *feuerborni*) and *Simulium asakoae* were found from middle to high elevations (711–1,813 m). *Simulium whartoni*, *Simulium brevipar* and *Simulium bishopi* were distributed widely from low to high elevations (159–1,813 m). Regression analysis between species richness and PCs revealed that the species richness was significantly associated with wider, deeper and faster streams at low elevation, water temperature 23°C–25°C, low conductivity, higher discharge, more canopy cover and riparian vegetation and with larger streambed particles. Forward logistic regression indicated four species were significantly related to the stream variables (*Simulium whartoni*, *Simulium* sp. (nr. *feuerborni*), *Simulium tani* and *Simulium angulistylum*). Canonical correspondence analysis indicated that the temperature, stream size and discharge were the most important factors contributing to the separation of the stream sites from different elevation and hence are the predictors for the distribution of black fly species assemblages. The present study has provided new insights into the black fly species composition and diversity in Peninsular Malaysia and these findings will be the stepping stone promoting more black fly studies in Malaysia as well as other countries in Southeast Asia.

ABSTRAK

Satu kajian menyeluruh ke atas lalat hitam telah jalankan di Semenanjung Malaysia (1) untuk melaporkan kekunci pengenalan dan huraian taksonomi yang terperinci, (2) untuk mentafsirkan corak taburan mereka di seluruh negara dan (3) untuk mengkaji pola taburan mereka di sepanjang kecerunan berbeza. Lima spesies baru, *Simulium vanluni*, *Simulium ledangense*, *Simulium pairoti*, *Simulium azhari* dan *Simulium johorense* telah ditemui dan dihuraikan berdasarkan larva matang, pupa, dewasa jantan dan betina. Sejumlah 47 spesies *Simulium* telah berjaya diselidiki melalui kajian yang jalankan di seluruh negara di Semenanjung Malaysia. Spesies paling kerap dikumpul ialah *Simulium trangense*, diikuti oleh *Simulium angulistylum*, *Simulium cheongi, Simulium tani*, *Simulium vanluni*, *Simulium sheilae* dan *Simulium bishopi*. *Simulium vanluni*, *Simulium trangense* dan *Simulium angulistylum* ialah spesies dengan kepadatan paling tinggi. Kekayaan spesies yang tinggi telah dikaitkan dengan habitat sungai yang memiliki aliran keluar lebih besar, lebih dalam, lebih laju dan dengan partikel batuan dasar sungai yang lebih besar, lebih banyak tumbuhan gigi air dan pH rendah. *Simulium vanluni* dan *Simulium tani* lebih memilih habitat seperti sungai bersaiz besar, aliran yang lebih pantas, pH tinggi, partikel sungai yang lebih besar dan banyak tumbuhan pinggiran tebing sungai. *Simulium bishopi* pula biasa dijumpai di tempat lebih tinggi, suhu sungai lebih sejuk, kekonduksian yang rendah dan lebih banyak pokok pinggiran tebing sungai. Sebaliknya, *Simulium sheilae* secara positif berkaitan dengan sungai di ketinggian rendah, suhu air sungai yang normal dengan kekonduksian yang rendah dan kurang pokok pinggiran tebing sungai. Di sudut yang berlainan, sejumlah 35 spesies lalat hitam telah direkodkan di sepanjang cerun ketinggian. Spesies paling kerap dikumpul adalah *Simulium tani* dan *Simulium whartoni*, manakala spesies yang agak biasa di temui ialah *Simulium* sp. (nr. *feuerborni*), *Simulium decuplum*, *Simulium angulistylum*, *Simulium bishopi* dan *Simulium izuae*. Enam spesies simuliid telah dijumpai pada taburan di bawah ketinggian 500 m, manakala lapan spesies pada taburan melebihi 1,400 m. *Simulium* sp. (nr. *feuerborni*) dan *Simulium asakoae* direkodkan di kawasan berketinggian sederhana ke tinggi (711−1,813 m). *Simulium whartoni*, *Simulium brevipar* dan *Simulium bishopi* dijumpai bertabur secara meluas dari kawasan rendah hingga ke kawasan tinggi (159−1,813 m). Kekayaan spesies berkait secara signifikan dengan habitat sungai yang lebih lebar, dalam, aliran lebih laju, di kawasan lebih rendah bersuhu sungai normal (23–25°C), rendah kekonduksian, pelepasan yang lebih tinggi, lebih banyak litupan dan tumbuhan pinggiran tebing sungai dan partikel sungai yang lebih besar. Regresi logistik ke hadapan menunjukkan empat spesies berkait secara signifikan dengan pembolehubah aliran (*Simulium whartoni*, *Simulium* sp. (nr. *feuerborni*), *Simulium tani* dan *Simulium angulistylum*). Kelajuan aliran dan pelepasan air adalah faktor paling penting yang menyumbang kepada pemisahan antara sungai dari ketinggian yang berbeza dan justeru menjadi penentu penting bagi corak taburan lalat hitam. Kajian ini telah menyediakan satu informasi baru mengenai komposisi dan kepelbagaian spesies lalat hitam di Semenanjung Malaysia dan penemuan ini diharap akan menjadi batu loncatan dalam menggalakkan lebih banyak kajian-kajian lalat hitam di Malaysia serta negara-negara lain di Asia Tenggara.

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LIST OF SYMBOLS AND ABBREVIATIONS

cm	centimeter
mm	millimeter
m	meter
m/s	meter per second
km	kilometer
mS/cm	micro Siemens per centimeter
mg/l	milligram per liter
h	hour
°C	degree Celsius
°	degree
'	minute
>	more than
<	less than
≤	less than or equal to
=	equal to
%	percent
et al.	et alia (“ and others”)
i.e.	Id est. (“that is”)
S.	*Simulium*
N	north
E	east
df	degree of freedom
F	regression analysis value
P	possibility value
sp. nov.	species nova (“new species”)
sp.	species (“singular”)
spp.	species (“plural”)
S. l.	sensu lato ("in the broad sense")
S. str.	sensu stricto ("in the narrow sense")
ca.	circa ("around/about")
n	number of sample size (“total”)
e.g.	exampli gratia ("for example")
pH	potential of Hydrogen
PCA	principle components analysis
PCs	principle components (“plural”)
PC-1	principle component 1
PC-2	principle component 2
PC-3	principle component 3
PC-4	principle component 4
PC-5	principle component 5
GPS	global positioning system
SE	standard error
±	plus or minus
FO	frequency of occurrence

LO	local occurrence
SO	stream occurrence
CCA	canonical correspondence analysis
min	minimum
max	maximum
asl	above sea level
Q	stream discharge
m^3/s^{-1}	cubic meter per second
D_1	stream depth
w	stream width
U	velocity

CHAPTER 1: GENERAL INTRODUCTION

1.1 SCOPE OF STUDY

Black flies (Diptera: Simuliidae) are well known for blood-sucking habits and capable of transmitting parasitic diseases to birds and mammals including human (Takaoka, 1996; Choochote *et al*., 2005; Currie & Adler, 2008). Because of female habit of taking blood-meal, certain female species are known to be the pest causing dermatitis and also playing role as vector of filarial parasites (Choochote *et al*., 2005). To date, 2,204 species of 26 genera and 15 fossil species from nine genera have been documented worldwide (Adler *et al*., 2016). About 98% of the black flies feed on a vertebrate blood (Adler *et al*., 2010). Over the past decades, black flies have received significant attention since they transmit filarial worms responsible for human onchocercosis or 'river blindness' (Adler *et al*., 2010; Tada, 2015). About 18 million people are infected by onchocercosis particularly in African and Latin American countries with approximately a quarter of a million cases of blindness (Fukuda *et al*., 2003; Tada, 2015).

Despite potential risk of disease transmission, the significance of Malaysian black flies have been little studied. A lack of taxonomic observation is indeed a leading cause of poor understanding on the ecology and biology of black flies. Hence, the current study was conducted to discover as many as species inhabiting Peninsular Malaysia, aiming to resolve the scarcity of black fly information through describing and illustrating new species.

Additionally, black flies are important components of the stream ecosystem (Pramual & Kuvangkadilok, 2009). The immature stages of black flies are present as a dominant component of the stream macroinvertebrates and play a crucial role in the aquatic habitat as an ideal bio-indicator for clean water quality. Polluted stream due to human disturbance could affect black fly species distribution (Zhang *et al*., 1998; Pramual & Kuvangkadilok, 2009). Thus, changing pattern in black fly distribution could reflect the level of stream habitat disturbance. Over the past few years, a number of studies have pointed out several factors influencing the immature stages of black fly distribution in Thailand (i.e., stream size, stream depth, stream cover and elevation) (Pramual & Kuvangkadilok, 2009; Srisuka *et al*., 2015) while no study has been conducted so far in other countries in Southeast Asia, including Malaysia. Hence, this study was the first attempt to obtain insight into the Malaysian black fly distribution and their associated ecological characteristics. This study aimed to investigate the nationwide distribution of preimaginal black fly species in relation to their breeding habitat preference across four geographic regions in Peninsular Malaysia.

Species community structure and distribution varied spatially in response to a broad range of environmental factors including elevation (Hodkinson, 2005; McCain & Grytnes, 2010). To date, the ecological studies of black flies have been given more attention on spatial distribution in response to habitat disturbance (Pramual & Kuvangkadilok, 2009; Couceiro *et al*., 2015), seasonal variation (McCreadie *et al*., 2005; Pramual & Wongpakam, 2010; Srisuka *et al*., 2015) and locality richness (McCreadie & Adler, 1998, 2006; Hamada & McCreadie, 1999; Hamada *et al*., 2002; McCreadie *et al*., 2005). Nevertheless, there has been limited research undertaken on the vertical distribution of preimaginal black flies and their associated ecological factors. Certain preimaginal stages of black flies would have a broad range of vertical distribution (generalist species) while others might show a specific range of distribution (specialist species). The specialist species may limit their distribution to certain preferred microhabitat/niche condition (i.e., water temperature) (Pramual & Kuvangkadilok, 2009; Pramual & Wongpakam, 2010). It has been hypothesized that those preferring cooler stream temperature are distributed at higher elevations (and vice versa), and those tolerable to a broad range of water temperatures are distributed from low to high elevations. Therefore, this study investigated the distribution pattern of black fly species assemblages and their associated environmental factors, for the first time, along an elevational gradient in Peninsular Malaysia.

1.2 PROBLEM STATEMENTS

Although black flies are well known for their medical and ecological importance, the significance of Malaysian black flies remains unexplored (Takaoka & Davies, 1995). The diversity of black flies are expected to increase with additional sampling effort, hence surveys have to be carried out to discover potential new species.

Studies of black fly ecology in Peninsular Malaysia have been totally neglected. As a consequence, no information available on species distribution pattern in relation to the associated environmental factors at various geographic scales.

1.3 SIGNIFICANCE OF STUDY

Vital findings from this study will assist public health stakeholders in establishing effective control and preventive strategies based on black fly distribution. Besides, it may provide interest to medical and veterinary entomologists, parasitologists, freshwater ecologists, cytologists, molecular biologists as well as the epidemiologists from different parts of the world. The black fly fauna in Peninsular Malaysia is supposed to be rich in species diversity because of its unique geographical situation. Black fly studies in this region may provide substantial evidence to explain its faunal relationship to surrounding regions, such as Thailand, Sumatra, Java, Sulawesi and the Philippines. The description of new species in Malaysia will provide the basis of key identification and allow a comparison with other taxa from other geographic regions. These data will be helpful in identifying species complex members with geographically widespread distribution. It surely merits and accelerates further development of research of various disciplines on black flies in Malaysia and also in neighboring countries.

Outcomes obtained from this study would provide the most comprehensive baseline data for future studies in related disciplines. Ecological information in relation to habitat preferences and variation in geographic factors revealed from this study would encourage further ecological and biological studies particularly on specific species in this region. Moreover, this study can be a stepping stone to promote research on the biting habit of adult black flies in Malaysia.

1.4 AIMS AND OBJECTIVES

The major aim of this study was to reveal the diversity of black flies in Peninsular Malaysia with special attention paid on their taxonomy and ecology. With these in mind, the present study was performed to address the following specific objectives:

1.4.1 To report detailed identification keys and taxonomic descriptions of new species.

i. To describe new species in the subgenus *Simulium*:

a) *Simulium* (*Simulium*) *vanluni* Ya'cob, Takaoka & Sofian-Azirun, 2016.

ii. To describe new species in the subgenus *Nevermannia*:

a) *Simulium* (*Nevermannia*) *pairoti* Ya'cob, Takaoka & Sofian-Azirun, 2016.

b) *Simulium* (*Nevermannia*) *ledangense* Ya'cob, Takaoka & Sofian-Azirun, 2014.

iii. To describe new species in the subgenus *Gomphostilbia*:

a) *Simulium* (*Gomphostilbia*) *azhari* Takaoka, Sofian-Azirun & Ya'cob, 2014.

b) *Simulium* (*Gomphostilbia*) *johorense* Takaoka, Sofian-Azirun & Ya'cob, 2014.

1.4.2 To elucidate the nationwide distribution of preimaginal black flies in Peninsular Malaysia.

i. To examine the distribution patterns of preimaginal black flies and their associated environmental factors.
ii. To determine the widespread and abundant species across four geographic regions.

1.4.3 To investigate the distribution pattern of preimaginal black flies (Diptera: Simuliidae) along an elevational gradient in Peninsular Malaysia.

i. To elucidate the fine-scale distribution patterns of preimaginal black flies and their associated environmental factors at 18 different elevations.
ii. To determine the generalist and specialist species along low to high elevations.

CHAPTER 2: LITERATURE REVIEW

2.1 BIOLOGY OF BLACK FLY (DIPTERA: SIMULIIDAE)

The life cycle and morphological features of the larva, pupa and adult are presented in Figures 2.1–2.3. Black flies are members of the Family Simuliidae in the Order Diptera. As other holometabolous insects, black flies pass through four distinct life stages to complete their developments to an adult. The immature stages: egg, larva and pupa are entirely aquatic. Unlike mosquito larvae, immature black flies only can survive in moving or running water (Crosskey, 1990; Currie & Adler, 2008).

The eggs are commonly laid in batches of 200−300, on substrata in or near running water or directly dropped into the water then settle into the sediments (Crosskey, 1990; Currie & Adler, 2008). Females of several species crawl up to 15 cm below the water surface to oviposit on submerged substrates. Eggs are 0.1 to 0.5 mm long, imperfectly ovoid, usually subtriangular from some viewpoint, most strongly convex on upper surface, without floats or other special morphological features, and surface is comparatively smooth. Freshly laid eggs are creamy-white covered with a gelatinous substance, changing to dark brown or black within 24 hours. The water temperatures at which eggs embryonate can be as low as a few degrees above freezing point in cool temperate or sub-polar regions to more than 30°C (Crosskey, 1990).

Life duration for egg stage is varying geographically, such as in warm tropical streams, the duration is as minimum as one day and maximum over a year in prolonged drought (Crosskey, 1990). The eggs with the shortest known duration are those of the tropical African *Simulium damnosum* complex, which at the prevailing natural water temperatures (about 20°C−30 °C) mostly hatch within 36−48 hours of being laid; in

Simulium sirbanum sibling, of the hot West African savannas, the eggs even hatch within 24 hours of being laid when the water temperature is up to 28.5°C−33 °C (Crosskey, 1990).

The egg hatches to produce a maggot-like larva, of which the thorax and posterior parts of the abdomen are broader than the anterior segments. The head bears a pair of large feeding fans open hemispherically, larval body with one proleg on the underside of the thorax and another on the hind end of the body, each crowned with hooks for attachment. They attached to immersed non-living objects or to vegetations, except a few species are phoretic on freshwater crabs and mayfly nymphs (Crosskey, 1990). Larvae may be found near the surface of the water or at depths of several meters in turbulent water. In normal feeding position the larvae are anchored posteriorly and extended in the direction of the current with the head downstream. A sticky secretion produced by the cibarial glands enables the fans to capture fine particles, which are transferred to the cibarium by the mandibular brushes. The aquatic larval stage involves several moults between successively larger substages (instars), and total number of instars ranging from six to nine depending upon species such as *Simulium vittatum* Zetterstedt had seven instars (Ross & Merritt, 1978). Life as a larva can be a minimum of four days in tropics and a maximum of several months if long cold winter to be outlasted. Larvae spin a silken cocoon onto an object in the current and pupate within this silken shroud.

Pupae are immobile, fixed in an underwater case (cocoon) by a precise arrangement of abdominal hooks, and with a pair of large thoracic gills issuing from the anterior of the thorax. Pupal stage can possibly last a minimum of two days and a maximum of about two or three weeks (Crosskey, 1990). The pupa, which does not feed, becomes progressively darker as the adult develops within. At emergence, the pupal skin splits, and the adult floats up to the surface in a bubble of air and immediately takes flight, or the newly emerged adult crawls up some emergent object to reach the air.

Adults escape the water in the daytime to a resting site on the vegetation at the margin of the water where their wings expand to full size and their bodies harden (not grow to a larger body size). In general, adult black flies are classified as backboneless (invertebrate) designed with hardened external skeleton, body length range 1.2 to 6 mm, segmented limbs with three pairs of legs, one pair of wings length range 1.4 to 6 mm accompanied behind by a pair of small balancer knobs (the modified hind wings or halteres), feet (tarsi) with five segments, mouthparts adapted for sucking liquid food, mandibles and maxillae of most females toothed at tips for cutting and piercing the skin of their host animal, cigarshaped antennae with 11 segments with very shorthair in both sexes, large wings which close fully over one another at rest. Head of female with frons (dichoptic) while male without frons (holoptic), eyes of male are divided into upper region of enlarged facets and lower region of small facets; eyes of female with uniformly small facets (Crosskey, 1990; Takaoka & Davies, 1995; Takaoka, 2012). Basically, adult life-span is about two or three months and probably normally about three or four weeks in the female and a few days in the male (Crosskey, 1990). Adult flight range is varying with sex and species where females of bloodsucking species for example *Simulium ochraceum*, *Simulium metallicum* and *Simulium callidum* with maximum flight range 10.1 km, 11.7 km and 11.9 km respectively (Gibson & Dalmat, 1952; Dalmat, 1955), however lesser in those of non-bloodsuckers (Crosskey, 1990). Moreover, understanding the flight range of the vector black fly is a crucial for vector control and epidemiological decisions. Annual generation for black flies is varying depending upon geographical or climatic zones, where it can be as high as 16 generations in permanent flow stream of lowland tropical regions and one or six in temperate or subarctic habitats with cold winters (Crosskey, 1990).

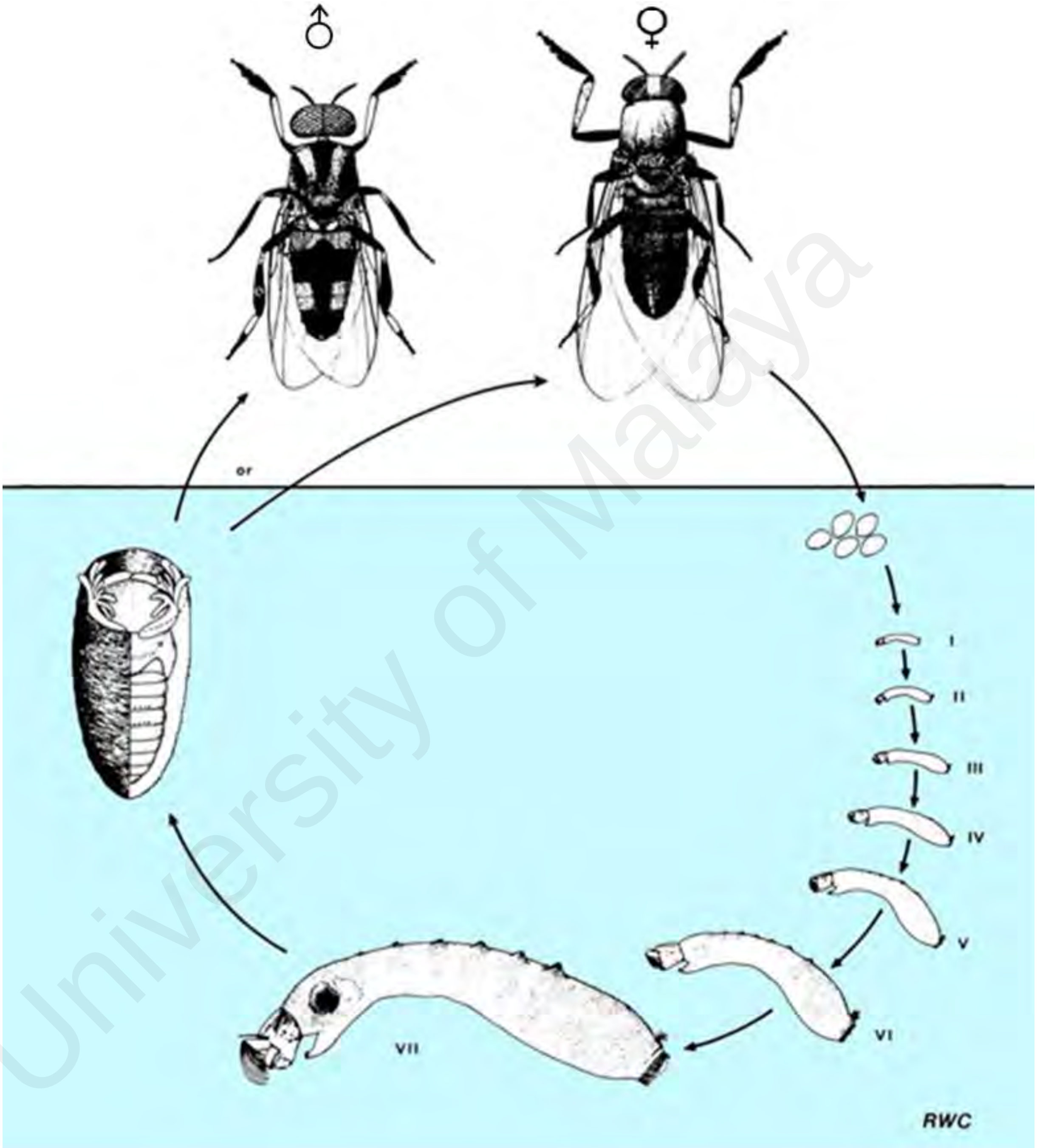

Figure 2.1: Life cycle of black flies adapted from Crosskey (1990).

2.2 TAXONOMIC BACKGROUND

Since Blacklock (1926a; 1926b) ratified that black flies transmit the filarial worms responsible for human onchocercosis, thousands of studies have been conducted on Simuliidae (Adler *et al*., 2010). Nonetheless, several onchocercosis control programs such as The Guatemala-Japan Cooperative Project in 1975 and Onchocerca Control Program (OCP) in 1973 have been instrumental promoting multidisciplinary research combining aspects of entomology, epidemiology, parasitology, immunology, dermatology, ophthalmology and human ecology (Moji, 2015). Consequently, morphological taxonomy increased rapidly with more than one thousand species being described and new ones still being found and named at the rate of about two hundred per decade (Crosskey, 1990).

To date, a total of 2,219 species (2,204 livings and 15 fossils) from 26 genera are listed worldwide (Adler & Crosskey, 2016). Of these, the genus *Simulium* is the largest and most widely distributed consisting of 37 subgenera (1,745 species) (Adler & Crosskey, 2016). Interestingly, *Simulium* is the only genus found in the Oriental Region, where a total of 524 species are recorded (Adler & Crosskey, 2016), which further classified in 10 subgenera. Of these, the subgenus *Simulium* is dominant, having 230 species (43.9%), followed by *Gomphostilbia* with 193 species (36.8%) and *Nevermannia* with 55 species (10.5%), while seven other subgenera consists of 1−16 species. The updated identification keys to all these subgenera of the genus *Simulium* are provided in section 3.1.1. The complete list for world's black flies is presented in Table 2.1.

Four subgenera, *Gomphostilbia* Enderlein, *Simulium* Latreille s. str., *Nevermannia* Enderlein and *Daviesellum* Takoaka and Adler, are reported from Malaysia comprising 86 nominal species in 19 species-groups (Adler & Crosskey, 2016). Peninsular Malaysia holds 56 species, of these, one in subgenus *Daviesellum*, 33 species (32 named and 1 unnamed) in the subgenus *Gomphostilbia*, three species in the subgenus *Nevermannia* and 19 species (17 named 2 unnamed) in the subgenus *Simulium* s. str. (Adler & Crosskey, 2016).

The most striking characteristics of the simuliid fauna in this region is the presence of the subgenus *Daviesellum*, first discovered in Peninsular Malaysia (Takaoka & Davies, 1995) then in Thailand (Takaoka & Adler, 1997). Apart from *Daviesellum*, the simuliid fauna of Malaysia is at the subgeneric level similar to those of the Asian countries (i.e., Thailand, India, Indonesia and Philippines). However, the fauna of Malaysia differs from that of Thailand by lacking two subgenera, *Asiosimulium* and *Montisimulium*, from India by lacking *Montisimulium*, from Indonesia by lacking *Morops* and *Wallacellum* and from the Philippines by lacking *Wallacellum* (Takaoka, 1983; 1995; 1996; 2003; Adler & Crosskey, 2016).

At the species-group level, the Peninsular Malaysia appears to play a role in bridging the species-group to extend their distribution from the Eurasian continent to southern and eastern insular regions or vice versa (Takaoka, 1996). Additionally, Peninsular Malaysia marks the distribution boundary for certain species group which is essentially continental distribution, and a westernmost boundary for *S. melanopus* species-group, which is representative of the insular groups. The *S. eximium* species group of the subgenus *Simulium* s. str. found in Sumatra, Java and Flores (Takaoka, 2003) did not reach the Peninsular Malaysia and East Malaysia (Sarawak and Sabah).

Table 2.1: Numbers of formally living and fossil species in the genera and subgenera level of world Simuliidae (Adler & Crosskey, 2016).

Genus	**Subgenus**	**Total species**	**Genus**	**Subgenus**	**Total species**
***Parasimulium* (4)**				*Similium*	30
	Astoneomyia	1		*Eusimulium*	39
	Parasimulium	3		*Freemanellum*	7
Gymnopais		12		*Gomphostilbia*	234
***Helodon* (35)**				*Hebridosimulium*	16
	Distosimulium	2		*Hellichiella*	23
	Helodon	30		*Inseliellum*	52
	Parahelodon	3		*Lewisellum*	9
Levitinia		3		*Meilloniellum*	5
Prosimulium		78		*Metomphalus*	33
Twinnia		10		*Montisimulium*	79
Urosimulium		3		*Morops*	75
Araucnephia		3		*Nevermannia*	230
Araucnephioides		1		*Notolepria*	9
***Austrosimulium* (31)**				*Phoretomyia*	15
	Austrosimulium	24		*Pomeroyellum*	47
	Novaustrosimulium	6		*Psaroniocompsa*	48
	[Unplaced to subgenus]	1		*Psilopelmia*	69
Cnephia		7		*Psilozia*	5
Cnesia		3		*Pternaspatha*	28
Cnesiamima		1		*Rubzovia*	5
Crozetia		2		*Schoenbaueria*	22
Ectemia		4		*Simulium*	482
Gigantodax		65		*Trichodagmia*	75
Greniera		13		*Wallacellum*	17
Lutzsimulium		4		*Wilhelmia*	28
Metacnephia		54		*Xenosimulium*	5
***Paracnephia* (19)**				[Unplaced to subgenus]	8
	Paracnephia	6	***Stegopterna***		14
	Procnephia	3	***Sulcicnephia***		23
	[Unplaced to subgenus]	10	***Tlalocomyia***		16
Paraustrosimulium		1	**TOTAL**		**2204**
Pedrowygomyia		4			
***Simulium* (1745)**				**Fossil species**	
	Afrosimulium	1	[*Kovalevimyia*]		1
	Anasolen	11	[*Simulimima*]		1
	Asiosimulium	4	[*Arachicnephia*]		1
	Aspthia	26	[*Baisomyia*]		1
	Boophthora	5	*Ectemnia*		2
	Boreosimulium	19	*Greniera*		5
	Byssodon	12	[*Gydarina*]		1
	Chirostilbia	15	*Simulium* (*Hellichiella*)		2
	Crosskeyellum	2	[*Simuliites*]		1
	Daviesellum	2	**TOTAL**		**15**

2.3 TAXONOMIC CHARACTERS

Taxonomy is usually defined as the theory and practice of describing, naming and classifying organisms (Crosskey, 1990). Morphotaxonomic characters of simuliids are the quantitative analysis of external and internal forms or structures obtained from many parts of the body from three life stages including larva, pupa, and adults male and female (Crosskey, 1990).

The common morphological features and taxonomic terms of adult, pupa and larva are presented in Figure 2.2 and Figure 2.3. Larvae and pupae have morphological features which offer good taxonomic characters and allow immature stages to be taken into account in constructing the taxonomic system. The most valuable characters (diagnostic) in larval taxonomy as shown in the Figure 2.2, lie in the shape of the body, cephalic apotome, postgenal cleft, hypostomial teeth, anal sclerite, the structure of the labral fans, mandibles and rectal organ, the pigmentation of the attachment sites of the head muscles (known as 'head-spots'), and the vestiture (if any) of the body cuticle. Pupal taxonomy uses principally variations in the shape and arrangement of the gill filament, shape and texture of the cocoon, form and disposition of hooks and spines on the abdomen (Crosskey, 1990; Takaoka & Davies, 1995; Takaoka, 2012)

The adult taxonomic characters are more numerous and varied. They include not only features common to both sexes of any species, such as the wing vein structure and number of antennal segments, but also an abundance of features residing in the primary (reproductive) and secondary (non-reproductive) sexual differences between male and female flies. The most significant of these unisexual characters are found in the copulatory organs (the male genitalia being specifically important in all levels of taxonomy), but other features of the body which differ sexually includes eyes, claws, certain leg structures, including colour patterns (Crosskey, 1990; Takaoka & Davies, 1995; Takaoka, 2012).

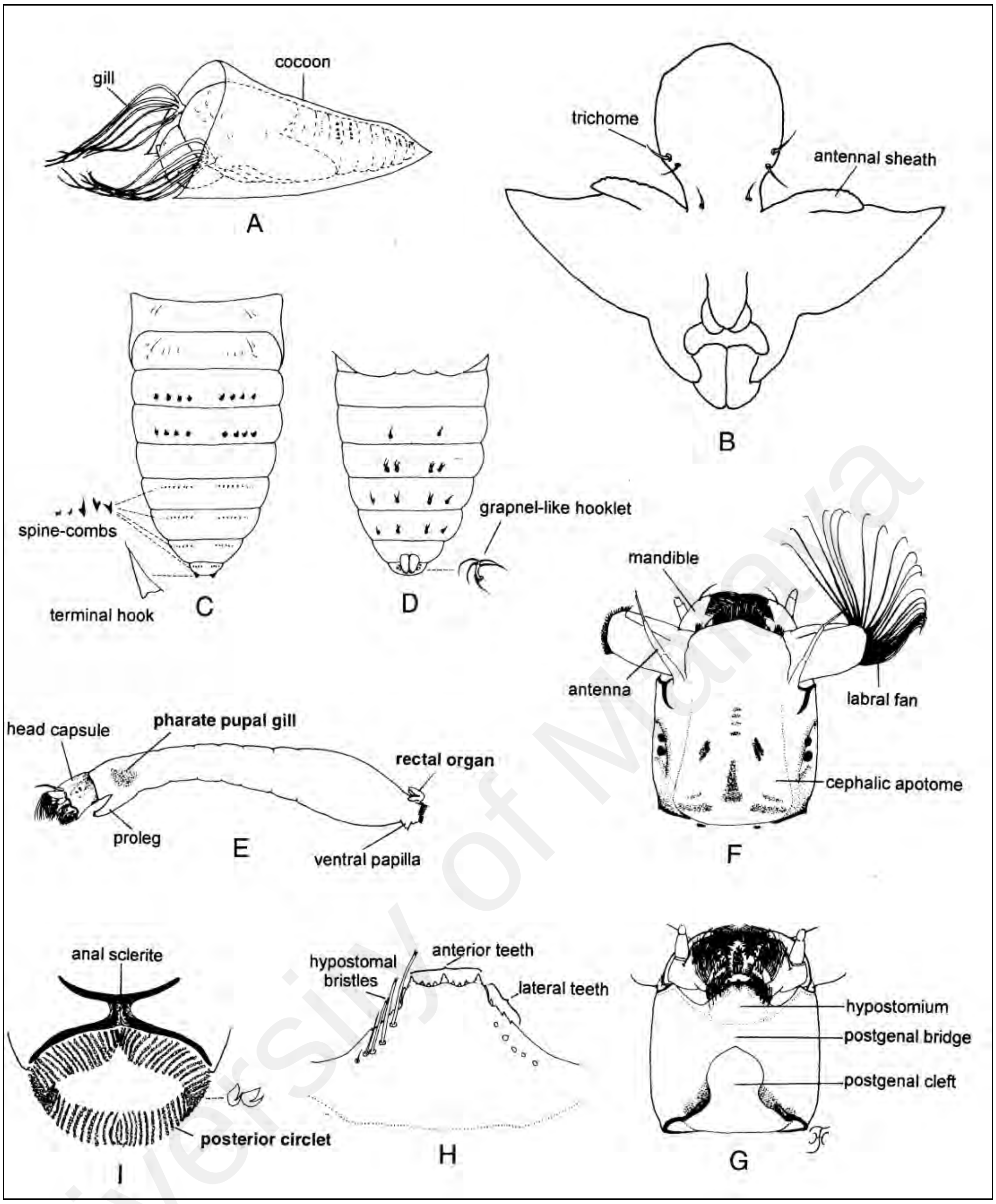

Figure 2.2: Morphological features and taxonomic terms of pupa (A−D) and larva (E–I) of the Simuliidae, adapted from Takaoka and Davies (1995). A, pupa in cocoon; B, head integument of pupa (front view, spread flattened); C and D, pupal abdomen (C, dorsal view; D, ventral view); E, mature larva (side view); F and G, larval head capsule (F, dorsal view; G, ventral view); H, hypostoma; I, posterior tip of larval abdomen (posterodorsal view).

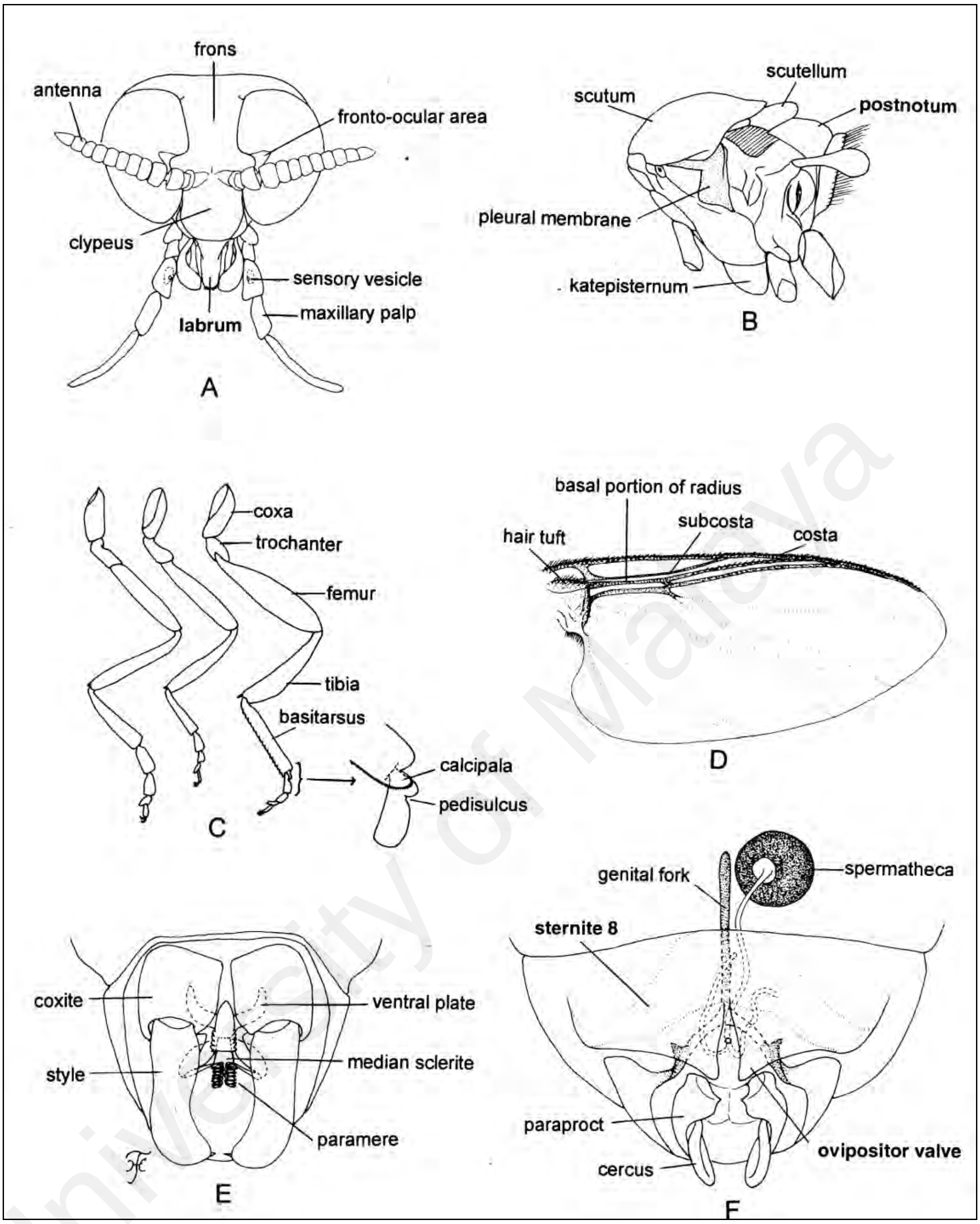

Figure 2.3: Morphological features and taxonomic terms of adult black flies cited from Takaoka and Davies (1995). A, female head (front view); B, thorax (side view); C, male legs (fore, mid and hind legs from left); D, wing; E, male genitalia (ventral view); F, female genitalia (ventral view).

2.4 ECOLOGY OF BLACK FLY

Black fly is a cosmopolitan insect, present almost in all zoogeographical regions over a wide range of elevation except Antarctica, deserts and islands without running waters (Crosskey, 1990; Currie & Adler, 2008; Adler *et al*., 2010). They are postulated to have evolved in cool and mountainous environments (Adler *et al*., 2010). The climate change during the glacial periods has driven the population expansion of simuliid along latitudinal and elevational gradients (Pramual *et al*., 2005; Adler *et al*., 2010; Low *et al*., 2014). However, in a warming trend, the widespread alpine taxa in the cooler regions could be fragmented and isolated as highly ecologically specialized taxa (high-elevation specialist) (Adler *et al*., 2010).

Black fly experiences very different ecological conditions which involve life under water and air. Preimaginal stages (egg, larva and pupa) are confined to unpolluted running waters ranging from small headwaters to large rivers (Currie & Adler, 2008). This absolute requirement for flowing water contrasts with the habitat requirements for another blood-feeding flies such as mosquitoes, which breed in still, standing water (Service, 2004). Black fly larvae are important components of the stream ecosystem (Hamada *et al*., 2002; Currie & Adler, 2008; Pramual & Kuvangkadilok, 2009). They act as the keystone species in the ecology of running water, because they are usually present as a major component of stream macroinvertebrates (Malmqvist *et al*., 2004) where the larvae can reach a density of a million per m^2 in some rivers (Currie & Adler, 2008). The larvae have an ability to filter dissolved organic matter and make it available in the food chain (Currie & Adler, 2008). According to Currie and Adler (2008), black flies are important in monitoring of freshwater contamination because larvae and pupae are susceptible to both organic and inorganic pollution (i.e., insecticides and fertilizers).

2.5 ECOLOGICAL STUDIES OF BLACK FLY

Ecological studies related to the non-living environment have concentrated more on the preimaginal stages than the adult, partly because it is much easier to handle and as target stage for the vector control. To date, the Palearctic Region has by far the largest number of described species (709 species) followed by Oriental Region (524 species), Neotropical Region (306 species), Nearctic region (265 species), Australasian Region (220 species) and Ethiopian Region (216 species). This considerable sufficient taxonomic information, stimulated entomologists to gain an insight into the ecology of black flies in response to their physical and biotic environments. The spatial distribution of preimaginal black flies has been well-studied in the temperate regions (McCreadie & Adler, 1998, 2006; McCreadie *et al*., 2005), the tropical regions of South America (Grillet & Barrera, 1997; Hamada & McCreadie, 1999; Hamada *et al*., 2002; McCreadie *et al*., 2004) and the Oriental regions, notably Thailand (Pramual & Kuvangkadilok, 2009; Pramual & Wongpakam, 2010; Srisuka *et al*., 2015). Black flies of Malaysia, however, remain unknown.

The main objectives of ecological study are to understand the community structure and its determinants (Hamada *et al*., 2002). These ubiquitous organisms have served as model subjects for the study of community structure (Adler & McCreadie, 1997; McCreadie & Adler, 1998; Pramual & Wongpakam, 2010; Couceiro *et al*., 2014). Study in Thailand by Pramual and Kuvangkadilok (2009) indicated that large stream, fast flowing, large streambed particles, and cool water at high altitude with cover, and riparian forest along the stream are the main physicochemical factors accommodate more black fly species. These are largely consistent with the pattern of other previous studies in same and other geographic regions; large, fast-flowing, high discharge streams, with lower temperature and conductivity (Pramual & Wongpakam, 2010); larger streams, cooler water temperature, faster flowing, and larger streambed particles

(Hamada *et al*., 2002); larger streams (Grillet & Barrera, 1997); and current velocity and size of streambed particle (Scheder & Waringer, 2002).

In addition, other associated factors in influencing black fly distribution have also been pointed out: water chemistry (Townsend *et al*., 1983; Jenkins *et al*., 1984; Erman & Erman, 1995; McCreadie *et al*., 2005), habitat disturbance (Erman & Erman, 1995; Palmer *et al*., 1995; Pramual & Kuvangkadilok, 2009; Couceiro *et al*., 2014), elevation (Tate & Heiny, 1995; Srisuka *et al*., 2015), intense riparian vegetation (Lautenschläger & Kiel, 2005) as well as the labral fan of black fly larvae (Pangjanda & Pramual, 2015).

2.5.1 Standard Sampling Protocol

An established protocol for collecting both larvae and stream data has been provided by McCreadie and Colbo (1991) and McCreadie *et al*., (2006). By following this standardization, data sets are comparable among various geographic regions. The Standardized protocols for stream physical and ecological parameters are as follows;

- Stream width (m) – Measured using a meter tape at a single point and will also serve as a plumb line to measure water depth and velocity.

- Stream depth (m) – Measured at equal distance points along the plumb line with a stout steel ruler. Two (streams less 1 m in width) to five (streams up to 100 m in width) measure of depth. Mean depth is then calculated and designated as D_1.

- Velocity (m/s) – At the same location where water depth is measured, by using stopwatch, water velocity can be estimated by the time cork takes to move in 1 m distance.

- Discharge (m^3/s^{-1}) – Depth (**D_1**), width (**w**) and velocity (**U**) measurements are then used to estimate discharge using the following formula:

$$\boxed{\mathbf{Q}\ m^3/s^{-1} = \mathbf{D_1} \times \mathbf{w} \times \mathbf{U}}$$

- Stream bed particle size classifications:

Table 2.2: Classification for stream bed particle size and the ranking system for the statistical analysis by (McCreadie & Colbo, 1991).

Category	Particle diameter (mm)	Rankings
Mud/Silt	<1	1
sand	1−2	2
Small Stone	2−32	3
Rubble	32−256	4
Boulder	>256	5
Bedrock	−	6

- Riparian vegetation classifications:

Table 2.3: Classification for riparian vegetation type and the rankings system for statistical analysis (McCreadie & Colbo, 1991).

Category	Vegetation form	Rankings
Open	Pasture, Grasslands, Bogs, Meadows	1
Brush	Extensive herbaceous growth, Scattered trees, Saplings	2
Forest	Continuous border of trees along stream banks	3

- Stream canopy cover classifications:

Table 2.4: Classification for stream canopy cover and the ranking system for the statistical analysis (McCreadie *et al*., 2006).

Category	Vegetation form	Rankings
Open	Vegetation extends over less than 10% of the stream	1
Brush	Vegetation extends over 10% of the stream	2
Forest	Vegetation from opposite sides of the bank touch over the stream or if vegetation is founds only on one side of the bank but it extends to the other side	3

2.6 MEDICAL AND VETERINARY IMPORTANCE OF BLACK FLY

Blood-feeding simuliid females are of considerable medical and veterinary relevance. A total of 97.6% of total nominal members worldwide are blood-feeding particularly on avian and mammalian (Adler *et al*., 2010). Of these, 31 (1.5%) species, mostly members of the genus *Simulium*, are vectors of human-disease agents (Adler *et al*., 2010). More than 25 black fly species are responsible for transmitting filarial nematode, *Onchocerca volvulus* (Adler *et al*., 2010), the causative agent of human onchocercosis or 'river blindness', the second ranking cause of infectious blindness occurring in tropical Africa and tropical Latin America (Adler *et al*., 2010). Approximately 17.7 million people (17.5 million in Africa, 30,000 in Arabia Peninsula and 140,000 in America) are estimated to be infected and 270,000 people are blind and another 500,000 have severe visual problems (Tada, 2015). *Simulium* spp. are also involved in the transmission of *Mansonella azzardi,* the causal agent of human mansonellosis with mild symptoms.

This filarial parasite is endemic to Central America, several places in South America and the Caribbean Island (Shelley & Coscaròn, 2001; Shelley *et al*., 2006; Cobo, 2014).

Black flies are also vectors of diseases of wild animals and livestock (Crosskey, 1990). It is a potential carrier of bovine onchocercosis caused by the filarioid nematode of the genus *Onchocerca*, including *O. dewittei japonica*, a common parasite of wild boar in Japan (Takaoka, 1994; Uni *et al*., 2001; Uni *et al*., 2015) and *O. linealis* of cattle in the United States (Lok *et al*., 1983). It is also a vector of dirofilariosis caused by *Dirofilaria ursi*, a filarial nematode of bears reported in Canada and the United States (Addison, 1980; Michalski *et al*., 2010) and Japan (Yokohata *et al*., 1990). It is an important vector of avian leucocytozoonosis caused by blood protozoa of the genus *Leucocytozoon* in Canada (James & Barrow, 1975; Greiner, 1991) where some species of simuliid may transmit more than one species of Leucocytozoon (Greiner, 1991). Moreover, its significance in the transmission of *Trypanosome confusum*, the causative agent of the avian trypanosomiasis has been acknowledged in North America (Mullen & Durden, 2009). Black flies also have been associated with arboviruses. Several North American black flies (e.g. *Simulium notatum* and *Simulium vittatum*) were reported to transmit vescular stomatitis virus to cattle, horse and pig (Schmidtmann *et al*., 1999; Adler & McCreadie *et al*., 2009; 2010). An African black fly (*Simulium damnosum*) was reported to transmit minute nematodes of the family Robertdollfusidae among wildlife (Bain & Renz, 1993). It is also a vector of other disease agents among wildlife such as bunyaviruses (Cupp, 1987), eastern equine encephalitis virus and snowshoe hare virus (Mullen & Durden, 2009). It is reported as a vector of myxomatosis among rabbit in South-Eastern Australia (Mykytowycz, 1957). In South East Asia (i.e., Thailand), three species of black flies namely, *Simulium asakoae*, *Simulium nodosum* and *Simulium nigrogilvum*, were reported to transmit the possible agents of zoonotic filariasis (Takaoka *et al*., 2003; Fukuda *et al*., 2003; Ishii *et al*., 2008). The host biting habits and vectorial capacity of Malaysian black flies, however, remain unknown.

The massive biting of black flies can trigger 'black fly fever' or simuliotoxicosis, a toxic effect of saliva during simuliid bites. Severe biting can bring to fatality as a result from acute toxemia and anaphylactic shock (Mullen & Durden, 2009). Deaths caused by the simuliotoxicosis have been reported among avian in Louisiana (Schnellbacher *et al*., 2012).

CHAPTER 3: MORPHOTAXONOMIC DESCRIPTION OF NEW BLACK FLY SPECIES OF THE GENUS *SIMULIUM* (DIPTERA: SIMULIIDAE) FROM PENINSULAR MALAYSIA

3.1 INTRODUCTION

The black flies of Malaysia have been little studied. Edwards (1928) recorded five new species of the subgenus *Simulium, S. argentipes*, *S. digrammicum*, *S. fuscopilosum*, *S. hackeri*, and *S. hirtinervis*. In 1973, Crosskey recorded seven species, *S.* (*Nevermannia*) *aureohirtum* Brunetti, *S.* (*Gomphostilbia*) *metatarsale* Brunetti, *S.* (*G.*) *sundaicum* Edwards, *S.* (*G.*) *varicorne* Edwards, *S.* (*G.*) *zonatum* Edwards, *S.* (*S.*) *nobile* de Meijere, *S.* (*S.*) *nitidithorax* Puri. Later, taxonomic revision made by Takaoka and Davies (1995) has confirmed all 12 species except *S.* (*G.*) *sundaicum* and three species, *S. metatarsale*, *S. zonatum*, and *S. nitidithorax*, all of which were treated as new species. Further revision was made by Takaoka and Davies (1995) upon samples collected by Dr. Douglas M. Davies (1975) and several other researchers namely Dr. John E. Bishop (1969−1970), Dr. John Smart (1964) and Dr. D. C. Kurtak (1975), revealed 28 new species which overall brings to 38 *Simulium* spp. validated in Peninsular Malaysia (Takaoka & Davies, 1995). Later, Takaoka *et al*., (2011, 2012, 2013, 2014) recorded 15 additional new species and three new records, increasing the number of species from Peninsular Malaysia from 38 to 56.

Simulium Latreille s. I., is the only genus recognized in the Oriental region, where a total of 524 species are recorded (Adler & Crosskey, 2016). Taxonomically, this genus is characterized by an adult antenna with 8 or 9 flagellomeres, anterior wing veins with spinules as well as hairs, radial sector not forked, well developed calcipala on the hind basitarsus, distinct pedisulcus on the 2nd hind tarsomere, pupa with a cocoon wall-pocket, shoe or boot shaped, hypostomal teeth of larvae rather low, median tooth not trifid, posterior arms of the anal sclerite subequal to or longer than anterior arms (Davies & Györkös, 1987).

Of 10 subgenera recognized in the Oriental Region, four are reported in Malaysia (*Daviesellum, Gomphostilbia, Nevermannia and Simulium*). Definitions of the genus *Simulium* and 10 subgenera follow those given by Crosskey (1969), Takaoka (2003, 2012), Takaoka and Adler (1997) and Takaoka and Choochote (2005). The updated identification keys to all these subgenera of the genus *Simulium* are presented in section 3.1.1. The subgenera and species-group known from Malaysia are shown in bold.

3.1.1 Identification keys to 10 subgenera of the genus *Simulium* in the Oriental Region. The subgenus and the species-group from Malaysia are shown in bold

Females

1	Katepisternum haired...	2
	Katepisternum bare..	3
2	Calcipala elongate, reaching apex of second tarsomere..................	*Wallacellum*
	Calcipala medium-long, not reaching apex of second tarsomere.....	***Gomphostilbia***
3	Pleural membrane haired...	4
	Pleural membrane bare..	5
4	Claw without tooth..	*Wilhelmia*
	Claw with small subbasal tooth...	*Simulium (S. ornatum* species-group*)*
5	Postnotum with hairs...	*Eusimulium*
	Postnotum without hairs..	6
6	Claw simple or with small subbasal tooth.......................................	7
	Claw with large or medium-sized basal tooth.................................	8
7	Paraproct with cluster of dark spines anteriorly..............................	***Daviesellum***
	Paraproct without cluster of dark spines anteriorly.........................	***Simulium*** (most species)
8	Basal section of radial vein bare...	*Byssodon*
	Basal section of radial vein haired...	9
9	Claw with medium-sized basal tooth..	*Montisimulium*
	Claw with large basal tooth..	10
10	Cibarium with numerous spinous processes medially....................	*Asiosimulium*
	Cibarium bare or with minute processes near ventral margin..........	***Nevermannia***

Males

1	Style longer than coxite..	2
	Style shorter than coxite..	3
2	Coxite much longer than wide..	***Daviesellum***
	Coxite as long as, or slightly shorter than, wide.............................	***Simulium***
3	Katepisternum haired...	4
	Katepisternum bare..	5
4	Calcipala elongate, reaching apex of second tarsomere..................	*Wallacellum*
	Calcipala medium-long, not reaching apex of second tarsomere.....	***Gomphostilbia***
5	Pleural membrane haired...	*Wilhelmia*
	Pleural membrane bare..	6
6	Basal section of radial vein bare...	*Byssodon*
	Basal section of radial vein haired...	7
7	Paramere without hooks...	*Asiosimulium*
	Paramere with parameral hook(s)..	8
8	Hind basitarsus slender; ventral plate with median keel.................	9
	Hind basitarsus enlarged; ventral plate without median keel...........	10
9	Ventral plate narrow, with divergent long arms..............................	*Eusimulium*
	Ventral plate broad, with parallel-sided short arms.........................	***Nevermannia*** (***S. ruficorne*** species-group)
10	Paramere with one long hook; median sclerite forked apically.......	***Nevermannia*** (***S. vernum*** species-group)
	Paramere with three or more hooks; median sclerite club-shaped, not forked apically………………………………………………..	*Montisimulium* & ***Nevermannia*** (***S. feuerborni*** species-group)

Pupae

1	Gill filaments with numerous minute black spots...........................	*Montisimulium*
	Gill filaments without minute black spots.......................................	2
2	Abdominal segments 5–9 without spine-combs dorsally................	3
	Abdominal segments with spine-combs dorsally (at least on segment 8)...	8
3	Gill with 19–33 filaments..	*Asiosimulium*
	Gill with three, four, six or eight filaments....................................	4
4	Gill with three filaments..	*Byssodon*
	Gill with four, six or eight filaments...	5
5	Gill with four filaments...	*Wallacellum*
	Gill with six or eight filaments..	6
6	Gill with eight filaments...	*Wilhelmia*
	Gill with six filaments..	7
7	Cocoon boot-shaped, with high neck..	***Daviesellum***
	Cocoon shoe-shaped, with low neck..	***Simulium*** (two species)
8	Frons with three pairs of trichomes; grapnel-shaped hooklets present on last abdominal segment (except a few species).............	***Gomphostilbia***
	Frons with two pairs of trichomes; grapnel-shaped hooklets absent on last abdominal segment..	9
9	Abdominal segments 7 and 8 (or only segment 8) with spine-combs dorsally; terminal hooks absent or small, cone-like, if present...	***Simulium***
	Abdominal segments 6–9 (or 5–9 or 6–8) with spine-combs dorsally; terminal hooks large, cone-like.......................................	10
10	Gill with inflated structure...	***Nevermannia*** (one species of ***S. vernum*** species-group)
	Gill with four, five, six or eight thread-like filaments.....................	11
11	Gill with four filaments..	*Eusimulium* & ***Nevermannia*** (most species of ***S. vernum*** species-group, one species of ***S. feuerborni*** species-group & one species of ***S. ruficorne*** species-group
	Gill with five, six or eight filaments…….......................................	12
12	Gill with eight filaments...	***Nevermannia*** (one species of ***S. ruficorne*** species-group)
	Gill with five or six filaments...	13
13	Gill with five filaments...	***Nevermannia*** (one species of ***S. feuerborni*** species-group)
	Gill with six filaments..	***Nevermannia*** (most species of ***S. feuerborni*** species-group & one species of ***S. ruficorne*** species-group)

Mature larvae

1	Hypostoma wide, with prominent median tooth accompanied by six small teeth on each side; posterior circlet with over 400 rows of hooklets..	***Daviesellum***
	Hypostoma with median tooth accompanied by one corner tooth and three intermediate teeth on each side; posterior circlet with fewer than 250 rows of hooklets..	2
2	Lateral margins of hypostoma smooth (except a few species); main tooth of mandibular serrations at an acute angle to mandible apically...	***Gomphostilbia***
	Lateral margins of hypostoma serrated; main tooth of mandibular serrations at right or obtuse angle to mandible apically..................	3
3	Mandibular serrations composed of two large teeth.........................	*Eusimulium* & ***Nevermannia*** (***S. ruficorne*** species-group)
	Mandibular serrations composed of one large tooth and one small tooth..	4
4	Postgenal cleft absent or vestigial...	*Montisimulium*
	Postgenal cleft small to large...	5
5	Antenna as long as stem of labral fan..	*Asiosimulium*
	Antenna longer than stem of labral fan...	6
6	Pectination of outermost primary ray of labral fan different from those of other primary rays..	*Wallacellum*
	Pectination of outermost primary ray of labral fan similar to those of other primary rays...	7
7	Ventral papillae large..	***Nevermannia*** (***S. vernum*** species-group & ***S. feuerborni*** species-group)
	Ventral papillae absent or small..	8
8	Rectal organ compound, each of three lobes with secondary lobules..	***Simulium***
	Rectal organ simple, each of three lobes without secondary lobules..	9
9	Histoblast of pupal gill with three filaments...................................	*Byssodon*
	Histoblast of pupal gill with eight filaments...................................	*Wilhelmia*

* The characters of the subgenus *Byssodon* used above are those of *S.* (*B.*) *languidum*, the only species of the subgenus in the Oriental Region, and are not necessarily shared with other species of the subgenus in other regions.

The morphotaxonomy is undeniably important for ecological, phylogenetic and chromosomal studies. All these aspects of black fly studies could not be reliably conducted without sufficient basic knowledge of the morphotaxonomy. Hence, the current study aimed to describe five new species using traditional but established important morphotaxonomy: *Simulium* (*Simulium*) *vanluni* **sp. nov**., *S.* (*Nevermannia*) *ledangense* **sp. nov**., *S.* (*N.*) *pairoti* **sp. nov**., *S. (G.) azhari* **sp. nov**., and *S.* (*G.*) *johorense* **sp. nov.**

3.2 MATERIALS AND METHODS

3.2.1 Sampling areas

Type localities of five new species are presented in Figure 3.1. During black fly surveys in Peninsular Malaysia, two new species were discovered from the state of Pahang: *Simulium vanluni* sp. nov. from Janda Baik (03º18.236'N 101º52.144'E) in the district of Bentong, and *S. pairoti* sp. nov. from Mount Brinchang (N04º31.461' E101º23.338') located in Cameron Highlands. *Simulium ledangense* sp. nov and *S. johorense* sp. nov were collected from Mount Ledang National Park (02°22.760'N 102°36.615'E and 02°19.705'N 103°37.410'E) in the state of Johor and *S. azhari* sp. nov. from Sedim (05°24.871'N 100°46.815'E) in the state of Kedah.

3.2.2 Sampling

Accessible streams and rivers supposed to be suitable for breeding of immature stages of black flies are surveyed. Larvae and pupae attached on aquatic substrates such as grasses, leaves and stems, twigs, plant roots and rocks were collected by hand using fine forceps. Pupae attached on similar substrates were individually kept alive in vials until emergence. The adults, together with their pupal exuviae and cocoons were preserved in 80% ethanol for identification at the subgenus, species-group or species level. The methods of collection and identification followed those of Takaoka (2003) and Adler *et al*. (2004).

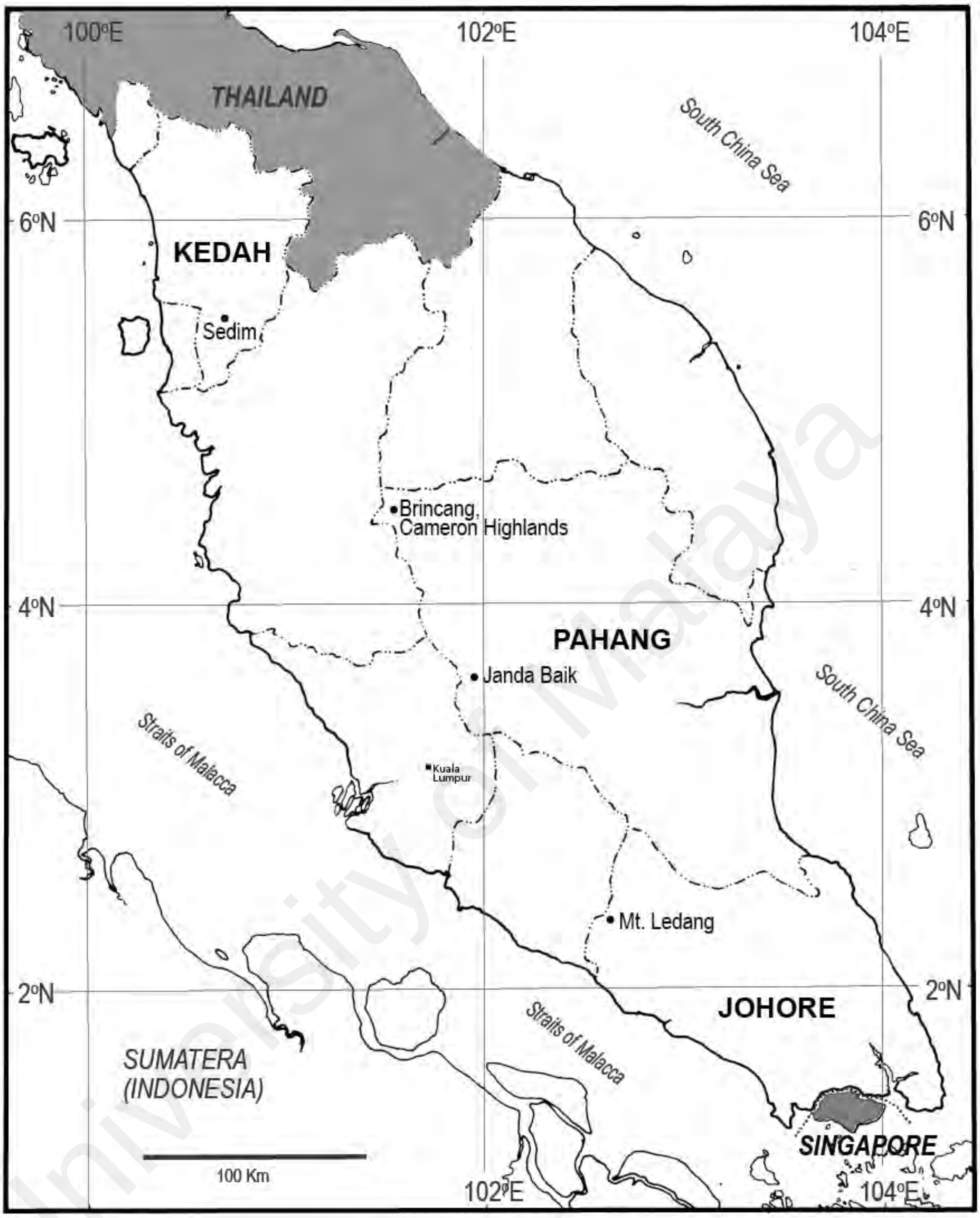

Figure 3.1: Map showing localities where the five new species were collected from Peninsular Malaysia.

3.2.3 Morphological observation

For taxonomic studies, adults, together with their pupal exuviae and cocoons are preserved in 80% ethanol and examined in a petri dish under a stereomicroscope (Nikon SMZ800) for identification at subgenus, species-group or species level. New species were described based on available specimens. Heads and abdominal tips of adult specimens were treated in 10% Potassium hydroxide (KOH) overnight to dissolve internal tissues and key characteristics observed and illustrated using compound microscope attached with a drawing apparatus (Nikon E200). The methods of description, illustration, and terms for morphological features used here, follow those of Takaoka (2003) and partially those of Adler *et al*. (2004). Observation of the morphological features involves all stages, adult, pupa and larva. Holotype and paratype specimens of the new species were deposited in the Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.

3.3 RESULTS AND DISCUSSION

3.3.1 *Simulium* (*Simulium*) *vanluni* sp. nov. Ya'cob, Takaoka and Sofian-Azirun 2016

Female (n=6). Body length 2.0−2.1 mm. ***Head***. Nearly as wide as thorax. Frons black, shiny, with several dark long stout hairs along each lateral margin; frontal ratio 1.24:1.00:1.35; frons:head ratio 1.0:4.4. Fronto-ocular area well developed, short, directed laterally and slightly upward. Clypeus black, white pruinose, slightly shiny when illuminated, moderately covered with dark long stout hairs along lateral and ventral margins and middle portion widely bare. Labrum 0.68−0.70 times as long as clypeus. Antenna composed of scape, pedicel and nine flagellomeres, yellow with apical two flagellomeres blackish; flagellomeres segments 1−7 often brownish dorsally, becoming darker apically with two or three apical segments darkest; first flagellomere 1.37 times length of second flagellomere. Maxillary palp with five segments, proportional lengths of third, fourth, and fifth segments 1.0:1.0−1.1:1.9−2.0; third segment (Fig. 3.2A) of normal size (not enlarged); sensory vesicle (Fig. 3.2A) small, ellipsoidal, with rugged surface, 0.22−0.24 times as long as third segment. Maxillary lacinia with 12 or 13 inner and 12 or 13 outer teeth. Mandible with 24 inner and 6 outer teeth. Cibarium with numerous well developed tubercles. ***Thorax***. Scutum black, shiny, not patterned, thinly gray pruinose and moderately covered with dark brown, recumbent pubescence. Scutellum brownish black, covered with dark upright long hairs. Postnotum bare, dark brown, shiny, silvery iridescent when illuminated. Pleural membrane bare. Katepisternum dark brown, longer than deep, bare. ***Legs***. *Foreleg*: coxa and trochanter pale yellow; femur dark yellow, gradually darkened towards apex, with pale scale-like hairs and usual straight hairs on outer surface;

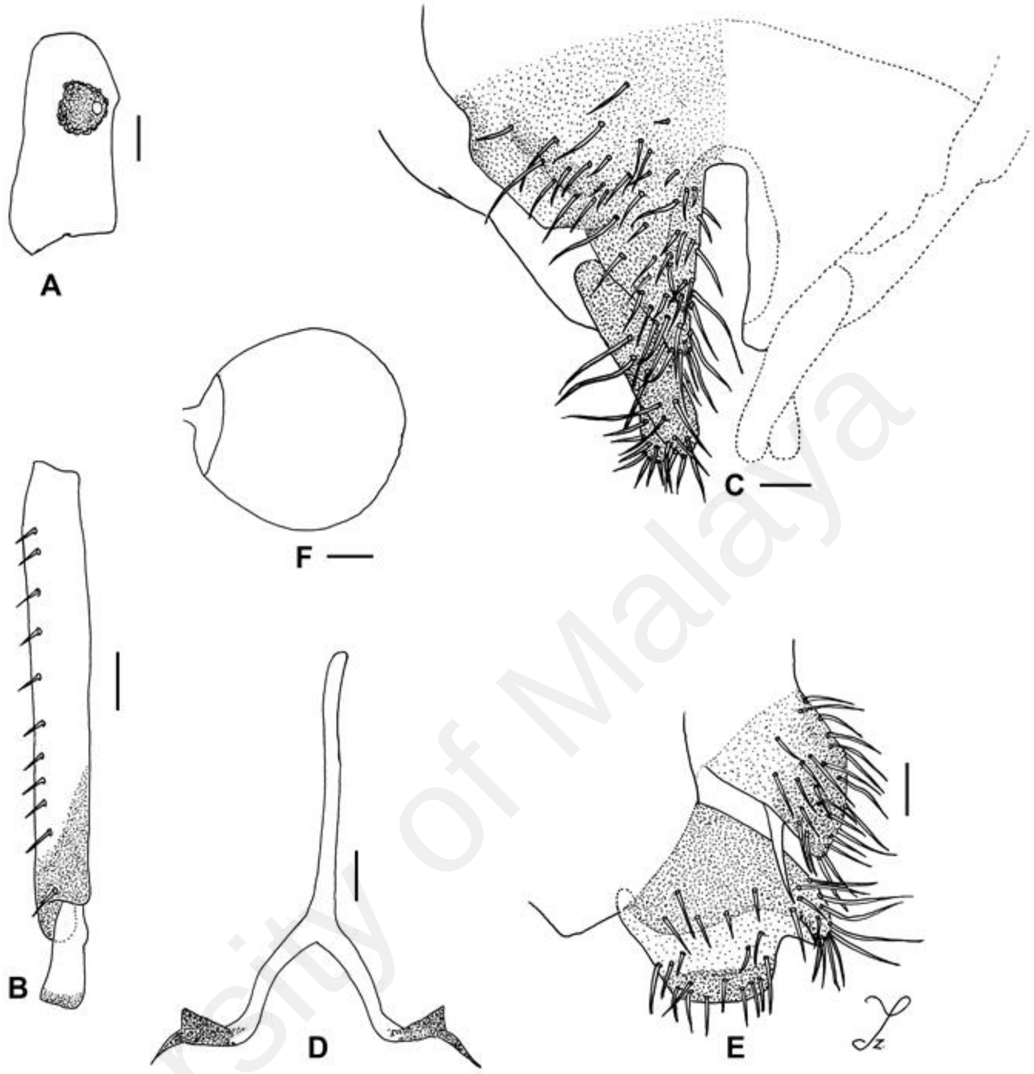

Figure 3.2: Female of *Simulium* (*Simulium*) *vanluni* **sp**. **nov**. (A) Third segment of right maxillary palp with sensory vesicle (front view). (B) Tibia of left hind leg (outer view). (C) Sternite 8 and ovipositor valves. (D) Genital fork (ventral view). (E) Right paraproct and cercus (lateral view). (F) Spermatheca. Scale bars = 0.1 mm (B) and 0.02 mm (A, C−F).

tibia brownish black, with brown scale-like hairs and large area of whitish sheen on outer surface; tarsus brownish black; fore basitarsus dilated, 4.0−4.1 times as long as greatest width. *Midleg*: coxa, trochanter and femur brownish black; tibia brownish black with large area of whitish sheen on posterior surface when illuminated; tarsus almost whitish except tarsomeres 4 and 5 light brown and medium brown respectively, and distal tip of basitarsus, tarsomeres 2 and 3 light brown. *Hind leg*: coxa brownish black; trochanter pale yellow; femur brownish black except base yellow, with brownish scale-like hairs and usual hairs on outer surface; tibia brownish black with brown scale-like hairs on outer surface with large area of whitish sheen on posterior surface when illuminated; basitarsus whitish except basal one-third brown, tarsomeres 4 and 5 medium brown and distal tips of tarsomeres 2 and 3 light brown; basitarsus (Fig. 3.2B) parallel-sided 8.0 times as long as it greatest width and 0.50 and 0.46 times as wide as greatest width of tibia and femur, respectively. Calcipala well-developed, 0.9 times as long as wide, 0.5 times as wide as basitarsal tip. Pedisulcus well developed. Claw with subbasal tooth. Wing. Length 1.6−2.0 mm; Costa with dark spinules and hairs; subcosta bare; basal section of radial vein bare; hair tuft on base of radial vein dark brown; basal cell absent. ***Abdomen***. Basal scale black with fringe of dark hairs; segment 2 dark brown with large, whitish, iridescent dorsolateral spots broadly connected in middle to each other; dorsal surface of abdomen dark brown to brownish black, with sparse dark hairs; tergites 6−8 shiny. ***Terminalia***. Sternite 8 (Fig. 3.2C) well sclerotized, bare medially, with dark medium-long to long stout hairs on each lateral surface. Ovipositor valves (Fig. 3.2C) produced backwardly fused with posterior margin of sternite 8 forming submedian lobes, each covered with numerous long and short hairs, bluntly pointed posteriorly; inner margin of lobes nearly parallel-sided, widely separated, each with nearly transparent projection sharply pointed backwardly (distinctively visible when viewed laterally). Genital fork (Fig. 3.2D) of inverted-Y form, with long well sclerotized stem; arms slender each with strongly sclerotized distal ridge. Paraproct (Fig. 3.2E) well sclerotized, much produced ventroposteriorly up to or slightly beyond level of anterior margin of cercus, with numerous long hairs ventrally and posteriorly; ventral surface narrow and rounded; paraproct subtriangular in lateral view (Fig. 3.2E), ca. 0.7 times as long as wide. Cercus rounded posteriorly, ca. 0.3 times as long as wide, covered with several short hairs. Spermatheca nearly (Fig. 3.2F) globular, well sclerotized except small area near tubal juncture, with weakly defined reticulate pattern, and minute internal setae.

Male (n=8). Body length 2.0–2.1 mm. ***Head***. Much wider than thorax. Upper eye brown, consisting of large facets in 13 or 14 vertical columns and in 13 or 14 horizontal rows. Clypeus black, white pruinose, iridescent when illuminated, sparsely covered with dark brown hairs along and near lateral margins (most of central portion bare). Antenna composed of scape, pedicel and nine flagellomeres, yellow with apical two flagellomeres (Fig. 3.3A) black; first flagellomere elongate (W:L = 1.0:1.8 in dorsal view), 1.29 times as long as second one. Maxillary palp composed of five segments, proportional lengths of third, fourth, and fifth segments 1.00:1.32:2.67−2.74; third segment (Fig. 3.3B) of normal size; sensory vesicle (Fig. 3.3B) small (0.22 times as long as third segment), globular, and with opening of moderate size. ***Thorax***. Scutum brownish-black to black, white pruinose (silver iridescent when illuminated) except two broad black bands each slanting across scutum from anteromedial portion to wing base forming inverted V-shaped; scutum moderately covered with recumbent scale-like brown hairs. Scutellum brownish-black, with several dark long upright hairs. Postnotum brownish-black, white pruinose, bare. Pleural membrane and katepisternum as in female. ***Legs***. Coloration of legs and shape of scale-like hairs on outer surface of femora and tibiae as in female.

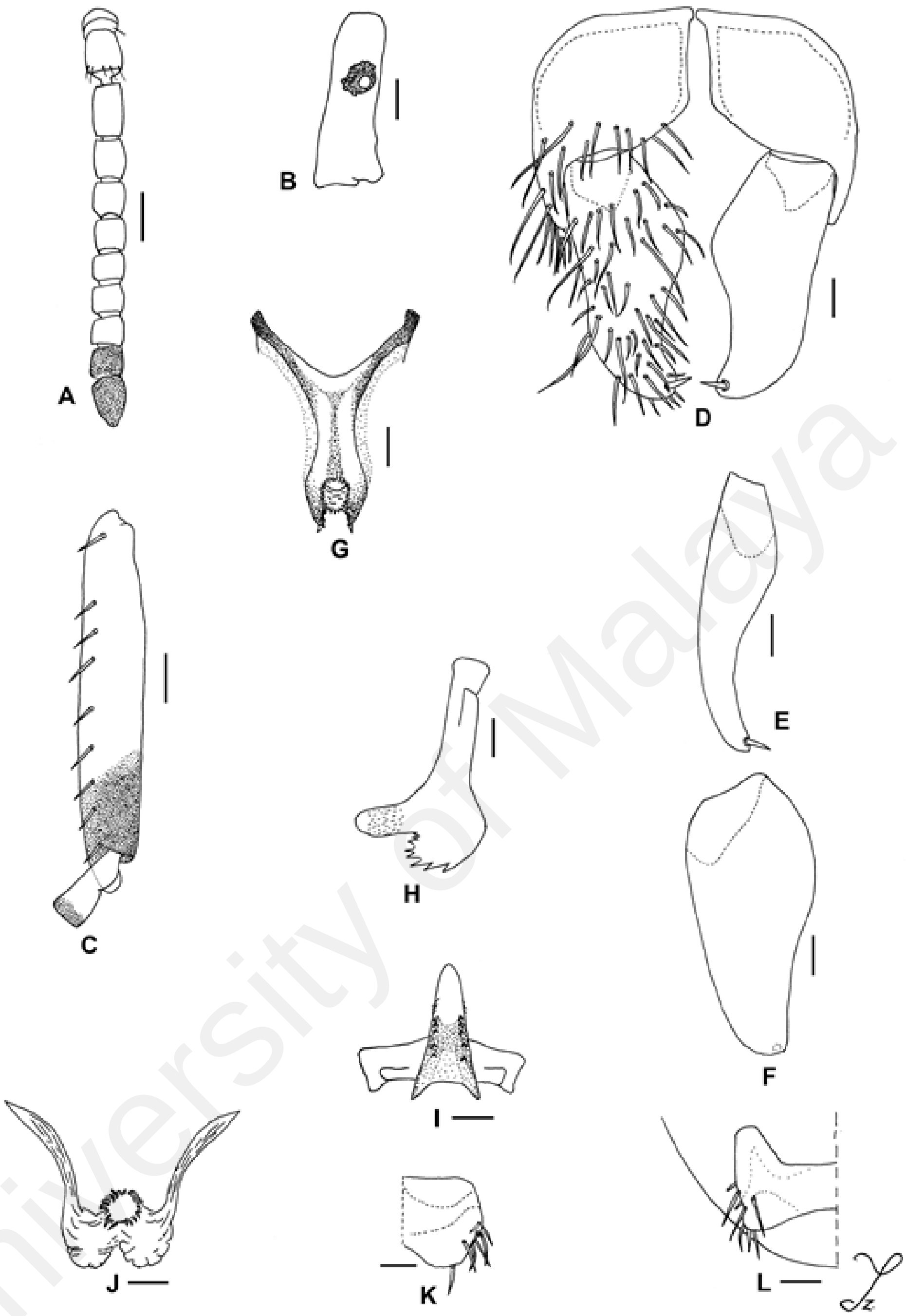

Figure 3.3: Male of *Simulium* (*Simulium*) *vanluni* **sp**. **nov**. (A) Antenna with apical two flagellar segments black. (B) Third segment of right maxillary palp with sensory vesicle having small-sized opening (front view). (C) Tibia of left hind leg (outer view). (D) Coxite, styles and (G) ventral plate (ventral view). (E) Left style (medial view). (F) Left style (ventrolateral view). (H) Ventral plate and median sclerite (lateral view). (I) Ventral plate (caudal view). (J) Parameres each with 5 hooks (end view). (K−L) Right cercus (lateral view). (Scale bars = 0.1 mm (C), 0.05 mm (A) and 0.02 mm (B, D−L).

Foreleg: basitarsus dilated, 4.5−4.6 times as long as its greatest width. *Hind leg*: basitarsus (Fig. 3.3C) parallel-sided, 6.1 times as long as its greatest width (in three males), and 0.62 and 0.56 times as wide as greatest widths of hind tibia and femur, respectively; calcipala well developed, as long as wide, 0.55 times as wide as basitarsal tip. Pedisulcus well developed. ***Wing***. Length 1.6−1.7 mm; other features as in female. ***Abdomen***. Basal scale blackish, with fringe of dark long hairs. Dorsal surface of abdomen black, with dark short hairs; segments 2 and 4–7 each with pair of silvery iridescent spots dorsolaterally, those on segment 2 broadly connected in middle to each other. ***Genitalia***. Coxite in ventral view (Fig. 3.3D) nearly quadrate, moderately covered with hairs on posterior half. Style in ventral view (Fig. 3.3D) elongate, widened to basal one-third then tapered towards apex with apical spine; style in ventrolateral view (Fig. 3.3F) elongate, 2.24 times as long as its greatest width at basal one-fourth, with inner margin slightly sinuous, slightly widened from base to basal one-fourth, then tapered toward apex; style in medial view (Fig. 3.3E) flattened dorsoventrally, without basal protuberance, 1.24 times as long as coxite. Ventral plate in ventral view (Fig. 3.3G) with elongate base, twice as long as wide, rounded posteriorly, much raised ventrally, bearing parallel rows of sharp teeth posteriorly; arms widely diverging; ventral plate in lateral view (Fig. 3.3H) with median process thumb-like, hairy anteriorly, projecting ventrally beyond dentate portion; ventral plate in end view (Fig. 3.3I) having body narrowed to apex with five to six teeth at two regular vertical rows on posterior surface. Median sclerite simple, narrow, plate-like, slightly widened apically. Paramere (Fig. 3.3J) elongate each with several small hooks. Aedeagal membrane short, moderately covered with minute setae. Abdominal segment 10 without any distinct hair on ventral and lateral surface. Cercus (Fig. 3.3K, L) small, rounded, with 8 or 9 distinct hairs.

Pupa (n=6). Body length 2.0–2.1 mm. ***Head***. Integument dark yellow except antennal sheaths yellow, sparsely covered with small minute tubercles; frons with two pairs of unbranched slender short trichomes; face with pair of unbranched medium-long trichomes. ***Thorax***. Integument dark yellow, unevenly covered with minute tubercles (sparsely on anterodorsal surface, moderately on peripheral and posterodorsal areas); thorax on each side with three medium-long trichomes mediodorsally, two medium-long trichomes anterolaterally, one medium-long trichome mediolaterally, three medium-long trichomes ventrolaterally; all unbranched. Gill (Fig. 3.4A) with six short filaments in three pairs arising from short common basal stalk, short-stalked except middle pair sessile, somewhat inflated basally, tapered apically; upper and middle paired filaments subequal in length (ca. 0.57 mm) and lower pair slightly longer (ca. 0.73 mm); cuticle of filaments with numerous transverse ridges sharply edged for the most part but weakly edged basally; densely covered with minute tubercles. ***Abdomen***. Dorsally, segment 1 weakly sclerotized, with one unbranched slender short seta on each side; segment 2 transparent, with one unbranched short seta and five minute setae on each side; segments 3 and 4 each with four hooks spines along posterior margin and one short seta on each side; segments 5−7 bare, without spine-comb and comb-like group of minute spines; segment 8 with transverse row of seven to eight spine-combs and one short seta on each side; segment 9 with comb-like groups of minute spines, lacking spine-combs and terminal hooks. Ventrally, segments 3–9 transparent; segment 5 with pair of bifid hooks close together submedially on each side; segments 6 and 7 each with pair of bifid hooks widely spaced on each side. Grapnel-shaped hooklets absent on each side of segment 9. ***Cocoon*** (Fig. 3.4B3.4C). Shoe-shaped with narrow anteroventral collar, tightly and thickly woven, light brown, moderately extending ventrolaterally; opening directed upward and forward, with thick margin; 2.5−2.8 mm long by 1.1−1.2 mm wide.

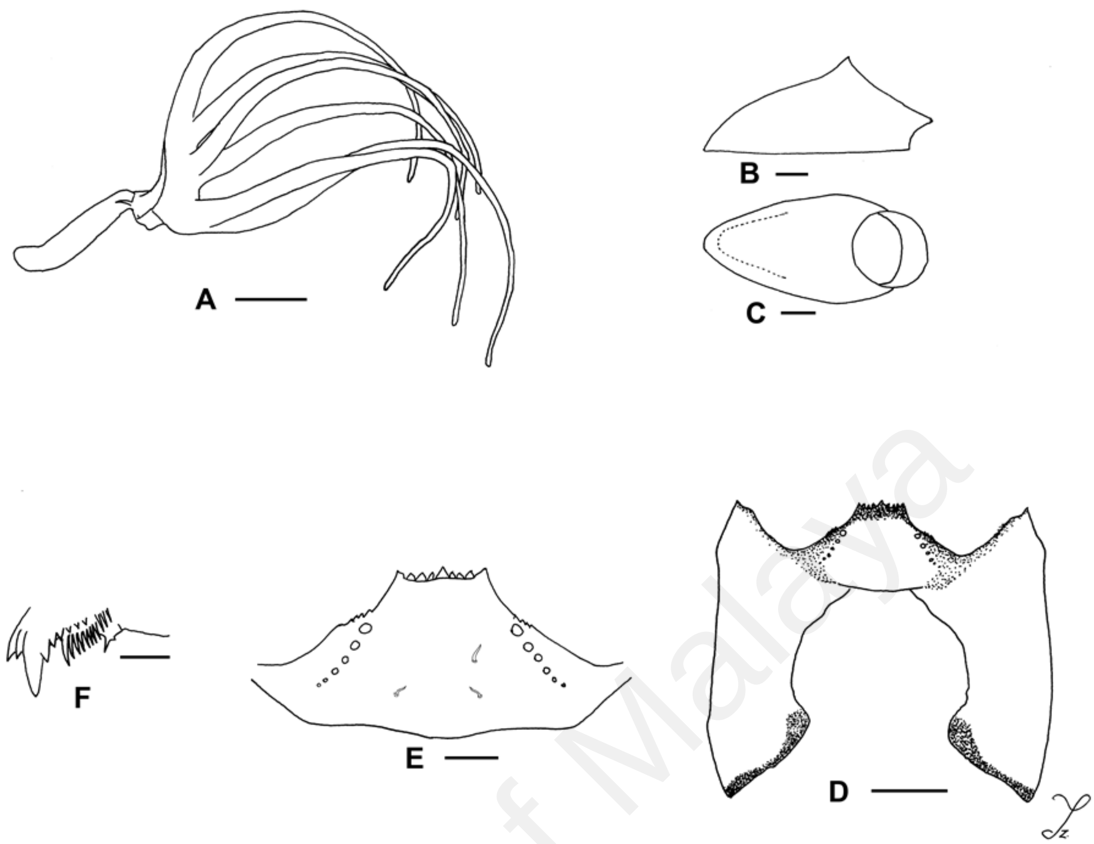

Figure 3.4: Pupa and larva of *Simulium* (*Simulium*) *vanluni* **sp**. **nov**. (A) Gill filaments (Right side, outer view). (B−C) Cocoon (lateral view and dorsal view). (D) Larva head capsule (ventral view). (E) Hypostoma. (F) Mandible. Scale bars = 0.1 mm (A−D), 0.02 mm (E−F).

Mature larva (n=14). Body length 3.7–4.2 mm. Body dark gray, banded on abdominal segments 1−4, and often with purplish markings on posterior abdominal segments. Cephalic apotome variable in markings (entirely pale yellowish with indistinct head spots in three larvae, more or less darkened partially or entirely on posterior two-fifths in five larvae, markedly dark submedially between anterolateral and posterolateral spots and along posterior border in six larvae). Ventral surface of head capsule (Fig. 3.4E) dark yellow to medium brown though area near posterior margin of hypostoma often yellowish narrowly, and each side of basal portion of postgenal cleft dark brown. Antenna composed of three segments and apical sensillum, slightly longer than stem of labral fan; length ratio of three segments (from base to tip) 1.00:0.98–1.04:0.60–0.65. Labral fan with 46−48 primary rays (46 primary rays in 5 larvae, 47 primary rays in 8 larvae and 48 primary rays in 1 larva). Mandible (Fig. 3.4F) with mandibular serration composed of two teeth (one medium-sized, one small); main tooth at obtuse angle against mandible on apical side; supernumerary serrations absent; comb-teeth decreasing in length from first to third. Hypostoma (Fig. 3.4G) with nine anterior teeth, of which median and corner teeth most prominent; three intermediate teeth on each side small, subequal in length to one another; lateral margins serrate apically; six hypostomal bristles divergent posteriorly from lateral border on each side. Postgenal cleft (Fig. 3.4E) deep, reaching posterior border of hypostoma. Histoblast of pharate pupal gill with six short filaments. Abdominal segments 1−5 with each with dorsal pair of conical protuberances decreasing in size forwardly; another dorsal pair of small protuberances present on metathoracic segment. Thoracic cuticle bare. Abdominal cuticle covered dorsally with spatulate setae moderately on segments 5−8, very sparsely on segments 2−4; segment 5 and adjacent segments covered with minute branched setae dorsally; few pale setae near each dorsal protuberance and numerous short, pale setae on sides of anal sclerite. Rectal papilla of three lobes, each with 5−11 finger-like secondary lobules.

Anal sclerite X-shaped, with broadened anterior arms, much shorter than posterior ones. Last abdominal segment bulged laterally, lacking ventral papillae. Posterior circlet with 96−99 rows with 15 or 17 hooklets per row.

Type specimens. HOLOTYPE: Male (with associated pupal exuviae and coccon) (preserved in 80% ethanol), reared from a pupa, manually collected from a moderate flowing stream (width 3.7 m, streambed rocky, depth 0.6 m, water temperature 22.8°C, partially shaded, altitude 481 m, 03º18.236'N 101º52.144'E), located at Janda Baik village, Pahang Province, Peninsular Malaysia, 26. XI. 2015, by Chin AC, Prakash BK, Fadil NM and Ya'cob Z. Paratypes: 8 males, 6 females (with associated pupal exuviae and coccons), and 14 mature larvae, preserved in 80% ethanol, same data and date as those of holotype.

Distribution. Peninsular Malaysia (Janda Baik, Pahang).

Etymology. The species name *vanluni* is in honor of Van Lun Low, PhD, University of Malaya, Kuala Lumpur, Malaysia, for his enthusiastic and tremendous contributions in molecular phylogenetics and systematics of black flies in Asia.

Biology. The pupae and larvae of *S.* (*S.*) *vanluni* sp. nov. were collected from artificial substrates, leaves and twigs of a fallen tree trailing in the water. The co-existing species were *S. duolongum* Takaoka and Davies and *S. jeffreyi* Takaoka and Davies.

Remarks. A recent phylogenetic study clearly separated *S. nobile* into three geographically based lineages–lineage A is composed of a population from Sabah, East Malaysia (Borneo); lineage B represents the type population from Java, Indonesia; and lineage C includes populations from the mainland of Southeast Asia (southern Thailand and Peninsular Malaysia) (Low *et al*., 2016). Hence, formal taxonomic description and naming of lineage B from the mainland population were performed in the present study. The *Simulium nobile* species complex provides a noteworthy example of cryptic species in the family Simuliidae. *Simulium vanluni* sp. nov. is almost identical with the re-description of *S. nobile* from Java (Takaoka & Davies, 1996) in most of the diagnostic characters of all life stages: enlarged upper-eye facets, scutal pattern and antenna darker on apical two flagellomeres in the male, the size and shape of the sensory vesicle, shape and size of genitalia (ventral plate, coxite and style), color pigmentation and sizes (length against its greatest width) of the legs in both male and female, number and arrangement of gill filament, shape of the cocoon in pupa, body pigmentation, number of teeth on the hypostoma and mandible, shape of the postgenal cleft, possession of dorsal protuberances and spatulate setae in the mature larva.

Morphologically re-examination of the larvae of *S. nobile* from Java and Borneo was also conducted in the present study. Several numerical differences were detected on the number of primary labral fans and row posterior hooklets among three lineages. *Simulium nobile* from Java and Borneo having reduced number of primary labral fans (32−38 and 40−43 primary rays respectively) and row of posterior hooklets (86−88 and 75−80 rows respectively). These characters are consistent with the previous re-description of *S. nobile* by Takaoka and Davies (1996) and original description of *S. kiuliense* (synonym for *S. nobile*) by Smart and Clifford (1968). The encountered numerical differences, however, are not reliable in distinguishing species because these variations might be influenced by the ecological factors (i.e., water flow, seston availability and habitat filtering) (Palmer & Craig, 2000; Pangjanda & Pramual, 2016). Presently, DNA sequences provide the most reliable diagnostic method for discriminating these three lineages. Specifically, the distinctiveness of *S. vanluni* sp. nov. is supported by the robust phylogenetic analyses (data not shown).

Six species of the *S. nobile* species-group from the Philippines (Takaoka, 1983; Adler & Crosskey, 2016) (*S. baltazarae* Delfinado, *S. cotabatoense* Takaoka, *S. leytense* Takaoka, *S. latistylum* Takaoka, *S. benquetense* Takaoka) differ from the new species by the absence of the dorsal pair of conical protuberances on larva abdominal segments 1−5 and on metathoracic segment and without spatulate setae mainly on posterior abdominal segments (Takaoka & Davies, 1996).

Two members of the same species-group from other countries, *S. asishi* Datta from India (Datta, 1988) and *S. nodosum* Puri from China, Myanmar, Taiwan, Thailand and Vietnam (Puri, 1933; Low *et al*., 2015; Adler & Crosskey, 2016), are easily distinguished from the new species by the pupa having three gill filaments per side. *Simulium timorense* described from Flores and West Timor, Indonesia (Takaoka *et al*., 2006), differs by the larva without paired dorsal protuberances on larva abdominal segments 1−5 (Takaoka *et al*., 2006).

3.3.2 *Simulium* (*Nevermannia*) *ledangense* sp. nov. Ya'cob, Takaoka and Sofian-Azirun 2014

Female (n = 3). Body length 2.1 to 2.5 mm. ***Head***. Slightly narrower than thorax. Frons dark brown, thinly grey pruinose, densely covered with yellow hairs and several dark longer hairs along each lateral margin. Frontal ratio 1.81–1.90:1.00:2.75–2.96. Frons:head ratio 1.00:4.76–4.79. Clypeus dark brown, whitish-gray pruinose and covered with whitish-yellow hairs and several dark longer hairs on lower half and bare in middle. Labrum 0.84–1.00 times length of clypeus. Antenna composed of scape, pedicel and 9 flagellomeres, dark brown except scape, pedicel and base of first flagellomere yellow; first flagellomere 2.50–2.62 times length of second flagellomere. Maxillary palp consisting of 5 segments, proportional lengths of third, fourth, and fifth segments 1.00:0.78–0.93:1.47–1.61; third segment much enlarged, sensory vesicle elongate (Fig. 3.5A), 0.64 times length of third segment, with medium-sized opening. Maxillary lacinia with 6 or 8 inner teeth and 13 or 14 outer teeth. Mandible with 19–20 inner and lacking outer teeth. Cibarium (Fig. 3.5B) with 40–44 dark minute conical processes with pointed apices as well as numerous minute spinous processes near lower margin. ***Thorax***. Scutum light to medium brown except anterolateral calli yellow, two submedian longitudinal vittae and narrow portion along lateral margin and prescutellar area dark brown, white pruinose except 3 longitudinal vittae (1 narrow median, 2 little wider submedian) and slightly shiny when illuminated at certain angles; scutum covered with yellow short hairs and several dark-brown longer hairs on prescutellar area. Scutellum dark yellow, slightly shiny when illuminated at certain angles, with whitish-yellow hairs and dark longer hairs. Postnotum medium to dark brown, thinly gray pruinose and slightly shiny when illuminated at certain angles.

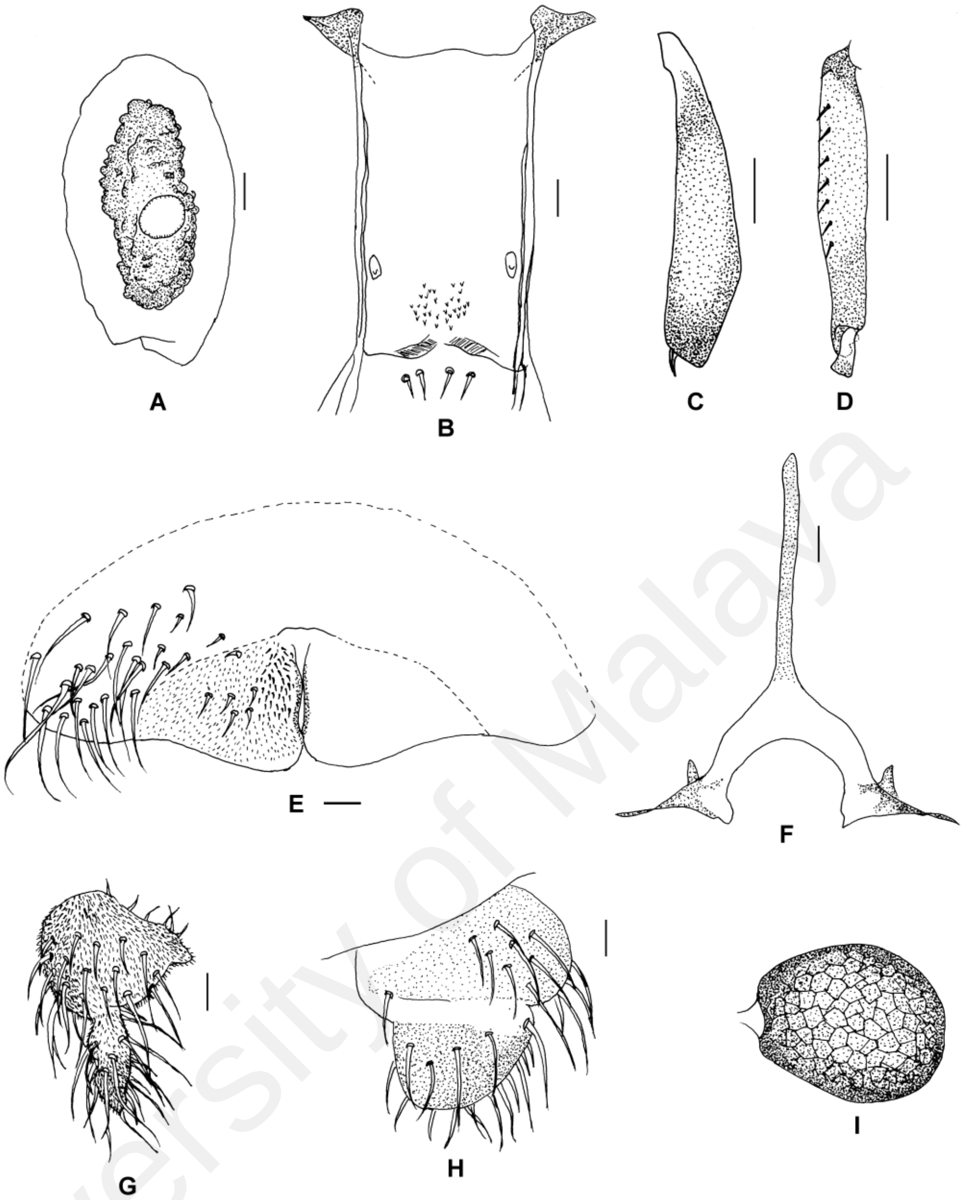

Figure 3.5: Female of *Simulium* (*Nevermannia*) *ledangense* **sp. nov**. (A) Third segment of left maxillary palp with sensory vesicle (front view). (B) Cibarium. (C) Tibia of left hind leg (outer view). (D) Basitarsus and second tarsomere of left hind leg (outer view). (E) Sternite 8 and ovipositor valves. (F) Genital fork (ventral view). (G) Right paraproct and cercus (ventral view). (H) Right paraproct and cercus (lateral view). (I) Spermatheca. Scale bars = 0.1 mm (C, D) and 0.02 mm (A, B, E–I).

Pleural membrane and ketepisternum medium brown, thinly grey pruinose and slightly shiny when illuminated at certain angles and bare; ***Halter***. White except basal portion light brown. ***Legs***. *Foreleg*: coxa yellow and trochanter yellow to light brown; femur yellow to light brown except apical cap dark brown; tibia dark brown except median large portion of outer surface light brown and basal tip yellow; tarsus dark brown. Basitarsus slender, slightly dilated, 7.64–7.73 times as long as its greatest width. *Midleg*: coxa medium brown except posterolateral surface dark brown; trochanter yellow; femur yellow to light brown except apical cap dark brown; tibia dark brown except medium large portion light brown; tarsus dark brown except fourth segment light brown with basal portion lighter. *Hind leg*: coxa light to median brown; trochanter yellow; femur yellow to light brown except apical cap dark brown; tibia (Fig. 3.5C) dark brown except median large portion of outer surface medium brown and basal tip yellow; tarsus dark brown except basal two-thirds of basitarsus (Fig. 3.5D) whitish-yellow though basal tip dark brown and basal half of second tarsomere whitish-yellow; basitarsus 7.64–7.73 times as long as it greatest width; basitarsus parallel-sided, 6.00–6.33 times as long as width, and 0.77 and 0.58–0.67 times as wide as greatest width of tibia and femur, respectively. ***Wing***. Length 2.2 mm. Costa with two parallel rows of dark-brown spinules as well as dark-brown hairs except sub-basal patch of hairs yellow. Subcosta with dark-brown hairs except near apex bare. Basal portion of radius fully haired; R_1 with dark brown spinules and hairs; R_2 with dark-brown hairs. Hair tuft on base of radial vein dark brown. Basal medial cell absent. ***Halter***. Entirely dark brown. ***Abdomen***. Basal scale light brown with fringe of whitish-yellow long hairs. Dorsal surface of abdomen medium to dark brown; tergites of segment 2 and 5 to 7 shiny. Abdomen whitish-yellow short hairs and brownish-black short hairs mixed with dark-brown long hairs. ***Genitalia***. Sternite 8 wide (Fig. 3.5E), bare medially and furnished with 14–21 medium to long hairs and few short hairs on each side. Ovipositor valves

(Fig. 3.5E) triangular (through posteromedial corner rounded), thin, membranous except inner margin narrowly sclerotized, densely covered with microsetae interspersed with 6 or 7 short hairs; inner margin sinuous, narrowly separated from each other. Genital fork (Fig. 3.5F) of inverted Y-form, with well sclerotized stem and relatively wide arms; each arm with lateral plate bearing triangular lobe-like projection directed medioposteriorly and short narrow stout projection directed anterodorsally. Paraproct (Fig. 3.5G) in ventral view subquadrate, slightly longer than its greatest width; anteromedial surface nearly transparent, with 5–10 sensilla; paraproct in lateral view (Fig. 3.5H) somewhat protruded ventrally beyond ventral margin of cercus, and with 21–24 medium to long hairs on ventral and lateral surfaces. Cercus in lateral view (Fig. 3.5H) rounded posteriorly, short, 0.62 times as long as basal width. Spermatheca (Fig. 3.5I) nearly ovoidal, strongly sclerotized except small area around juncture with duct and duct itself unsclerotized, with distinct reticulate surface pattern and without internal setae; main spermathecal duct narrow, somewhat narrower than both accessory ducts.

Male (n = 5). Body length 2.1–2.5 mm. ***Head***. Slightly wider than thorax. Holoptic; upper eye consisting of large facets in 13 or 14 vertical columns and 15 horizontal rows. Clypeus dark brown, gray pruinose when illuminated at certain angles, moderately covered with yellow hairs intermixed with dark-brown longer hairs except medial portion widely bare. Antenna medium brown except scape and pedicel dark yellow to light brown and base of first flagellomere whitish-yellow. Maxillary palp composed of 5 segments, light dark except third segment dark brown. Proportional length of third, fourth and fifth segments 1.00:0.96–1.04:1.79–1.93; third segment (Fig. 3.6A) of moderate size; sensory vesicle (Fig. 3.6A, B) small, ellipsoidal, 0.16–0.18 times length of third segment. ***Thorax***. Scutum light to medium brown except anterolateral calli yellow, submedian longitudinal vittae and narrow portion along lateral margin and prescutellar area dark brown, whitish pruinose except 3 longitudinal vittae (1 narrow median, 2 little wider submedian) non-pruinose and slightly shiny when illuminated at certain angles, densely covered with yellow short hairs and several dark brown longer hairs on prescutellar area. Scutellum dark yellow, slightly shiny when illuminated at certain angles, with whitish-yellow hairs and dark longer hairs. Postnotum medium to dark-brown, thinly gray pruinose and slightly shiny when illuminated at certain angles. Pleural membrane and ketepisternum medium brown, thinly gray pruinose, slightly shiny when illuminated at certain angles and bare. ***Legs***. *Foreleg*: coxa yellow; trochanter yellow with somewhat dark portion on outer surface; femur dark yellow to light brown except apical cap dark brown; tibia dark brown though median large area on outer surface light to median brown; tarsus entirely brownish-black; basitarsus slender, slightly dilated, 7.8–8.8 times as long as its greatest width. *Midleg*: coxa medium brown though dark brown on posterolateral surface; trochanter yellow with somewhat dark portion on outer surface; femur dark yellow to light brown with apical cap dark brown; tibia dark brown with median large portion; tarsus dark brown. *Hind leg*: coxa light to medium brown; trochanter yellow; femur dark yellow to light brown with apical cap dark brown; tibia dark brown with extreme base dark yellow and median large portion light to medium brown; tarsus medium brown except base and apical portion of basitarsus dark brown, and basal half of basitarsus and second tarsomere light brown; basitarsus (Fig. 3.6C) enlarged, spindle-shaped, 4.46–4.64 times as long as its greatest width, and 0.96–1.00 and 0.89–0.93 times as wide as greatest widths of hind tibia and femur, respectively; calcipala well developed, nearly as long as basal width and 0.37 times as wide as greatest width of basitarsus; pedisulcus moderately developed.

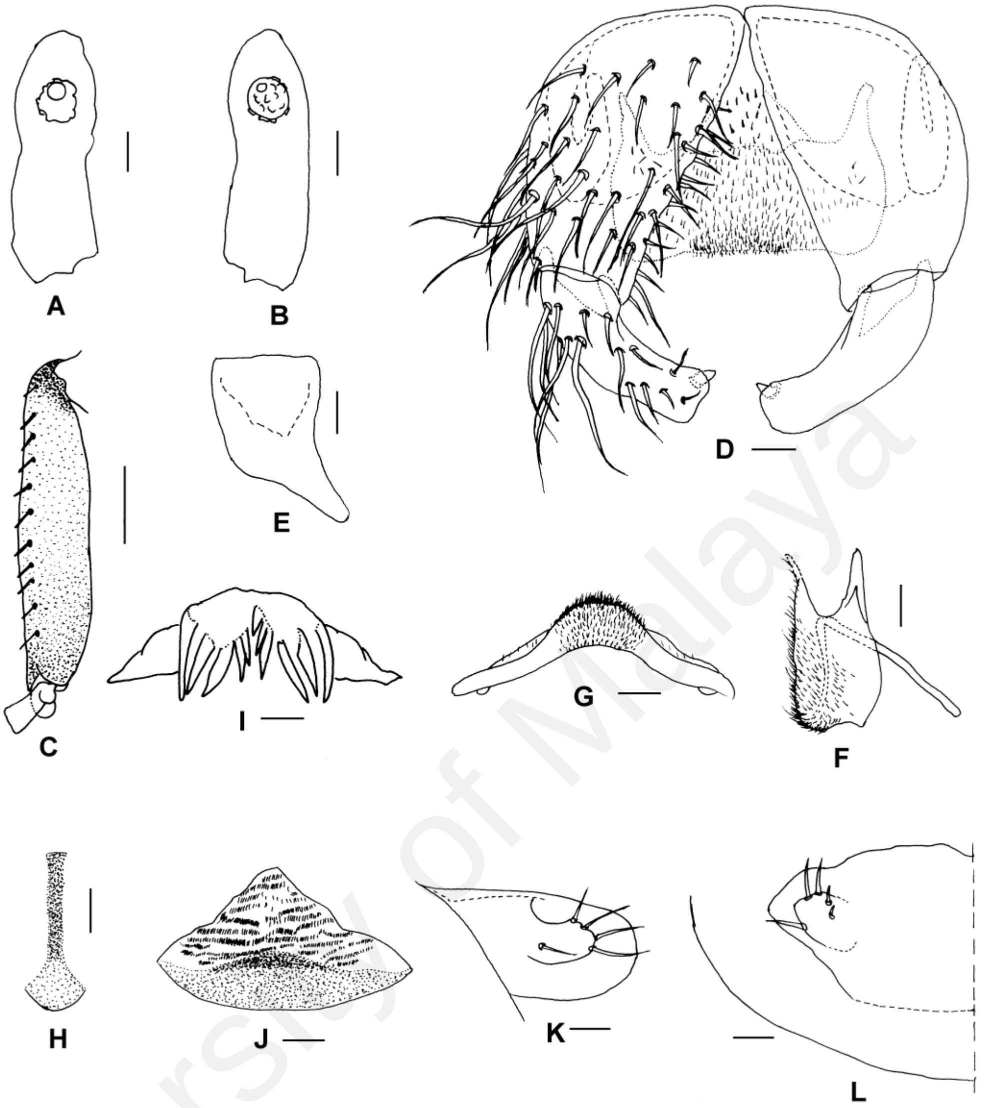

Figure 3.6: Male of *Simulium* (*Nevermannia*) *ledangense* **sp. nov**. (A) Third segment of right maxillary palp with sensory vesicle having moderate-sized opening (front view). (B) Third segment of left maxillary palp with sensory vesicle of small-sized opening (front view). (C) Basitarsus and second tarsomere of left hind leg showing calcipala and pedisulcus (outer view). (D) Coxites, styles and ventral plate (ventral view). (E) Left style (ventrolateral view). (F) Ventral plate and median sclerite (lateral view). (G) Ventral plate (caudal view). (H) Median sclerite ventral view). (I) Parameres each with 5 or 6 hooks (end view). (J) Aedeagal membrane and dorsal plate (end view). (K) 10th abdominal segment and cercus (right side; lateral view). (L) Right cercus (end view). Scale bars = 0.1 mm (C) and 0.02 mm (A, B, D–L).

Wing. As in female except subcosta bare or 1–7 hairs; length 2.4–2.5 mm. ***Halter***. Entirely dark brown. Abdomen. Basal scale brownish-black, with fringe of dark and pale long hairs. Dorsal surface of abdominal segments entirely dark brown to brownish-black, and moderately covered with light brown to black short to long hairs mixed with brassy short hairs. Segments 2–8 each with pair of white pruinose dorsolateral patches which are shiny when illuminated at certain angles. ***Genitalia***. Coxite in ventral view (Fig. 3.6D) rectangular, 1.78 times as long as its greatest width. Style in ventral view (Fig. 3.6D) short, 0.70 times as long as coxite, bent inwardly, with outer margin angled medially and with short stout spine apically; style in medial view gently curved dorsally and nearly parallel-sided; style in ventrolateral view (Fig. 3.6E) broad, nearly parallel-sided from base to little beyond middle, then abruptly tapered apically; style in end view tapered inward, with round apex. Ventral plate in ventral view (Fig. 3.6D) lamellate, subquadrate, 0.52 times as long as its greatest width, well sclerotized except anteromedian portion unsclerotized, with posterior margin slightly concave medially and submedially, and moderately covered with fine short setae on ventral surface except lateral portion widely bare; arms short, slender, directed anteriorly; ventral plate in lateral view (Fig. 3.6F) with ventral margin nearly straight and arm short, tapered anterodorsally; ventral plate in caudal view (Fig. 3.6G) with dorsal margin markedly concave, with fine short setae centrally on posterior surface. Median sclerite (Fig. 3.6H) simple, club-shaped, narrow and strongly sclerotized except weakly sclerotize apical portion. Paramere (Fig. 3.6I) with 5 or 6 hooks decreasing in length toward apex. Aedeagal membrane (Fig. 3.6J) moderately covered with minute setae; dorsal plate triangular in shape, thin, weakly sclerotized. Ventral surface of segment 10 without any distinct hairs near each posterolateral corner. Cercus (Fig. 3.6K, L) small, rounded and encircled by 5–9 simple hairs.

Pupa (n = 8). Body length 3.0 mm. ***Head***. Integument yellow to light brown, moderately covered with round tubercles of various sizes on frons and sparsely covered with similar tubercles of various sizes on lateral surface of face; antennal sheath bare; frons with pair of short minute trichomes on each side; face with 1 medium-long unbranched trichome on each side. ***Thorax***. Integument yellow to light brown, moderately covered with round tubercles of different sizes on dorsal and lateral surfaces of anterior half and sparsely covered with smaller ones on dorsal surface of posterior half; thorax on each side with 3 long trichomes with coiled apices mediodorsally (Fig. 3.7G), 2 trichomes (1 long with coiled apex, 1 medium-long with uncoiled apex) anterolaterally, 1 medium-long trichome with uncoiled apex mediolaterally and 3 short trichomes with uncoiled apices ventrolaterally (Fig. 3.7H), all trichomes unbranched. Gill (Fig. 3.7A, B) with 6 long thread-like slender filaments, arranged as [1 + (2+ 1)] + 2 filaments in vertical plane from dorsal to ventral; common basal stalk of moderate length, subequal in length to interspiracular trunk, and stalk of ventral pair 0.52 times total length of ventral paired filaments including common basal stalk (varying from 1.05 mm to 1.50 mm in individual pupae); stalk of middle pair short; all filaments tapered toward tip, subequal in length (4.4–5.5 mm) and thickness to one another except 2 filaments of ventral pair somewhat shorter (3.5–4.3 mm) and slightly thinner than others; cuticular surface with distinct annular ridges and furrows though becoming less distinct near apex, and densely covered with minute tubercles. ***Abdomen.*** Dorsally, all segments weakly yellowish, moderately and neatly covered with minute tubercles; segment 1 with 1 long-medium slender setae on each side; segment 2 with 1 hair-like seta and 5 short setae, of which 4 stout and spinous and 1 slender on each side; segments 3 and 4 each with 4 hooks and 1 very short spinous seta on each side; segment 5 bare; segments 6–8 each with spine-combs directed backwards in transverse row and comb-like group of minute spines on each side; segment 9 with pair of distinct conical terminal hooks as well as comb-like groups of minute spines. Ventrally, segments 3–8 with comb-like groups of minute spines; segment 4 with 1 simple hooklet and few short unbranched slender setae on each side; segment 5 with pair of bifid hooks submedially and few very short slender setae on each side; segments 6 and 7 each with pair of bifid inner and unbranched outer hooks and few slender very short setae on each side. ***Cocoon***. Simple, wall-pocket shaped, compactly woven without open spaces in web, with anterior margin thickly woven, without anterodorsal projection; individual threads invisible; 3.7 mm long by 2.5 mm wide.

Mature larva (n = 3). Body length 5.1 mm. Body (Fig. 3.7C) creamy, with reddish-brown markings on abdominal segments 3–4. Cephalic apotome whitish-yellow to yellow; head spots very faintly positive. Lateral surface of head capsule yellow except eye-spot region white; eyebrow indistinct though 1 dark sport present medially; 2 large spot and 1 small spot near posterior margin and 2 small spots below eye-spot region faintly to moderately positive. Ventral surface of head capsule (Fig. 3.7D) yellow except medial large portion darkened, and basal area on each side of postgenal cleft dark brown; 1 elongate and 1 round spot on each side of postgenal cleft markedly positive. Cervical sclerite compose of 2 faint small elliptical pieces, not fused to occiput, widely separated medially from each other. Antennae consisting of 3 articles and apical sensilium, much longer than stem of labran fan; proportional lengths of first, second, and third articles 1.00:0.97:0.64. Labran fan with 19 main rays. Mandible (Fig. 3.7F) with serrations consisting of 2 teeth (1 large and 1 small); large tooth at obtuse angle with mandible on apical side; comb-teeth composed of 3 teeth shortened from first to third; supernumerary serration absent.

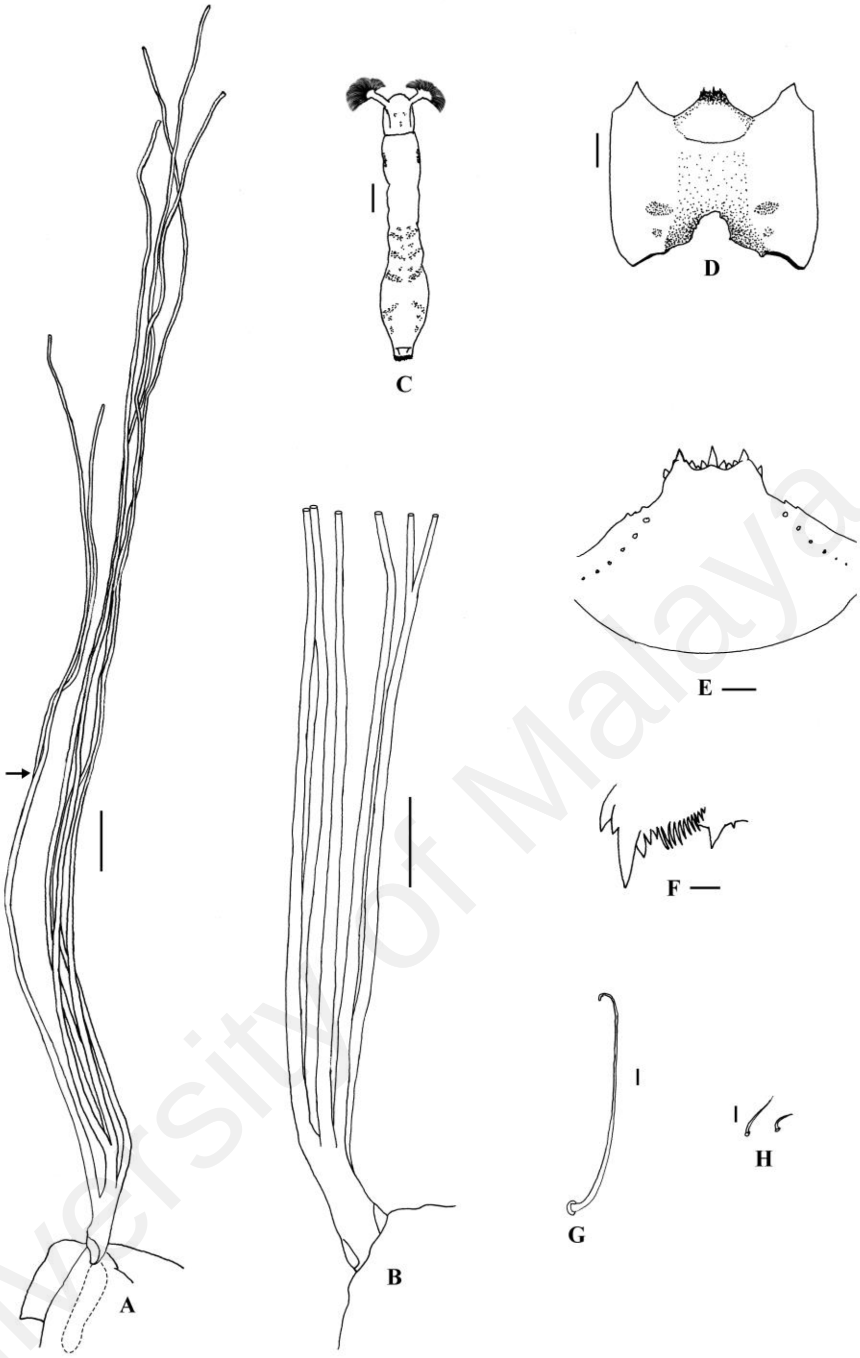

Figure 3.7: Pupa and larva of *Simulium* (*Nevermannia*) *ledangense* **sp. nov**. (A) Gill filaments (left side, outer view) (arrow showing the beginning of the branching). (B) Basal portion of gill filaments (left side, dorsal view). (C) Larva body (dorsal view). (D) Larva head capsule (ventral view). (E) Hypostoma. (F) Mandible. (G, H) Thoracic trichomes (G, mediodorsal; H, ventrolateral). Scale bars = 0.2 mm (A, B), 0.1 mm (C, D), 0.01 mm (H, G) and 0.02 mm (E, F).

Hypostoma (Fig. 3.7E) with 9 apical teeth in row; median and corner teeth well developed; lateral margin nearly smooth except apical portion with 2 or 3 weakly developed teeth; 3 or 4 hypostomal bristles lying slightly divergent posteriorly from lateral margin on each side. Postgenal cleft small, 0.73 times length of postgenal bridge. Thoracic cuticle bare. Histoblast of pharate pupal gill with 6 slender thread-like filaments. Abdominal cuticle bare except both sides of anal sclerite moderately covered with unbranched colorless setae and each lateral surface just above ventral papilla also sparsely covered with unbranched colorless setae. Rectal scales minute, colorless. Secondary lobules of rectal papillae uncountable. Anal sclerite of usual X-form, with anterior arms nearly as long as posterior ones, broadly sclerotized at basal juncture; sensilla absent on and just posterior to basal juncture area; accessory sclerite absent. Last abdominal segment much expanded ventrolaterally forming large ventral papilla on each side. Posterior circlet with 82 rows of up to 18 hooklets per row.

Type specimens. HOLOTYPE: male (with associated pupal exuviae and coccon) (preserved in 80% ethanol), reared from pupa, manually collected from a slow running small stream (width 0.5–0.6 m, depth 12 cm, water temperature 18.9°C, completely shaded, altitude 1000 m, 02°22′45.6″N, 102°36′36.9"E), Mount Ledang, or originally known as Mount Ophir National Park, located in Ledang District, Johor Province, Peninsular Malaysia, 11. III. 2013, by Ya'cob Z, Sofian-Azirun M and Azhar-Jamil N. Paratypes: 5 males, 3 females (with associated pupal exuviae and coccons), 1 male without associated pupal exuviae and coccon, and 3 mature larvae, preserved in 80% ethanol, same data and date as those of holotype.

Distribution. Peninsular Malaysia (Mount Ledang, Johor).

Biological notes. The pupae and larvae of this new species were collected together with *Simulium* (*Gomphostilbia*) *roslihashimi* Takaoka and Sofian-Azirun. The habit of biting of the female remains unknown.

Etymology. The species name *ledangense* refers to the name of the mountain, Mount Ledang, where this new species was collected.

Remarks. *Simulium* (*N*.) *ledangense* sp. nov. is readily assigned to the *S. feuerborni* species-group redefined by Takaoka (2003) by the combination of the following characteristics: male genitalia with a simple lamellate ventral plate, a short inwardly-twisted style, a simple narrow median sclerite and several parameral hooks (Fig. 3.5D–H); pupal gill with six long thread-like filaments per side (Fig. 3.7A, B); and larval head with a small short postgenal cleft (Fig. 3.7D). The pupa of this new species is easily distinguished from those of known species within this species-group by having a very long stalk of the ventral paired gill filaments which is almost five times longer than the interspiracular trunk (Fig. 3.7A, B) and by the unique arrangement of gill filaments (i.e., arranged as [1+ (1+2)] +2 filaments from dorsal to ventral, of which two individual filaments arise close together with the stalk of the middle pair). The pupa of this new species is similar to that of *S*. (*N*.) *qiongzhouense* Chen, Zhang and Yang from China in having a very long stalk of ventral paired gill filaments. However, *S*. (*N*.) *qiongzhouense* is distinguished from *S*. (*N*.) *ledangense* sp. nov. by the different arrangement of the gill filaments: i.e., 6 filaments in 3 pairs (2 + 2 + 2), male upper-eye enlarged facets in 15 vertical columns and 16 horizontal rows, and parameres with three hooks.

3.3.3 *Simulium* (*Nevermannia*) *pairoti* sp. nov. Ya'cob, Takaoka and Sofian-Azirun 2016

Female (n = 3). Body length 2.8–3.1 mm. ***Head***. Slightly narrower than thorax. Frons brownish black, whitish-gray pruinose, densely covered with whitish-yellow hairs and several dark longer hairs along each lateral margin, shiny when illuminated at certain angles. Clypeus brownish-black, whitish-gray pruinose and covered with whitish-yellow hairs and several dark longer hairs on lower half and bare in middle. Frontal ratio1.8:1.0:2.7–2.8. Frons-head ratio 1.0:4.9–5.6. Labrum 1.0–1.2 length of clypeus. Antenna composed of scape, pedicel and 9 flagellomeres, brown except scape, pedicel and base of first flagellomere yellow; first flagellomere 1.7–2.1 times length of second flagellomere. Maxillary palp brownish black consisting of 5 segments, proportional lengths of 3rd, 4th, and 5th segments 1.0:0.7–0.9:1.3–1.6; third segment much enlarged, sensory vesicle elongate (Fig. 3.8A), 0.6–0.7 times length of third segment, with medium-sized opening. Maxillary lacinia with 7 or 9 inner teeth and 12 or 14 outer teeth. Mandible with 22 inner teeth and lacking outer teeth. Cibarium with 22 dark minute spines with pointed apices as well as numerous minute spinous processes near lower margin. ***Thorax***. Scutum light to medium brown except anterolateral calli yellow, two submedian longitudinal vittae and narrow portion along lateral margin and prescutellar area dark brown, white pruinose except 3 longitudinal vittae (1 narrow median, 2 little wider submedian); scutum covered with yellow short hairs and several dark brown longer hairs on prescutellar area, and slightly shiny when illuminated at certain angles. Scutellum light brown, whitish-gray pruinose, slightly shiny when illuminated at certain angles, with whitish-yellow hairs and dark longer hairs. Postnotum brown, whitish-gray pruinose, bare and slightly shiny when illuminated at certain angles. Pleural membrane and ketepisternum bare;

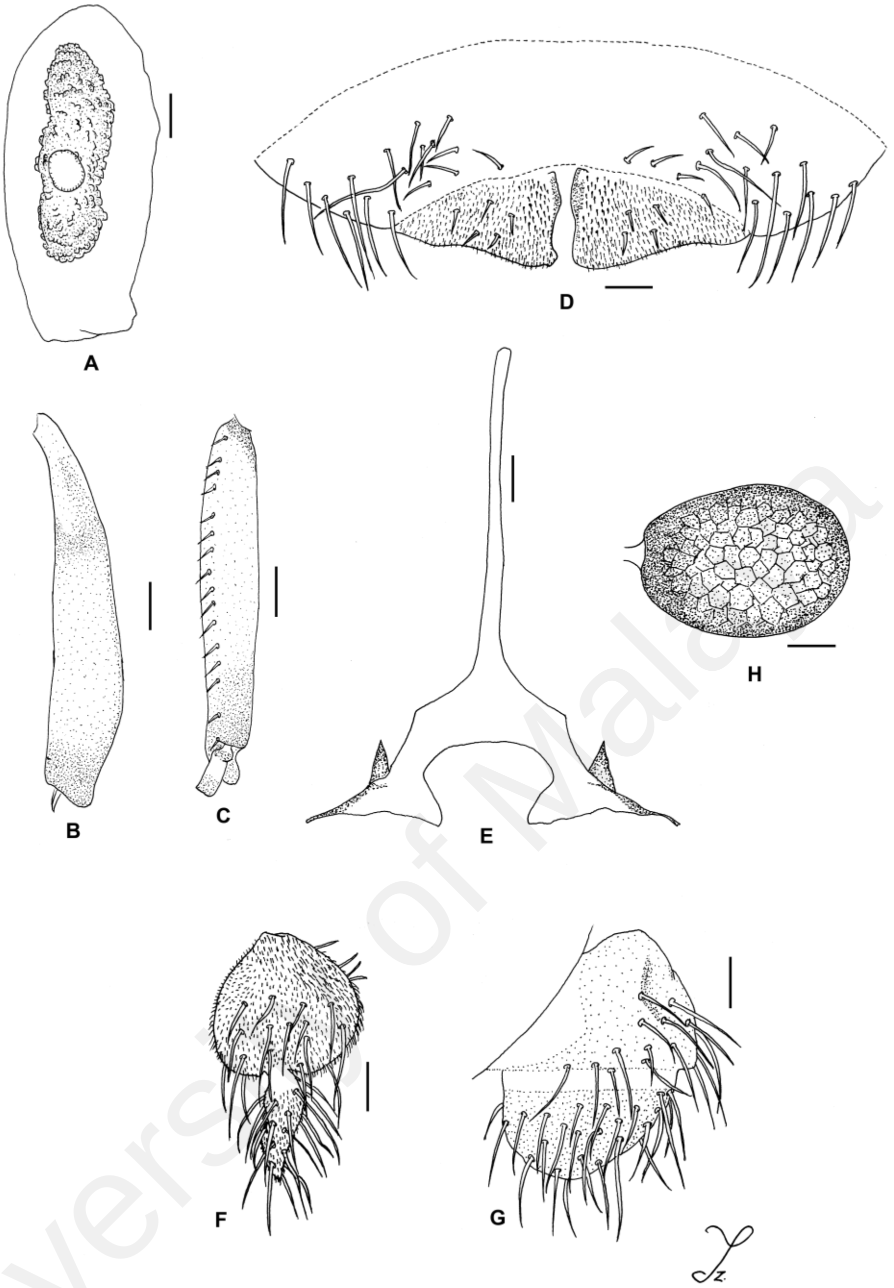

Figure 3.8: Female of *Simulium* (*Nevermannia*) *pairoti* **sp**. **nov**. (A) Third segment of right maxillary palp with sensory vesicle (front view). (B) Tibia of left hind leg (outer view). (C) Basitarsus and second tarsomere of left hind leg (outer view). (D) Sternite 8 and ovipositor valves. (E) Genital fork (ventral view). (F) Right paraproct and cercus (ventral view), (G) Right paraproct and cercus (lateral view). (H) Spermatheca. Scale bars = 0.1 mm (B, C) and 0.02 mm (A, D−H).

Halter. White except grayish near apex. ***Legs***. *Foreleg*: coxa and trochanter yellow; femur yellow with apical cap tip brown; tibia brown except median large portion of outer surface dark yellow to light brown and basal tip yellow; tarsus dark brown. Basitarsus slender, cylindrical, 7.5–8.3 times as long as its greatest width. *Midleg*: coxa brown except posterolateral surface dark brown; trochanter yellow; femur yellow except apical cap brown; tibia brown except medium large portion dark yellow to light brown; tarsus dark brown. *Hind leg*: coxa and trochanter yellow; femur yellow except apical cap dark brown; tibia (Fig. 3.8B) brown except median large portion of outer surface dark yellow to light brown; tarsus dark brown except basal two-thirds of basitarsus (Fig. 3.8C) grayish though extreme base dark brown and basal half of second tarsomere yellow; femur slightly wider than tibia; basitarsus parallel-sided, 6.3–7.0 times as long as it greatest width and 0.7–0.8 and 0.6–0.7 times as wide as greatest width of tibia and femur, respectively. Calcipala 0.7 times as long as width at base, and 0.5 as wide as greatest width of basitarsus; pedisulcus moderately developed. Claws each with large basal tooth, 0.46 times length of claw. ***Wing***. Length 2.8–3.2 mm. Costa with two parallel rows of short spines as well as hairs. Subcosta and basal portion of radius fully haired; R_1 with dark brown spinules and hairs; R_2 with dark-brown hairs; hair tuft on base of radial vein dark brown. Basal medial cell absent. ***Abdomen***. Basal scale light brown with fringe of whitish-yellow long hairs. Dorsal surface of abdomen brown except segment 2 pale; tergites of segments 6–9 shiny. Abdomen with whitish-yellow and brownish-black short hairs. ***Terminalia***. Sternite 8 wide (Fig. 3.8D), bare medially and furnished with 17–21 medium to long hairs and few short hairs on each side. Ovipositor valves (Fig. 3.8D) triangular (through posteromedial corner rounded), thin, membranous except inner margin narrowly sclerotized, densely covered with microsetae interspersed with 4 or 5 short setae; inner margins sinuous, narrowly separated from each other. Genital fork (Fig. 3.8E) of inverted Y-form, with well sclerotized stem and relatively wide arms; each arm with lateral plate bearing triangular lobe-like projection directed posteromedially and short narrow stout projection directed anterodorsally. Paraproct in ventral view (Fig. 3.8F) subquadrate, as long as its greatest width; anteromedial surface nearly transparent, with 5–9 sensilla; paraproct in lateral view (Fig. 3.8G) somewhat protruded ventrally beyond ventral margin of cercus, and with 21–23 medium to long hairs on ventral and lateral surfaces. Cercus in lateral view (Fig. 3.8G) rounded posteriorly, short, 0.5 times as long as basal width. Spermatheca (Fig. 3.8H) nearly ovoidal, strongly sclerotized except small area around juncture with duct and duct itself unsclerotized, with distinct reticulate surface pattern and without internal setae; main spermathecal duct narrow, somewhat narrower than both accessory ducts.

Male (n = 5). Body length 2.8–3.2 mm. ***Head***. Slightly wider than thorax. Holoptic; upper eye consisting of large facets in 15−16 vertical columns and 16−17 horizontal rows. Clypeus brownish black, whitish-gray pruinose when illuminated at certain angles, moderately covered with yellow hairs intermixed with dark-brown longer hairs except medial portion widely bare. Antenna dark yellow or light brown except scape and pedicel and base of first flagellomere light yellow. Maxillary palp composed of 5 segments dark brown except third segment dark brown. Proportional length of third, fourth and fifth segments 1.0:1–1.1:1.9–2.0; third segment (Fig. 3.9A) of moderate size; sensory vesicle (Fig. 3.9A, B) small, ellipsoidal, 0.16–0.20 times length of third segment. ***Thorax***. Scutum medium brown except anterolateral calli yellow, submedian longitudinal vittae and narrow portion along lateral margin and prescutellar area dark brown, whitish pruinose except 3 longitudinal vittae non-pruinose (1 narrow median, 2 slightly wider submedian), densely covered with yellow short hairs and several dark longer hairs on prescutellar area. Scutellum dark yellow, several yellow hairs and dark longer hairs.

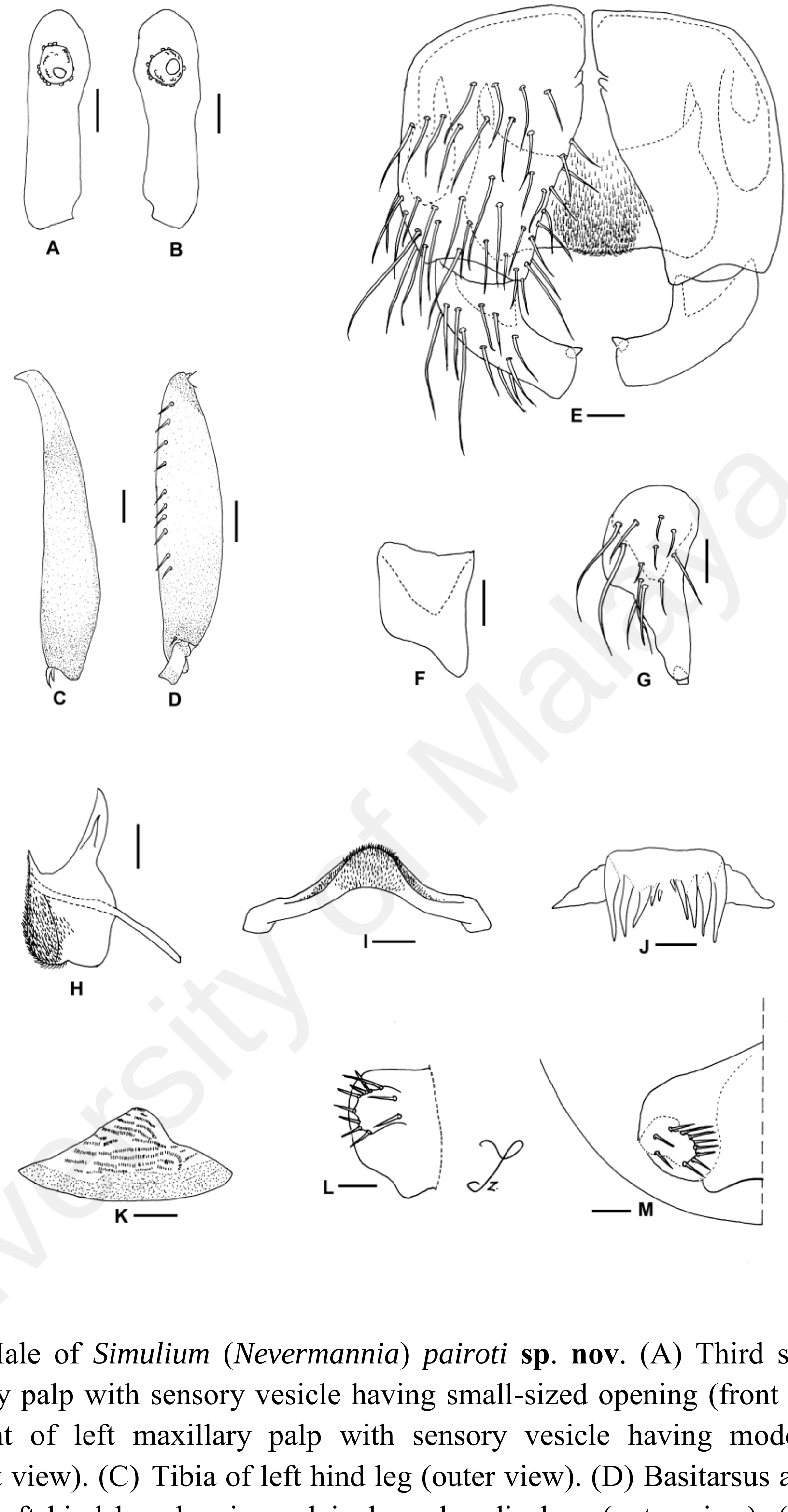

Figure 3.9: Male of *Simulium* (*Nevermannia*) *pairoti* **sp**. **nov**. (A) Third segment of right maxillary palp with sensory vesicle having small-sized opening (front view). (B) Third segment of left maxillary palp with sensory vesicle having moderate-sized opening (front view). (C) Tibia of left hind leg (outer view). (D) Basitarsus and second tarsomere of left hind leg showing calcipala and pedisulcus (outer view). (E) Coxite, styles and ventral plate (ventral view). (F) Left style (ventrolateral view). (G) Left style (end view). (H) Ventral plate and median sclerite (lateral view). (I) Ventral plate (caudal view). (J) Parameres each with 5 hooks (end view). (K) Aedeagal membrane and dorsal plate (end view). (L) 10th abdominal segment and cercus (right side; lateral view). (M) Right cercus (end view). Scale bars = 0.1 mm (C, D) and 0.02 mm (A, B, E−M).

Postnotum dark brown, gray pruinose and slightly shiny when illuminated at certain angles and bare. Pleural membrane medium brown and bare; ketepisternum dark brown, thinly gray pruinose, slightly shiny when illuminated at certain angles and bare. ***Legs***. *Foreleg*: coxa and trochanter yellow; femur yellow except apical cap dark brown; tibia dark brown with median large area on outer surface light brown and base yellow; tarsus entirely brownish black; basitarsus cylindrical, slightly dilated, 8.5–9.3 times as long as its greatest width. *Midleg*: coxa yellow with posterolateral surface dark brown; trochanter yellow; femur yellow with apical cap dark brown; tibia dark brown with median large portion somewhat greyish with extreme base yellow; tarsus dark brown. *Hind leg*: coxa and trochanter yellow; femur yellow with apical cap dark brown; tibia (Fig. 3.9C) dark brown with extreme base yellow and median large portion somewhat greyish; tarsus dark brown with median large portion of basitarsus greyish brown, and basal half of basitarsus and second tarsomere light brown; basitarsus (Fig. 3.9D) enlarged, spindle-shaped, 4.5–5.1 times as long as its greatest width, nearly as wide as width of hind tibia, but slightly narrower than width of hind femur or 0.9–1.1 and 0.8–1.0 times as wide as greatest widths of hind tibia and femur, respectively; calcipala well developed, nearly as long as basal width and 0.45−0.46 times as wide as greatest width of basitarsus; pedisulcus moderately developed. ***Wing***. As in female except subcosta bare or 4–5 hairs; length 2.8–3.1 mm. ***Halter***. As in female. ***Abdomen***. Basal scale brown, with fringe of long yellow hairs. Dorsal surface of abdominal segments dark brown, not shiny, moderately covered with short yellow and dark hairs. Segments 2 white pruinose, shiny dorsolaterally when illuminated at certain angles. ***Genitalia***. Coxite in ventral view (Fig. 3.9E) rectangular, 1.61 times as long as its greatest width. Style in ventral view (Fig. 3.9E) short, 0.65 times as long as coxite, bent inwardly, with outer margin angled medially and with short stout spine apically; style in medial view gently curved dorsally and nearly parallel-sided; style in ventrolateral view (Fig. 3.9F) broad, nearly parallel-sided from base to little beyond middle, then abruptly tapered apically; style in end view (Fig. 3.9G) tapered inward, with round apex. Ventral plate in ventral view (Fig. 3.9E) lamellate, subquadrate, 0.69 times as long as its greatest width, well sclerotized except anteromedian portion unsclerotized, with posterior margin slightly concave medially and submedially, and moderately covered with fine short setae on ventral surface except lateral portion widely bare; arms short, slender, directed anteriorly; ventral plate in lateral view (Fig. 3.9H) with ventral margin nearly straight and arm short, tapered anterodorsally; ventral plate in caudal view (Fig. 3.9I) with dorsal margin markedly concave, with fine short setae centrally on posterior surface. Median sclerite simple, club-shaped, narrow and strongly sclerotized except weakly sclerotize apical portion. Paramere (Fig. 3.9J) with 5 hooks decreasing in length toward apex. Aedeagal membrane (Fig. 3.9K) moderately covered with minute setae; dorsal plate triangular in shape, thin, weakly sclerotized. Ventral surface of segment 10 without any distinct hairs near each posterolateral corner. Cercus (Fig. 3.9L, M) small, rounded and encircled by 10 simple hairs.

Pupa (n = 8). Body length (excluding gill filaments) 3.0–3.1 mm. ***Head***. Integument light medium brown, moderately covered with round tubercles of various sizes on frons and sparsely covered with similar tubercles of various sizes on lateral surface of face; antennal sheath bare; frons with pair of short minute trichomes on each side; face with 1 medium-long unbranched trichome on each side. ***Thorax***. Light brown to medium brown, moderately covered with round tubercles of different sizes on dorsal and lateral surfaces of anterior half and sparsely covered with smaller ones on dorsal surface of posterior half; thorax on each side with 3 long trichomes with coiled apices mediodorsally, 2 trichomes (1 long with coiled apex, 1 medium-long with uncoiled apex) anterolaterally, 1 medium-long trichome with uncoiled apex mediolaterally and 3 short trichomes with uncoiled apices ventrolaterally (Fig. 3.10H), all trichomes unbranched. Gill (Fig. 3.10A−E) with 6 long thread-like slender filaments; filaments arranged in two groups (1 ventral group consisting of paired filaments varying from short to long stalk, and dorsal group consisting of four filaments); branching of 4 filaments of dorsal group somewhat variable from outer to inner by pupae and also by gills of the same pupae e.g., 23 pupae examined, 16 pupae arranged as (1+ 1+1+1) (Fig. 3.10B), 2 pupae arranged as (2 + 1+ 1) (Fig. 3.10C), 2 pupa arranged as (2 + 2) (Fig. 3.10D) and 3 pupae arranged as (1+2+1) (Fig. 3.10E); common basal stalk 0.7−0.9 times length of interspiracular trunk (0.7−0.9), stalk of ventral pair (varying from 0.14 mm to 0.88 mm in individual pupae) 0.01 to 0.24 times total length of ventral paired filaments (including common basal stalk) varying from 3.2 mm to 5.1 mm in individual pupae; dorsal inner and outer paired filaments subequal in length (varying from 3.3 mm to 5.3 mm in individual pupae), all filaments tapered toward tip; cuticle surface with distinct annular ridges and furrows though becoming less distinct near apex. ***Abdomen***. Dorsally, all segments weakly yellowish, moderately covered with minute tubercles; segment 1 with 1 long-medium slender setae on each side; segment 2 with 1 unbranched or bifid hair-like seta and 6 short setae, of which 5 stout and spinous and 1 slender on each side; segments 3 and 4 each with 4 hooks and 2 very short spinous seta on each side; segment 5 with few unbranched short slender setae and number of spine-combs varying from 0 to 2 on each side e.g., of 13 pupae examined, 3 pupae without spine-comb, 1 pupa with two spine-combs on each side, 4 pupae with one spine-comb on each side, 2 pupae with 1 spine-comb on right or left side only, 2 pupae with 2 spine-combs on left or right side only and 1 pupa with 2 spine-combs on left side and one on the right side; segments 6–8 each with spine-combs directed backwards in transverse row and comb-like group of minute spines on each side; segment 9 with pair of distinct conical terminal hooks (Fig. 3.10F) as well as comb-like groups of minute spines. Ventrally, segments 3–8 each with comb-like groups of minute spines; segment 4 with 1 simple hooklet and few short unbranched slender setae on each side; segment 5 with pair of bifid hooks submedially and few very short slender setae on each side; segments 6 and 7 each with pair of bifid inner and unbranched outer hooks and few slender very short setae on each side. ***Cocoon***. Wall-pocket shaped (Fig. 3.10G−3I), 2.5−4.4 mm long by 3.5−4.4 mm wide, thinly woven, extending ventrolaterally, with anterodorsal projection varying in length from 0.0 mm to 0.7 mm.

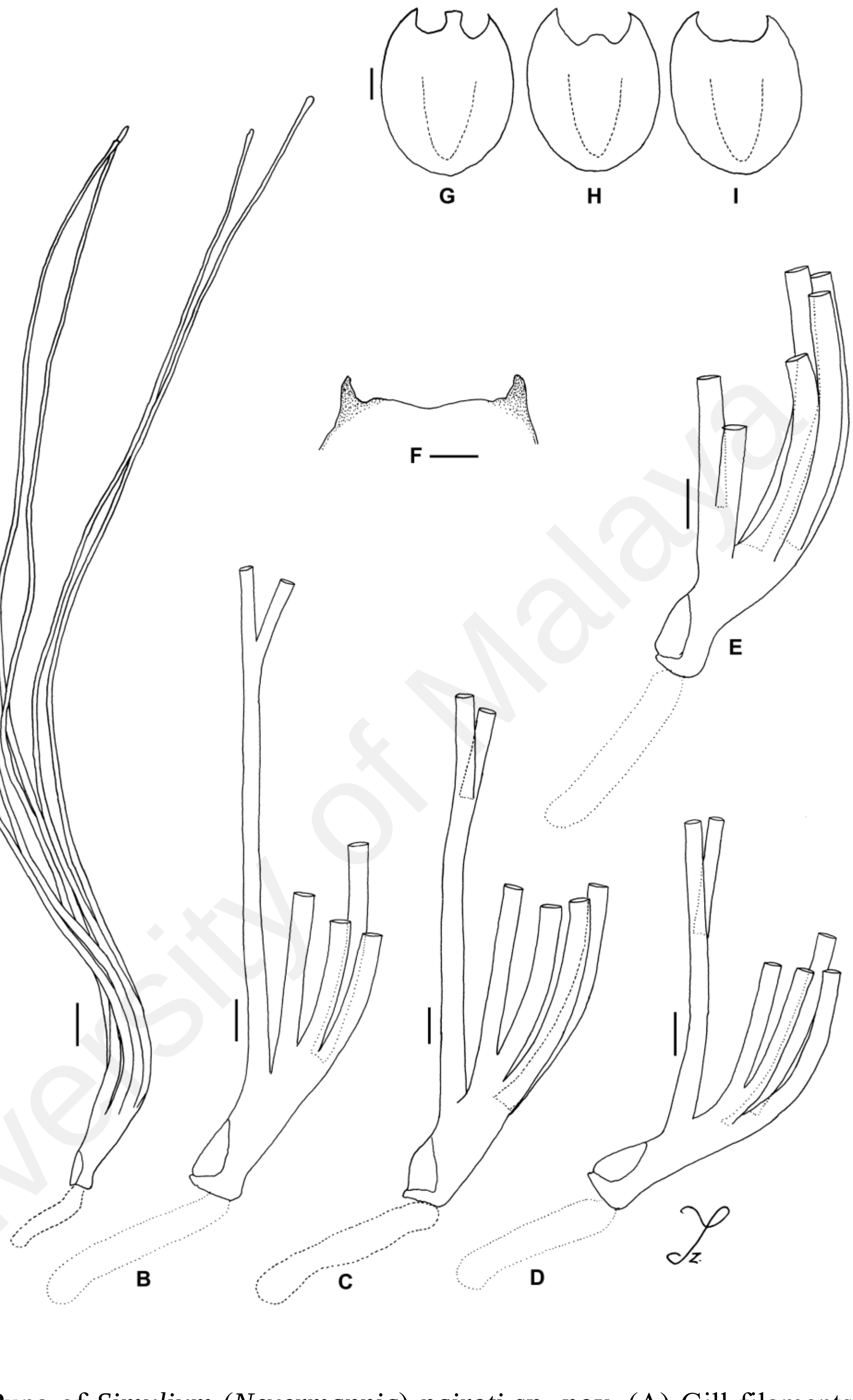

Figure 3.10: Pupa of *Simulium* (*Nevermannia*) *pairoti* **sp. nov**. (A) Gill filaments (left side, outer view) (arrow showing the beginning of the branching). (B−E) Gill filaments (left side, outer view) (showing different length of the stalk of the ventral paired filaments). (F) Terminal hooks (caudal view). (G) Cocoon with long anterodorsal projection (dorsal view). (H) Cocoon with moderate-sized of anterodorsal projection (dorsal view). (I) Cocoon without anterodorsal projection. Scale bars = 0.2 mm (A), 0.1 mm (B−E, G−I) and 0.02 mm (F).

Mature larva (n = 3). Body length 7.0−8.0 mm. Cephalic apotome whitish-yellow to yellow; head spots markedly brownish. Lateral surface of head capsule yellow except eye-spot region white with two large dark spots behind eye-spots and 4 isolated dark spots (1 in eye brow, 3 below eye-spots region); ventral surface of head capsule (Fig. 3.11A) yellow except medial large portion darkened, and basal area on each side of postgenal cleft dark brown; 1 elongate and 1 round spot on each side of postgenal cleft markedly positive. Cervical sclerite composes of 2 faint small elliptical pieces, not fused to occiput, widely separated medially from each other. Antennae consisting of 3 articles and apical sensilium, much longer than stem of labran fan; proportional lengths of first, second, and third articles 1.00:1.20−1.22:1.04−1.08. Labran fan with 34−36 main rays. Mandible (Fig. 3.11B) with serrations consisting of 2 teeth (1 large and 1 small); large tooth at obtuse angle with mandible on apical side; comb-teeth composed of 3 teeth shortened from first to third; supernumerary serration absent. Hypostoma (Fig. 3.11C) with 9 apical teeth in row; median and corner teeth well developed; lateral margin nearly smooth except apical portion with 2 or 3 weakly developed teeth; 5 or 6 hypostomal bristles lying slightly divergent posteriorly from lateral margin on each side. Postgenal cleft small, 0.41 times length of postgenal bridge. Body creamy with characteristic color markings dorsally and laterally (thorax with brownish transverse band just after head capsule; abdominal segments 1 and 2 each with pair of reddish-brown small spots laterally; segments 3 and 4 each with pair of large reddish-brown markings of rather irregular form dorsally, sometimes divided into anterior and posterior portions plus with pair of reddish brown spots laterally sometimes contiguous to dorsal spots; segment 5 dorsally with pair of large reddish-brown markings close together near base and pair of large markings somewhat spaced posteriorly; segment 6 dorsally with 2 pairs of large spots, each anteriorly and posteriorly; segment 7 and 8 usually pale above with faint sub-lateral longitudinal markings, all these markings of variable intensity, although those on abdominal segments 3 and 4 often stronger than others). Thoracic cuticle bare. Histoblast of pharate pupal gill with 6 slender thread-like filaments. Abdominal cuticle bare except sides on anal sclerite of last segment covered with numerous unbranched colorless setae. Rectal papilla compound, colorless, each of 3 lobes with 11−13 slender, finger-like secondary lobules. Anal sclerite of usual X-form, with anterior arms slightly shorter than posterior ones, broadly sclerotized at basal juncture; Ventral papillae well developed. Posterior circlet with 94 rows of up to 13−14 hooklets per row.

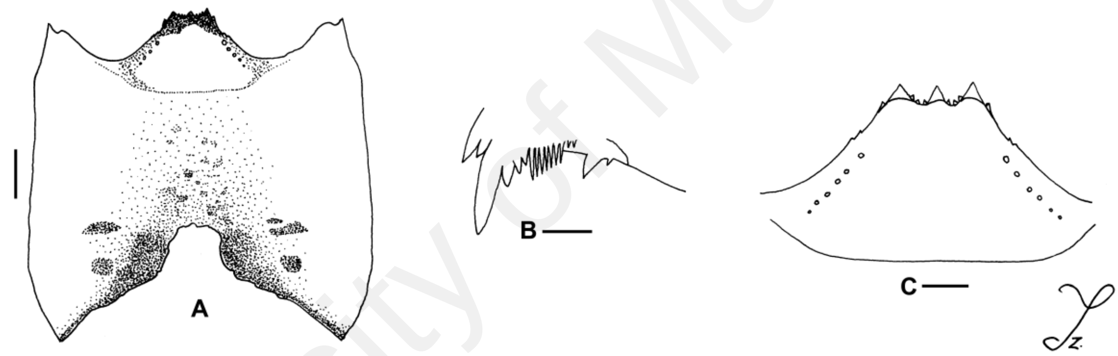

Figure 3.11: Larva of *Simulium* (*Nevermannia*) *pairoti* **sp. nov**. (A) Larva head capsule (ventral view). (B) Mandible. (C) Hypostoma. Scale bars = 0.1 mm (A), 0.02 mm (B−C).

Type specimens: HOLOTYPE: Male (with associated pupal exuviae and coccon) (preserved in 80% ethanol), reared from pupa, manually collected from a slow running small stream (width 0.2–0.75 m, depth 0.1−0.12 m, water temperature 17.4°C, completely shaded, altitude 1,813 m, N04º31.461' E101º23.338'), Mount Brinchang, located in the district of Cameron Highland, Pahang Province, Peninsular Malaysia, 26. I. 2014, by Ya'cob Z, Sofian-Azirun M and Azhar-Jamil N. Paratypes: 7 males, 7 females (with associated pupal exuviae and coccons), and 7 mature larvae, preserved in 80% ethanol, same data and date as those of holotype.

Distribution. Peninsular Malaysia (Cameron Highland, Pahang)

Biological notes. The pupae and larvae of this new species were collected together with *Simulium brevipar* Takaoka and Davies, *S. bishopi* Takaoka and Davies, *S. tani* Takaoka and Davies, *S. whartoni* Takaoka and Davies and *S. asakoae* Takaoka and Davies, *S. brinchangense* Takaoka, Sofian-Azirun, Ya'cob and Hashim, *S. aureohirtum* Brunetti, *S. kurtaki* Takaoka and Davies and *S. caudisclerum* Takaoka and Davies. The biting habit of the female remains unknown.

Etymology. The species name *pairoti* is in honour of Associate Professor Pairot Pramual, PhD, Mahasarakham University, Thailand, who greatly contributed to the genetics, cytology, and ecology of black flies in Asia.

Remarks. The distinctiveness of *Simulium pairoti* sp. nov. was supported by molecular and chromosomal evidence (Pramual *et al*., 2015). Accordingly, the present study formally described the S. sp. (nr. *feuerborni*) from Cameron Highlands as new species based on morphotaxonomical approach. *Simulium pairoti* sp. nov. is assigned to the *Simulium feuerborni* species-group by the characters of male genitalia with simple lamellate ventral plate (Fig. 3.9E), short inwardly-twisted styles (Fig. 3.9F−G), several parameral hooks (Fig. 3.9J), and a simple narrow median sclerite (Fig. 3.9H). *Simulium* *feuerborni* Edwards originally described from East Java, Indonesia (Edwards, 1934) differs from the new species by having smaller number of parameral hooks (3 or 4 hooks), sternite 8 with ca. 30 stout hairs on each side, reduced number of posterior circlet as well as number of hooklets per rows e.g. ca. 90 rows up to 16 hooklets per row, stalk of ventral paired gill filaments (0.6−1.0 mm long) and anterodorsal projection of the cocoon of moderate size (Fig 3.10A−E, G−I). This species also has a reduced number of the labral fan with 22−26 main rays. A total of four species of the same species-group were recorded in Malaysia. Of these, *S.* (*N.*) *ledangense* was recorded from Peninsular Malaysia, but it differs from the new species by having larger number of minute spines on middle of cibarium (e.g. ca. 42−44), male with reduced number of enlarged upper-eye facets, i.e. ca. 13 or 14 vertical columns and 15 horizontal rows and stalk of ventral paired filaments very long, about half the total length of filaments; *S.* (*N.*) *borneoense* described from Sabah, East Malaysia (Takaoka, 2001), also differs by having parameres each with 8 distinct hooks, cerci with reduced number of simple hairs e.g. ca. 6 to 7, and pupa with 4 gill filaments per side with distinct horn-shaped terminal hooks; *S. fuscinervis*, described from Sabah (Edwards, 1933) based only on adult male, is different by having reduced number of enlarged upper-eye facets i.e. ca. 12 vertical columns and 12−14 horizontal rows and parameres with 10 hooks per side (Takaoka, 2001). Other members of the same species group from different geographic regions including; *S. maeaiense* described from Chiang Mai, Thailand (Takaoka & Srisuka, 2011) is different by having a narrow horizontal bars on each arm of genital fork, 40−44 dark minute spines on middle of cibarium, arrangement of the pupa gill filaments 2+1+2+1 in horizontal plane from inside to outside, anterodorsal projection of the cocoon much longer e.g. ca. 1.2−1.5 mm and sometimes bent downward, reduced number of the posterior circlet e.g. ca. 75−86 rows, and labral fan with 23−25 main rays; *S. fangense* described from Thailand (Takaoka & Choochote, 2006) is different by having the claw with larger basal tooth (ca. 0.50 times as long as claw), sternite 8 with 25−32 short and long hairs on each side, enlarged upper-eye facets in 21 vertical columns and 21 horizontal rows, labral fan with 26−32, and the posterior circlet with 68−74 rows; *S. fruticosum* originally described from northern Thailand (Takaoka & Choochote, 2005) is also different by having parameral hooks with 6−7 hooks, labral fan with 26−29, posterior circlet with 86 rows up to 16 hooklets per row and the cocoon without an anterodorsal projection.

3.3.4 Keys to separate four species of the *Simulium feuerborni* species-group in Malaysia

Female*

1	Number of minute spines on middle of cibarium 42–44......................	***S. ledangense* sp. nov**
	Number of minute spines on middle of cibarium 22............................	***S. pairoti* sp. nov**

**Female of S. borneoense and S. fuscinervis are unknown.*

Male

1	Upper-eye large facets in 15 vertical columns and 17 horizontal rows...	*S. borneoense*
	Upper-eye large facets in 12–14 vertical columns and 12–15 horizontal rows...	2
2	Paramere with 10 hooks..	*S. fuscinervis*
	Paramere with 4–6 hooks..	3
3	Paramere with 4 hooks..	***S. pairoti* sp. nov**
	Paramere with 5 or 6 hooks...	***S. ledangense* sp. nov**

Pupa*

1	Gill with 4 filaments..	*S. borneoense*
	Gill with 6 filaments..	2
	Cocoon with anterodorsal projection..	***S. pairoti* sp. nov**
	Cocoon without anterodorsal projection..	***S. ledangense* sp. nov**

**Pupa of S. fuscinervis is unknown.*

Mature larva*

1	Pharate gill with 5 filaments..	*S. borneoense*
	Pharate gill with 6 filaments..	2
2	Stalk of ventral paired of pharate gill filaments very long, about half of total length of filaments..	***S. ledangense* sp. nov**
	Stalk of ventral paired of pharate gill filaments short, as long as or shorter than common basal stalk..	***S. pairoti* sp. nov**

**Larva of S. fuscinervis is unknown.*

3.3.5 *Simulium* (*Gomphostilbia*) *azhari* sp. nov. Takaoka, Sofian-Azirun and Ya'cob 2014

Female. Body length 2.2−2.4 mm. ***Head***. Nearly as wide as thorax. Frons (Fig. 3.12A) narrow, black, moderately covered with whitish-yellow scale-like recumbent short hairs interspersed with several dark un-branched longer hairs along each lateral margin; frontal ratio 1.72:1.00:4.35; frons:head ratio 1.00:7.13. Fronto-ocular area well developed, narrow, directed dorsolaterally. Clypeus black, gray pruinose, densely covered with yellow hairs interspersed with several dark longer hairs on each side. Labrum 0.64 times length of clypeus. Antenna composed of scape, pedicel, and nine flagellomeres, medium brown except scape, pedicel and base of first flagellomere whitish yellow. Maxillary palp composed of five segments, light to medium brown, proportional lengths of third, fourth, and fifth segments 1.00:1.26:3.04; third segment (Fig. 3.12B) widened apically; sensory vesicle (Fig. 3.12B) small, ellipsoidal, 0.28−0.30 times length of third segment and with medium-sized opening. Maxillary lacinia with 11 inner and 14 or 15 outer teeth. Mandible (Fig. 3.12C) with 23 or 24 inner and 6 or 7 outer teeth. Cibarium (Fig. 3.12D) medially forming sclerotized plate folded forward from posterior margin, with strongly sclerotized medial longitudinal ridge. ***Thorax***. Scutum brownish-black to black, shiny when illuminated at certain angles, densely covered with whitish and whitish-yellow scale-like recumbent hairs except median and submedian longitudinal vittae bare, interspersed with dark brown upright hairs on prescutellar area. Scutellum brownish-black, covered with whitish-yellow short hairs and dark brown upright long hairs along posterior margin. Postnotum brownish-black, shiny when illuminated at certain angles, and bare. Pleural membrane bare. Katepisternum dark brown to brownish-black, longer than deep, shiny when illuminated at certain angles, moderately covered with short hairs.

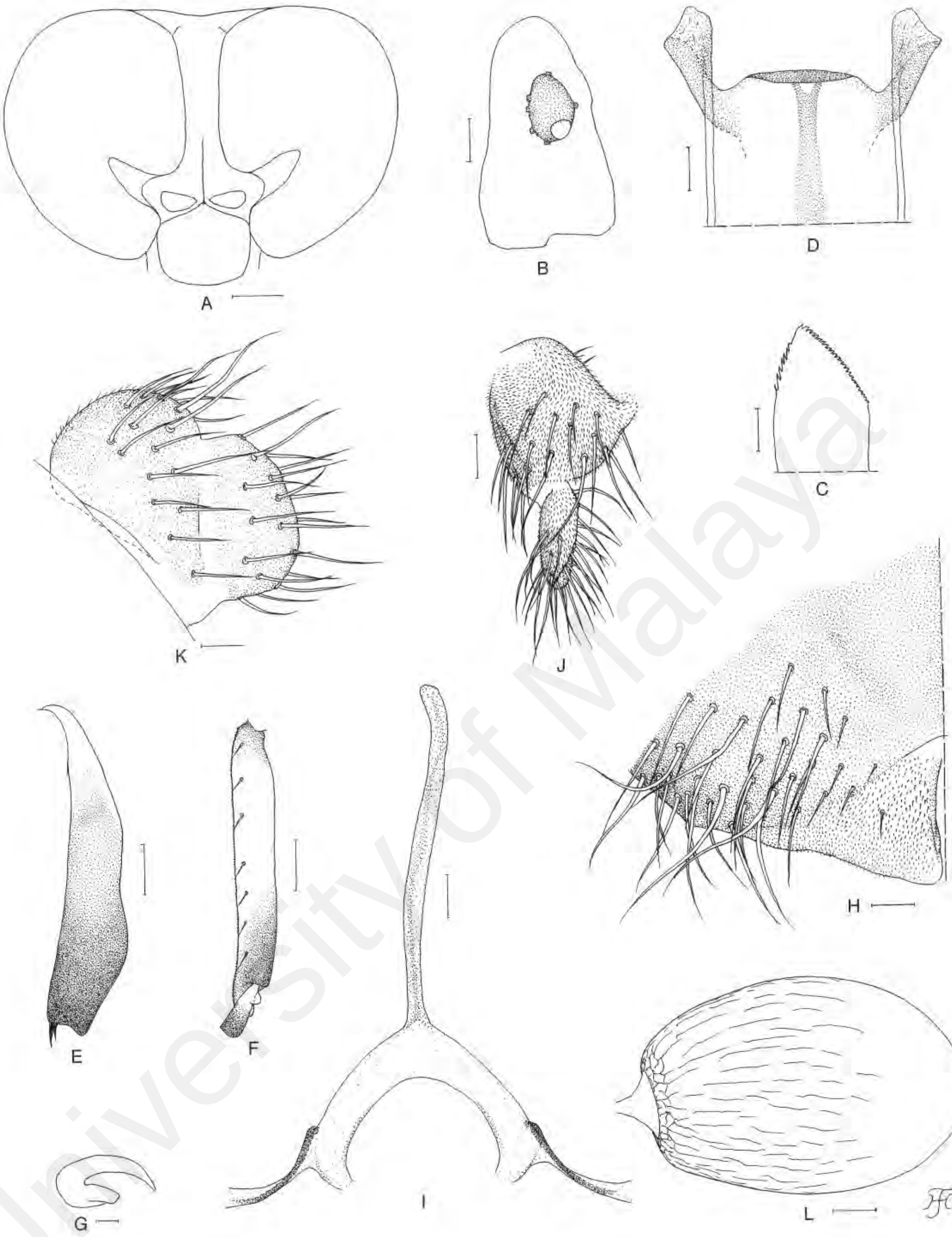

Figure 3.12: Female of *Simulium* (*Gomphostilbia*) *azhari* sp. nov. (A) Head showing narrow frons (frontal view). (B) Third segment of right maxillary palp with sensory vesicle (front view). (C) Tip of left mandible. (D) Cibarium. (E) Left hind tibia (outer view). (F) Basitarsus and second tarsomere of left hind leg showing calcipala and pedisulcus (outer view). (G) Claw. (H) Sternite 8 and ovipositor valve (only right half shown; ventral view). (I) Genital fork (ventral view). (J) Right paraproct and cercus (ventral view). (K) Right paraproct and cercus (lateral view). (L) Spermatheca. Scale bars = 0.1 mm (A, E, F), 0.02 mm (B−D, H−L) and 0.01 mm (G).

Legs. *Foreleg*: coxa yellow; trochanter light brown; femur medium brown except apical tip yellowish; tibia medium to dark brown except basal tip yellow; tarsus brownish-black, with moderate dorsal hair crest; basitarsus somewhat dilated, 5.5 times as long as its greatest width. *Midleg*: coxa light brown except posterolateral portion dark brown; trochanter dark yellow; femur medium brown except apical cap dark brown (although tip yellowish); tibia medium brown except apical cap dark brown and basal tip yellow, and with dark brown subbasal band; tarsus dark brown except base of basitarsus somewhat lighter. *Hind leg*: coxa light brown; trochanter yellow; femur medium brown except base yellow, and apical cap dark brown (although tip yellowish); tibia (Fig.3.12E) medium brown except basal one-fifth yellow and apical cap dark brown, and with dark brown subbasal spot; tarsus (Fig. 3.12F) dark brown except basal three-fifths of basitarsus (although base light brown) and basal half of second tarsomere whitish-yellow; basitarsus (Fig. 3.12F) narrow, nearly parallel-sided, 6.2 times as long as wide, and 0.63 and 0.53 times as wide as greatest width of tibia and femur, respectively; calcipala (Fig. 3.12F) well developed, 1.4 times as long as wide, and 0.5 times as wide as greatest width of basitarsus; pedisulcus (Fig. 3.12F) well developed; claw (Fig. 3.12G) with large basal tooth 0.51 times length of claw. ***Wing***. Length 1.8−1.9 mm. Costa with dark brown spinules and light brown hairs except basal patch of hairs yellow. Subcosta with hairs except bare near apex. Hair tuft on base of radial vein dark brown. Basal portion of radius fully haired. Basal cell absent. Halter. Dull white except basal stem partially darkened. ***Abdomen***. Basal scale light brown, with fringe of yellowish- white hairs. Dorsal surface of abdomen dark brown to brownish-black except basal half of segment 2 yellowish, moderately covered with dark short to long hairs; tergites of segments 2 and 6−9 shiny when illuminated at certain angles. Ventral surface of abdomen medium brown except base of segment 2 whitish; sternal plate on segment 7 undeveloped. ***Genitalia***. Sternite 8 (Fig. 3.12H) bare medially, with

28−31 medium-long to long hairs together with three or four slender short hairs on each side. Ovipositor valves (Fig. 3.12H) triangular (although medioposterior corners rounded), thin, membranous, moderately covered with microsetae interspersed with one short hair; inner margins slightly concave, somewhat sclerotized, and somewhat separated from each other. Genital fork (Fig. 3.12I) of usual inverted-Y form, with slender stem; arms of moderate width, moderately folded medially. Paraproct in ventral view (Fig. 3.12J) with anterolateral tip unsclerotized, and anteromedian margin darkened, with three sensilla on anteromedial surface; paraproct in lateral view (Fig. 3.12K) somewhat produced ventrally, 0.78 times as long as wide, with 20−23 medium long to long hairs on ventral and lateral surfaces. Cercus in lateral view (Fig. 3.12K) short, rounded posteriorly, 0.50 times as long as wide. Spermatheca (Fig. 3.12L) oblong, 1.50 times as long as greatest width, well sclerotized except duct and small area near juncture with duct unsclerotized, and with several fissures on surface; internal setae absent; both accessory ducts slender, subequal in diameter to major one.

Male. Body length 2.2 mm. ***Head***. Wider than thorax. Upper eye medium to dark brown, consisting of 11 vertical columns and 11 or 12 horizontal rows of large facets. Face brownish-black, shiny, whitish-gray pruinose. Clypeus brownish-black, shiny, whitish-gray pruinose, moderately covered with yellow short hairs interspersed with dark brown longer hairs. Antenna composed of scape, pedicel, and nine flagellomeres, light to dark brown except scape and pedicel yellow, and base of first flagellomere whitish-yellow; first flagellomere elongate, 1.58 times length of second one. Maxillary palp with five segments, light brown, proportional lengths of 3rd, 4thth, and 5th segments 1.00:1.09:3.00; third segment (Fig. 3.13A) widened apically; sensory vesicle (Fig. 3.13A) ellipsoidal, small, 0.17 times length of third segment, and with small opening.

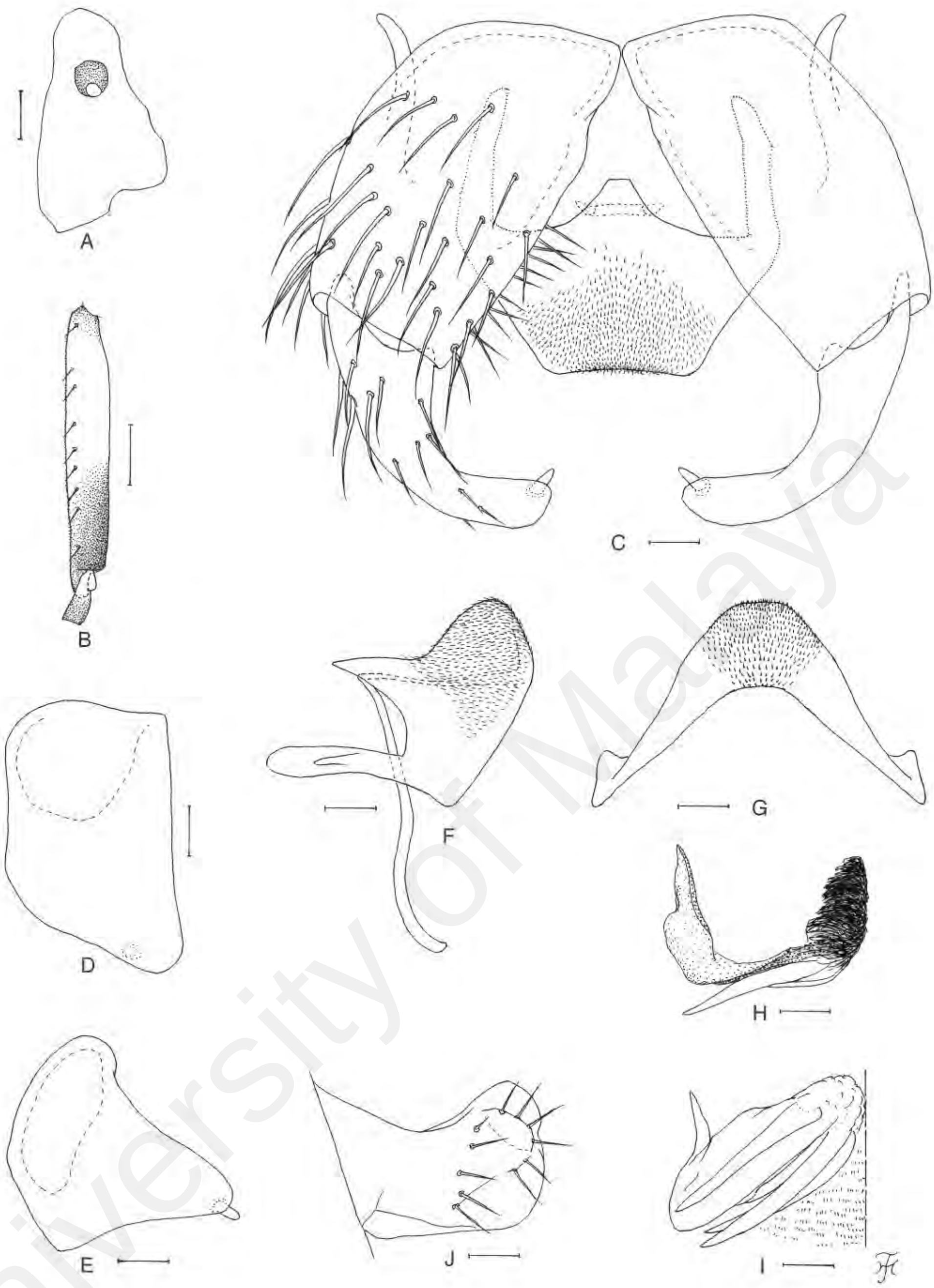

Figure 3.13: Male of *Simulium* (*Gomphostilbia*) *azhari* sp. nov. (A) Third segment of right maxillary palp with sensory vesicle (front view). (B) Basitarsus and second tarsomere of left hind leg showing calcipala and pedisulcus (outer view). (C) Coxites, styles, and ventral plate (ventral view). (D, E) Right styles with one apical spine (D, ventrolateral view; E, caudal view). (F) Ventral plate and median sclerite (lateral view). (G) Ventral plate (caudal view). (H) Right paramere (ventral view). (I) Right paramere and aedeagal membrane (caudal view). (J) Right cercus (lateral view). Scale bars = 0.1 mm (B), and 0.02 mm (A, C−J).

Thorax. Brownish-black, shiny, thinly gray pruinose, moderately covered with yellow short hairs (hairs on large central areas lost). Scutellum dark brown, somewhat shiny when illuminated at certain angles, with yellow short hairs and dark brown upright long hairs. Postnotum dark brown, somewhat shiny when illuminated at certain angles, and bare. Pleural membrane and katepisternum as in female. ***Legs***. Color nearly as in female. Fore basitarsus somewhat dilated, 6.67 times as long as its greatest width. Hind basitarsus (Fig. 3.13B) narrow, nearly parallel-sided (although slightly tapered from middle to apex), 6.2 times as long as wide, and 0.63 and 0.53 times as wide as greatest width of tibia and femur, respectively; calcipala (Fig. 3.13B) well developed, 1.3 times as long as wide, and 0.45 times as wide as greatest width of basitarsus; pedisulcus (Fig. 3.13B) well developed. ***Wing***. Length 1.5 mm. As in female except subcosta without hairs. ***Halter***. As in female. ***Abdomen***. Basal scale medium brown, with fringe of long hairs. Dorsal surface of abdomen dark brown to black except basal half or more (including tergal plates) of segment 2 yellow, covered with dark brown short to long hairs; segments 2 and 5−8 each with pair of shiny dorsolateral or lateral patches; ventral surface of abdomen dark brown except basal five-sixths of segment 2 whitish. ***Genitalia***. Coxite in ventral view (Fig. 3.13C) nearly rectangular, 1.72 times as long as its greatest width. Style in ventral view (Fig. 3.13C) 0.88 times as long as coxite, slender, somewhat tapered toward apex, markedly curved inward, with apical spine; style in ventrolateral view (Fig. 3.13D) wide, nearly parallel-sided from base to apical one-third (0.63 times as wide as its length), then tapered toward blunt apex; style in caudal view (Fig. 3.13E) curved inward, nearly tapered toward apex. Ventral plate in ventral view (Fig. 3.13C) transverse, 0.60 times as long as wide, with body widest at base, markedly tapered posteriorly, with anterior margin produced anteromedially, posterior margin nearly straight or shallowly concave, posterolateral corners angulate, and densely covered with microsetae on ventral surface except wide areas along anterior margin bare; basal arms of moderate length, directed forward although slightly convergent; ventral plate in lateral view (Fig. 3.13F) with posterior portion of body roundly produced ventrally; ventral plate in end view (Fig. 3.13G) inverted-V shaped, rounded ventrally (width: height ratio 1.00:0.61), moderately covered with microsetae on central portion of posterior surface. Median sclerite (Fig. 3.13F) thin, plate like, wide, connected to anterior one-eighth of ventral plate. Paramere (Figs. 3.13H, I) slender, with three long distinct hooks and several much smaller ones directed dorsally near apex. Aedeagal membrane (Fig.3.13I) moderately covered with microsetae, and with no sclerotized dorsal plate. Ventral surface of abdominal segment 10 without distinct hairs near posterior margin on each side. Cercus (Fig. 3.13J) not well defined, with 11 or 12 hairs.

Pupa. Body length ≈ 2.5 mm. ***Head***. Integument yellow, moderately and elaborately covered with small round tubercles; antennal sheath without any protuberances; frons with three pairs of unbranched long trichomes with coiled or uncoiled apices; face with pair of unbranched long trichomes with coiled apices; three frontal trichomes on each side arising close together, subequal in length to one another and somewhat longer than facial one. ***Thorax***. Integument yellow, moderately covered with small round tubercles, with three unbranched long mediodorsal trichomes with coiled apices, two unbranched anterolateral trichomes (one long with coiled apex, one medium long with uncoiled apex), one unbranched (or bifid) short to medium-long mediolateral trichome with uncoiled apex, and three unbranched ventrolateral trichomes with uncoiled apices (one long, two short) on each side. Gill (Fig. 3.14A) composed of eight slender thread-like filaments, arranged as (2 + 1) + (1 + 2) + 2 filaments from dorsal to ventral, with short common basal stalk having somewhat swollen basal fenestra ventrally at base; common basal stalk short, 0.51−0.59 times as long as interspiracular trunk; dorsal and middle triplets not sharing stalk and each triplet composed of one single and two paired filaments, with short primary and secondary stalks; length of primary and secondary stalks of middle triplet combined much shorter than stalk of ventral pair (Fig. 3.14A) in four pupae but as long as or slightly longer than stalk of ventral pair (Fig. 3.14B) in one pupa; stalk of ventral pair medium-long to long, 1.06−2.41 times length of common basal stalk and 0.56−1.26 times length of interspiracular trunk; stalk of ventral pair as thick as or slightly thicker than primary stalk of middle triplet, which is as thick as or slightly thicker than primary stalk of dorsal triplet; primary stalk of dorsal triplet lying against stalk of lower pair at angle of ≈ 90 degrees when viewed laterally; all filaments grayish light brown, gradually tapered toward apex, increasing in length from dorsal to ventral; only two pupal exuviae were available for measurement of total length of filaments: dorsal triplet 1.5−1.7 mm, middle triplet 1.8−2.0 mm, and ventral paired filaments 2.0−2.1mm in one pupal exuviae (female), and dorsal triplet 1.2−1.3 mm, middle triplet 1.5−1.6 mm, and ventral paired filaments 1.7 mm in another pupal exuviae (male); cuticle of all filaments with well-defined annular ridges and furrows although gradually becoming indistinct from middle to apex, densely covered with minute tubercles. ***Abdomen***. Dorsally, all segments nearly transparent except segment 9 yellowish; segments 1 and 2 without tubercles; segment 1 with one unbranched slender short hair-like seta on each side; segment 2 with one unbranched slender short hair-like seta and five short somewhat spinous setae submedially near posterior margin on each side; segments 3 and 4 each with four hooked spines and one short somewhat spinous seta near posterior margin on each side; segments 6−8 each with spine-combs in transverse row, comb-like groups of minute spines near anterior margin and two unbranched short setae near posterior margin on each side; segment 9 with spine-combs in transverse row, comb-like groups of minute spines near anterior margin on each side and pair of cone-like terminal hooks (Fig. 3.14C).

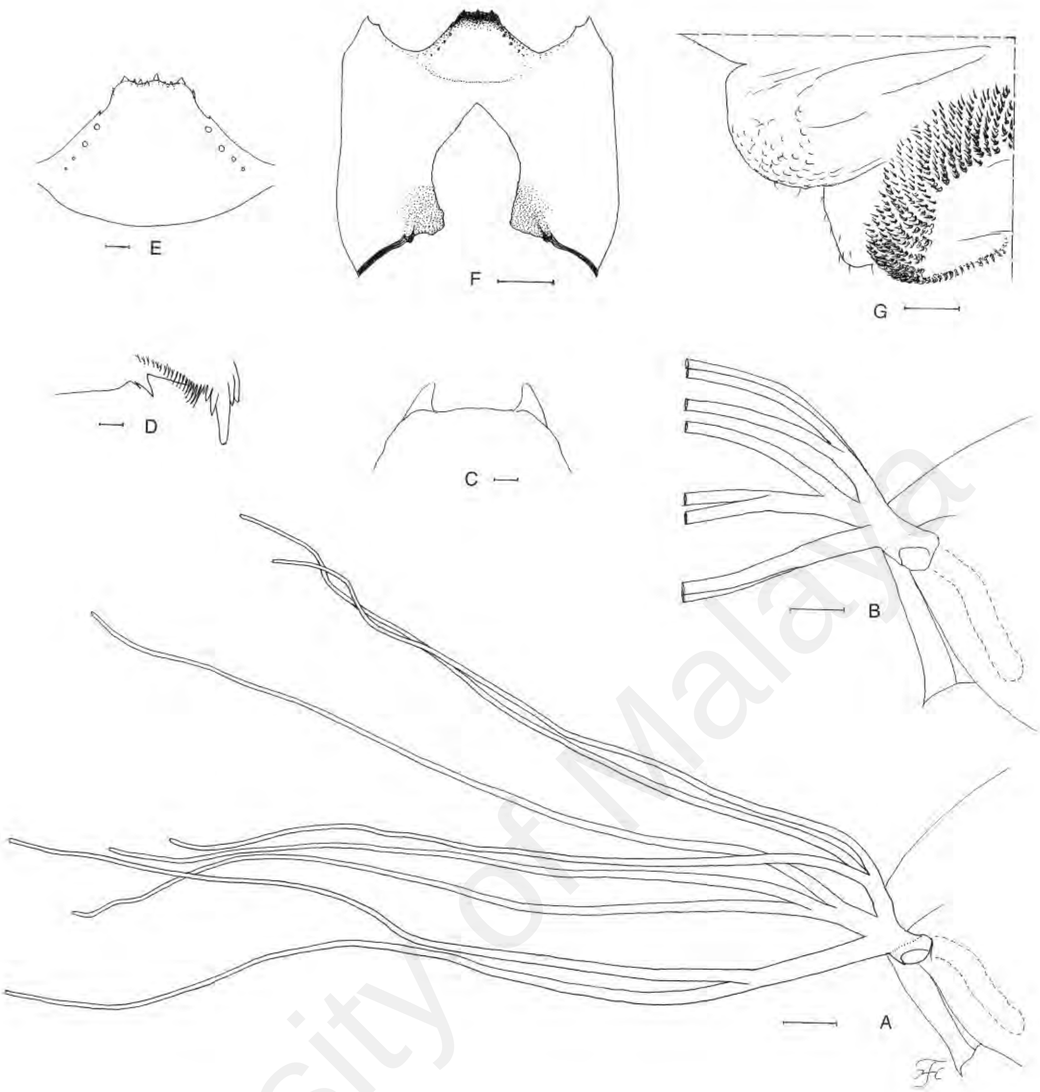

Figure 3.14: Pupa and larva of *Simulium* (*Gomphostilbia*) *azhari* **sp. nov**. (A−C) Pupa. (D−G) Larva. (A) Left gill (outer view). (B) Left gill showing different arrangement (only basal portion shown, outer view). (C) Terminal hooks (caudal view). (D) Mandible (lateral view). (E) Hypostoma (ventral view). (F) Head capsule showing postgenal cleft (ventral view). (G) Last abdominal segment showing bulge with small rounded protuberances (only right half shown, ventral view). Scale bars = 0.1 mm (A, B and F), 0.05 mm (G), 0.02 mm (E), and 0.01 mm (C and D).

Ventrally, segment 4 with one bifid hook and few unbranched short setae on each side; segment 5 with pair of bifid hooks submedially and few unbranched short slender setae on each side; segments 6 and 7 each with pair of bifid inner and unbranched outer hooks somewhat spaced from each other and few unbranched short slender setae on each side; segments 4−8 with comb-like groups of minute spines. Each side of segment 9 with three or four grapnel-shaped hooklets. ***Cocoon***. Wall pocket-shaped, moderately woven, somewhat extended ventrolaterally; anterior margin somewhat thickly woven, with dorsal portion not produced anteriorly when viewed dorsally, and anteroventral tips approaching each other to varying extent, thus narrowly connected to each other in one cocoon; posterior half with floor roughly or moderately woven; individual threads visible; 3.4−3.6 mm in length by 1.6−2.3 mm in width.

Mature Larva. Body length 4.2 mm. Body mostly dark grayish, with reddish-brown color markings as follows: thoracic segment 1 encircled with reddish brown broad transverse band (although disconnected ventromedially), thoracic segments 2 and 3 thinly reddish-brown dorsally, abdominal segments 1−5 each encircled with reddish-brown broad transverse band (although disconnected dorsally and ventrally on segment1, ventrally on segment 5, and faintly on dorsal surface of segments 2 and 3), abdominal segments 6−8 each with reddish-brown areas of various extent dorsomedially and laterally, and abdominal segment 7 with reddish-brown transverse band ventrally. Cephalic apotome yellow; head spots faintly positive except posteromedian and posterolateral spots indistinctive or even negative. Lateral surface of head capsule yellow except eye-spot region whitish; eyebrow moderately visible; two relatively large spots and two small spots near posterior margin indistinct; one small spot below eye-spot region faintly positive or indistinct. Ventral surface of head capsule yellow except darkened area near posterior margin on each side of postgenal cleft; one elongate and one round spot on each side of postgenal cleft indistinct. Antenna composed of three segments and apical sensillum, somewhat longer than stem of labral fan; proportional lengths of 1st, 2nd, and 3rd segments 1.00:0.94−1.03:0.82−0.85. Labral fan with 39 main rays. Mandible (Fig. 3.14D) with three comb-teeth decreasing in length from 1st tooth to 3rd; mandibular serration composed of two teeth (one medium-sized and one small); major tooth at acute angle against mandible on apical side; supernumerary serrations absent. Hypostoma (Fig. 3.14E) with row of eight apical teeth, of which median tooth most prominent, followed by each corner tooth; intermediate teeth on right side three in number but those on left side two; lateral margin smooth; four hypostomal bristles per side lying parallel to lateral margin. Postgenal cleft (Fig. 3.14F) arrow-head shaped, long, 5.8 times length of postgenal bridge. Cervical sclerites small, pale, not fused to occiput, widely separated medially from each other. Thoracic cuticle almost bare. Abdominal cuticle almost bare except few posterior segments moderately covered with unbranched or branched (bifid to quadrifid) minute colorless or slightly darkened setae dorsally and dorsolaterally, and last segment densely covered with unbranched colorless setae on each side of anal sclerite. Rectal scales not observable. Rectal papillae compound, each of three lobes with six or seven finger-like secondary lobules. Anal sclerite of usual X-form, with anterior arms 0.93 times length of posterior ones, broadly sclerotized at base; accessory sclerite absent. Last abdominal segment expanded ventrolaterally forming double bulges on each side, visible as large conical ventral papillae when viewed from side; middle portion of bulge near base of ventral papillae with small round protuberances and sparsely covered with unbranched minute setae (Fig. 3.14G). Posterior circlet with 77 rows of up to 13 hooklets per row.

Type Materials. HOLOTYPE: Female (with associated pupal exuviae and cocoon; preserved in 80% ethanol) reared from pupa, MALAYSIA: Sungai Sedim, Kulim, Kedah Province, 18-III-2012 and 4-III-2013, Sofian-Azirun M, Ya'cob Z, and Azhar-Jamil N. Paratypes: One male (with associated pupal exuviae and cocoon), and one pupal exuviae and cocoon, all preserved in 80% ethanol, same data as those of the holotype; one female (with associated pupal exuviae and cocoon), one pupal exuviae and cocoon, and one mature larva, all preserved in 80% ethanol, collected from a stream near Sungai Sedim, other data as those of the holotype.

Distribution. Peninsular Malaysia (Sedim, Kedah).

Biological notes. Two pupae and one pupal exuviae of this new species were collected from dead tree leaves in the water of a small shallow stream (width 1.0 m, depth 5 cm, bottom muddy, water temperature 23.0°C, shaded, altitude 106 m; 05° 24’52.3”N, 100° 46’48.9”E) flowing slowly in a natural forest in a swampy area; and one pupa, one pupal exuviae, and one mature larva were collected from dead tree leaves in the water of a small stream (width 56 cm, depth 2 cm, bottom sandy, water temperature 24.0°C, partially shaded, altitude 118 m; 05°24'42.6" N, 100°46'50.2" E) flowing slowly at the edge of a natural forest in a swampy area. Associated species were *Simulium* (*Gomphostilbia*) sp. A Takaoka and Davies, *Simulium* (*Gomphostilbia*) *cheongi* Takaoka and Davies, *Simulium* (*Gomphostilbia*) *terengganuense* Takaoka, Sofian-Azirun and Ya’cob, *Simulium* (*Gomphostilbia*) sp. (ceylonicum species group) from Sungai Sedim; *Simulium* (*Gomphostilbia*) *angulistylum* Takaoka and Davies, *S*. (*G*.) *cheongi*, *Simulium* (*Gomphostilbia*) *gombakense* Takaoka and Davies, *S*. (*G*.) *terengganuense*, *S*. (*G*.) sp. (*ceylonicum* species group), *Simulium* (*Gomphostilbia*) *whartoni* Takaoka and Davies from another stream.

Etymology. The species name *azhari* is in honor of Nor Azhar Jamil, University of Malaya, who helped our field surveys.

Remarks. This new species is assigned to the *S. batoense* species group of the subgenus *Gomphostilbia* as redefined by Takaoka (2012) in having the antenna with nine flagellomeres, the pleural membrane bare, hind tibiae mostly darkened (Fig. 3.12E), female claws with a large basal tooth (Fig. 3.12G), the spermatheca without sclerotized neck (Fig. 3.12L), male hind basitarsi slender and parallel-sided (Fig. 3.13B), the ventral plate moderately produced ventrally (Fig. 3.13G) (its ratio of the height against the greatest width is 0.61, which is, although, slightly greater than the maximum value [0.59] of the *S. batoense* species group given by Takaoka [2012]), the paramere with hooks (Fig. 3.13I), and the pupal gill with eight filaments (Fig. 3.14A). Among six subgroups of the *S. batoense* species group (Takaoka, 2012), *S.* (*G.*) *azhari* sp. nov. is assigned to the *parahiyangum* subgroup (11 known species included) because of having the pupal gill with eight filaments that are shorter than the pupal body (Fig.3.14A). This new species is characterized in the female by the narrow frons (frons:head ratio 1.00:7.13; Fig. 3.12A). None of the nine known species of the *parahiyangum* subgroup, of which the female is known, has such an arrow frons except *Simulium* (*Gomphostilbia*) *krombeini* Davies and Györkös (1987) from Sri Lanka, from which this new species is distinguished by the claw with an average size of the tooth (claw:tooth ratio1.00:0.51) [cf. 1.00:0.66 in *S.* (*G.*) *krombeini*] (Davies & Györkös, 1987). Females of *Simulium* (*Gomphostilbia*) *miyagii* Takaoka, from Sulawesi, Indonesia (Takaoka, 2003), and *Simulium* (*Gomphostilbia*) *dentistylum* Takaoka & Davies, from Peninsular Malaysia and Thailand (Takaoka & Davies, 1995), are not known. The male of this new species is characterized by the genitalia: in particular, the broad style (Fig. 3.13D) and the ventral plate that is much produced ventrally (Fig. 3.13G). Both characters are not seen in any of the nine known species of the subgroup for which the male is known [*Simulium* (*Gomphostilbia*) *bhutanense* Takaoka and Somboon from Bhutan and Nepal, and *Simulium* (*Gomphostilbia*) *chuzargangense* Takaoka and Somboon from Bhutan, are known only from females] (Takaoka & Somboon, 2008). The style of the male genitalia is usually slender and tapered toward the apex, and the broad style as observed in this new species is rare among the subgenus *Gomphostilbia*, being recorded in only five species: *Simulium* (*Gomphostilbia*) *auratum* Takaoka from Sarawak, Malaysia, *S.* (*G.*) *angulistylum* from Peninsular Malaysia and Thailand, *Simulium* (*Gomphostilbia*) *epistum* Delfinado from Palawan, Philippines, and *Simulium* (*Gomphostilbia*) *otsukai* Takaoka and Choochote from Thailand, all of the epistum species group (Takaoka, 1983; 2009; Takaoka & Davies, 1995; Takaoka *et al.*, 2009), and *Simulium* (*Gomphostilbia*) *charlesi* Takaoka, of the *varicorne* species group (Takaoka, 2008). However, the pupa of this new species is similar to *S.* (*G.*) *krombeini* in many characters including the arrangement of the eight gill filaments: i.e., stalks of the dorsal and middle triplets and the ventral pair, all arising at the same level from the short common basal stalk at an angle of ≈ 90 degrees when viewed laterally (Fig. 3.14A). The pupae of eight other known species of the *parahiyangum* subgroup are easily distinguished from this new species by having following characters: a long common basal stalk [*S.* (*G.*) *miyagii*], a short stalk of the ventral pair [*Simulium* (*Gomphostilbia*) *brevitruncatum* Takaoka from Sulawesi, Indonesia, *S.*(*G.*) *dentistylum*, *Simulium* (*Gomphostilbia*) *dola* Davies and Györkös from Sri Lanka, *Simulium* (*Gomphostilbia*) *parahiyangum* Takaoka and Sigit from Indonesia (Java, Sumatra), Malaysia (Peninsular Malaysia, Sabah, Sarawak), Thailand and India (Assam), *Simulium* (*Gomphostilbia*) *pattoni* Senior-White from Sri Lanka], dorsal and middle triplets sharing a common stalk [*Simulium* (*Gomphostilbia*) *brivetruncum* Chen and Chen and *Simulium* (*Gomphostilbia*) *nigrofemoralum* Chen and Zhang, both from Hainan, China] (Davies & Györkös, 1987; Takaoka & Sigit, 1992; Takaoka & Davies, 1995; Chen & Chen, 2000; Chen & Zhang, 2001; Takaoka, 2003). In this study, only one mature larva of this new species was available. The hypostoma of this unique larva apparently is aberrant, lacking one of three intermediate teeth on the left side (Fig. 3.14E). This larva is characterized by the presence of small round protuberances on the surface of each ventrolateral bulge of the last abdominal segment (Fig. 3.14G), which have not been reported in any

Gomphostilbia species. Further study will be needed to confirm whether this character is also recognized in other larval individuals of this new species.

3.3.6 *Simulium* (*Gomphostilbia*) *johorense* sp. nov. Takaoka, Sofian-Azirun and Ya'cob 2014

Female. Body length 1.8−2.0 mm. ***Head***. Nearly as wide as thorax. Frons black, shiny, moderately covered with whitish yellow scale-like recumbent shorthairs interspersed with several dark simple longer hairs along each lateral margin; frontal ratio 1.74:1.00:2.46; frons:head ratio 1.00:4.82. Fronto-ocular area well developed, narrow, directed dorsolaterally. Clypeus black, shiny, gray pruinose, densely covered with yellow hairs interspersed with several dark longer hairs on each side. Labrum 0.58 times length of clypeus. Antenna composed of scape, pedicel and nine flagellomeres, medium brown except scape, pedicel and basal one-third to one-half of 1st flagellomere, which is whitish-yellow when viewed dorsally, (although first flagellomere almost entirely whitish-yellow when viewed ventrally). Maxillary palp composed of five segments, light to medium brown, proportional lengths of 3rd, 4th, and 5th segments 1.00:1.09:2.64; third segment (Fig. 3.15A) of moderate size; sensory vesicle (Fig. 3.15A) of medium size, ellipsoidal, 0.41 times length of third segment and with medium-sized opening. Maxillary lacinia with 8−10 inner and 11 outer teeth. Mandible with 20 inner and 10 outer teeth. Cibarium (Fig. 3.15B) medially forming sclerotized plate folded forward from posterior margin, with strongly sclerotized medial longitudinal ridge. ***Thorax***. Scutum black, shiny when illuminated at certain angles, thinly gray pruinose, appearing to have three (median and submedian) non pruinose longitudinal vittae, densely covered with whitish and whitish-yellow scale-like recumbent hairs except three longitudinal vittae with dark recumbent hairs and prescutellar area with few dark upright hairs. Scutellum brownish-black, slightly shiny when illuminated at certain angles, covered with dark short hairs and dark brown upright long hairs along posterior margin. Postnotum brownish-black and bare. Pleural membrane bare. Katepisternum brownish-black to black, longer than deep, shiny when illuminated at certain angles, moderately covered with yellow short hairs. ***Legs***. *Foreleg*: coxa whitish-yellow; trochanter medium brown except dorsal surface whitish-yellow; femur medium brown with apical cap dark brown; tibia medium brown with apical cap brownish-black, moderately covered with white (shiny under illumination) short hairs on outer surface; tarsus black, with moderate dorsal hair crest; basitarsus somewhat dilated, 4.93−5.18 times as long as its greatest width. *Midleg*: coxa brownish-black; trochanter medium brown except base yellow; femur medium brown except apical cap dark brown; tibia medium brown except apical cap dark brown and basal tip dark yellow to light brown, and with white (shiny under illumination) short hairs on outer and posterior surfaces of basal two-thirds; tarsus dark brown except basal half of basitarsus dark yellow to light brown. *Hind leg*: coxa medium brown; trochanter yellow; femur medium to dark brown except base yellow and apical cap brownish-black; tibia (Fig. 3.15C) medium brown except base yellowish-white and apical two fifths dark brown, and with dark brown subbasal spot; tibia moderately covered with white (shiny under illumination) short hairs on outer and posterior surfaces of basal two-thirds; tarsus (Fig. 3.15D) brownish-black except basal two-thirds of basitarsus (although base light brown) and basal half of second tarsomere whitish; basitarsus (Fig. 3.15D) narrow, nearly parallel-sided, 6.13−6.39 times as long as wide, and 0.57−0.64 and 0.48−0.50 times as wide as greatest width of tibia and femur, respectively; calcipala (Fig.3.15D) well developed, 1.43 times as long as wide, and 0.44 times as wide as greatest width of basitarsus;

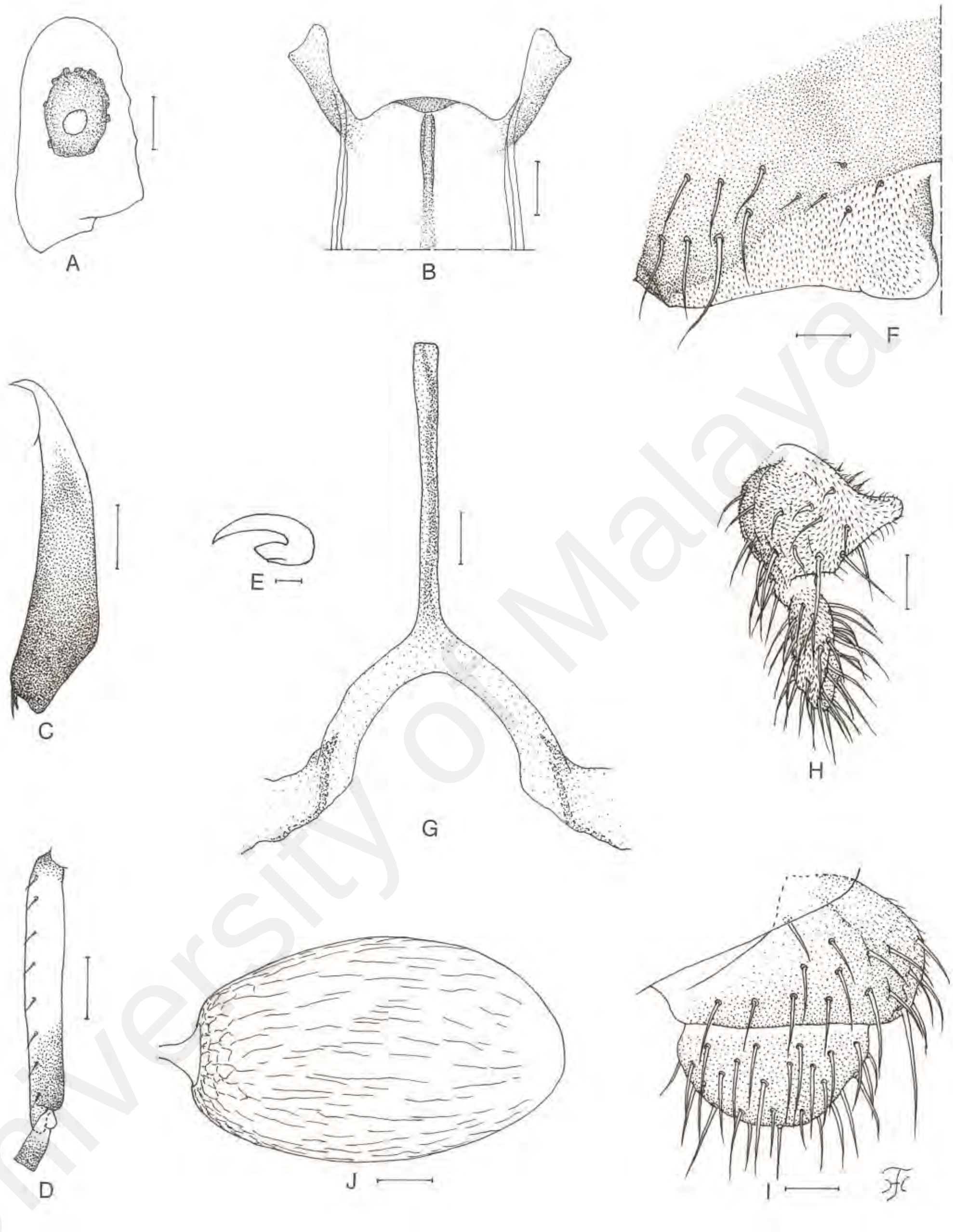

Figure 3.15: Female of *Simulium* (*Gomphostilbia*) *johorense* **sp. nov**. (A) Third segment of right maxillary palp with sensory vesicle (front view). (B) Cibarium. (C) Left hind tibia (outer view). (D) Basitarsus and second tarsomere of left hind leg showing calcipala and pedisulcus (outer view). (E) Claw. (F) Sternite 8 and ovipositor valve (only right half shown; ventral view). (G) Genital fork (ventral view). (H) Right paraproct and cercus (ventral view). (I) Right paraproct and cercus (lateral view). (J) Spermatheca. Scale bars = 0.1 mm (C and D), 0.02 mm (A and F−J) and 0.01 mm (E).

pedisulcus (Fig. 3.15D) well developed; claw (Fig. 3.15E) with large basal tooth 0.50 times length of claw. ***Wing***. Length 1.5 mm. Costa with dark brown spinules and pale hairs including basal patch of whitish hairs. Subcosta bare in 3 females, bare on right side and with one hair on left side in one female, with five hairs in middle on right side and one hair on left side in one female, and with three hairs on right side and five hairs on left side in one female. Hair tuft on base of radial vein dark brown. Basal portion of radius fully haired. Basal cell absent. ***Halter***. Clear white except basal stem partially darkened. ***Abdomen***. Basal scale medium brown, with fringe of yellowish-white hairs. Dorsal surface of abdomen dark brown to black except basal half of segment 2 dark yellow to light brown, moderately covered with dark short to long hairs; tergites of segments 2 and 6−9 shiny when illuminated at certain angles. Ventral surface of abdomen dark brown except segments 2 and 3 creamy to gray; sternal plate on segment 7 undeveloped. ***Genitalia***. Sternite 8 (Fig. 3.15F) bare medially, with seven to nine medium-long to long hairs together with one to four slender short hairs on each side. Ovipositor valves (Fig. 3.15F) triangular (although medioposterior corners rounded), tapered laterally, thin, membranous, moderately covered with microsetae interspersed with one to three short hair; inner margins sinuous, moderately sclerotized, and somewhat separated from each other. Genital fork (Fig.3.15G) of usual inverted-Y form, with slender stem; arms of moderate width, moderately folded medially, without posteromedial lobe or projection. Paraproct in ventral view (Fig. 3.15H) with anterolateral tip unsclerotized and anteromedian margin darkened, with three or four sensilla on anteromedial surface; paraproct in lateral view (Fig. 3.15I) somewhat produced ventrally, 0.63 times as long as wide, with 17−19 medium−long to long hairs on ventral and lateral surfaces. Cercus in lateral view (Fig. 3.15I) short, rounded posteriorly, 0.51 times as long as wide. Spermatheca (Fig.3.15J) oblong, 1.63−1.66 times as long as greatest width, well sclerotized except duct and small area near juncture with duct unsclerotized, and with many fissures on surface; internal setae absent; both accessory ducts slender, subequal in diameter to major one.

Male. Body length 2.3 mm. ***Head***. Wider than thorax. Upper eye medium to dark brown, consisting of 13 vertical columns and 14 horizontal rows of large facets. Face brownish-black, shiny, whitish-gray pruinose. Clypeus brownish-black, shiny, whitish-gray pruinose, moderately covered with yellow short hairs interspersed with dark brown longer hairs. Antenna composed of scape, pedicel and nine flagellomeres, light to dark brown except scape and pedicel yellow to dark yellow, and base of 1st flagellomere whitish-yellow; 1st flagellomere elongate, 1.44 times length of second one. Maxillary palp with five segments, light brown, proportional lengths of 3rd, 4th, and 5th segments 1.00:1.19:2.68; 3rd segment (Fig. 3.16A) somewhat widened apically; sensory vesicle (Fig. 3.16A) ellipsoidal, small, 0.17 times length of 3rd segment, and with small opening. ***Thorax***. Scutum black, shiny, thinly gray pruinose variously (i.e., three males gray pruinose on shoulders, along lateral margins and on prescutellar area leaving large central area non-pruinose [Fig. 3.16B], one male with non-pruinose medial vitta and two lateral spots [Fig. 3.16C], and one male with non-pruinose medial spot extending anteriorly as narrow line and two small lateral spots [Fig. 3.16D]), densely covered with golden yellow short hairs except non-pruinose area or spots covered with brassy shorthairs, and prescutellar area with few dark upright hairs. Scutellum dark brown, somewhat shiny when illuminated at certain angles, with dark short hairs and dark brown upright long hairs. Postnotum brownish black, somewhat shiny and gray pruinose when illuminated at certain angles, and bare. Pleural membrane and katepisternum as in female. ***Legs***. Color nearly as in female except fore coxa yellow except anterior surface somewhat darkened, mid basitarsus with basal one-third light brown, and hind tibia with narrower whitish basal portion (Fig. 3.16E).

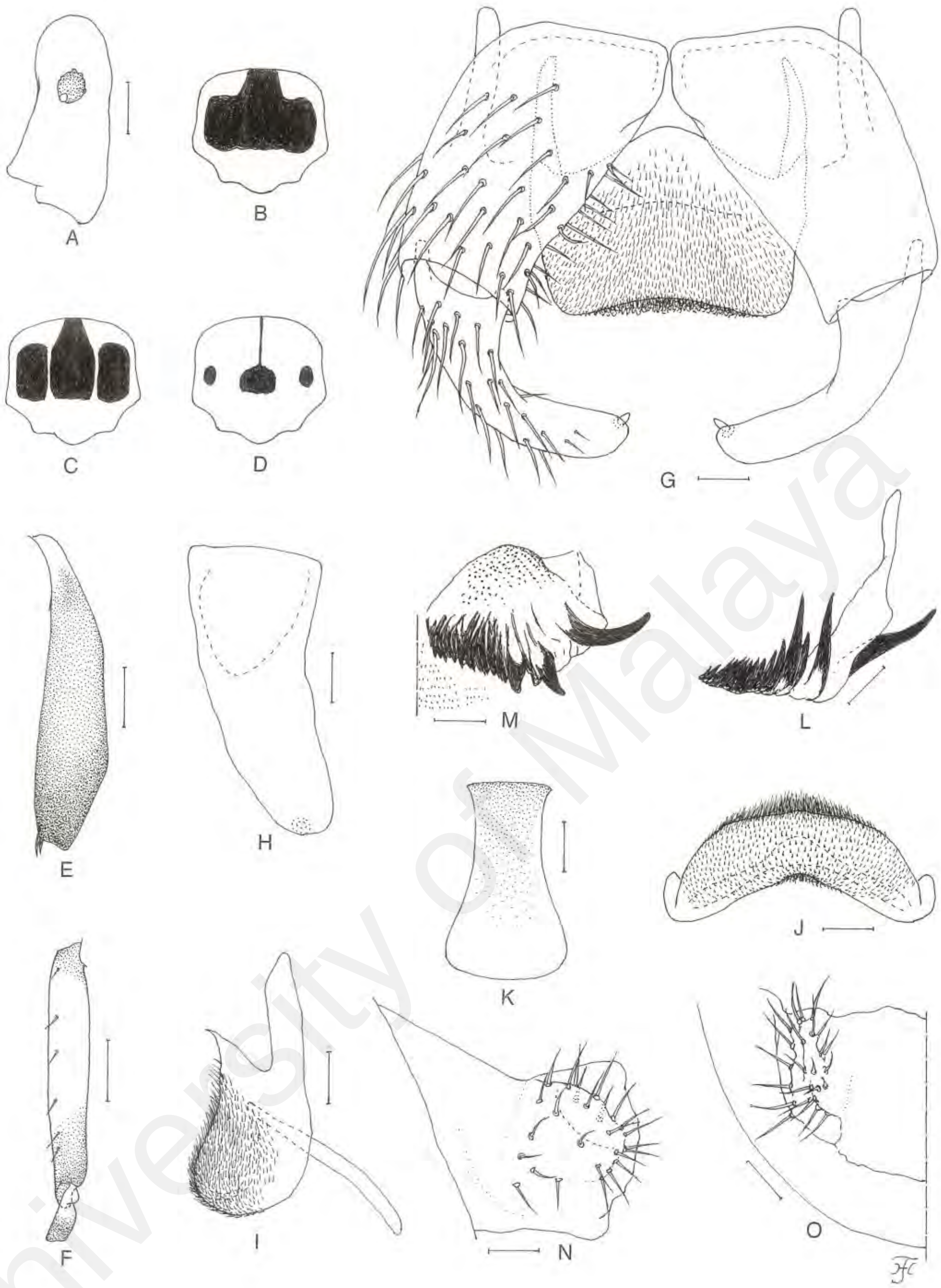

Figure 3.16: Male of *Simulium* (*Gomphostilbia*) *johorense* **sp. nov**. (A) Third segment of left maxillary palp with sensory vesicle (front view). (B−D). Scutum showing different patterns formed by pruinose and nonpruinose areas (dorsal view). (E) Hind tibia (left side; outer view). (F) Basitarsus and second tarsomere of left hind leg showing calcipala and pedisulcus (outer view). (G) Coxites, styles and ventral plate (ventral view). (H) Right style (ventrolateral view). (I) Ventral plate and median sclerite (lateral view). (J) Ventral plate (caudal view). (K) Median sclerite (posteroventral view). (L) Left paramere (dorsal view). (M) Left paramere and aedeagal membrane (caudal view). (N, O) Right cerci (N, lateral view; O, caudal view). Scale bars = 0.1 mm (E and F), and 0.02 mm (A and G−O).

Fore basitarsus somewhat dilated, 6.75 times as long as greatest width. Hind basitarsus (Fig. 3.16F) narrow, nearly parallel-sided (although slightly tapered from middle to apex), 5.33times as long as wide, and 0.60 and 0.42 times as wide as greatest width of tibia and femur, respectively; calcipala (Fig. 3.16F) well developed, nearly as long as wide, and 0.39 times as wide as greatest width of basitarsus; pedisulcus (Fig. 3.16F) well developed. ***Wing***. Length 1.5 mm. As in female except subcosta without hairs. ***Halter***. Grayish-white with basal portion darkened. ***Abdomen***. Basal scale dark brown, with fringe of light brown long hairs. Dorsal surface of abdomen dark brown to black except basal one-third of segment 2 yellow, covered with dark brown short to long hairs; segments 2 and 5−7 each with pair of shiny dorsolateral patches. ***Genitalia***. Coxite in ventral view (Fig. 3.16G) nearly rectangular, 1.81 times as long as its greatest width. Style in ventral view (Fig. 3.16G) 0.83 times length of coxite, slender, somewhat tapered toward middle, then nearly parallel-sided, curved inward, with apical spine; style in ventrolateral view (Fig. 3.16H) gradually tapered from base to blunt apex. Ventral plate in ventral view (Fig. 3.16G) transverse, 0.64 times as long as wide, with posterior half of body slightly narrower than anterior half, with anterior margin roundly produced anteromedially, posterior margin shallowly concave, and densely covered with microsetae on ventral surface except areas near anterior margin bare; basal arms of moderate length, directed anteriorly, nearly parallel-sided although slightly convergent; ventral plate in lateral view (Fig. 3.16I) with posterior portion of body somewhat produced ventrally; ventral plate in end view (Fig. 3.16J) rounded ventrally (width:height ratio 1.00:0.38), moderately covered with microsetae on most of posterior surface. Median sclerite (Figs. 3.16I, K) thin, plate-like, wide, connected to slightly anterior to middle of ventral plate. Paramere (Figs. 3.16L, M) with three long distinct hooks (one directed laterally, two directed anteriorly) and several smaller ones directed anteriorly. Aedeagal membrane (Fig. 3.16M) moderately covered with microsetae, and with no sclerotized dorsal plate. Ventral surface of abdominal segment 10 somewhat sclerotized basally, without distinct hairs near posterior margin on each side. Cercus (Figs. 3.16N, O) rounded, somewhat produced ventrally, with 20−26 hairs.

Pupa. Body length 2.0−2.4 mm. Similar to pupa of *S*. (*G*.) *azhari* sp. nov. except for the following characters. ***Head***. Integument dark yellow. ***Thorax***. Integument dark yellow, moderately or densely covered with small round tubercles. Gill (Fig. 3.17A) composed of eight slender thread-like filaments, arranged as (2+1) + (1+ 2) + 2 filaments or 3 + (1 + 2) + 2 filaments (Fig. 3.17A), or 2 + 1 + 3 + 2 filaments (Fig.3.17B; only in right gill of one pupa), from dorsal to ventral, with short common basal stalk having somewhat swollen basal fenestra ventrally; common basal stalk short, 0.46 times length of interspiracular trunk; dorsal and middle triplets not sharing stalk; dorsal triplet composed of one single and two paired filaments with short primary and secondary stalks, or three single filaments arising at same level from short stalk; three filaments of dorsal triplet subequal in length (0.9−1.6 mm) and thickness to one another; middle triplet composed of one single and two paired filaments subequal in length (1.2−1.8 mm) and thickness to one another (although one of paired filaments slightly shorter and thinner than other two filaments); two filaments of ventral pair longest and thickest of all, although inner filament (2.5−2.8 mm) is somewhat longer and thicker than outer filament (2.1−2.7 mm); stalk of ventral pair long, 2.19−4.44 times length of primary and secondary stalks of middle triplet combined, 2.50−3.33 times length of common basal stalk, and 1.08−1.54 times length of interspiracular trunk; stalk of ventral pair 1.07−1.54 times as thick as primary stalk of middle triplet, which is slightly thicker than primary stalk of dorsal triplet; primary stalk of dorsal triplet lying against stalk of lower pair at angle of 90−110 degrees when viewed laterally; all filaments light to medium brown, gradually tapered toward apex;

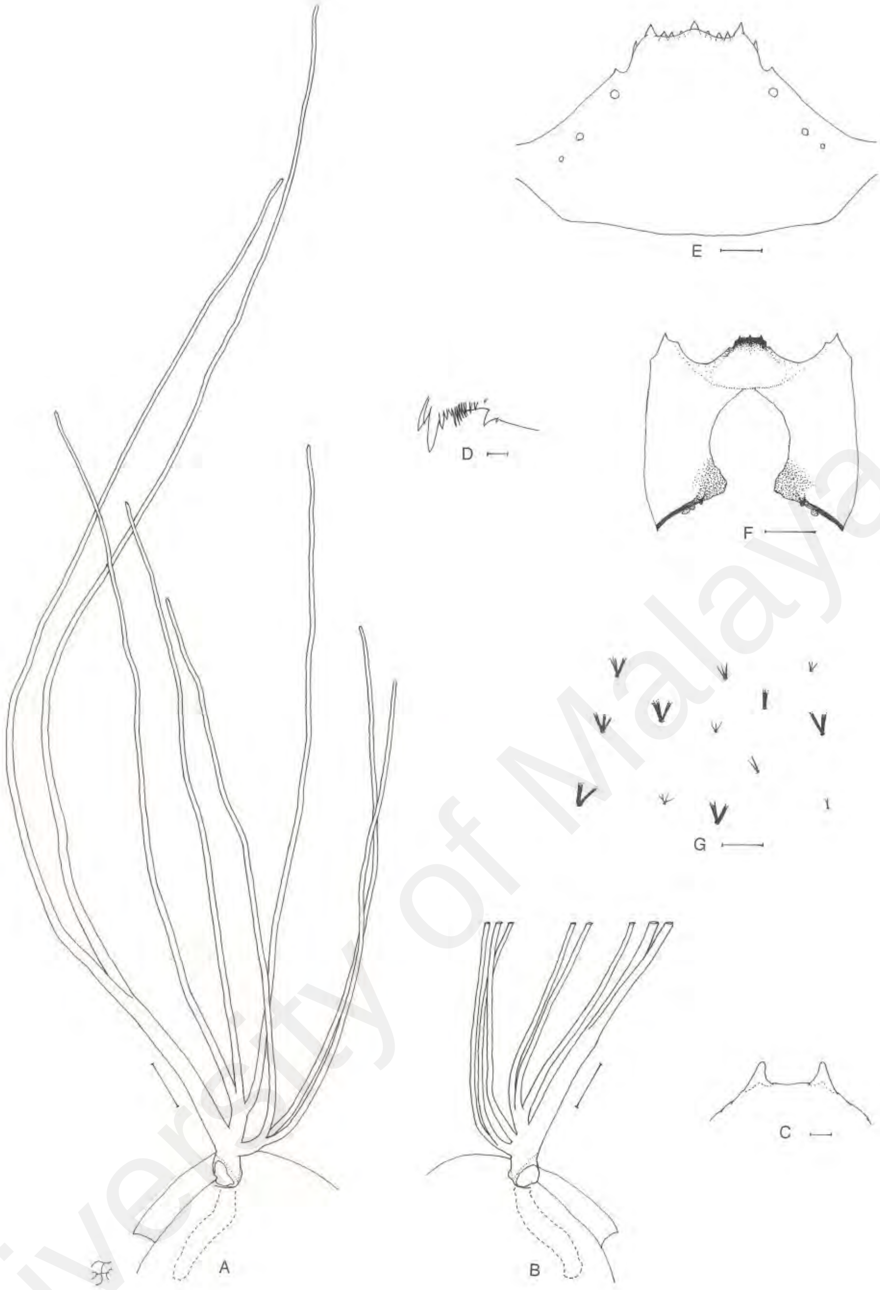

Figure 3.17: Pupa and larva of *Simulium* (*Gomphostilbia*) *johorense* **sp. nov**. (A−C) Pupa. (D−G) Larva. (A) Left gill (outer view). (B) Right gill showing different arrangement (only basal portion shown, outer view). (C) Terminal hooks (caudal view). (D) Mandible (lateral view). (E) Hypostoma (ventral view). (F) Head capsule showing postgenal cleft (ventral view). (G) Black spinous setae on dorsal surface of abdominal segment 8. Scale bars = 0.1 mm (A, B and F), 0.05 mm (G), 0.02 mm (E and G), and 0.01 mm (C and D).

cuticle of all filaments nearly smooth except basal half or more of ventral paired filaments with annular ridges and furrows, and basal portions of some of six filaments of dorsal and middle triplets with weakly defined furrows, and densely covered with minute tubercles. ***Abdomen***. Dorsally, all segments nearly transparent except segments 1−4 gray-pigmented, although pigmented area limited along anterior margin on segments 3 and 4; terminal hooks cone-like (Fig. 3.17C). Ventrally, segment 4 with one unbranched or bifid hook and few unbranched short setae on each side. ***Cocoon***. Anteroventral tips not approaching each other; 2.4−3.0 mm in length by 1.2−2.0 mm in width.

Mature Larva. Body length 3.6−4.0 mm. Body whitish, with color markings as follows: thoracic segment 1 encircled with gray or grayish-green broad transverse band (although disconnected ventromedially) and dark gray on anterior surface of proleg; thoracic segments 2 and 3 light gray (in two larvae) or not (in one larva) on dorsal surface, and dark grayish-green on ventral surface, abdominal segments 1−4 each encircled with gray or grayish-green broad transverse band (although less distinct on dorsomedially in two larvae); abdominal segments 5−8 each with gray or grayish-green areas of various extent dorsomedially and laterally (in one larva, abdominal segments 7 and 8 with purple-colored areas dorsolaterally and abdominal segment 9 with purple-colored area dorsomedially), abdominal segments 5 and 6 with light gray areas ventrolaterally, and abdominal segment 7 with light gray area ventromedially; in one larva from a different stream, body with reddish-brown as well as grayish markings as follows: dark grayish on anterior surface of proleg, on ventral surfaces of thoracic segments 2 and 3, and thinly grayish on ventral surfaces of abdominal segments 1−5; reddish-brown transverse band on thoracic segment 1 although disconnected ventromedially, reddish-brown small spot on dorsolateral surface of each side of thoracic segments 2 and 3, reddish brown transverse bands each on abdominal segments

1−5 although disconnected dorsomedially and ventrally, reddish-brown areas of varying extent on dorsal and dorsolateral surfaces of abdominal segments 6−8. Cephalic apotome whitish or whitish-yellow; head spots indistinct or posteromedian and posterolateral spots faintly negative. Lateral surface of head capsule white or whitish-yellow except eye-spot region whitish; eyebrow, two relatively large spots and two small spots near posterior margin indistinct; one small spot below eye-spot region indistinct. Ventral surface of head capsule whitish-yellow except darkened area near posterior margin on each side of postgenal cleft; one elongate spot and one round spot on each side of postgenal cleft indistinct. Antenna composed of three segments and apical sensillum, somewhat longer than stem of labral fan; proportional lengths of 1st, 2nd, and 3rd segments 1.00:0.92−0.96:0.92−0.96. Labral fan with 29−34 main rays. Mandible (Fig. 3.17D) with three comb-teeth decreasing in length from 1st tooth to 3rd; mandibular serration composed of two teeth (one medium-sized and one small); major tooth at acute angle against mandible on apical side; supernumerary serrations absent. Hypostoma (Fig. 3.17E) with row of nine apical teeth, of which median tooth and each corner tooth are prominent; lateral margin smooth; three or four hypostomal bristles per side lying parallel to lateral margin. Postgenal cleft (Fig. 3.17F) deep, reaching posterior margin of hypostoma (although in one larva, postgenal cleft deep but not reaching posterior margin of hypostoma leaving a very short postgenal bridge). Cervical sclerites small, pale, not fused to occiput, widely separated medially from each other. Thoracic cuticle and abdominal cuticle covered with dark branched spinous setae (Fig. 3.17G) dorsally and dorsolaterally, sparsely with relatively smaller setae on thorax and abdominal segments 1−3, moderately or densely with relatively larger and smaller setae on abdominal segments 4−9; and last abdominal segment also moderately covered with simple colorless setae on each side of anal sclerite. Rectal scales not observable. Rectal papillae compound each of three lobes with six to nine finger-like secondary lobules.

Anal sclerite of usual X-form, with anterior arms 0.81−0.83 times as long as posterior ones, broadly sclerotized at base; accessory sclerite absent. Last abdominal segment expanded ventrolaterally forming double bulges on each side, visible as large conical ventral papillae when viewed from side. Posterior circlet with 64−70 rows of up to 13 or 14 hooklets per row.

Type Materials. HOLOTYPE: Female (with associated pupal exuviae and cocoon; preserved in 80% ethanol)reared from pupa, MALAYSIA: foothill of Mount Ledang, State of Johor, 12-IV-2013, Sofian-Azirun M., Ya'cob Z., and Azhar-Jamil N. Paratypes: Four females, two males (all with associated pupal exuviae and cocoon),one pharate male, and three mature larvae, all preserved in 80% ethanol, same data as the holotype; two females and one male (with associated pupal exuviae and cocoon), same data as the holotype except date 12-III-2013; and one pharate male and one mature larva, all preserved in 80% ethanol, collected from a different stream, foothill of Mount Ledang, State of Johor, 12-III-2013, Sofian-Azirun M, Ya'cob Z and Azhar-Jamil N.

Distribution. Peninsular Malaysia (Mount Ledang, Johor).

Biology. The pupae and larvae of this new species were collected from dead tree leaves in the water of a small shallow stream (width 0.2−0.5 m, depth 10 cm, bottom sandy, water temperature 28.5 °C, exposed to sun, altitude 79 m; 02°19'42.3"N, 103°37'24.6"E) flowing slowly along the edge of an oil palm plantation; and from trailing grass in the water of a small stream (width 0.5−3.0 m, depth 14−20 cm, bottom sandy, water temperature 27.8 °C, exposed to sun, altitude 67 m; 02°19'15.7"N, 102°37'19.8"E), flowing moderately at the edge of an oil palm plantation. Associated species were *S.* (*G.*) *angulistylum*, *S.* (*G.*) sp. (ceylonicum species group), and *Simulium* (*Nevermannia*) *aureohirtum* Brunetti, 1911.

Etymology. The species name *johorense* refers to the name of the state, Johor, where this new species was collected.

Remarks. This new species is assigned to the *S. batoense* species group of the subgenus *Gomphostilbia* as redefined by Takaoka (2012) in having the antenna with nine flagellomeres, the pleural membrane bare, hind tibiae mostly darkened (Fig. 3.15B), female claws with a large basal tooth (Fig. 3.15D), the spermatheca without a sclerotized neck (Fig. 3.15I), male hind basitarsi slender, parallel-sided (Fig. 3.16F), the ventral plates lightly produced ventrally (Fig. 3.16J; its ratio of the height against the greatest width is 0.38), the paramere with hooks (Figs. 3.16L and M), and the pupal gill with eight filaments (Fig. 3.17A). Among the six subgroups of the *S. batoense* species group (Takaoka, 2012), *S.* (*G.*) *johorense* sp. nov. is assigned to the *duolongum* subgroup (22 known species included) by having the pupal gill with eight filaments, of which two filaments of the ventral pair, although slightly different in length and thickness from each other, are longer than the pupal body, and at least one of two ventral pared filaments is 1.5 times as long as other six filaments of the dorsal and middle triplets (Fig. 3.17A). This new species is characterized in the female by the subcosta, which is bare or with one to five hairs, a character reported only in a few species [i.e., *Simulium* (*Gomphostilbia*) *kolakaense* Takaoka and *Simulium* (*Gomphostilbia*) *singgihi* Takaoka, both described from Sulawesi, Indonesia (Takaoka, 2003), both of the *duolongum* subgroup, *Simulium* (*Gomphostilbia*) *binuanense* Takaoka and Tenedero, from Palawan, Philippines (Takaoka & Tenedero, 2008), of the *binuanense* subgroup, and *S.* (*G.*) *chuzargangense* Takaoka and Somboon, described from Bhutan (Takaoka & Somboon, 2008), of the *parahiyangum* subgroup]. However, the female of *S.* (*G.*) *johorense* sp. nov. is distinguished from those of the former three known species by the more dilated fore basitarsus (ratio of the length against the greatest width 4.93−5.18in this new species vs. 5.52 in *S.* (*G.*) *chuzargangense*, 6.06 in

S. (*G*.) *kolakaense*, and 6.11 in *S*. (*G*.) *singgihi*), and darker fore and mid femora (mostly medium brown in this new species but mostly grayish-yellow or dark yellow to light brown in the three known species), and from *S*. (*G*.) *chuzargangense* also by the length ratio of the labrum against the clypeus (0.58 in this new species vs. 0.68 in the latter), and from the remaining known species, *S*. (*G*.) *binuanense*, by the length ratio of the sensory vesicle against the third maxillary palpal segment (0.41 in this new species vs.0.56 in *S*. (*G*.) *binuanense*). The male of this new species appears to be similar to those of *S*. (*G*.) *kolakaense* and *S*. (*G*.) *singgihi* in having the same number of enlarged upper-eye facets (i.e., 13 vertical columns and 14 horizontal rows), but differs from both species by the narrower hind basitarsus relative to the hind femur (0.42 in this new species vs. 0.51 and 0.58 in the latter two species), and the number of hairs on the cercus (20−26 in this new species vs. 10−14 and 12 or 13 in the latter two species). The numbers of large male upper-eye facets of 17 of the remaining 20 species of the *duolongum* subgroup are fewer (i.e., 10−12 vertical columns and 10−13 horizontal rows) or greater (i.e., 14−17 vertical columns and 15−17 horizontal rows) than that of *S*. (*G*.) *johorense* sp. nov. Among three other species, the male of *Simulium* (*Gomphostilbia*) *tahanense* Takaoka and Davies described from Peninsular Malaysia (Takaoka & Davies, 1995) was unknown, but the number of large male upper-eye facets of this species is in 11 vertical columns and 11 horizontal rows according to male specimens reared from pupae recently collected from Peninsular Malaysia (H. T., unpublished data).The male of *Simulium* (*Gomphostilbia*) *jinbianense* Zhang and Chen described from China (Zhang & Chen, 2004) is unknown. Information is not available on the upper-eye facets of the type male of *Simulium* (*Gomphostilbia*) *friederichsi* Edwards, originally described from a single male collected from East Java (Edwards, 1934; Takaoka & Davies, 1996). However, upper-eye facets are in 15 or 16 vertical and 15 or 16 horizontal rows according to males emerged from pupae collected from

Sumatra and tentatively identified as *S.* (*G.*) *friederichsi* (Takaoka *et al*., 2000; H. T. unpublished data). The pupa and larva of this new species are similar to those of *Simulium* (*Gomphostilbia*) *siamense* Takaoka and Suzuki described from Thailand in many characters including the arrangement of the pupal gill filaments (Fig. 3.17A) and the shapes of the dark spinous setae on the dorsal surface of the larval abdomen (Fig.3.16G) (Takaoka & Suzuki, 1984). However, the female and male of *S.* (*G.*) *johorense* sp. nov. are easily distinguished from those of *S.* (*G.*) *siamense* by the absence of hairs or reduced number of hairs (one to five hairs) on the female subcosta (cf., the subcosta almost fully haired except bare near the apex in *S.* (*G.*) *siamense*), and the smaller number of male upper-eye facets in 14 horizontal rows (cf. 16 horizontal rows in *S.* (*G.*) *siamense*). In the females of both species, there are also differences in the relative length of the sensory vesicle against the 3rd maxillary palpal segment (i.e., 0.41 in this new species vs. 0.38 in *S.* (*G.*) *siamense*) and the fore basitarsus (i.e., ratio of the length against the greatest width 4.93−5.18 in this new species vs. 5.8 in *S.* (*G.*) *siamense*). This new species is distinguished from *S.* (*G.*) *azhari* sp. nov. and eight other known species of the *S. batoense* species-group recorded from Peninsular Malaysia as shown in the following keys.

3.3.7 Keys to separate 10 species of the *Simulium batoense* species group in Peninsular Malaysia. New species described in this study are shown in bold

Females*

1	Posterior surface of hind tibia whitish on basal 1/2.................................	*S. terengganuense*
	Posterior surface of hind tibia mostly darkened except basal tip............	2
2	Basal tooth of claw much shorter than half the length of claw; sensory vesicle 0.2 times the length of the third maxillary palpal segment..........	*S. tahanense*
	Basal tooth of claw about half the length of claw; sensory vesicle 0.3 or more times the length of the third maxillary palpal segment..................	3
3	Sensory vesicle 0.5 or more times the length of third maxillary palpal segment..	4
	Sensory vesicle 0.41 or less times the length of third maxillary palpal segment..	5
4	Mandible without teeth on outer margin..	*S. parahiyangum*
	Mandible with three teeth on outer margin at some distance from apex..	*S. decuplum*
5	Frons widely bare except several hairs along both lateral margin and above lower margin..	*S. sextuplum*
	Frons moderately covered with hairs except central portion bare and varying extent..	6
6	Frons:head ratio 1.0:7.1...	***S. azhari* sp. nov**
	Frons:head ratio 1.0:4.8−5.0...	7
7	Sensory vesicle 0.41 times the length of the third maxillary palpal segment..	***S. johorense* sp. nov**
	Sensory vesicle 0.3 times the length of the third maxillary palpal segment..	*S. duolongum*

*Females of *S. dentistylum* and S. sp. A are unknown.

Males*

1	Posterior surface of hind tibia whitish on basal 1/2...........................	*S. terengganuense*
	Posterior surface of hind tibia mostly darkened except basal tip.......	2
2	Abdominal segments 2, 6 and 7 each with pair of shiny patches dorsolaterally...	3

	Abdominal segments 2, 5, 6 and 7 (or and 8) each with pair of shiny parches dorsolaterally..	4
3	Fore coxa light to medium brown..	*S. decuplum***
	Fore coyellow..	*S. parahiyangum*
4	Style broad from base to middle or more...	***S. azhari* sp. nov**
	Style tapered from base to apex..	5
5	Upper-eye facets in 15 vertical columns and 15 horizontal rows..	*S. duolongum*
	Upper-eye facets in 11−14 vertical columns and 11−14 horizontal rows..	6
6	Upper-eye facets in 11 vertical columns and 11 horizontal rows..........	*S. tahanense***
	Upper-eye facets in 13 or 14 vertical columns and 14 horizontal rows..	7
7	Dorsal surface of abdomen dark except abdominal segment 2 and 3 yellow...	*S. dentistylum*
	Dorsal surface of abdomen dark except basal one-third of abdominal segment 2 yellow..	***S. johorense* sp. nov**

*Males of *S. sextuplum* and S. sp. A are unknown.
** Information about males of *S. decuplum* and
S. tahanense is based on recently collected
reared material from Peninsular Malaysia
(H. T., unpublished data).

Pupae

1	Gill with six filaments...	*S. sextuplum*
	Gill with eight or 10 filaments...	2
2	Gill with 10 short filaments..	*S. decuplum*
	Gill with eight short to long filaments..	3
3	All gill filaments (length 1.0 mm) much shorter than pupal body; antennal sheath with tubercles..	4
	All gill filaments (length of ventral paired filaments 1.7 mm) subequal to or longer than pupal body; anntenal sheath bare................	5
4	Gill filaments arranged as 3+3 +2 filaments from dorsal to ventral..	*S. parahiyangum*
	Gill filaments arranged as 2+1+3+2 filaments from dorsal to ventral..	*S. dentistylum*

5	Terminal hook with round, flat apex, appearing mushroom when viewed laterally..	*S. terengganuense*
	Terminal hook cone-like, tapered apically....................................	6
6	Dorsal and middle triplets sharing common stalk.........................	7
	Dorsal and middle triplets not sharing common stalk...................	8
7	One single and two paired filaments of dorsal group sharing common basal stalk..	S. sp. A
	One single and two paired filaments of dorsal group not sharing common basal stalk..	*S. tahanense*
8	Ventral pair of filaments >2.5 times the length of other filaments..	*S. duolongum*
	Ventral pair of filaments <2X the length of other filaments..........	9
9	Ventral pair of filaments slightly longer than middle triplets filaments..	***S. azhari* sp. nov**
	Ventral pair of filaments 1.5 times the length of middle triplets filaments..	***S. johorense* sp. nov**

Mature Larvae

1	Body length 5.3−6.0 mm..	*S. terengganuense*
	Body length 3.5−4.6 mm...	2
2	Abdomen markedly constricted between segments 4 and 5...........	3
	Abdomen not so constricted between segments 4 and 5...............	4
3	Abdominal segments 1−5 each with two pairs of dorsal protuberances..	*S. parahiyangum*
	Abdominal segments 1−5 without such dorsal protuberances.......	*S. dentistylum*
4	Postgenal cleft approaching or reaching posterior margin of hypostoma leaving no or very short postgenal bridge...........................	5
	Postgenal cleft not reaching posterior margin of hypostoma leaving distinct postgenal bridge..	6
5	Dorsal surface of abdominal segments 5−8 covered with dark spinous setae with 10−14 branches..	*S. decuplum*
	Dorsal surface of abdominal segments 5−8 covered with dark spinous setae with 2−4 branches..	***S. johorense* sp. nov**
6	Dorsal surface of abdominal segments 5−8 covered with colorless setae..	7
	Dorsal surface of abdominal segments 5−8 covered with dark spinous setae..	8

7	Thoracic segment 1 and abdominal segment 1 each with encircled with grayish black transverse band..	*S. tahanense*
	Thoracic segment 1 and abdominal segment 1 (together with segments 2−5) each encircled with reddish-brown transverse band (although disconnected ventromedially)..	***S. azhari*** **sp. nov**
8	Thoracic segment 1, abdominal segments 1, 4, 5, 7 and 8 each with sepia-colored tranverse band..	*S. sextuplum*
	Thoracic segment 1 and each abdominal segment with brown transverse band...	9
9	Rectal papillae with 4 or 5 secondary lobules per lobe..........................	S. sp. A
	Rectal papillae with 7−9 secondary lobules per lobe............................	*S. duolongum*

CHAPTER 4: NATIONWIDE DISTIBUTION PATTERN OF PREIMAGINAL BLACK FLIES OF THE GENUS *SIMULIUM* (DIPTERA: SIMULIIDAE) IN PENINSULAR MALAYSIA

4.1 INTRODUCTION

Adult black flies (Diptera: Simuliidae) are one of the most important groups of blood-sucking Diptera (Takaoka, 1983). The females of certain species, when they bite and draw blood, not only cause severe skin diseases to humans and animals, but also serve as a vector of filarial parasites (Ishii *et al*., 2008). The Simuliidae is widely distributed across all zoogeographical regions and the preimaginal black flies generally inhabit unpolluted running water (Takaoka, 1995; Currie & Adler, 2008). In fact, preimaginal black flies are important components of the stream ecosystem (Hamada *et al*., 2002; Currie & Adler, 2008; Pramual & Kuvangkadilok, 2009). They act as the keystone species in the ecology of running water, because they are usually present as a major component of stream macroinvertebrates (Malmqvist *et al*., 2004) and have an ability to filter dissolved organic matter and make it available in the food chain (Currie & Adler, 2008). Black flies are also important in the monitoring of freshwater contamination because larvae and pupae are susceptible to both organic and inorganic pollution (i.e., insecticides and fertilizers) (Currie & Adler, 2008).

Although black flies are well known for their medical and ecological importance, the significance of Malaysian black flies remains unexplored. By contrast, the spatial distribution of preimaginal black flies has been well-studied in many parts of the world, for example in the temperate regions (McCreadie & Adler, 1998, 2006; McCreadie *et al*., 2005), the tropical regions of South America (Grillet & Barrera, 1997; Hamada & McCreadie, 1999; Hamada *et al*., 2002; McCreadie *et al*., 2004) and the Oriental regions, notably Thailand (Pramual & Kuvangkadilok, 2009; Pramual & Wongpakam, 2010). These previous studies reported that the species richness and distribution of preimaginal black flies have been primarily associated with stream physicochemical conditions.

Ecological studies of black flies in the Oriental region, particularly South East Asia are still scarce except in Thailand, where the advances in simuliid morphotaxonomy (Takaoka & Choochote, 2004), cytotaxonomy (Phasuk *et al*., 2005; Kuvangkadilok *et al*., 2008; Tangkawanit *et al*., 2009; ; Pramual *et al*., 2012; Pramual & Adler, 2014) and phylogenetics (Pramual *et al*., 2011; Pramual & Adler, 2014) have allowed researchers to gain a better understanding on their ecology (Pramual & Kuvangkadilok, 2009; Pramual & Wongpakam, 2010). Many countries in Southeast Asia are experiencing extremely slow growth in the taxonomic knowledge of black flies, as a consequence, nothing is known about their ecology, biology and other related information. In Malaysia, morphotaxonomical study of black flies has been progressing well in recent years, (Takaoka & Davies, 1995; Takaoka, 2012) thus, allowing this study as the first attempt to obtain insight into the black fly ecology in association with their habitat characteristics. Exploring the fauna of black flies is a prerequisite for larval habitat control programs. Hence, the current study aims to investigate the nationwide distribution of black flies and their associated environmental factors, for the first time, across four geographical regions in Peninsular Malaysia.

4.2 MATERIALS AND METHODS

4.2.1 Study sites

This study was carried out at 180 stream points across Peninsular Malaysia, encompassing four geographical regions and 10 states namely East coast (Kelantan, Terengganu and Pahang), Northern (Perlis, Kedah, Penang and Perak), Central (Selangor) and Southern (Negeri Sembilan and Johore) (Figure 4.1). The sampling sites included forests, recreational areas, agricultural sites (oil palm and rubber plantations), and residential areas. Details on sampling sites and collections are presented in Table 4.1.

4.2.2 Preimaginal sampling and identification

Samplings were conducted from March 2013 until February 2015 (Table 4.1). There is no distinct wet or dry season throughout the year and rain is experienced every single month in Malaysia with an average 2000 to 3500 mm per year. Seasonal rainfall variation occurred in every state of Malaysia during the northeast and northwest monsoon seasons (Low *et al*., 2012). The sampling periods were free from its influence. All potential breeding sites for black flies were chosen based on accessibility and the presence of flow (i.e., streams, waterfalls and ditches).

Each stream was sampled once from downstream to upstream (30 m), for approximately 1 hour, by two people. Larvae and pupae attached on aquatic substrates such as grasses, leaves and stems, twigs, plant roots and rocks were collected by hand using fine forceps. These sampling protocols could represent the species occurrence in a locality (McCreadie & Colbo, 1991; McCreadie *et al*., 2005). Pupae attached on similar substrates were individually kept alive in vials until emergence. The adults, together with their pupal exuviae and cocoons were preserved in 80% ethanol for identification at the subgenus, species-group or species level. The methods of collection and identification followed those of Takaoka (2003) and Adler *et al*. (2004).

4.2.3 Physicochemical measurement

The following stream physicochemical parameters were measured at the time of each collection: width (m), depth (m), velocity (m/s) (one to three measurements along the collection path), temperature (°C), acidity (pH), electric conductivity (ms) and dissolved oxygen (mg/l). The values of pH, temperature, electric conductivity and dissolved oxygen were taken using a portable multi probe parameter (Hanna HI 9828). Meter tape and steel ruler were used to measure stream width and depth, respectively, while a cork and a timer watch were used to measure stream velocity; the time taken for a cork to move one meter in distance. Velocity, depth and width measurements were used to estimate discharge (McCreadie *et al*., 2006). The physicochemical measurements protocols including those for major streambed particles, riparian vegetation, and canopy cover followed those of McCreadie *et al*. (2006). For each study site, the latitude and longitudinal co-ordinates were taken and recorded using a hand held Global positioning system (GPS) instrument (Garmin International Inc., Olathe, KS).

4.2.4 Data analyses

Frequency of occurrence (FO) and local occurrence (LO) were designated in percentages (Table 4.2), FO was calculated by the total number of a species occurrence, divided by the total number of collections (n=180) (Hamada et al., 2002). Local occurrence (LO) was calculated by the number of states from a species was taken divided by total number of states sampled (n=10). A rarefied species accumulation curve of individuals was created for all samples to determine if species in the site were adequately sampled (Brühl *et al*., 1998). The expected richness (First Order Jackknife and Chao estimates) was obtained to predict the possible number of species occurring in all fixed-stream sites. Species diversity estimations were calculated to determine the efficiency of the sampling by dividing the number of actual species collected by the number of estimated species (Brühl, 2001). The presence or absence of a species was expressed on a binary scale (0 = absent, 1 = present), as in previous studies (Hamada & McCreadie, 1999; McCreadie *et al*., 2004 and Pramual & Kuvangkadilok, 2009). Because stream variables are inter-correlated, Principal Components Analysis (PCA) was used to reduce the number of variables into groups of independent components. Principal Components (PCs) with eigenvalues greater than 1.0 were retained as variables. To interpret the PCs, Spearman's rank correlations were used to detect the relationship between principal components and stream variables using a significance level of $P<0.001$. Forward logistic regression analysis was used to examine the relationships between spatial distribution and the PCs. Only species that occurred at more than 10% of the sites were considered in regression analyses (Hamada *et al*., 2002) because those present at a lower frequency have resulted in the lack of statistical power (large number of zero values were observed) (McCreadie *et al*., 2005). Linear regression was used to test the relationship between species richness (i.e., number of species in each sampling site) and the stream variables of the sampling sites (i.e. PC scores). All collections (180 stream points) were subjected to PCA, and the PC scores were used for regression analysis. Species Diversity and Richness (SDR) version 4 (Seaby & Henderson, 2006), the SPSS statistical package, version 16.0, Chicago, IL, were employed for diversity and statistical analyses respectively.

Table 4.1: Local and regional richness of black flies in Peninsular Malaysia

Region	GPS coordinates	Collecting date	Total collections	Regional richness	Mean local richness (±SE)
Northern					
Perak			*21*	*23*	*3.95±0.39*
Batang Padang	04.24−04.58°N 101.71−101.72°E	February 2015	4	16	6.25±0.48
Bukit Larut	04.86−04.87°N 100.76−100.89°E	February 2015	2	4	2.00±1.00
Pulau Pangkor	04.24−04.25°N 100.56−100.57°E	February 2015	2	3	2.00±0.00
Lenggong	05.17−05.18°N 100.93−101.10°E	February 2015	4	8	3.50±0.29
Kinta	04.35−04.71°N 100.07−101.24°E	February 2015	6	14	3.40±0.55
Kuala Kangsar	04.64−04.83°N 100.85−100.87°E	February 2015	3	9	4.33±0.88
Kedah			*30*	*20*	*2.57±0.23*
Langkawi	06.37−06.42°N 099.67−099.87°E	March 2013	17	10	2.06±0.22
Kulim	05.40−05.41°N 101.76−101.78°E	March 2013	6	8	2.80±0.49
Kuala Muda	05.41−05.81°N 100.44−101.43°E	March 2013	2	3	2.50±0.50
Yan	05.79°N 100.40°E	March 2013	1	6	-
Padang Terap	06.37°N 100.56°E	March 2013	1	4	-
Baling	05.72−05.99°N 100.81−100.90°E	March 2013	3	7	3.00±0.00
Perlis			*5*	*5*	*1.81±0.37*
Kaki Bukit	06.42-06.98°N100.02−100.11°E	March 2013	4	5	2.00±0.41
Mata Ayer	06.55°N 100.17°E	March 2013	1	1	-
Penang					
Balik Pulau	05.01−05.57°N 100.09−100.98°E	December 2014	4	5	2.00±0.58
Southern					
Johore			*16*	*16*	*2.81±0.27*
Ledang	02.07−02.36°N 102.53−102.64°E	March 2014	9	12	2.56±0.34
Kota Tinggi	02.37−02.83°N 102.61−103.87°E	March 2014	3	8	3.67±0.67
Pontian	01.37−01.83°N 103.51−103.87°E	March 2014	3	4	3.33±0.33
Mersing	01.81°N 103.87°E	March 2014	1	1	-
Negeri Sembilan					
Jelebu	02.83−03.02°N 102.03−102.04°E	January 2014	3	7	2.67±0.33
Central					
Selangor			*12*	*15*	*3.42±0.70*
Hulu Langat	03.21−03.89°N 101.41−102.31°E	April 2015	4	7	2.25±0.95
Hulu Selangor	03.33−03.67°N 101.68−101.75°E	April 2015	8	14	4.00±0.90
East coast					
Kelantan			*21*	*18*	*3.81±0.55*
Gua Musang	04.68−04.73°N 101.52−101.68°E	April 2013	7	7	2.00±0.49
Jeli	05.09−05.11°N 101.50−101.56°E	February 2015	4	11	5.75±1.60
Pasir Puteh	05.74°N 102.37°E	April 2013	1	5	-
Kuala Krai	05.31°N 102.29°E	April 2013	1	4	-
Tanah Merah	05.12−05.14°N 101.51−101.57°E	February 2015	8	12	4.25±0.88
Terengganu			*12*	*13*	*2.83±0.32*
Dungun	04.54−04.59°N 102.94−102.96°E	August 2013	7	8	2.86±0.50
Hulu Terengganu	04.77−04.97°N 102.75−102.84°E	August 2013	5	8	2.80±0.34
Pahang			*56*	*37*	*2.67±0.22*
Jerantut	03.02−04.93°N 102.12−102.89°E	May & April 2014	30	19	2.67±0.34
Lipis-Merapoh	04.40−04.65°N 102.14−102.40°E	September 2013	19	11	2.53±0.28
Raub	03.45−03.72°N 101.71−101.72°E	April 2015	4	13	3.75±1.49
Cameron Highland	04.40−04.58°N 101.34−101.38°E	September 2014	3	11	3.00±1.00

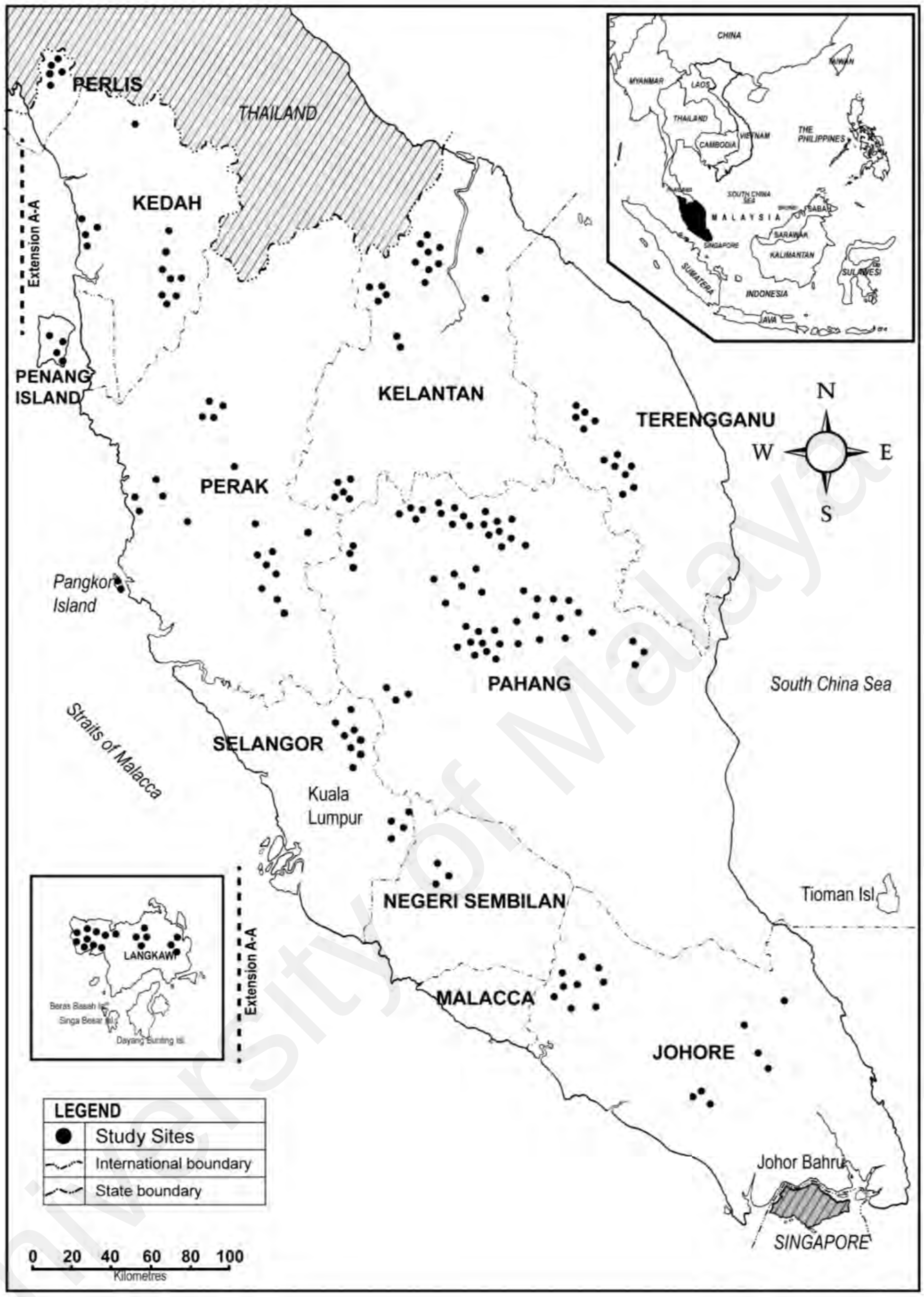

Figure 4.1: Map showing the location of sampling points in Peninsular Malaysia, small maps showing the Southeast Asian countries (top right) and Langkawi Island in the state of Kedah (bottom left). Details of all locations are presented in Appendix A.

4.3 RESULT

4.3.1 Black fly species composition

Forty-seven species were found from 180 sampled streams throughout Peninsular Malaysia (Table 4.2). At the subgeneric level, *Gomphostilbia* (31 species) was the largest subgenus in this study, followed by *Simulium* s. str. (12 species) and *Nevermannia* (4 species). The current study also successfully revealed 16 out of the 18 species-groups recorded in Malaysia. The *Simulium batoense* species-group (9 species) was the most abundant, followed by the *S. asakoae* species-group (7 species), the *S. ceylonicum* species-group and the *S. epistum* species-group (4 species each). Other species-groups were represented by one to three species.

Frequency of occurrence is shown Table 4.2. The predominant species were *S. trangense* (36.7%) and *S. angulistylum* (33.3%). Relatively common species were *S. cheongi* (29.4%), *S. tani* (25.6%), *S. vanluni* (16.2%), *S. sheilae* (14.5%) and *S. bishopi* (10.6%). Other species were collected at frequencies lower than 10%. Based on the current results, 85.1% (40 species) of total collected species had frequency of occurrence less than 10% and were considered as rare. Of these, 12 species were collected only once (0.6%).

Species richness and estimated richness are presented in Table 4.1 and Table 4.3 respectively. The maximum number of black fly species collected in a single stream for all samples (180 sampling points) was 10 and minimum was one with the mean number per stream for the total sample sites was 3.0 ± 0.1 (SE). The total estimated species richness ranged between 52 to 58, which yielded more than 80% sampling efficiency. Species accumulation curve is shown in Figure 4.2. Species reaching asymptote after approximately 105 sampling were performed.

Diversity indices are presented in Figure 4.3. Majority of surveyed streams (100 streams or 55.6%) had diversity values ranged from 0.20 to 0.59. A total of 11 streams (6.1%) showed highest diversity index ranging from 0.60 to 0.77. In contrast, dominance index was highest in 48 streams (or 26.7%) where each stream represented by a single species.

Table 4.2: Frequency of occurrence (FO) and local occurrence (LO) for 47 black fly species of the genus *Simulium* recorded from 180 sampling points across Peninsular Malaysia.

Species	% FO	% LO[#]
S. (*Gomphostilbia*) *adleri* Jitklang & Kuvangkadilok, 2008	0.6	10
S. (*Gomphostilbia*) *angulistylum* Takaoka & Davies, 1995	33.3	70
S. (*Gomphostilbia*) *asakoae* Takaoka & Davies, 1995	1.1	20
S. (*Gomphostilbia*) *azhari* Takaoka, Sofian-Azirun & Ya'cob, 2014	0.6	10
S. (*Gomphostilbia*) *aziruni* Takaoka, Hashim & Chen, 2012	0.6	10
S. (*Gomphostilbia*) *bishopi* Takaoka & Davies, 1995	10.6	60
S. (*Gomphostilbia*) *brinchangense* Takaoka, Sofian-Azirun & Ya'cob, 2014	0.6	10
S. (*Gomphostilbia*) *burtoni* Takaoka & Davies, 1995	1.7	20
S. (*Gomphostilbia*) *charlesi* Takaoka, 2008	1.1	10
S. (*Gomphostilbia*) *cheongi* Takaoka & Davies, 1995	29.4	70
S. (*Gomphostilbia*) *decuplum* Takaoka & Davies, 1995	6.7	40
S. (*Gomphostilbia*) *duolongum* Takaoka & Davies, 1995	5	60
S. (*Gomphostilbia*) *gombakense* Takaoka & Davies, 1995	6.1	40
S. (*Gomphostilbia*) *izuae* Takaoka, Sofian-Azirun & Hashim 2013	0.6	10
S. (*Gomphostilbia*) *jerantutense* Takaoka, Sofian-Azirun & Ya'cob, 2014	1.1	10
S. (*Gomphostilbia*) *johorense* Takaoka, Sofian-Azirun & Ya'cob, 2014	0.6	10
S. (*Gomphostilbia*) *kisapense* Takaoka, Sofian-Azirun & Ya'cob, 2012	0.6	10
S. (*Gomphostilbia*) *leparense* Takaoka, Sofian-Azirun & Ya'cob, 2014	1.1	10
S. (*Gomphostilbia*) *longitruncum* Takaoka & Davies, 1995	1.1	10
S. (*Gomphostilbia*) *roslihashimi* Takaoka & Sofian-Azirun, 2011	7.8	70
S. (*Gomphostilbia*) *sheilae* Takaoka & Davies, 1995	14.4	90
S. (*Gomphostilbia*) *sofiani* Takaoka & Hashim, 2011	1.7	30
S. (*Gomphostilbia*) sp. *(nr. parahiyangum)**	9.4	50
S. (*Gomphostilbia*) *tahanense* Takaoka & Davies, 1995	8.9	30
S. (*Gomphostilbia*) *tanahrataense* Takaoka, Sofian-Azirun & Hashim 2014	0.6	10
S. (*Gomphostilbia*) *tekamense* Takaoka, Sofian-Azirun & Ya'cob, 2014	1.1	10
S. (*Gomphostilbia*) *terengganuense* Takaoka, Sofian-Azirun & Ya'cob, 2012	2.2	10
S. (*Gomphostilbia*) *trangense* Jitklang et al. 2008	36.7	90
S. (*Gomphostilbia*) *whartoni* Takaoka & Davies, 1995	8.9	70
S. (*Gomphostilbia*) *langkawiense* Takaoka, Sofian-Azirun & Ya'cob, 2013	2.8	10
S. (*Gomphostilbia*) *lurauense* Takaoka, Sofian-Azirun & Hashim 2013	3.3	40
S. (*Gomphostilbia*) sp. *(nr. pegalanense)**	7.2	50
S. (*Nevermannia*) *caudisclerum* Takaoka & Davies, 1995	0.6	10
S. (*Nevermannia*) *kurtaki* Takaoka & Davies, 1995	0.6	10
S. (*Nevermannia*) *ledangense* Ya'cob, Takaoka & Sofian-Azirun, 2014	0.6	10
S. (*Nevermannia*) sp. (*nr. feuerborni*)*	1.7	20
S. (*Simulium*) *hackeri* Edwards, 1928	1.1	10
S. (*Simulium*) *yongi* Takaoka & Davies, 1997	0.6	10
S. (*Simulium*) *aureohirtum* Brunetti, 1911	6.7	50
S. (*Simulium*) *grossifilum* Takaoka & Davies, 1995	2.2	30
S. (*Simulium*) *hirtinervis* Edwards, 1928	3.9	40
S. (*Simulium*) *jeffreyi* Takaoka & Davies, 1995	4.4	40
S. (*Simulium*) *malayense* Takaoka & Davies, 1995	3.3	40
S. (*Simulium*) *vanluni* Ya'cob, Takaoka & Sofian-Azirun, 2017	16.1	100
S. (*Simulium*) sp. *(nr. grisescens)**	7.8	30
S. (*Simulium*) *brevipar* Takaoka & Davies, 1995	7.2	40
S. (*Simulium*) *tani* Takaoka & Davies, 1995	25.6	90

*undetermined species; FO was calculated by the total number of a species occurrence, divided by the total number of collections (n=180); LO calculated by the number of states from a species was taken divided by total number of states sampled (n=10).

Table 4.3: Actual and estimated species richness of black flies in Peninsular Malaysia.

Estimators	Actual species observed	Estimated richness	% of sampling
	47	-	-
Chao estimate		57.0 ± 4.17	82.5
Bootstrap		52.5 ± 4.90	89.5
First order Jackknife		58.0 ± 4.13	81.0

*Sampling efficiency was calculated by dividing the number of actual species collected by the number of estimated species (Brühl, 2001).

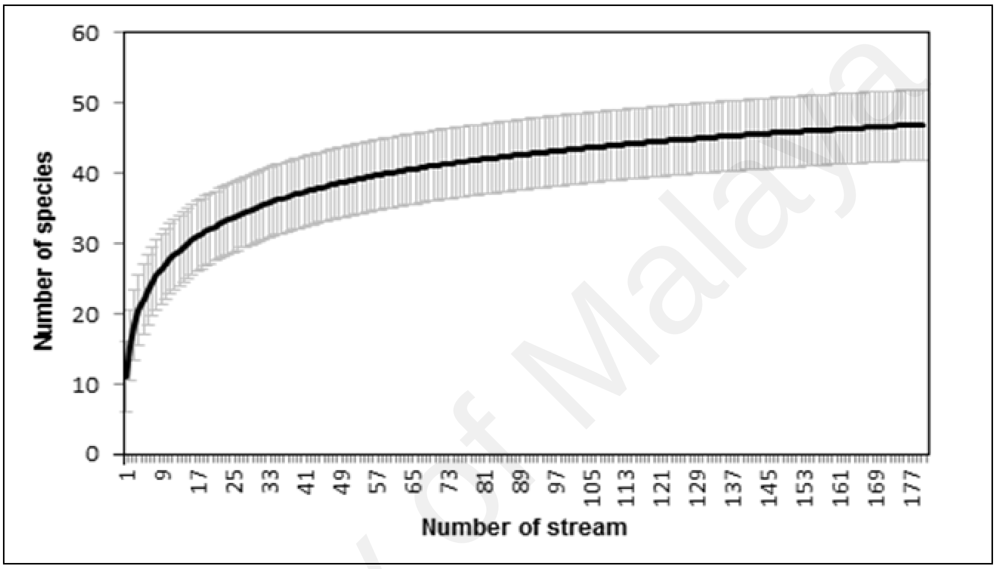

Figure 4.2: Accumulation curve with error bars for overall 180 collections across Peninsular Malaysia.

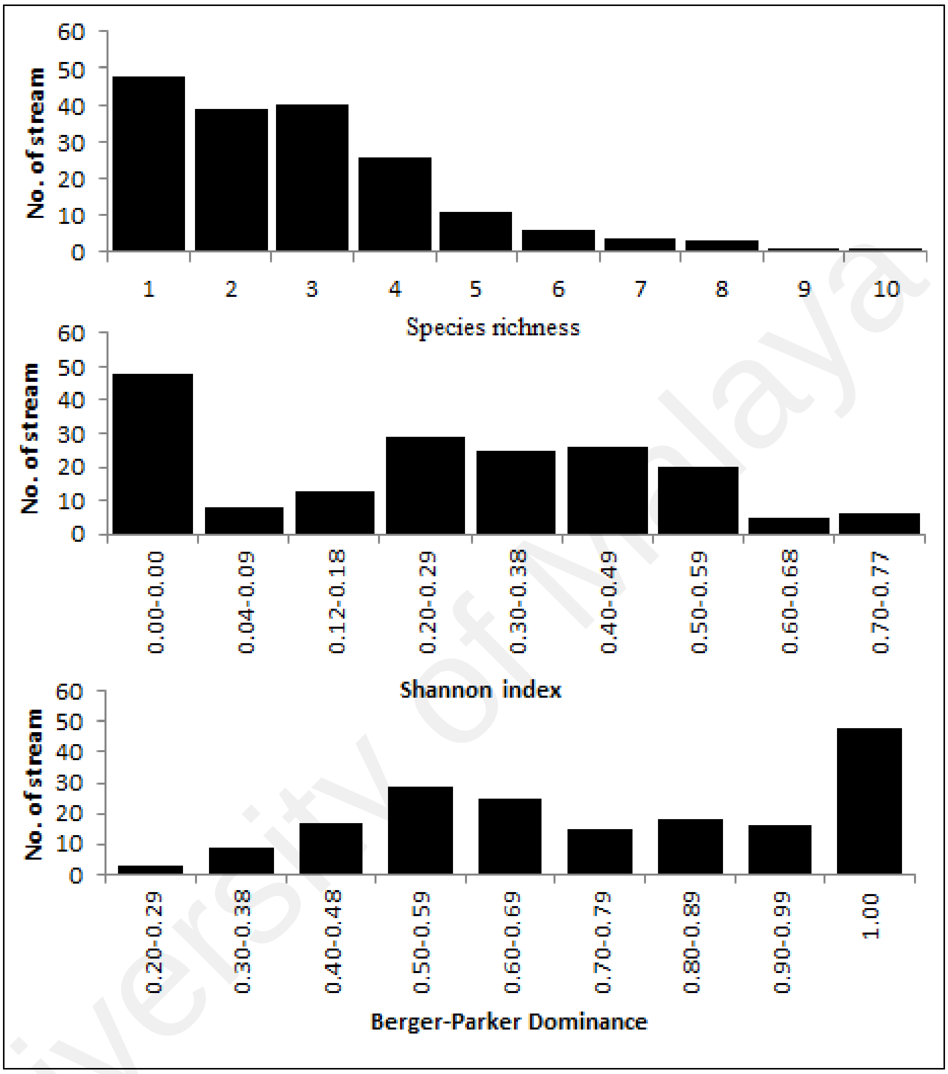

Figure 4.3: Diversity indices for black fly species from 180 sampling points across Peninsular Malaysia.

4.3.2 Spatial distribution pattern

Simulated and observed values of the C-score are presented in Figure 4.4. Based on current results, there was pattern of co-occurrence of species in the streams (C-score $_{(observed)}$ = 77.84, mean of simulated C-score = 76.66, variance of simulated indices = 0.33; $P_{(observed \leq expected)} = 0.00$, $P_{(observed \geq expected)} = 1.00$), suggesting that the assemblage of black flies is deterministic pattern or not distributed at random.

Local occurrence (LO) for each black fly species is presented in Figure 4.5 until Figure 4.22. *Simulium vanluni* was found in all states (100%), followed by *S. trangense*, *S. tani* and *S. sheilae* each occurred in nine states (90%), *S. angulistylum*, *S. cheongi* and *S. whartoni* each were occurred in seven states (70%) and *S. bishopi* in six states (60%). In contrast, eight black fly species (*S. hackeri, S. leparense*, *S. caudisclerum, S. adleri, S. kurtaki, S. asakoae, S. tanahrataense* and *S. brinchangense*) were collected only in the state of Pahang. *Simulium ledangense* and *S. johorense* were collected only in the state of Johor while, *S. azhari* and *S. kisapense* were collected from the state of Kedah. Details for locations and ecological conditions of all stream points (n=180) in Peninsular Malaysia are presented in Appendix A.

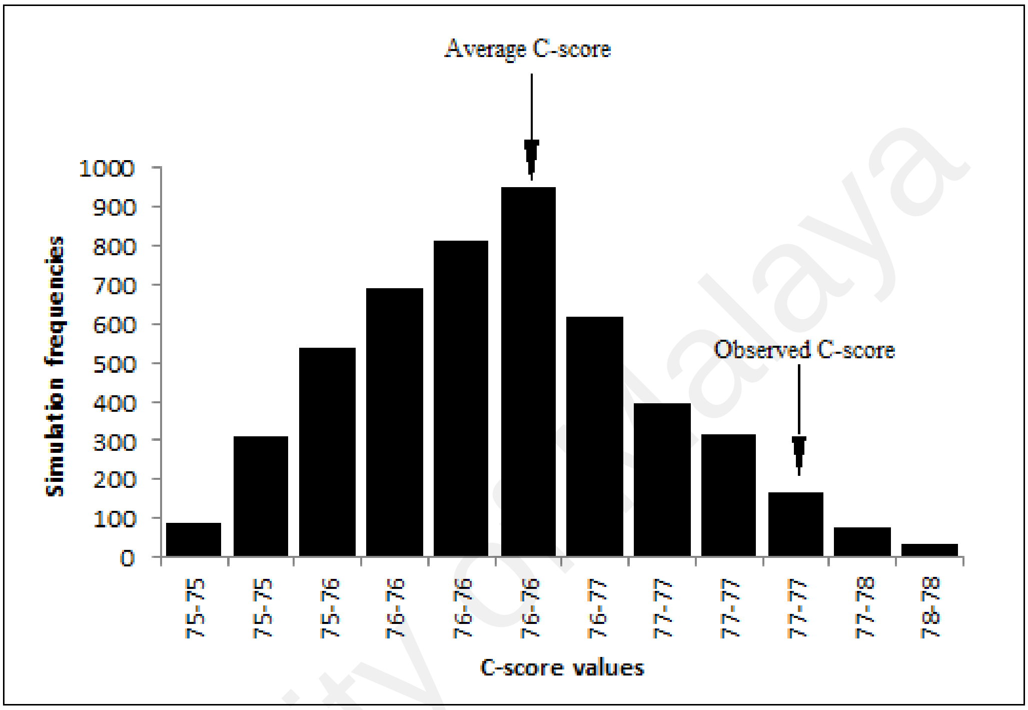

Figure 4.4: Simulated and observed values of C-score in the analysis of co-occurrence of black fly species collected in streams in Peninsular Malaysia.

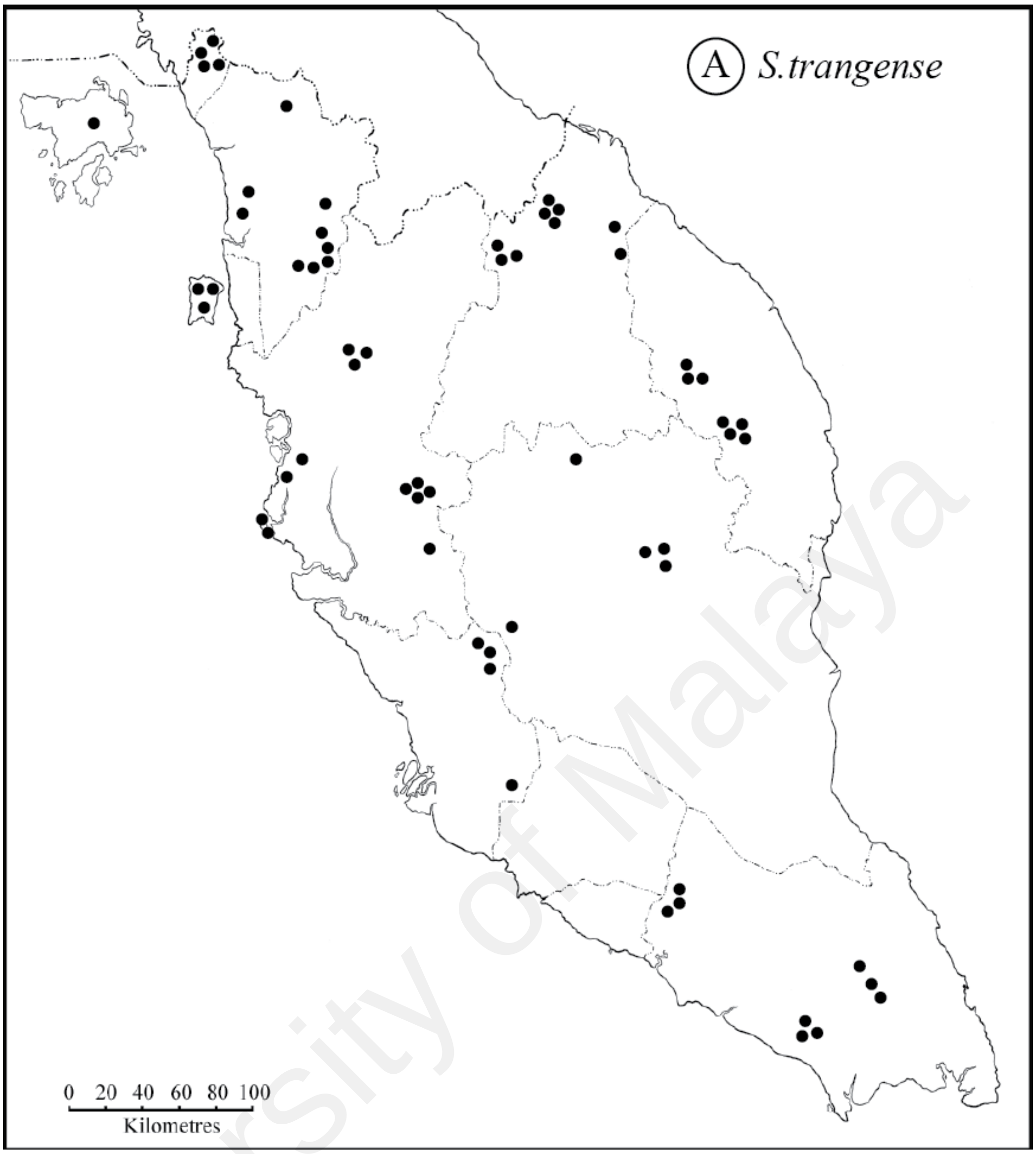

Figure 4.5: (**A**) ***S. trangense*** (PAHANG: Kota Gelanggi = KG1, Lepar/Tekam = LT1, Jerantut = JT4, Mount Tahan = MT13, Fraser Hill = FH1; TERENGGANU: Pasir Raja = PR2, PR4, PR6, Tasik Kenyir = TK2, TK4, TK5; KELANTAN: Jeram Pasu = JP3, Lata Rek = LR5, Batu Gajah = BG1, BG2, BG3, BG10, BG11, Jeli = JL6, JL7, JL8, JL9; KEDAH: Sungai Sedim = SS5, SS4, SS8, Langkawi = LK8, Gunung Jerai = GJ15, GJ16, Yan Besar = YB18, Puncak Janing = PJ1, Weng = WG2, Lata Bayu = LB3, Bukit Hijau = BH4; PERAK: Tapah = TP2, Pulau Pangkor = PP1, PP2, Bukit Larut = BL6, Lenggong = LG7, LG9, LG10, Batu Hampar = BH12, Ulu Chepor = UC14, UC15, UC16, Gopeng = GP18; PERLIS: Mata Ayer = BM1, Gua Kelam = GK2, State Park = SP3, SP4; P. PINANG: Youth Park = YP1, Botanical Garden = BG2, Balik Pulau = BP4; JOHOR: Gunung Ledang = GL4, GL5, GL10, Kota Tinggi = KT11, KT12, Gunung Panti = GP13, Gunung Pulai = GP14, GP15, GP16; SELANGOR: Hulu Langat = HL5, Ulu Yam = UY1, UY2, UY3).

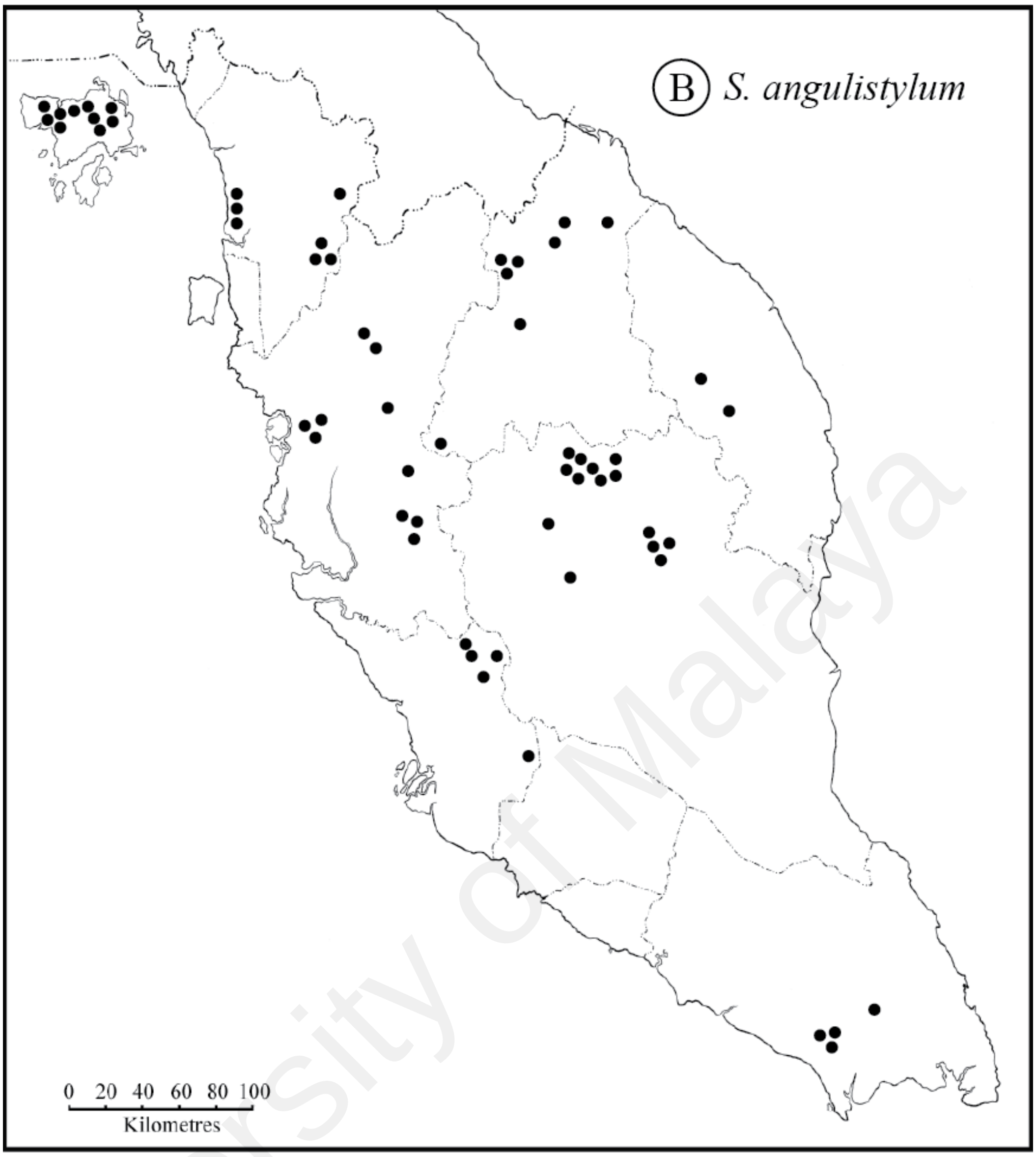

Figure 4.6: (**B**) ***S. angulistylum*** (PAHANG: Kota Gelanggi = KG1, KG2, Lepar/Tekam = LT3, LT4, Mount Tahan = MT8, MT9, MT10, MT11, MT12, MT13, MT15, MT17, Gunung Ais = GA2, Raub = RBS17; TERENGGANU: Pasir Raja = PR6, Tasik Kenyir = TK5; KELANTAN: Gunung Stong = GS1, Jeram Pasu = JP3, Lojing = LO2, Batu Gajah = BG4, BG11, Jeli = JL6, JL8, JL9; KEDAH: Langkawi = LK3, LK5, LK6, LK10, LK12, LK13, LK14, LK15, LK16, LK17, Sungai Sedim = SS2, SS7, SS5, Gunung Jerai = GJ15, GJ16, Yan = YB18, Weng = WG2, PERAK: Chenderiang = CD19, Gopeng = GP18, Jeliang = JG13, Batu Hampar = BH11, BH12, Lenggong = LG8, LG10, Bukit Larut = BL3, Tapah = TP2, TP4; JOHOR: Gunung Pulai = GP14, GP15, GP16, Gunung Panti = GP13; SELANGOR: Hulu Langat = HL5, Semangkok = SM6, Ulu Yam = UY1, UY2, UY3).

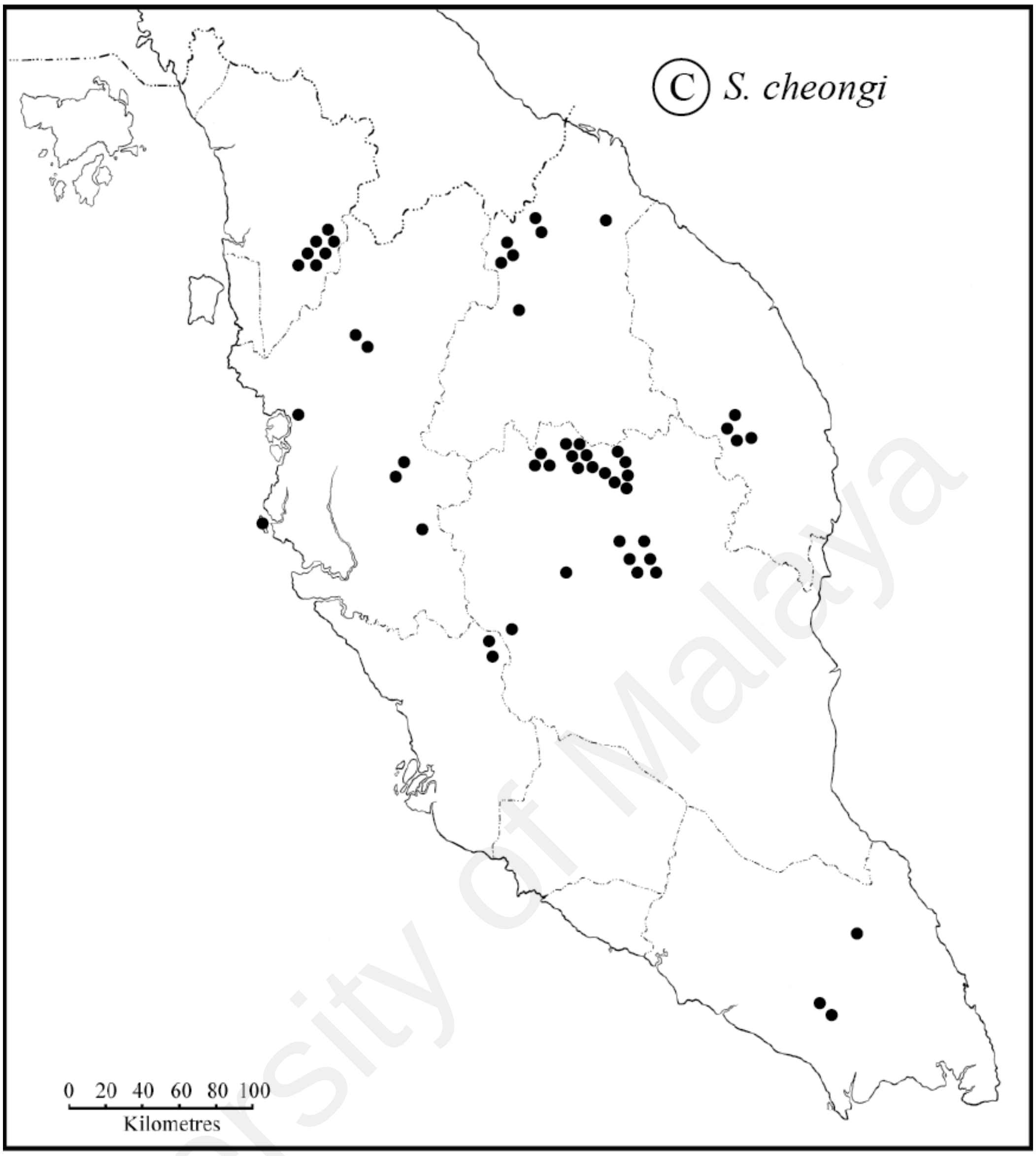

Figure 4.7: (**C**) ***S. cheongi*** (PAHANG: Kota Gelanggi = KG3, KG4, KG6, Lepar/Tekam = LT3, LT4, LT6, Mount Tahan = MT1, MT7, MT8, MT10, MT11, MT12, MT13, MT14, MT15, MT17, MT18, MT19, MT20, MT21, MT22, Gunung Ais = GA3, Fraser Hill = FH1; TERENGGANU: Pasir Raja = PR1, PR2, PR5, PR6; KELANTAN: Batu Gajah = BG10, BG11, Jeli = JL6, JL8, JL9, Jeram Pasu = JP3, Gunung Stong = GS1; KEDAH: Sungai Sedim = SS2, SS3, SS4, SS5, SS7, SS8, Bukit Hijau = BH4; PERAK: Chenderiang=CD19, Ulu Chepor = UC14, UC16, Batu Hampar = BH12, Lenggong = LG9, LG10, Pulau Pangkor = PP2; JOHOR: Gunung Pulai = GP16, GP15, Kota Tinggi = KT11; SELANGOR: Ulu Yam = UY2, UY3).

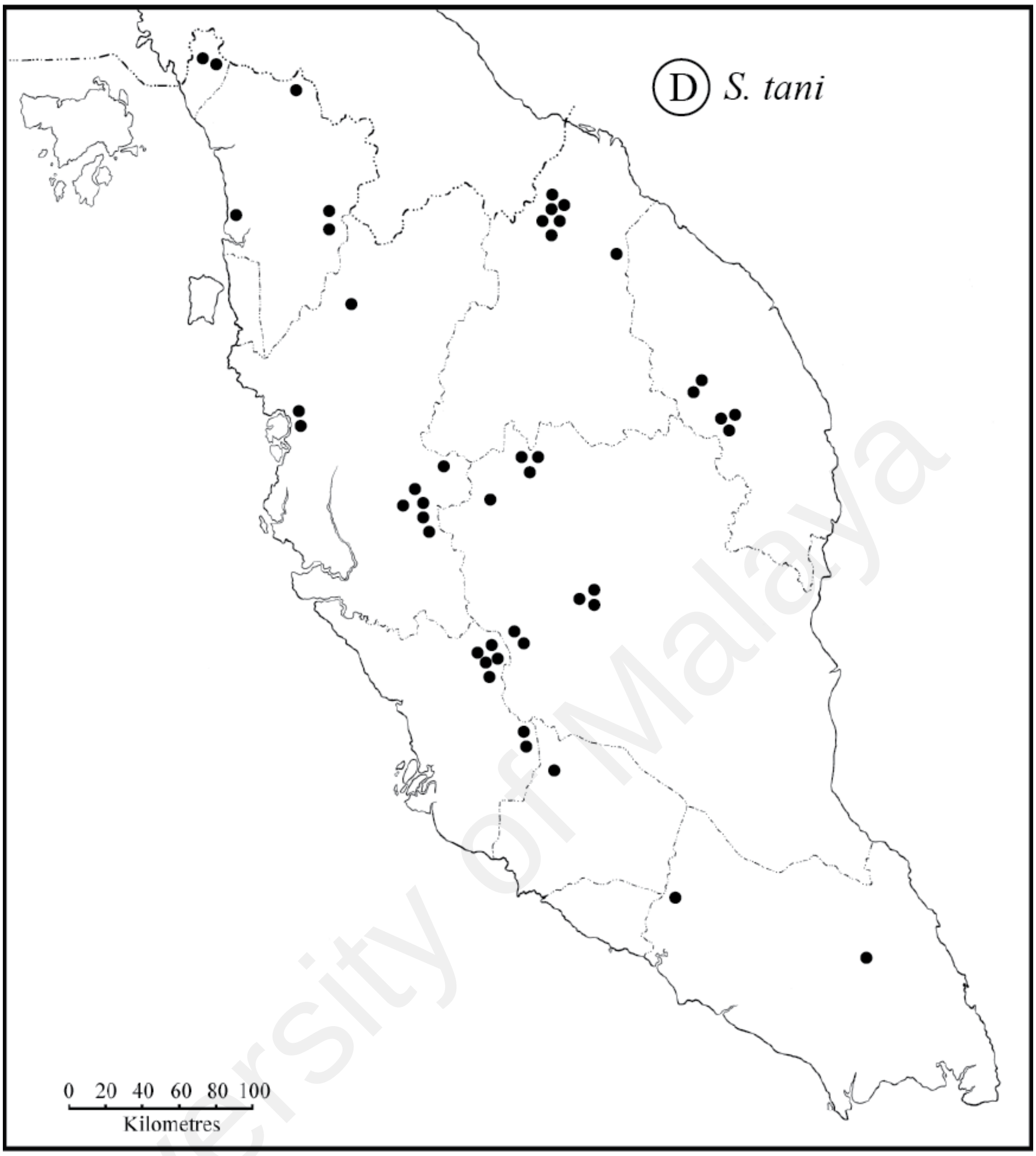

Figure 4.8: (**D**) ***S. tani*** (PAHANG: Cameron Highland = CH11, Fraser Hill = FH2, FH3, Mount Tahan = MT2, MT6, MT12, Jerantut = JT3, JT4, JT6; TERENGGANU: Pasir Raja = PR5, PR6, PR7, Tasik Kenyir = TK2, TK5; KELANTAN: Lata Rek = LR5, Batu Gajah = BG1, BG3, BG5, BG10, BG11, BG12; KEDAH: Sungai Sedim = SS7, Yan = YB18, Puncak Janing = PJ1, Lata Bayu = LB3; PERAK: Simpang Pulai = SP1, Tapah = TP2, TP3, TP4, Lenggong = LG8, Batu Hampar = BH11, BH12, Ulu Chepor = UC14, Chenderiang = CD19; PERLIS: State Park = SP3, SP4; JOHOR: Gunung Ledang = GL4, Kota Tinggi = KT12; N. SEMBILAN: Jelebu = JB3; SELANGOR: Hulu Langat = HL5, Ulu Yam = UY1, UY2, Sungai Sendat = SD5, Semangkok = SM8, SM9).

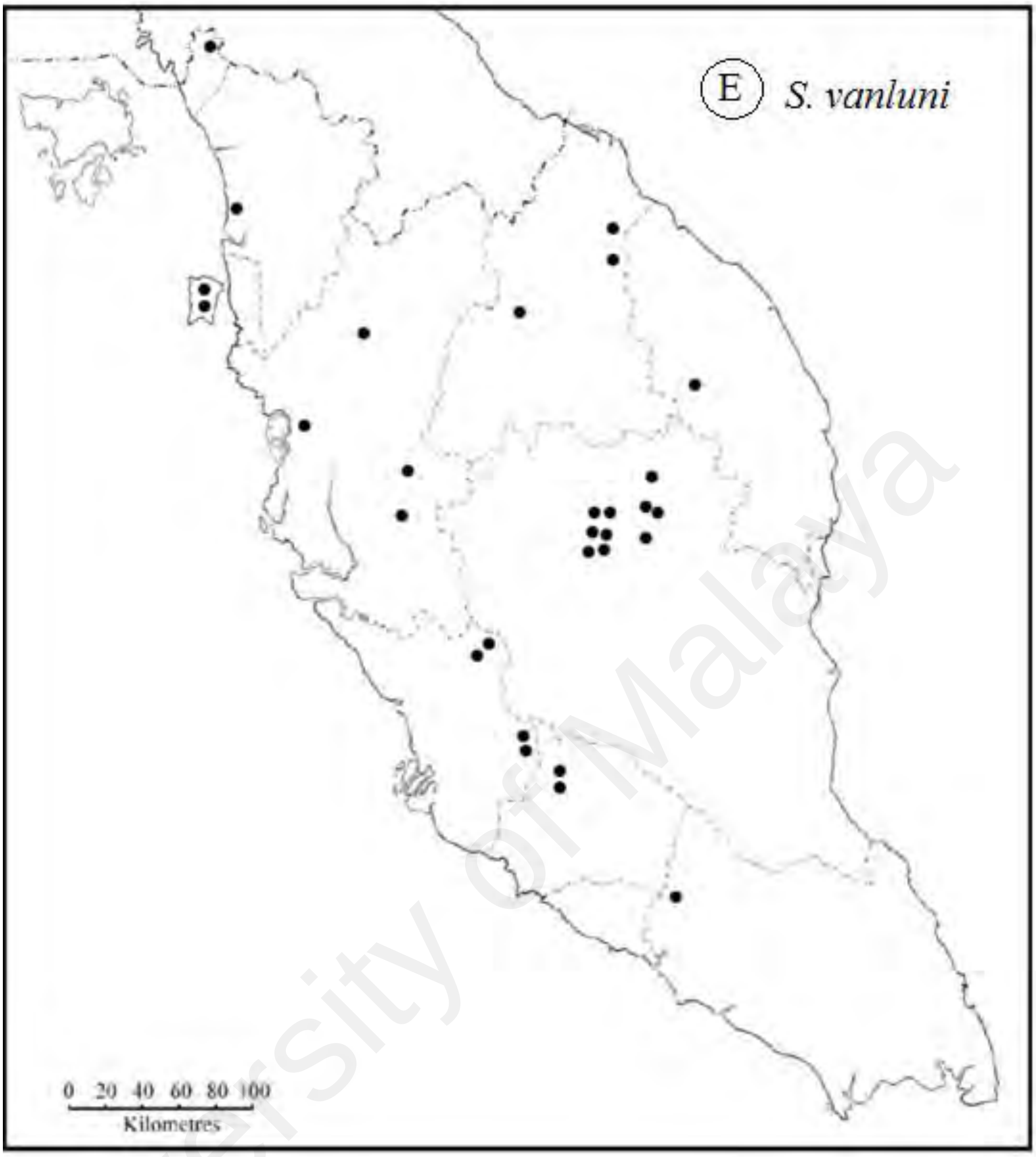

Figure 4.9: (**E**) ***S. vanluni*** (PAHANG: Kota Gelanggi = KG5, Lepar/Tekam = LT5, LT6, Jerantut = JT1, JT3, JT4, JT6, Mount Tahan = MT22, Gunung Ais = GA1, GA2; TERENGGANU: Tasik Kenyir = TK1, KELANTAN: Gunung Stong = GS1, Jeram Pasu = JP3, Lata Rek = LR5; KEDAH: Yan = YB18, PERAK: Tapah = TP3, Lenggong = LG7, Batu Hampar = BH11, Gopeng = GP17; PERLIS: Wang Kelian = WK5; P. PINANG: Sungai Ara = SA3, Balik Pulau = BP4; JOHOR: G. Ledang = GL2; N. SEMBILAN: Jelebu = JB2, JB3; SELANGOR: Hulu Langat = HL7, HL8, Ulu Yam = UY1, Sungai Sendat = SD5).

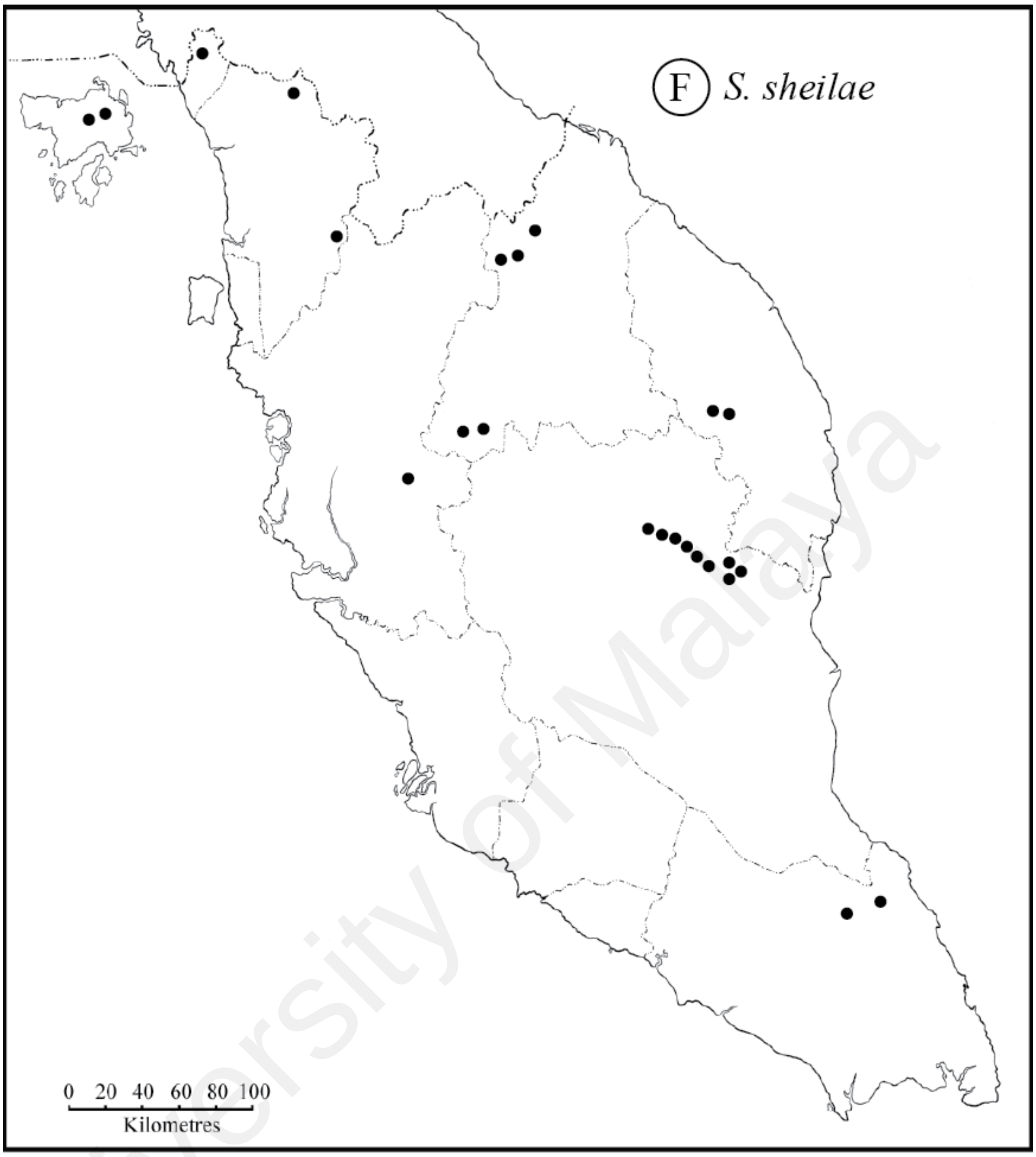

Figure 4.10: (**F**) ***S. sheilae*** (PAHANG: Kota Gelanggi = KG3, KG6, KG7, KG8, KG9, Lepar/Tekam = LT6, LT7, LT8, LT9; TERENGGANU: Pasir Raja=PR4, PR5; KELANTAN: Lojing = LO2, LO3, Jeli = JL6, JL8, Batu Gajah = BG10; KEDAH: Langkawi = LK9, LK17, Puncak Janing = PJ1, Bukit Hijau = BH4; PERAK: Ulu Chepor = UC15; PERLIS: Wang Kelian = WK5; JOHOR: Kota Tinggi = KT11, Mersing = MR17; N. SEMBILAN: Jelebu = JB2; SELANGOR: Ulu Yam = UY2).

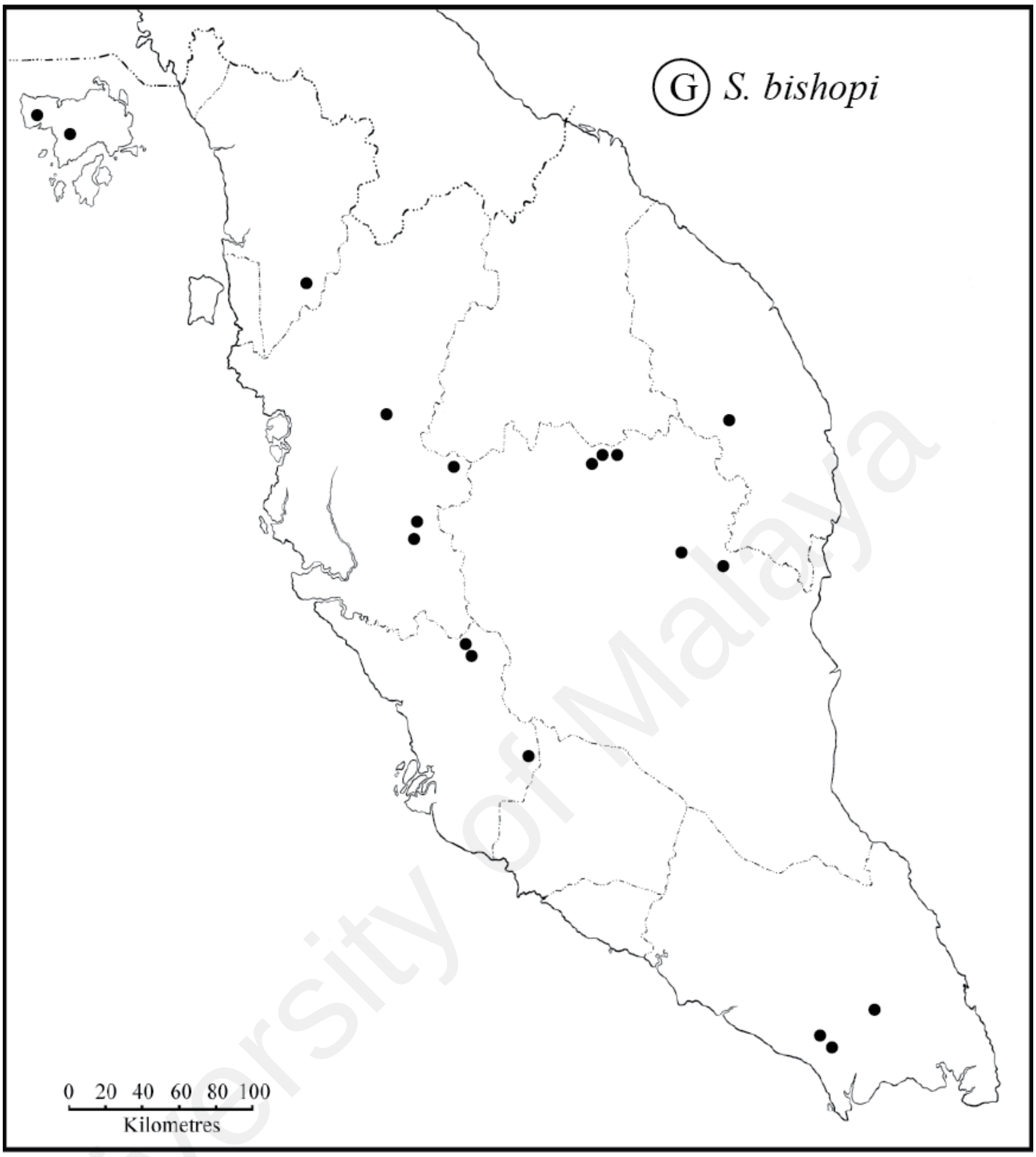

Figure 4.11: (**G**) ***S. bishopi*** (PAHANG: Kota Gelanggi = KG4, Lepar/Tekam = LT4, Mount Tahan = MT10, MT12; TERENGGANU: Pasir Raja = PR6; KEDAH: Langkawi = LK2, LK16, Sungai Sedim = SS7; PERAK: Simpang Pulai = SP1, Tapah = TP2, Jeliang = JG13, Chenderiang = CD19; JOHOR: Gunung Panti = GP13, Gunung Pulai = GP14, GP15; SELANGOR: Hulu Langat = HL9, Ulu Yam = UY1, Semangkok=SM9).

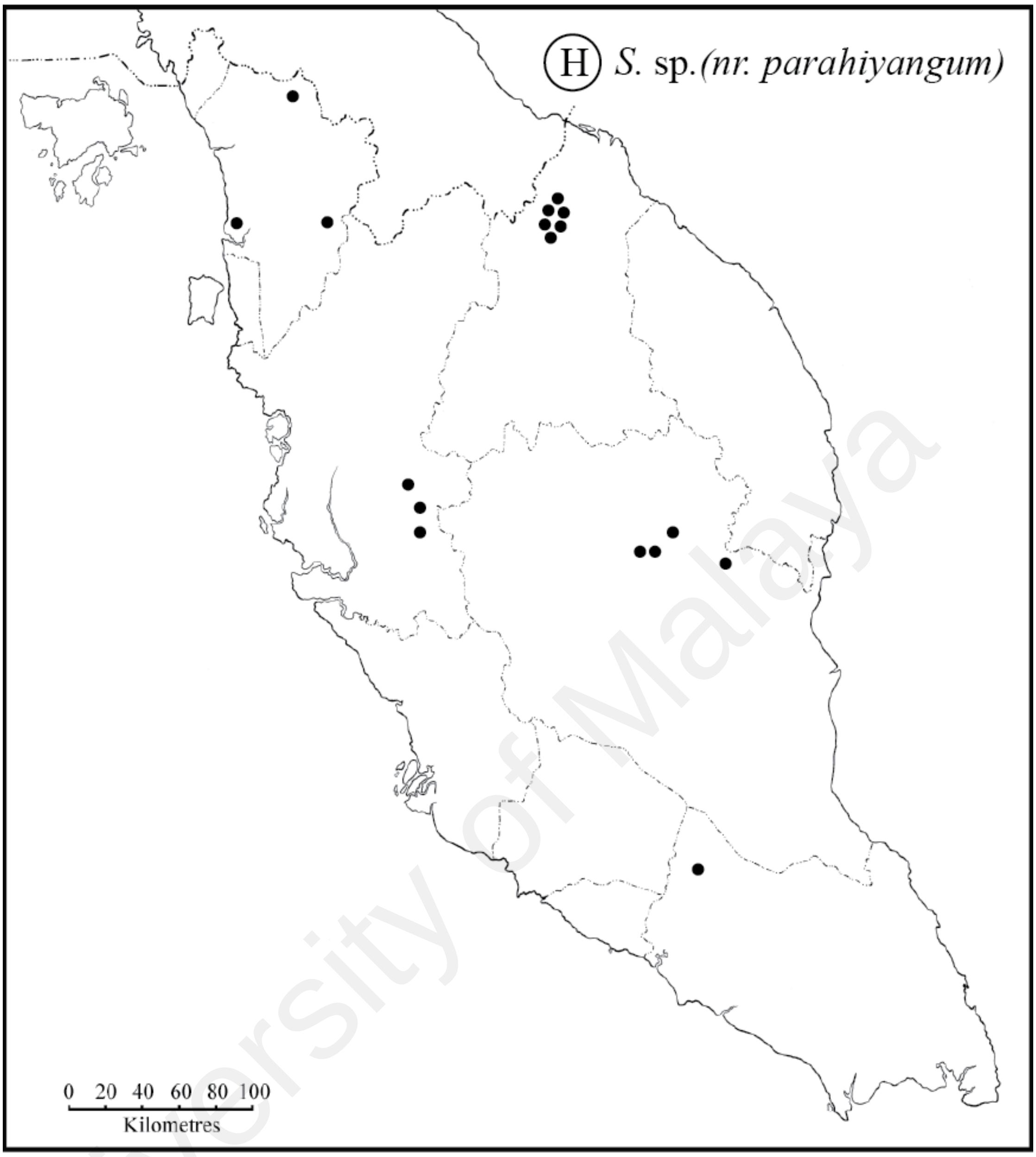

Figure 4.12: (**H**) ***S*. sp**. (**nr. *parahiyangum***) (PAHANG: Kota Gelanggi = 5, Lepar/Tekam = LT5, Jerantut = JT3, JT4; KELANTAN: Batu Gajah = BG1, BG3, BG5, BG10, BG11, BG12; KEDAH: Yan = YB18, Puncak Janing = PJ1, Lata Bayu = LB3; PERAK: Tapah = TP4, Ulu Chepor = UC14, Chenderiang = CD19; JOHOR: Gunung Ledang = GL10).

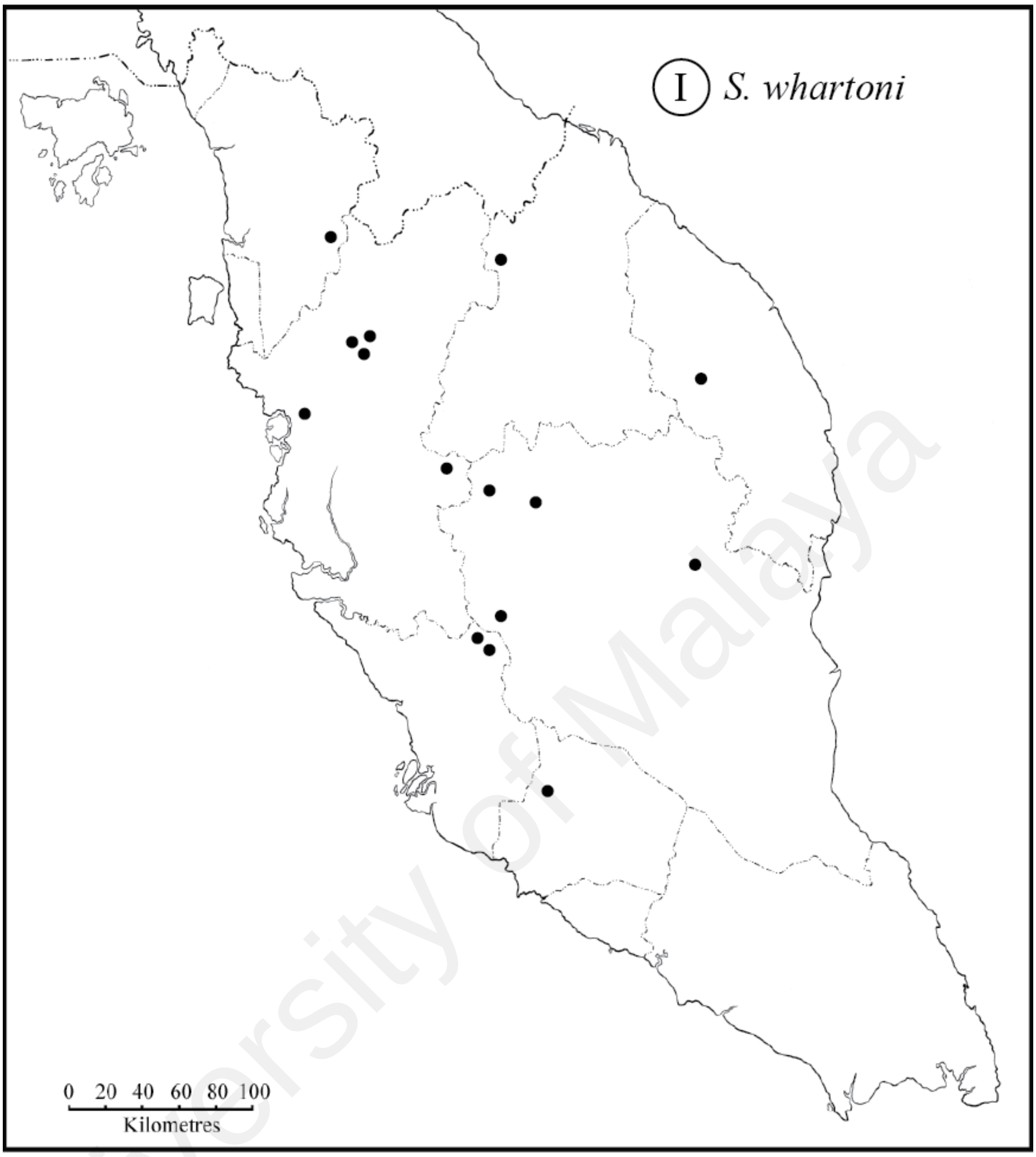

Figure 4.13: (**I**) ***S. whartoni*** (PAHANG: Kota Gelanggi = KG3, Fraser Hill = FH2, Cameron Highland = CHS11, Raub = RBS17; TERENGGANU: Tasik Kenyir = TK5; KELANTAN: Jeli = JL6; KEDAH: Sungai Sedim = SS8; PERAK: Pulau Pangkor = PP1, Bukit Larut = BL3, Lenggong = LG8, LG9, LG10, Simpang Pulai = SP1; N. SEMBILAN: Jelebu = JB1; SELANGOR: Ulu Yam = UY3, Semangkok=SM9.

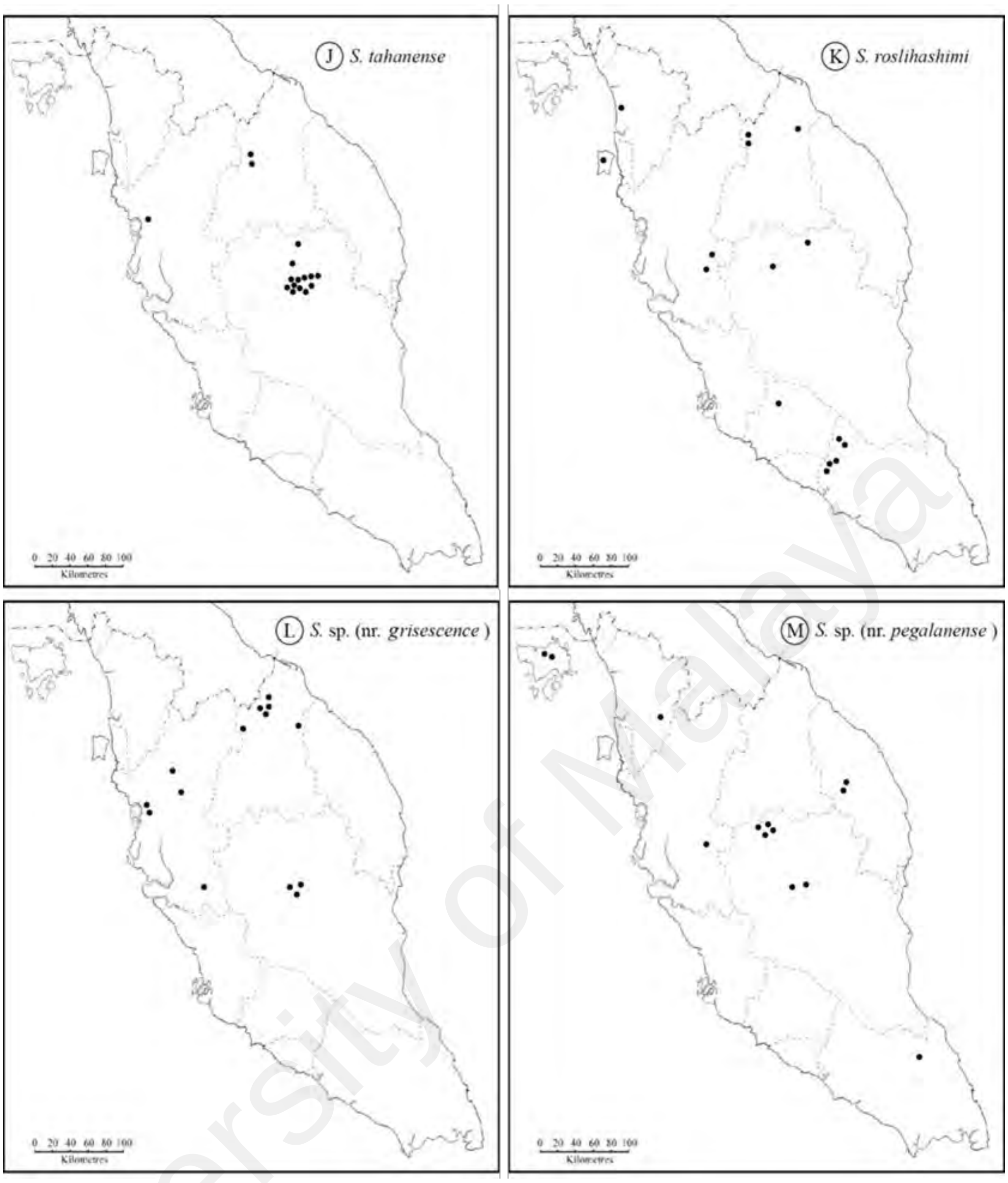

Figure 4.14: (**J**) ***S. tahanense*** (PAHANG: Kota Gelanggi = KG5, KG10, KG11, Lepar/Tekam = LT5, LT10, LT11, Jerantut = JT2, JT3, JT4, JT6, JT7, Mount Tahan = MT11, Gunung Ais = GA3; KELANTAN: Gunung Stong = GS1, GS2; PERAK: Batu Hampar = BH12). (**K**) ***S. roslihashimi*** (PAHANG: Mount Tahan = MT18, Raub = RBS17; KELANTAN: Jeram Pasu = JP3, Jeli = JL8, JL9; KEDAH: Gunung Jerai = GJ15; PERAK: Tapah = TP2, Gopeng = GP18; P. PINANG: Youth Park = YP1; JOHOR: Gunung Ledang = GL3, GL6, GL7, GL8, GL9; N. SEMBILAN: Jelebu = JB1). (**L**) ***S*. sp**. (**nr. *grisescens***) (PAHANG: Jerantut = JT3, JT4, JT5; KELANTAN: Lata Rek = LR5, Batu Gajah = BG1, BG10, BG11, BG12, Jeli = JL6, PERAK: Lenggong = LG7, Batu Hampar = BH11, BH12, Jeliang = JL13, Chenderiang = CD19). (**M**) ***S*. sp**. (**nr. *pegalanense***) (PAHANG: Kota Gelanggi = KG6, Lepar/Tekam = LT6, Mount Tahan = MT1, MT17, MT19, MT20; TERENGGANU: Tasik Kenyir = TK3, TK4; KEDAH: Langkawi = LK9, LK17, Sungai Sedim=SS8; PERAK: Ulu Chepor = UC16; JOHOR: Kota Tinggi = KT11).

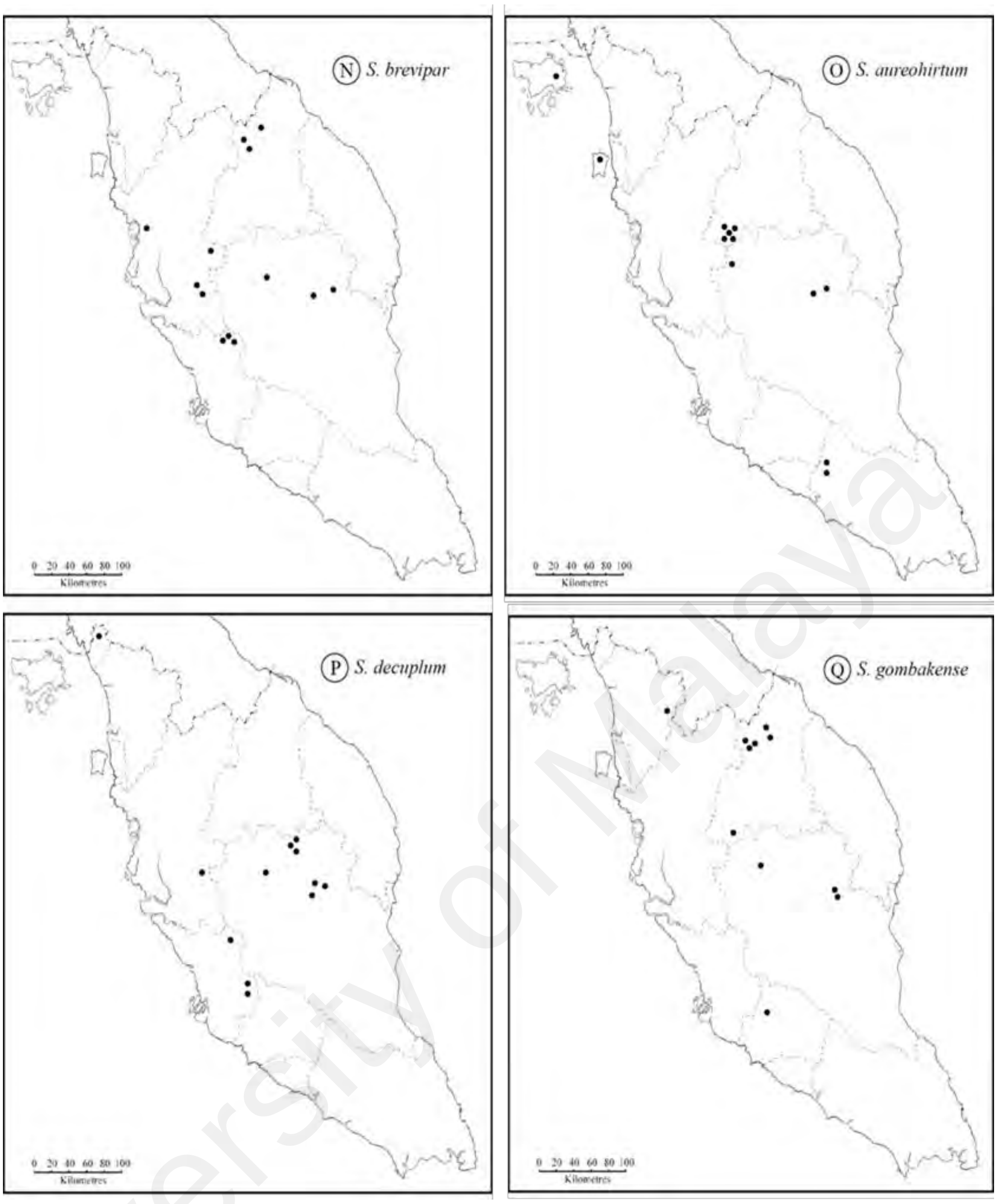

Figure 4.15: (**N**) ***S. brevipar*** (PAHANG: Kota Gelanggi = KG4, Lepar/Tekam = LT4, Raub = RBS17; KELANTAN: Batu Gajah = BG4, Jeli = JL6, JL8; PERAK: Simpang Pulai = SP1, Tapah = TP2, Bukit Larut = BL3, Chenderiang = CD19; SELANGOR: Ulu Yam = UY3, Semangkok = SM6, SM9). (**O**) ***S. aureohirtum*** (PAHANG: Kota Gelanggi = KG8, Lepar/Tekam = LT8, Cameron Highland = CH14; KELANTAN: Lojing = LO1, LO2, LO3, LO4, LO5; KEDAH: Langkawi = LK4, P. PINANG: Youth Park = YP1, JOHOR: Gunung Ledang = GL2, GL5). (**P**) ***S. decuplum*** (PAHANG: Kota Gelanggi = KG4, Lepar/Tekam = LT3, LT4, Mount Tahan = MT11, MT13, MT17; PERAK: Tapah = TP2, PERLIS = State Park = SP3, SELANGOR: Hulu Langat = HL9, HL5, Ulu Yam = UY3). (**Q**) ***S. gombakense*** (PAHANG: Kota Gelanggi = KG1, KG2, Raub = RBS17; KELANTAN: Lojing = LO3, Batu Gajah = BG4, BG10, Jeli = JL6, JL8, JL9; KEDAH: Weng = WG2; N. SEMBILAN: Jelebu = JB1).

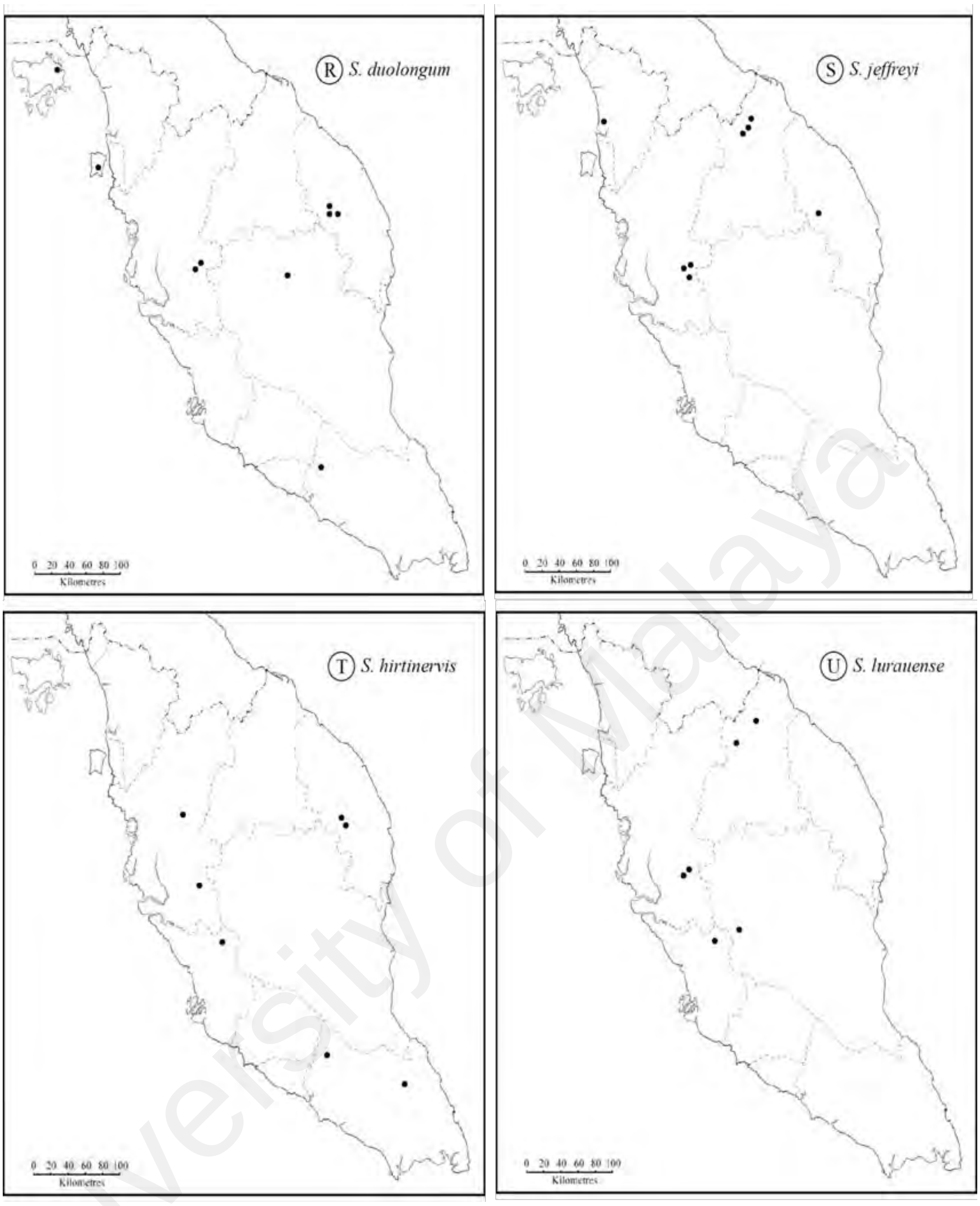

Figure 4.16: (**R**) ***S. duolongum*** (PAHANG: Gunung Ais = GA3; TERENGGANU: Tasik Kenyir = TK2, TK3, TK4; KEDAH: Langkawi = LK1; PERAK: Tapah = TP2, TP4; P. PINANG: Balik Pulau = BP4; JOHOR: Gunung Ledang = GL2.). (**S**) ***S. jeffreyi*** (TERENGGANU: Tasik Kenyir = TK1, KELANTAN: Batu Gajah = BT1, BG10, BG12; KEDAH: Yan = YB18; PERAK: Tapah = TP3, TP4, Chenderiang = CD19). (**T**) ***S. hirtinervis*** (TERENGGANU: Pasir Raja = PR5, PR7; PERAK: Jeliang = JG13, Chenderiang = CD19; JOHOR: Gunung Ledang = GL4, Kota Tinggi = KT12; SELANGOR: Ulu Yam = UY1.) (**U**) ***S. lurauense*** (PAHANG: Fraser Hill = FH1; KELANTAN: Jeli = JL9, Batu Gajah = BG4; PERAK: Tapah = TP3, TP4; SELANGOR: Semangkok = SM7).

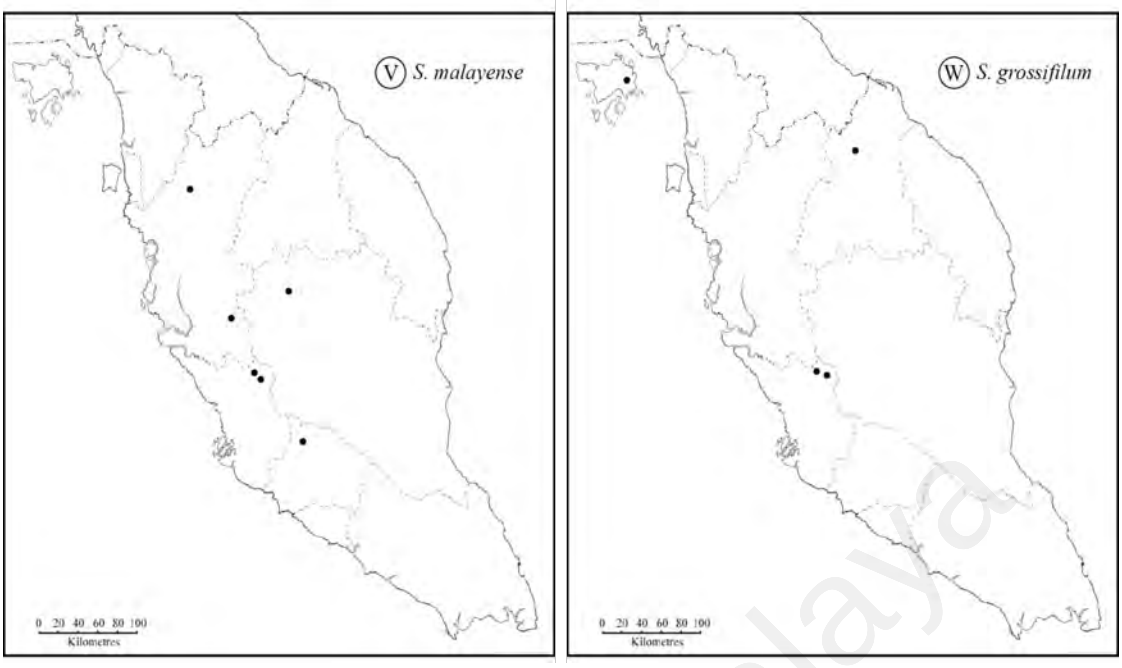

Figure 4.17: (**V**) ***S. malayense*** (PAHANG: Raub = RBS17; PERAK: Lenggong = LG8, Chenderiang = CD19; N. SEMBILAN: Jelebu = JB2; SELANGOR: Ulu Yam = UY3, Semangkok = SM9). (**W**) ***S. grossifilum*** (KELANTAN: Batu Gajah = BG10, KEDAH: Langkawi = LK2, SELANGOR: Ulu Yam = UY1, UY3).

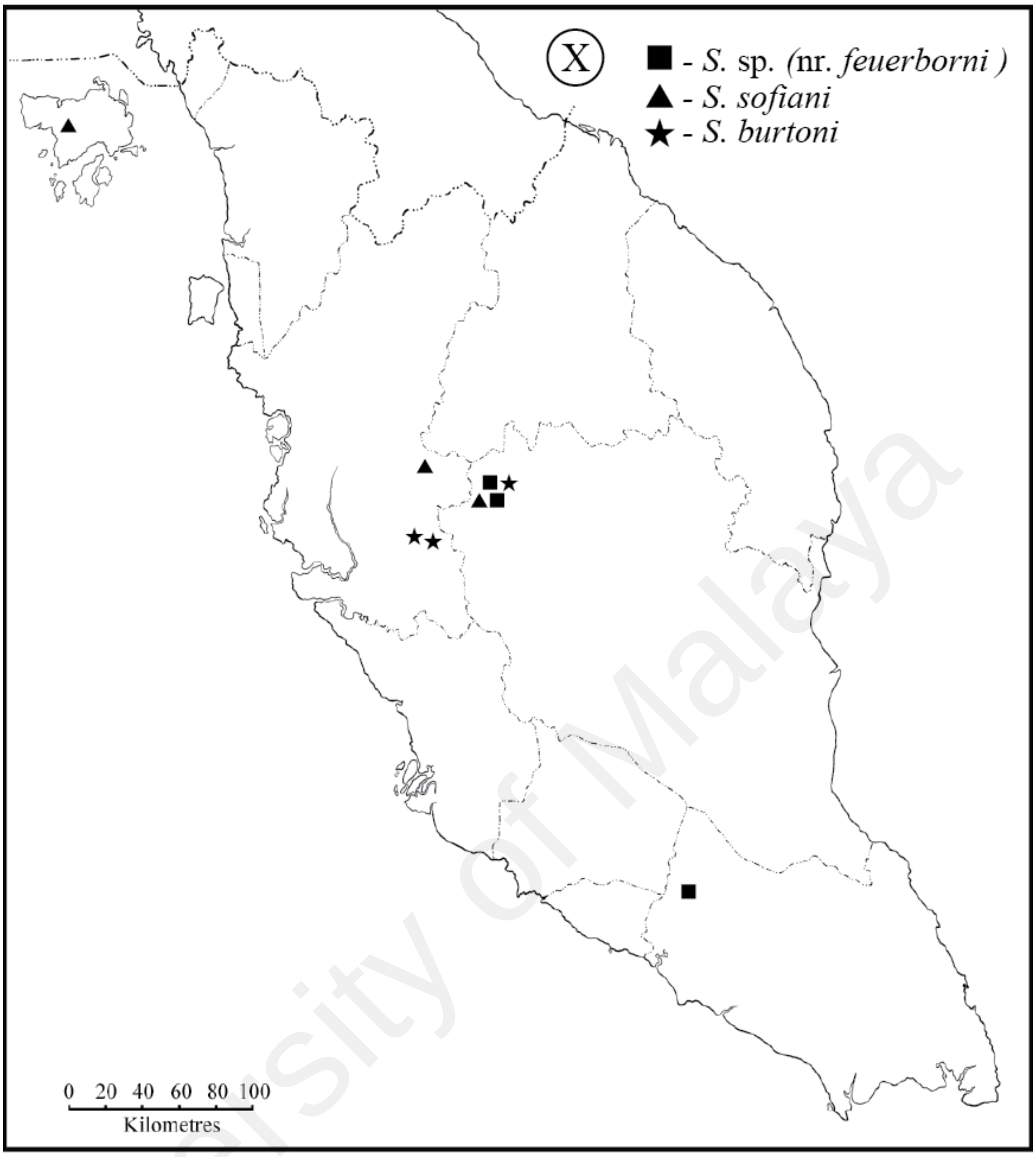

Figure 4.18: (■) **S. sp**. (**nr. *feuerborni***) (PAHANG: Cameron Highland = CHS11, CHS15; JOHOR: Gunung Ledang = GL6), (▲) ***S. sofiani*** (PAHANG: Cameron Highland = CHS11; KEDAH: Langkawi = LK6; PERAK: Simpang Pulai = SP1), (★) ***S. burtoni*** (PAHANG: Cameron Highland = CHS11; PERAK: Tapah = TP3, TP4).

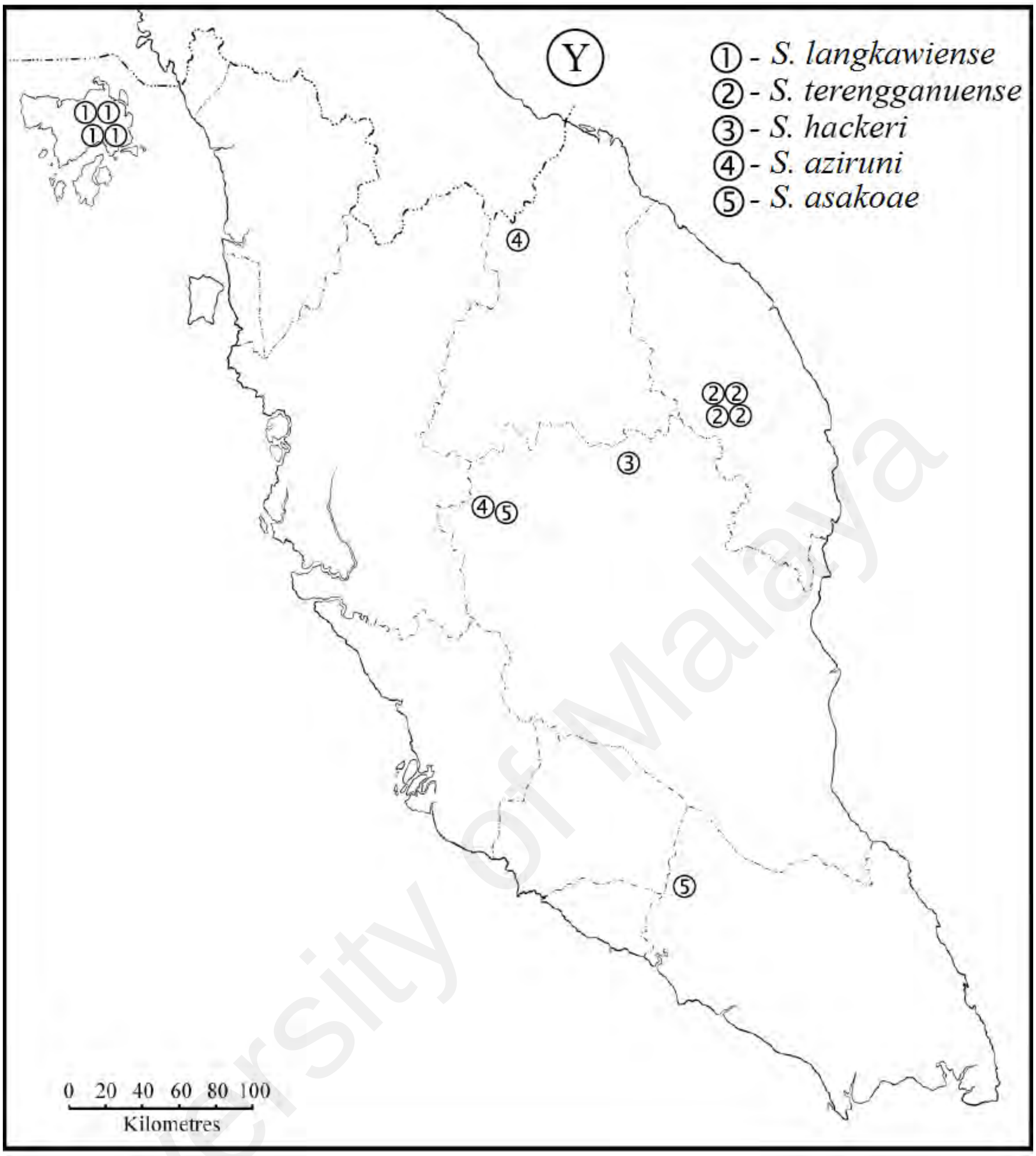

Figure 4.19: (**1**) ***S. langkawiense*** (KEDAH: Langkawi = LK3, LK7, LK11, LK12, LK14). (**2**) ***S. terengganuense*** (TERENGGANU: Pasir Raja = PR1, PR2, PR3, PR4). (**3**) ***S. hackeri*** (PAHANG: Cameron Highland = CHS11, Mount Tahan = MT19). (**4**) ***S. aziruni*** (KELANTAN: Jeli = JL9; PAHANG: Cameron Highland = CHS11). (**5**) ***S. asakoae*** (PAHANG: Cameron Highland = CHS14; JOHOR: Gunung Ledang = GL6).

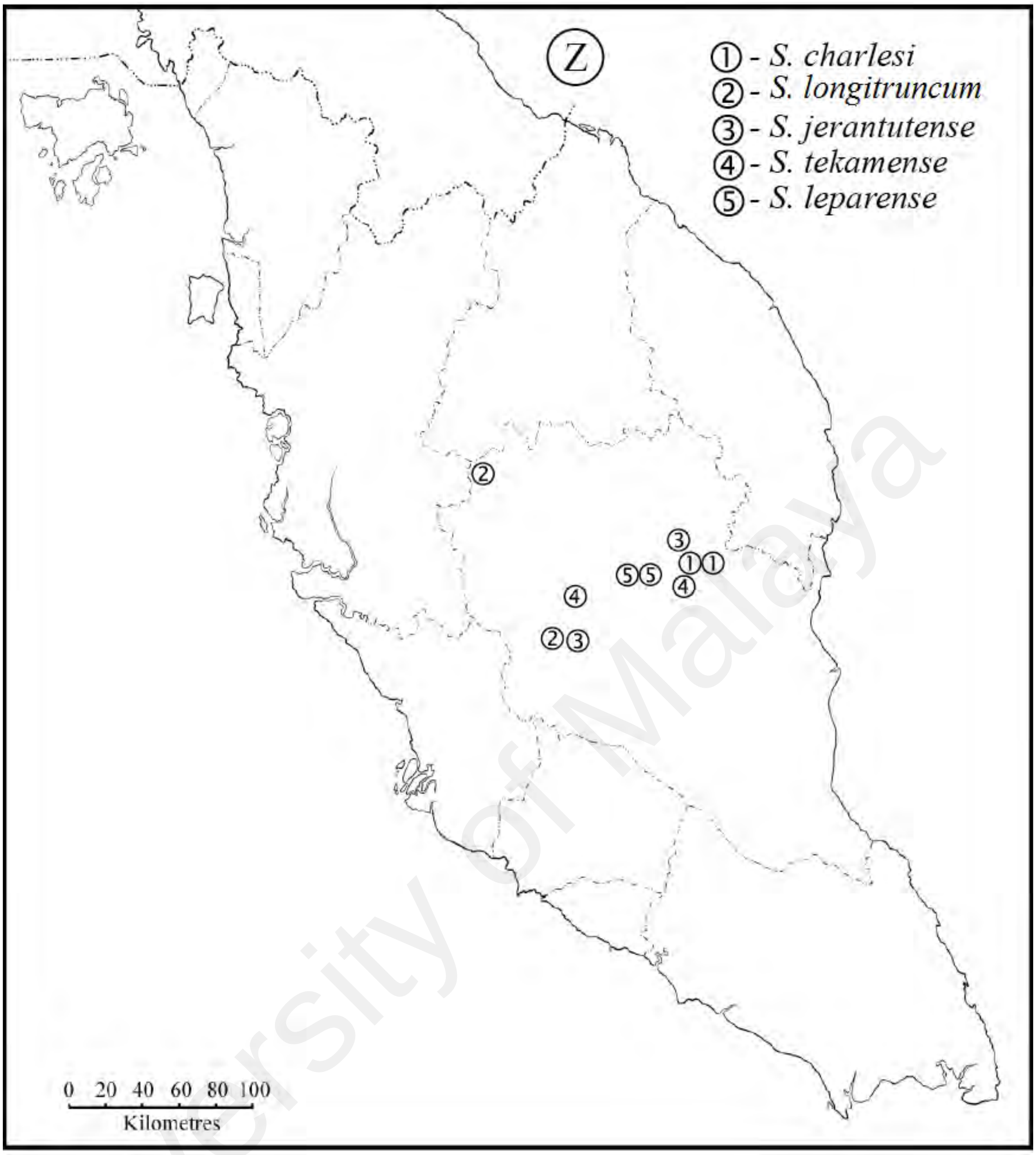

Figure 4.20: (**1**) ***S. charlesi*** (PAHANG: Kota Gelanggi = KG6, Lepar/Tekam = LT5). (**2**) ***S. longitruncum*** (PAHANG: Fraser Hill = FH1, Cameron Highland = CHS15). (**3**) ***S. jerantutense*** (PAHANG: Lepar/Tekam = LT1, Fraser Hill = FH1). (**4**) ***S. tekamense*** (PAHANG: Lepar/Tekam = LT1, LT8). (**5**) ***S. leparense*** (Lepar/Tekam = LT1, Jerantut = JT3).

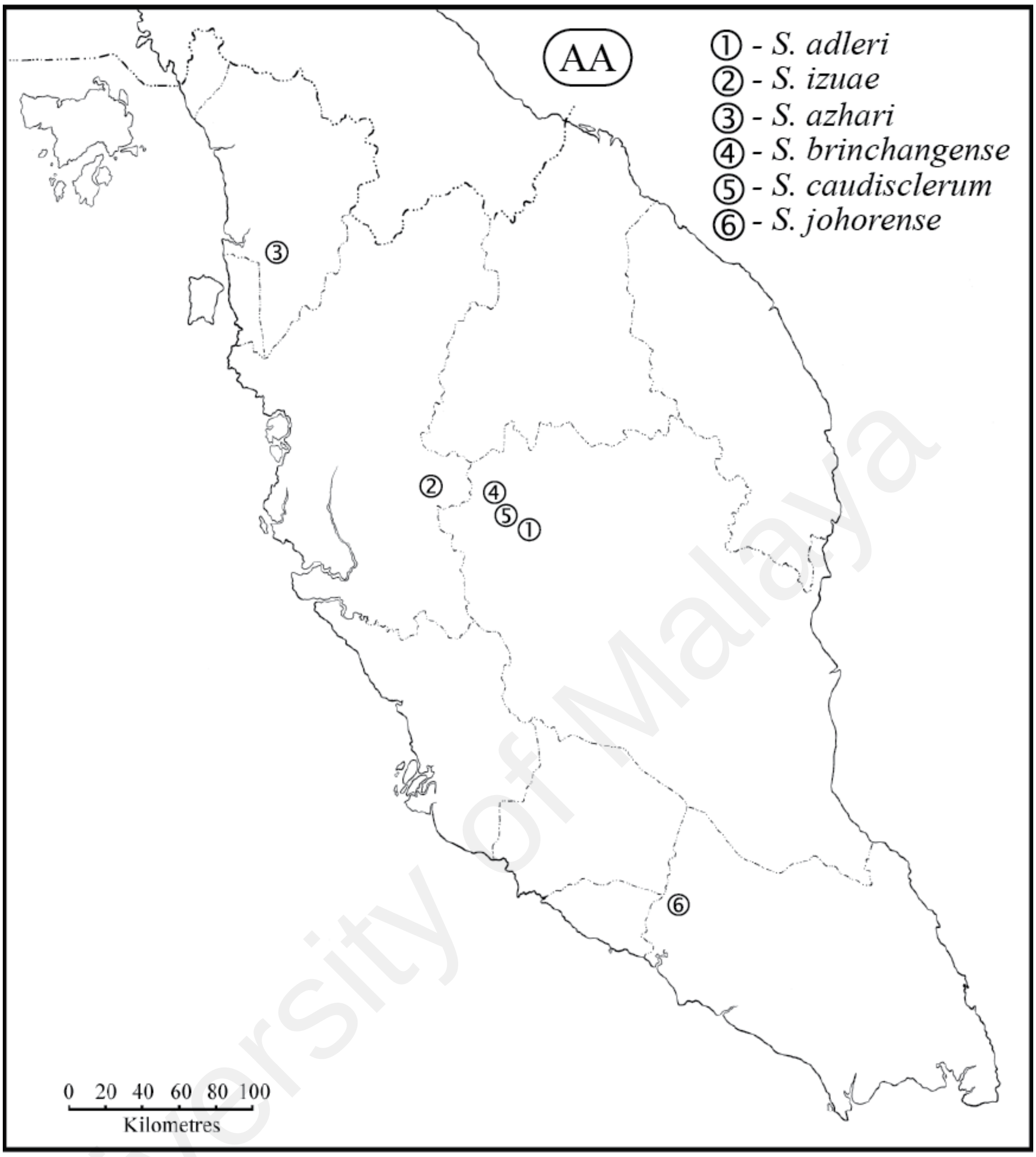

Figure 4.21: (**1**) ***S. adleri*** (PAHANG: Raub = RBS17). (**2**) ***S. izuae*** (PERAK: Simpang Pulai=SP1). (**3**) ***S. azhari*** (KEDAH: Sungai Sedim = SS3) (**4**) ***S. brinchangense*** (PAHANG: Cameron Highland = CHS15) (**5**) ***S. caudisclerum*** (PAHANG: Cameron Highland = CHS15). (**6**) ***S. johorense*** (JOHOR: Gunung Ledang = GL7).

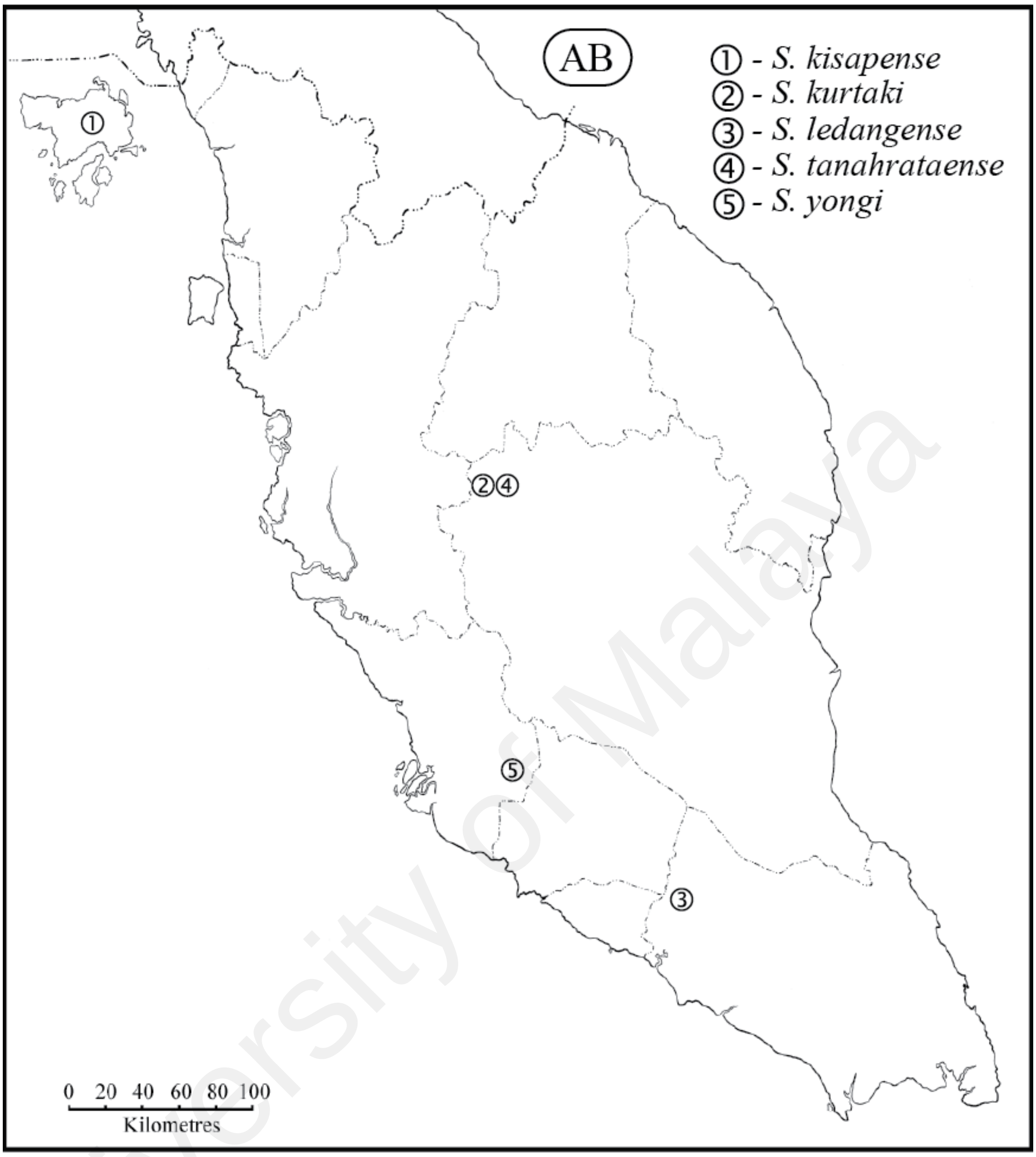

Figure 4.22: (**1**) ***S. kisapense*** (KEDAH: Langkawi = LK1) (**2**) ***S. kurtaki*** (PAHANG: Cameron Highland = CHS15) (**3**) ***S. ledangense*** (JOHOR: Gunung Ledang = GL3). (**4**) ***S. tanahrataense*** (PAHANG: Cameron Highland = CHS11) and (**5**) ***S. yongi*** (SELANGOR: Hulu Langat = HL5).

4.3.3 Breeding habitat preference

The PCA of all collections (180 sampling points) revealed four PCs which had eigenvalues >1.0, accounted for 69.3% of the total intersite variance of the stream condition variables (Table 4.4). Spearman's rank correlations revealed that sites with a higher PC-1, which explained 28.4% of the total variance, were wider, deeper, faster and had low pH, higher discharge, larger streambed particle size, with more riparian vegetation. PC-2 explained 18.3% of the total variance. Sites with a higher PC-2 score were at higher elevations, had cooler stream temperatures, low conductivity, higher dissolved oxygen, with more canopy cover and riparian trees. PC-3 accounted for 12% of the total variance. Sites with a higher PC-3 score had higher dissolved oxygen and pH with more stream cover. PC-4 accounted for 10.6% of the total variance. Sites with a higher PC-4 were at higher elevations, had cooler water, and larger streambed particles. This PC explains similar stream variables to PC-2 because both significantly related to elevation and water temperature. However, PC-2 was also significantly related to water conductivity and dissolved oxygen while these variables were not related to PC-4. Similarly, PC-4 was significantly related to streambed particle while PC-2 did not. Therefore, these two principal components were explained different stream variable.

Regression analysis between species richness and PCs indicated that the species richness was positively and significantly associated with PC-1 ($F = 22.7$, $df = 1, 173$; $P<0.001$). Therefore, more species were found in streams which were larger, deeper, and flowing faster with higher discharge, larger streambed particles, more riparian vegetation and high pH.

Table 4.4: Results of principal component analysis (PCA) and Spearman's rank correlation coefficient between stream variables and principal components (PCs) for 180 collections in Peninsular Malaysia.

Variable	Stream site				Principle components			
	Min	Max	Mean	±SE	PC-1	PC-2	PC-3	PC-4
Elevation (m)	10.00	1,345.00	235.4	17.00	−0.176	0.392**	0.167	0.551**
Temperature (°C)	16.30	30.90	23.8	0.13	0.052	−0.402**	−0.043	−0.315**
Width (m)	0.05	15.00	2.54	0.20	0.844**	−0.045	0.185	0.084
Depth (m)	0.01	0.70	0.14	0.009	0.787**	−0.137	0.071	0.031
Velocity (m/s)	0.12	1.10	0.43	0.01	0.808**	−0.085	0.161	0.142
Conductivity (mS/cm)	0.0115	1.83	0.306	0.02	0.222	−0.476**	0.225	0.231
Dissolved Oxygen (mg/l)	1.60	18.40	6.77	0.27	−0.005	0.293**	0.690**	0.03
pH	4.35	8.43	6.41	0.05	0.292**	−0.157	0.709**	0.14
Discharge (m^3/s)	0.0003	3.15	0.34	0.04	0.914**	−0.102	0.149	0.095
Stream-bed particle	Sand	Bedrock	3ǂ		0.628**	0.128	0.14	0.633**
Canopy cover	Open	Forest	2ǂ		−0.128	0.700**	0.351**	−0.021
Riparian vegetation	Open	Complete	3ǂ		0.336**	0.678**	0.062	−0.102
% Variance explained in PCA								
Proportion					28.4	18.3	12.0	10.6
Cumulative					28.4	46.7	58.7	69.3

** $P < 0.001$

ǂMedian values given for riparian vegetation (1=open, 2= brush and 3=forest), streambed-particle size (min; 1= mud/silt and max; 6= bedrock), and canopy cover. (1 = open, 2 = partial and 3 = complete). Rankings followed McCreadie *et al*. (2006).

Regression analysis for four black fly species is presented in Table 4.5. Forward logistic regression analysis was conducted for seven species (*S. trangense*, *S. angulistylum*, *S. cheongi*, *S. bishopi*, *S. vanluni*, *S. tani* and *S. sheilae*) which were found in more than 10% of the sampling sites. All of the regression models of species distribution, except *S. trangense*, *S. angulistylum* and *S. cheongi*, were significant at $P<0.001$ with correct classification varying from 76 to 86.1%. PC-1 was significantly correlated with the occurrence of two species (*S. tani* and *S. vanluni*). Occurrence of *S. sheilae* was negatively correlated with PC-2, and in contrast, *S. bishopi* was positively correlated with PC-2. The occurrences of *S. tani* and *S. vanluni* was positively associated with PC-1. The PC-3 and PC-4 were not significantly related to any species.

Table 4.5: Regression analysis for the distribution of preimaginal black fly species in Peninsular Malaysia

Species	Regression coefficient					p	Correct (%)
	K	PC-1	PC-2	PC-3	PC-4		
Simulium bishopi	−2.275	-	0.716	-	-	<0.001	89.1
Simulium vanluni	−1.927	1.030	-	-	-	<0.001	84.6
Simulium tani	−1.344	0.773	0.551	-	-	<0.001	76.0
Simulium sheilae	−1.876	-	−0.618	-	-	<0.001	85.1

Only species that were present at >10% of the sampling sites were analyzed. *K* = the intercept

4.4 Discussion and Conclusion

This comprehensive black flies survey was conducted for the first time in Peninsular Malaysia and yielded 75.8% of current total black fly species inhabiting Peninsular Malaysia (62 species) (Adler & Crosskey, 2016). These results indicate that all sampled streams are the natural breeding habitats for black flies, although some of the sampling points may have been affected by intensive agricultural practices (e.g. in Cameron Highlands). In fact, the black flies could serve as the bioindicator for environmental quality assessment, where the immature stages have specific tolerance to habitat disturbance (Docile *et al*., 2015). A thorough examination of the association between black fly distribution and degree of impacted habitat would deepen the knowledge on the biology and ecology of black fly.

Species accumulation curve calculated from streams across Peninsular Malaysia almost reaching asymptote after approximately 105 streams were sampled. The species estimation also indicates that the sampling efficiency falls above 80% throughout the study, supporting the efficiency of the sampling method applied in this study. Current result revealed more than half of the black fly species (40 species) assemblages in Peninsular Malaysia are rare species (FO<10%). High number of rare species also recorded in most of the black fly studies in other geographic regions (Hamada *et al*., 2002; McCreadie & Adler, 2008; Couceiro *et al*., 2014). Rare species are important as part of organism influencing the function of ecosystem (Lyons *et al*., 2005). The species occurrence pattern which largely influenced by factors such as species dispersal ability and the stream ecological conditions (Adler *et al*., 2004; Pramual & Wongpakam, 2010; Couceiro *et al*., 2014) are the important factors determining whether or not the species is rare.

In contrast, 8.5% (four species) of total collected species (i.e., *S. trangense*, *S. angulistylum*, *S. tani*, and *S. cheongi*) had frequencies of occurrence ranging from 25.6% to 36.7% of total sampled streams. Regards to frequency of local occurrence, of total 10 states, *S. vanluni* found in all states, while species such as *S. tani*, *S. trangense*, *S. whartoni*, *S. roslihashimi*, *S. cheongi* and *S. angulistylum*, were found distributed ranging from seven to nine states. Black fly species that are widely distributed and adaptable in various physicochemical conditions are likely to be a species complex (Adler & McCreadie, 1997). This situation has been highlighted in previous studies where *S. tani* and *S. angulistylum* were found as the cytological species complexes (Tangkawanit *et al*., 2009; Pramual & Kuvangkadilok, 2012). The present results revealed that this species was found in a wide range of stream conditions in Peninsular Malaysia. Therefore, additional cryptic diversity might be found in Malaysian specimens and further cytogenetic studies would help to clarify this hypothesis.

The species richness observed in this study showed left skewed distribution with a mean of 3.0 (±0.7), minimum of one and maximum of 10 species per stream. Species richness is strongly interconnected with the habitat characteristics (Hamada *et al*., 2002). This study found that species richness was significantly associated stream size, flow, streambed particles, and riparian vegetation. These results are largely consistent with the pattern of species richness found in previous studies: large, fast flowing streams, large streambed particles, and cool water at high elevation with cover, and riparian forest along the stream (Pramual & Kuvangkadilok, 2009); large, fast-flowing, high discharge streams, with lower temperature and conductivity (Pramual & Wongpakam, 2010); larger streams, cooler water temperature, faster flowing, and larger streambed particles (Hamada *et al*., 2002); larger streams (Grillet & Barrera, 1997); and current velocity and size of streambed particle (Scheder & Waringer, 2002). Moreover, regression analysis revealed that distribution of four common black fly species (*S. tani,* *S. vanluni, S. sheilae* and *S. bishopi*) are related to stream size, velocity, and riparian vegetation. Current study revealed the probability of predicting the species-habitat specific of the most-common species varied from 76% to 89.1%. The probability of predicting falls above 70% and this pattern corroborates with the previous studies (Hamada & McCreadie, 1999; Hamada *et al*., 2002; Pramual & Kuvangkadilok, 2009; Pramual & Wongpakam, 2010).

In addition to the mentioned stream conditions, other associated factors in influencing black fly ecology have also been pointed out: water chemistry (Townsend *et al*., 1983; Jenkins *et al*., 1984; Erman & Erman, 1995; McCreadie *et al*., 2005), habitat disturbance (Erman & Erman, 1995; Palmer *et al*., 1995; Pramual & Kuvangkadilok, 2009, Couceiro *et al*., 2014), elevation (Tate & Heiny, 1995; Srisuka *et al*., 2015), intense riparian vegetation (Lautenschläger & Kiel, 2005) as well as the labral fan of black fly larvae (Pangjanda & Pramual, 2015).

Co-occurrence with deterministic distribution pattern is produced by the closely similar assemblages under similar environmental variables (Leibold *et al*., 2004; Logue *et al*., 2011). Current result and other previous studies have corroborated this pattern (Hamada & McCreadie, 1999; Hamada *et al*., 2002; McCreadie *et al*., 2004; Landeiro *et al*., 2009; Figueiró *et al*., 2012). This is further supported by species assemblage's pattern showed by Shannon diversity index, where 17% of total sampled streams had diversity values above 50%, indicating the ability of stream to accommodate more than one species in a single stream. High co-occurrence suggesting that most of sampled streams have high environmental heterogeneity such as larger stream with larger streambed particles provides more than one microhabitat/niches which suited different species. Furthermore, more riparian vegetation provides more fallen leaves into the stream and consequently acts as potential substrates for preimaginal attachments.

Moreover, these identified environmental variables are the well-known factors determining black fly species occurrence (Grillet & Barrera, 1997; Hamada *et al*., 2002; Pramual & Kuvangkadilok, 2009). In contrast, co-occurrence of species also found in stream with low environmental heterogeneity which provides more homogenous stream ecology such as uniform in velocity and streambed particles (i.e., smaller particles or bedrock). Species co-occur in this particular streams require similar microhabitat preference and eventually will have an overlapped niche (Pramual *et al*., 2012). This situation will promote interspecific competitions and species potentially compete for resources which lead to the stronger ones to survive (Morin, 1999; Pramual *et al*., 2012). Result on the Berger-Parker Dominance proved this situation, of total streams with co-occurrence, 54% showed higher Dominance index (D) with the values falls between 50 to 99%.

In conclusion, this large-scale black fly survey has provided new insight into black fly species composition in Peninsular Malaysia. Current study has revealed that both ecological and physicochemical are the important factors in influencing the breeding habitat preference of simuliid species in tropical streams. These data can be useful for formulating effective prevention and vector control programs (i.e., environmental manipulation and use of biological control agents based on black fly species and density). Additionally, this first survey will be the stepping stone promoting more black fly studies in Malaysia as well as other countries in Southeast Asia.

CHAPTER 5: DISTIBUTION PATTERN OF PREIMAGINAL BLACK FLIES OF THE GENUS *SIMULIIUM* (DIPTERA: SIMULIIDAE) ALONG AN ELEVATIONAL GRADIENT IN PENINSULAR MALAYSIA

5.1 INTRODUCTION

Species community structure and distribution vary spatially in response to a broad range of environmental factors including elevation (Hodkinson, 2005; McCain & Grytnes, 2010). Towards high elevation, the main general changes observed involve stream size (Henriques-Oliveira & Nessimian, 2010), stream depth (Tomanova *et al*., 2007), temperature, precipitation (i.e., snow and rain), partial pressure of atmospheric gases, atmospheric turbulence and wind speed, and radiation input, including short-wave ultraviolet radiation at different wavelengths (Barry, 1992; Hodkinson, 2005). Consequently, all these changes create a barrier to species and drive to community diversification (McCain & Grytnes, 2010). Moreover, communities appear to have been gradually decreasing in taxa richness with increasing elevation (Wolda, 1987; McCoy, 1990).

Black fly larvae are dominant inhabitants of unpolluted streams and rivers over a wide range of elevations (Currie & Adler, 2008). They are postulated to have evolved in cool and mountainous environments (Adler *et al*., 2010). The climate change during the glacial periods has driven the population expansion of simuliids along latitudinal and elevational gradients (Pramual *et al*., 2005; Adler *et al*., 2010; Low *et al*., 2014). However, as climate changes, the widespread alpine taxa in the cooler regions could be fragmented and isolated as specialised taxa or high-elevation specialists (Adler *et al*., 2010).

Black fly assemblage is a colony of different species occurring in similar ecological or habitat requirements. Defining this assemblage is of paramount importance in evaluating the comparative richness of populations, and the effect of isolation or fragmentation (Dufrene & Legendre, 1997). Numerous factors have been linked to black flies assemblage, these include competition (Hart, 1986), food availability (Colbo & Porter, 1979), substrate type (Ciborowski & Adler, 1990, Halgos *et al*., 2001), water current velocity (McCreadie & Colbo, 1993; Figueiró *et al*., 2008), water temperature (McCreadie *et al*., 2005; Nascimento *et al*., 2007) and elevation (Tate & Heiny, 1995; Srisuka *et al*., 2015). To date, the ecological studies of black flies have been given more attention on spatial distribution in response to habitat disturbance (Pramual & Kuvangkadilok, 2009; Couceiro *et al*., 2014), seasonal variation (McCreadie *et al*., 2005; Pramual & Wongpakam, 2010; Srisuka *et al*., 2015), and locality richness (McCreadie & Adler, 1998, 2006; Hamada & McCreadie, 1999; Hamada *et al*., 2002; McCreadie *et al*., 2005). However, there has been limited research undertaken on the vertical distribution of preimaginal black flies and their associated ecological factors. The knowledge of distribution patterns related to elevation could contribute to the understanding of the geographical distribution of many species as well as their local diversity.

It has been hypothesized that stream conditions vary according to the elevation, thus black fly diversity and assemblages are expected to change along the elevational gradient. Certain preimaginal stages of black flies would have a broad range of vertical distribution (i.e. generalist species), while others might show a specific range of distribution (i.e. specialist species). The specialised taxa may limit their distribution to certain preferred microhabitat/niche conditions (Pramual & Kuvangkadilok, 2009; Pramual & Wongpakam, 2010). To test this hypothesis, this study represents the first attempt to investigate the distribution pattern of black flies and their associated environmental factors, along an elevational gradient in Peninsular Malaysia.

5.2 MATERIALS AND METHODS

5.2.1 Study sites

A total of 18 stream points were selected as fixed sites for sampling. Streams were chosen according to their accessibility for collection, elevation and the presence of flow. These stream locations were divided into three categories: (1) low elevation (100–500 m), (2) middle elevation (501–1,000 m), (3) high elevation (1,001–1,813 m). A total of six streams were assigned as low elevation: (E1–E6), five streams as middle elevation: (E7–E11) and seven streams as high elevation (E12–E18). Seven streams are located in the state of Pahang (Cameron Highland, 04º28.738'N 101º22.979'E and Lipis, 04º23.715'N 101º36.443'E) while 11 streams are located in the state of Perak (Tapah, 04º14.203'N 101º18.354'E and Simpang Pulai, 04º34.956'N 101º20.717'E). Location details for 18 fixed-stream sites and associated ecological characteristics are presented in Figure 5.1 and Table 5.1.

5.2.2 Black fly sampling and identification

Overall, a total of 432 collections were performed in this study (24 at each 18 fixed-stream site at monthly intervals) from February 2012 to January 2014. These extensive repeated samplings were expected to reveal the most accurate species number at all surveyed streams. Thus, sampling bias due to seasonal fluctuations in physicochemical parameters was minimized in this study. Each stream was sampled from downstream to upstream (20 m), for approximately 1 hour, by two people. Larvae and pupae attached on aquatic substrates such as grasses, leaves and stems, twigs, plant roots and rocks were collected by hand using fine forceps. These sampling protocols could represent the species occurrence in a locality (McCreadie & Colbo, 1991; McCreadie *et al*., 2005). Pupae attached on similar substrates were individually kept alive in vials until emergence. The adults, together with their pupal exuviae and cocoons were preserved in 80% ethanol for identification at the subgenus, species-group or species level. The methods of collection and identification followed those of Takaoka (2003) and Adler *et al*. (2004).

Table 5.1: Locations of 18 fixed-streams and associated ecological characteristics.

Stream/ elevation	Locality	Elevation (m)	Code	GPS		Canopy cover	Riparian vegetation	Streambed
Low								
E1	Tapah	159	TPS1	04º14.203'N	101º18.354'E	Partial	Forest	Rubble
E2	Tapah	224	TPS3	04º16.522'N	101º18.996'E	Complete	Forest	Rubble
E3	Tapah	235	TPS2	04º16.316'N	101º19.022'E	Open	Forest	Boulder
E4	Tapah	337	TPS4	04º18.420'N	101º19.658'E	Complete	Forest	Bedrock
E5	Lipis	388	RBS18	04º23.715'N	101º36.443'E	Open	Brush	Sand
E6	Lipis	423	RBS17	04º23.715'N	101º36.443'E	Partial	Brush	Bedrock
Middle								
E7	Cameron Highland	711	CHS5	04º22.220'N	101º21.512'E	Partial	Forest	Bedrock
E8	Cameron Highland	737	CHS6	04º22.660'N	101º21.902'E	Complete	Forest	Small stone
E9	Cameron Highland	872	CHS7	04º23.165'N	101º22.334'E	Open	Forest	Boulder
E10	Cameron Highland	992	CHS9	04º24.274'N	101º22.572'E	Partial	Forest	Bedrock
E11	Cameron Highland	1,000	CHS8	04º24.112'N	101º22.356'E	Complete	Forest	Sand
High								
E12	Cameron Highland	1,160	CHS10	04º26.723'N	101º22.979'E	Partial	Forest	Sand
E13	Simpang Pulai	1,315	SPS12	04º34.956'N	101º20.717'E	Open	Brush	Bedrock
E14	Simpang Pulai	1,345	SPS13	04º34.760'N	101º20.507'E	Complete	Brush	Sand
E15	Cameron Highland	1,405	CHT11	04º28.738'N	101º22.979'E	Partial	Forest	Rubble
E16	Cameron Highland	1,602	CHS14	04º31.258'N	101º24.247'E	Open	Brush	Boulder
E17	Cameron Highland	1,750	CHS16	04º31.519'N	101º23.365'E	Partial	Forest	Boulder
E18	Cameron Highland	1,813	CHS15	04º31.461'N	101º23.338'E	Complete	Brush	Small stone

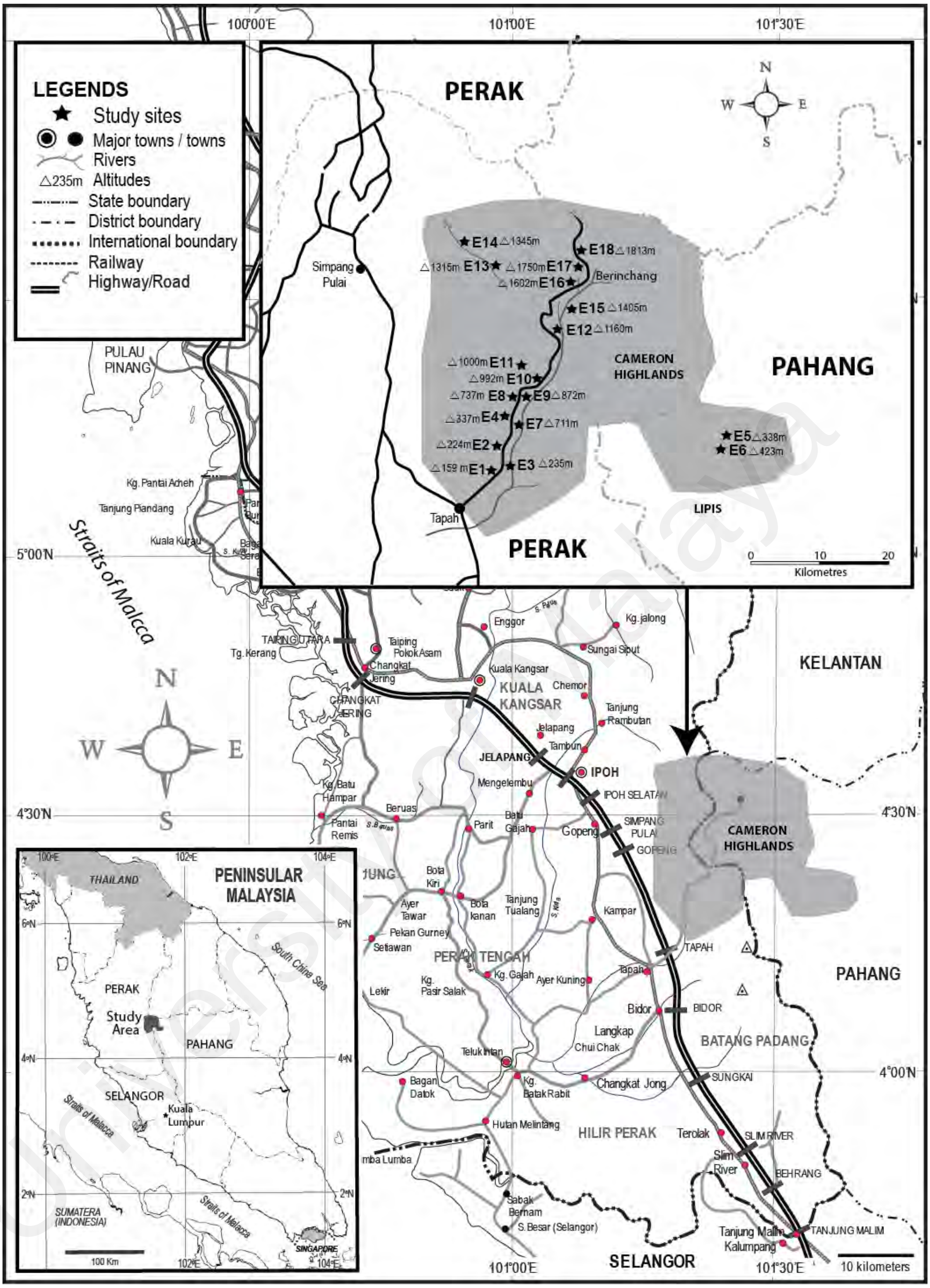

Figure 5.1: Map showing 18 fixed-streams (E1−E18) located in the states of Pahang and Perak in Peninsular Malaysia (top right), small map showing the Peninsular Malaysia (bottom left).

5.2.3 Physicochemical measurements

Protocols were described in detail in section 4.2.3.

5.2.4 Data analyses

Protocols were described in detail in section 4.2.4. Frequency of occurrence (FO) was designated in percentages (Table 6.2), calculated by the total number of a species occurrence, divided by the total number of collections (n=432) (Hamada *et al*., 2002). Stream occurrence (SO) presented in percentages was calculated by the number of sites where a species was taken, divided by the total streams (n=18). Cluster analysis based on Sorenson's coefficient was used to compare the percentage of site similarity in species composition for each site. Canonical correspondence analysis (CCA) was used to investigate the relationship between environmental variables and species assemblages. CCA was analysed using the combined data set (n=432 collections). The CCA was conducted using the program PC-ORD (version 5.14) (McCune & Mefford, 2006). Species Diversity and Richness (SDR) version 4 (Seaby & Henderson, 2006), the SPSS statistical package, version 16.0, Chicago, IL, were employed for diversity and statistical analyses respectively.

5.3 Results

5.3.1 Black fly species composition

Thirty-five species were collected from 24 samplings at each of 18 fixed-stream sites (Table 5.2). The most frequently collected species (FO) were *S. tani* (31.7%) and *S. whartoni* (21.5%). Relatively common species were *S.* sp. (nr. *feuerborni*) (16.2%), *S. decuplum* (15.5%), *S. angulistylum* (14.8%), *S. bishopi* (13.2%) and *S. izuae* (11.8%). Other species were collected at a frequency lower than 10% and considered as rare. In terms of total individuals collected, *S. tani*, *S. asakoae*, *S. whartoni* and *S.* sp. (nr. *feuerborni*) were the four most abundant species. Based on stream occurrence (SO), *S. whartoni, S. bishopi* and *S. brevipar* were the widest distributed species (14 streams or 77.8% each).

At the subgeneric level, *Gomphostilbia* was the most diverse subgenus found (19 species), followed by the *Simulium* s. str. (12 species) and the least was *Nevermannia* (four species). Of 18 species-groups in Malaysia, 17 were recorded in this study. The most abundant group was the *S. asakoae* species-group (six species), followed by the *S. epistum* species-group (4 species). Other species-groups were represented by one or two species.

Species richness and estimated richness are presented in Table 5.3. The maximum number of black fly species collected per total collections was 11 and the mean number was 3.2 ± 0.1 (SE). Total estimated species richness ranged between 39.8 to 41.3, which yielded more than 80% sampling efficiency, while the estimated species richness for each stream ranged between 4.1 to 24.0, which falls above 60% sampling efficiency. Species reaching asymptote after approximately 54 samplings were performed, supporting the efficiency of the sampling method used in this study (Figure 5.2).

Table 5.2: Abundance, frequency of occurrence (FO) and stream occurrence (SO) of 35 black fly species of the genus *Simulium* from 24 collections at 18 fixed-streams in Peninsular Malaysia.

Species	Number of specimens	%	
		FO	SO
S. (*Gomphostilbia*) *adleri* Jitklang & Kuvangkadilok, 2008	3	0.7	5.6
S. (*Gomphostilbia*) *angulistylum* Takaoka & Davies, 1995	347	14.8	55.6
S. (*Gomphostilbia*) *asakoae* Takaoka & Davies, 1995	837	7.6	55.6
S. (*Gomphostilbia*) *brinchangense* Takaoka *et al.* 2014	11	0.7	11.1
S. (*Gomphostilbia*) *burtoni* Takaoka & Davies, 1995	19	3.0	16.7
S. (*Gomphostilbia*) *cheongi* Takaoka & Davies, 1995	12	2.3	22.2
S. (*Gomphostilbia*) *decuplum* Takaoka & Davies, 1995	176	15.5	61.1
S. (*Gomphostilbia*) *duolongum* Takaoka & Davies, 1995	3	0.7	11.1
S. (*Gomphostilbia*) *gombakense* Takaoka & Davies, 1995	117	10.0	50.0
S. (*Gomphostilbia*) *izuae* Takaoka *et al.* 2013	333	11.8	55.6
S. (*Gomphostilbia*) *longitruncum* Takaoka & Davies, 1995	1	0.2	5.6
S. (*Gomphostilbia*) *lurauense* Takaoka *et al.* 2013	26	3.2	33.3
S. (*Gomphostilbia*) *roslihashimi* Takaoka & Sofian-Azirun, 2011	112	9.3	55.6
S. (*Gomphostilbia*) *sheilae* Takaoka & Davies, 1995	120	4.4	44.4
S. (*Gomphostilbia*) *sofiani* Takaoka & Hashim, 2011	31	3.2	16.7
S. (*Gomphostilbia*) sp. (nr. *parahiyangum*)*	54	6.5	38.9
S. (*Gomphostilbia*) *tanahrataense* Takaoka *et al.* 2014	1	0.2	5.6
S. (*Gomphostilbia*) *trangense* Jitklang *et al.* 2008	312	6.7	38.9
S. (*Gomphostilbia*) *whartoni* Takaoka & Davies, 1995	709	21.5	77.8
S. (*Nevermannia*) *aureohirtum* Brunetti, 1911	103	4.9	27.8
S. (*Nevermannia*) *caudisclerum* Takaoka & Davies, 1995	17	0.7	5.6
S. (*Nevermannia*) sp. (nr. *feuerborni)* *	688	16.2	44.4
S. (*Nevermannia*) *kurtaki* Takaoka & Davies, 1995	1	0.2	5.6
S. (*Simulium*) *bishopi* Takaoka & Davies, 1995	305	13.2	77.8
S. (*Simulium*) *brevipar* Takaoka & Davies, 1995	122	10.6	77.8
S. (*Simulium*) *digrammicum* Edwards, 1928	1	0.2	5.6
S. (*Simulium*) *grossifilum* Takaoka & Davies, 1995	106	8.3	50.0
S. (*Simulium*) *hackeri* Edwards, 1928	96	3.7	11.1
S. (*Simulium*) *hirtinervis* Edwards, 1928	63	3.0	33.3
S. (*Simulium*) *jeffreyi* Takaoka & Davies, 1995	442	8.3	11.1
S. (*Simulium*) *malayense* Takaoka & Davies, 1995	188	8.6	38.9
S. (*Simulium*) *vanluni* Ya'cob, Takaoka & Sofian-Azirun, 2017	42	1.6	5.6
S. (*Simulium*) sp. (nr. *grisescens*)*	7	0.2	5.6
S. (*Simulium*) *tani* Takaoka & Davies, 1995	2,669	31.7	66.7
S. (*Simulium*) *yongi* Takaoka & Davies, 1997	11	1.9	16.7

*Undetermined species, which probably are new species.

Table 5.3: Actual and estimated species richness for 18 fixed-streams along an elevational gradient in Peninsular Malaysia. Numbers in parentheses indicate sampling efficiency*

Elevation	Actual species	Mean richness (± SE)	Chao estimates	First Order Jackknife
All	*35*	*3.15 ± 2.00*	*41.3 ± 2.08 (84.7)*	*39.8 ± 1.95 (87.9)*
Low elevation				
E1	13	2.52 ± 0.35	13.7 ± 1.10 (94.8)	16.8 ± 1.78 (77.4)
E2	15	3.43 ± 0.31	24.0 ± 7.69 (62.5)	21.7 ± 3.22 (69.1)
E3	15	3.50 ± 0.28	23.0 ± 8.31 (65.2)	19.8 ± 1.90 (75.7)
E4	8	3.21 ± 0.41	8.4 ± 0.95 (95.1)	8.95 ± 0.96 (89.4)
E5	12	4.30 ± 0.52	12.1 ± 0.92 (99.2)	12.9 ± 0.96 (93.0)
E6	14	4.64 ± 0.48	16.0 ± 3.01 (87.5)	16.9 ± 1.59 (82.8)
Middle elevation				
E7	16	5.35 ± 0.67	18.0 ± 3.01 (88.8)	17.9 ± 1.33 (89.4)
E8	12	2.33 ± 0.40	16.1 ± 11.5 (74.5)	17.7 ± 2.07 (67.8)
E9	14	2.65 ± 0.33	20.3 ± 5.89 (68.9)	18.8 ± 1.95 (74.5)
E10	5	2.67 ± 0.67	7.0 ± 3.01 (71.4)	7.8 ± 2.87 (64.1)
E11	11	3.21 ± 0.32	13.6 ± 7.78 (80.9)	13.9 ± 1.59 (79.1)
High elevation				
E12	11	3.44 ± 0.35	13.4 ± 0.60 (82.1)	12.9 ± 1.32 (85.2)
E13	16	3.34 ± 0.43	22.3 ± 5.89 (71.7)	20.8 ± 2.40 (76.9)
E14	13	3.36 ± 0.50	16.5 ± 5.66 (78.8)	17.8 ± 2.39 (73.0)
E15	13	2.83 ± 0.28	14.7 ± 6.17 (88.4)	20.7 ± 3.29 (62.8)
E16	4	1.69 ± 0.15	4.1 ± 0.37 (97.5)	4.09 ± 0.09 (97.8)
E17	7	1.29 ± 0.18	11.0 ± 1.03 (63.6)	11.2 ± 3.83 (62.5)
E18	10	1.76 ± 0.42	14.2 ± 6.82 (70.4)	16.1 ± 1.95 (62.1)

*Sampling efficiency was calculated by dividing the number of actual species sampled by the number of estimated species (Brühl, 2001).

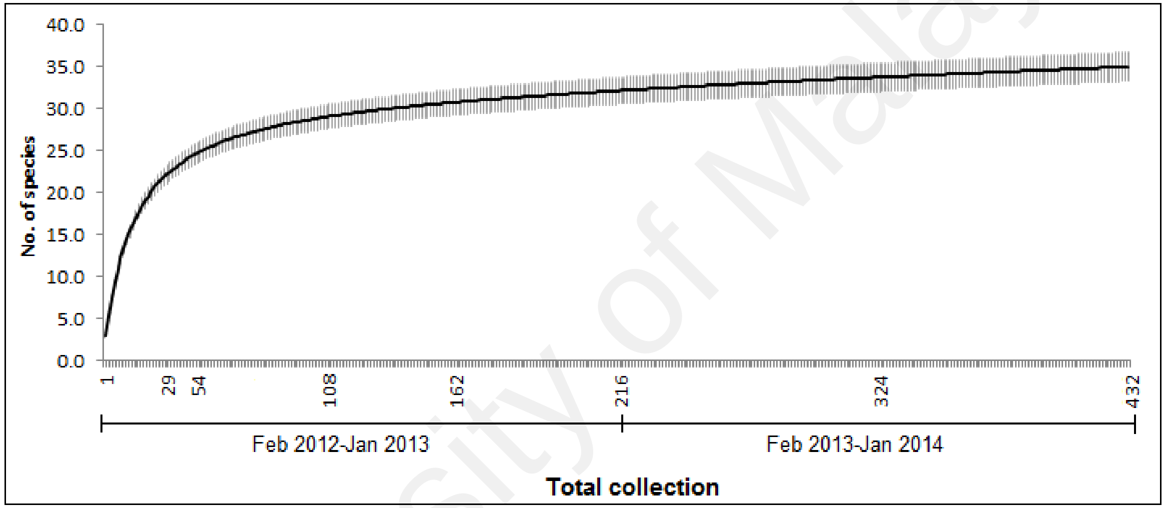

Figure 5.2: Species accumulation curve with error bars for overall 432 collections at 18 fixed-streams along an elevational gradient in Peninsular Malaysia.

5.3.2 Species diversity and distribution patterns

Diversity indices are presented in Figure 5.3. Species diversity and richness increased with elevation and declined at 1,600 m and above. Diversity value was highest at stream E8 (0.87) followed by streams E12 (0.81), E5 (0.79) and E13 (0.78). The values were lower at streams E17 (0.35), E18 (0.33), E16 (0.27) and E15 (0.25). In contrast, dominance index (D) was highest at 1,400 m and above (E15–E18).

Frequency of occurrence for distribution of 35 black fly species is presented in Table 5.4. Five patterns of species distribution were observed: (1) six species distributed at low elevation (159–423 m), (2) three species distributed from low to middle elevations (159–1,000 m), (3) two species distributed from middle to high elevations (711–1,813 m) (4) eight species distributed at high elevations (1,405–1,813 m) and (5) 16 species distributed from low to high elevations.

Of total collections (n=432), 76.6% (or 331) showed co-existence of species. Simulated and observed values of the C-score are presented in Figure 5.4. The null model for co-occurrence indicates the observed index is above the simulated indices [observed index = 572.01, mean of simulated indices = 558.83, variance of simulated indices = 2.75, P (observed $\leq$ expected) = 1.00, P (observed $\geq$ expected) = 0.00], therefore, the distributional patterns of species were not considered random.

Stream similarity based on species composition is presented in Figure 5.5. Streams were clustered into two main groups with similarity value at 18%: (1) ≤1,345 m (E1–E14) and (2) ≥1,405 m (E15–E18).

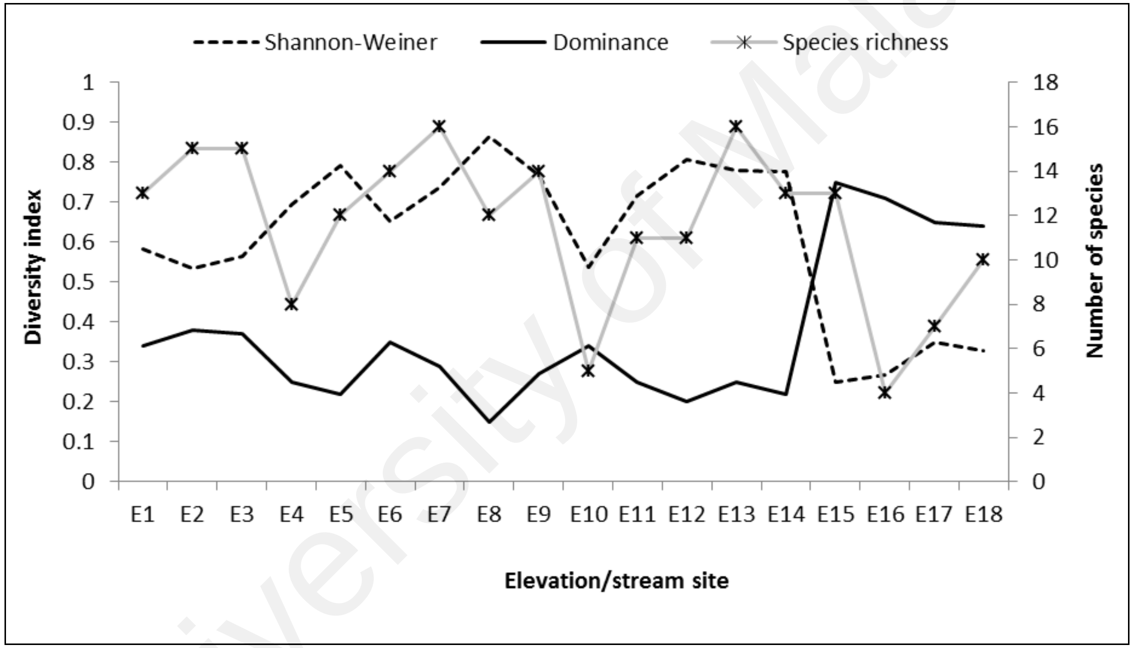

Figure 5.3: Diversity indices for black fly species along an elevational gradient in Peninsular Malaysia.

Table 5.4: Frequency of occurrence per 24 collections for 35 black fly species of the genus *Simulium* along an elevational gradient in Peninsular Malaysia

Species	Site/elevation																	
	Low						Middle					High						
	E1 (159)	E2 (224)	E3 235)	E4 (337)	E5 (388)	E6 (423)	E7 (711)	E8 (737)	E9 (872)	E10 (992)	E11 (1000)	E12 (1160)	E13 (1315)	E14 (1345)	E15 (1405)	E16 (1602)	E17 (1750)	E18 (1813)
S. sp. (*nr. grisescens*)		1																
S. duolongum	2	1																
S. vanluni			7															
S. cheongi	2	1	3		5													
S. adleri						3												
S. jeffreyi		16	20															
S. lurauense		2	2		3	1	5	1										
S. yongi				3			2		3									
S. sheilae	7	2	1	1	3	2	1				2							
S. angulistylum	16	2	4	5	13	18	2			1	2	1						
S. sp. (*nr. parahiyangum*)	1	15	6		3	1			1				1					
S. roslihashimi	6		1		1	5	7	4	1		12	1		2				
S. trangense	15	4	4			3		1	1					1				
S. grossifilum	1	1	2	8			16	1	6				1	1				
S. malayense		1				5	18	6	5				1	1				
S. izuae	1					1	7	8	2		13	10	2	6	1			
S. burtoni		5	7												1			
S. hirtinervis		1	1				6						1		1	3		
S. decuplum	2		2		16	14	10		3			9	6	2	1		1	
S. gombakense					12	11	2	3	1	25	6						1	
S. brevipar	2			2	2	8	3	2	5	3	2	5	3	7			1	1
S. bishopi	1			9	9	4	11	1	1	1	1	4	8	5	1			1
S. aureohirtum	2												4		4	10		1
S. tani		22	21	14	3		21	1	11		1	4	14	5	20			1
S. whartoni		1	1	3	14	19	6	3	2	1	15	9	5	11	1		1	1
S. asakoae							1	1	1		1	3	2		1	21	2	2
S. sp. (*nr. feuerborni*)											2	6	4	4	19	6	14	15
S. digrammicum													1					
S. longitruncum														2				
S. hackeri													9		7			
S. sofiani													4	1	9			
S. tanahrataense															1			
S. brinchangense																	2	1
S. kurtaki																		1
S. caudisclerum																		3

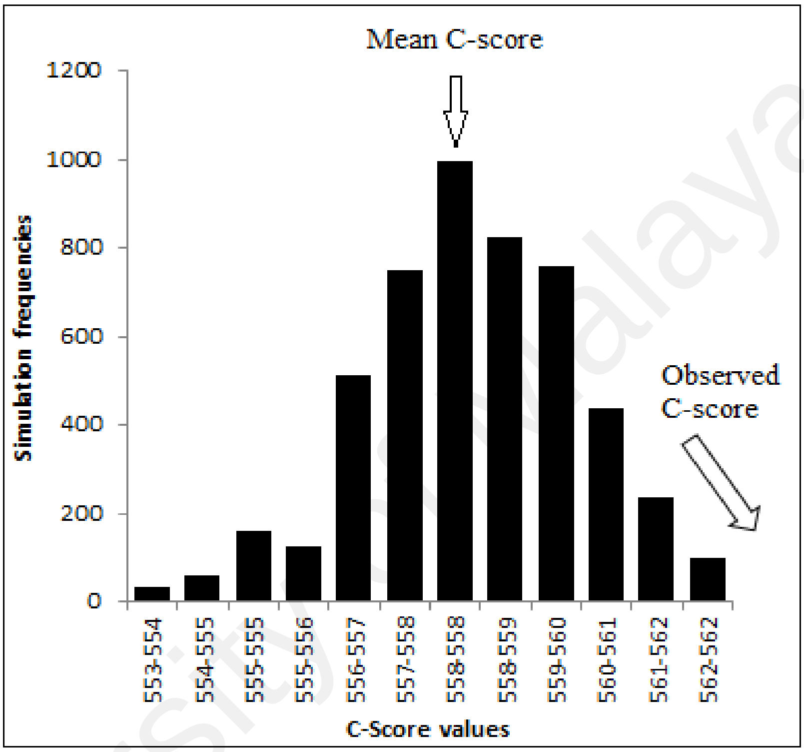

Figure 5.4: Simulated and observed values of the C-score for the co-occurrence of simuliid species collected at 18 fixed-streams along an elevational gradient in Peninsular Malaysia.

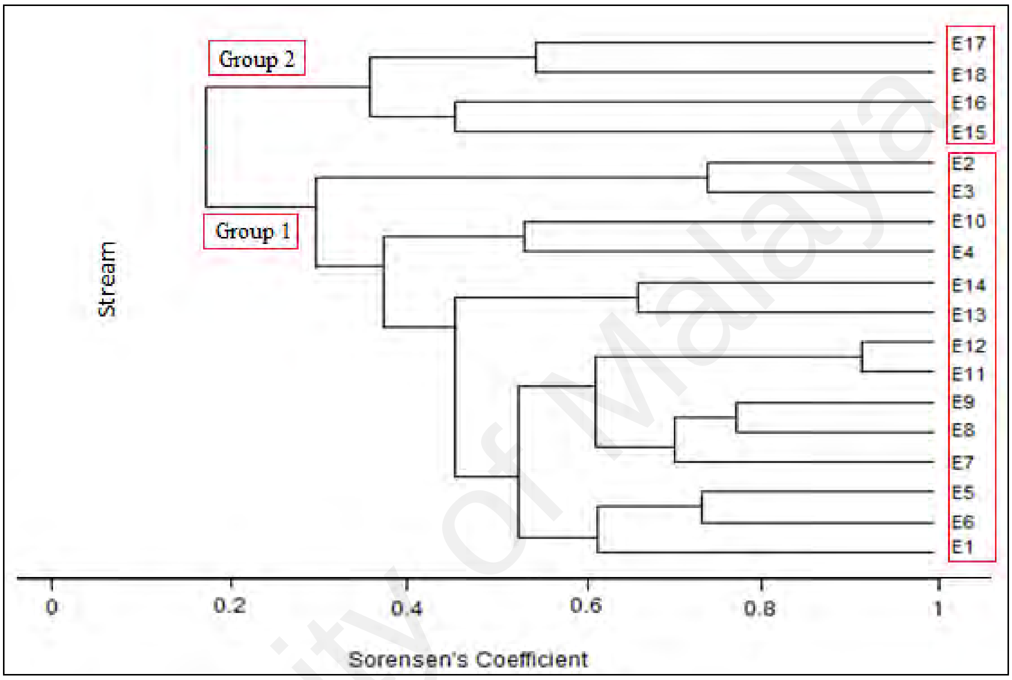

Figure 5.5: Cluster analysis based on Sorenson's coefficient for site similarity along an elevational gradient in Peninsular Malaysia.

5.3.3 Spatial distribution

Means and coefficient of variations for eight measured physicochemical variables of all collections are presented in Table 5.5. PCA of all collections revealed five PCs, which have eigenvalues >1.0 accounted for 71.2% of total inter-site variance of the physicochemical conditions (Table 5.6). Spearman's rank correlations revealed that sites with higher PC-1 (which explained 24.8% of the total variance) were at low elevation, normal water temperature (23°C–25°C), wider, deeper, faster, low conductivity, higher discharge, more canopy cover and riparian vegetation and with larger streambed particles. Sites with higher PC-2 (which explained 15.1% of the total variance) scores were at high elevation, cooler stream, wider, faster, higher dissolved oxygen with high discharge, less canopy cover and riparian vegetation. PC-3 accounted for 13.8% of the total variance. Sites with higher PC-3 scores were at high elevation, cooler stream, smaller, slower, low conductivity and pH with low discharge and smaller streambed particles. PC-4 explained 9.3% of the total inter-site variance. Sites with higher PC-4 scores were at high elevation, cooler stream, high dissolved oxygen and pH with more canopy covers and larger streambed particles. PC-5 accounted for 8.4% of the total variance. Streams with higher PC-5 scores were faster, high conductivity, low oxygen, more stream canopy covers and larger streambed particles. Regression analysis revealed that species richness was significantly associated with PC-1 ($F=20.8$, $df = 1, 422$, $P<0.001$).

Table 5.5: Physicochemical characteristics of all study sites presented as mean and coefficient of variation (CV)

	Site	Parameter	Temperature (°C)	Width (m)	Depth (m)	Velocity (m/s)	Conductivity (mS/cm)	Do (mg/l)	pH	Discharge (m^3/s)
Low elevations	E1	mean	25.03	0.89	0.14	0.42	0.15	4.84	6.51	0.07
		CV	4.89	106.67	47.46	14.73	19.77	76.62	10.8	157.53
	E2	mean	24.13	5.38	0.18	0.47	0.2	5.54	6.79	0.47
		CV	3.12	16.83	31.99	27.11	18.72	68.29	8.88	55.1
	E3	mean	24.26	5.7	0.16	0.44	0.18	5.67	6.93	0.41
		CV	11.24	28.31	42.37	21.51	23.96	71.67	12.39	73.48
	E4	mean	22.55	3.15	0.19	0.65	0.14	5.37	6.99	0.36
		CV	5.09	49.38	39.05	32.43	11.39	53.42	11	61.65
	E5	mean	23.92	0.61	0.12	0.59	0.44	5.94	6.85	0.04
		CV	4.12	30.35	34.65	21.93	16.23	33.45	9.65	52.67
	E6	mean	23.39	0.88	0.1	0.52	0.45	5.84	7.21	0.05
		CV	5.33	30.8	59.13	30.36	21.99	30.43	8.08	78.22
Middle elevations	E7	mean	21.68	1.64	0.22	0.53	0.34	5.54	7.31	0.23
		CV	5.91	38.3	66.78	17.85	18.73	39.59	6.62	130.2
	E8	mean	21.55	0.52	0.05	0.34	0.6	5.48	7.34	0.01
		CV	4.09	38.99	29.14	23.56	13.75	38.92	7.12	53.62
	E9	mean	20.21	1.81	0.14	0.52	0.33	6.35	7.24	0.15
		CV	5.15	50.59	41.25	31.59	14.7	35.53	7.8	84.73
	E10	mean	20.42	0.93	0.14	0.38	0.25	7.52	7.52	0.07
		CV	2.98	61.12	44.8	6	50	7.63	5.13	73.4
	E11	mean	19.36	0.37	0.09	0.35	0.43	5.18	7.06	0.01
		CV	3.97	57.45	88.63	23.1	25.51	43.15	8.04	116.13
High elevations	E12	mean	17.71	0.56	0.09	0.34	0.17	3.89	7	0.02
		CV	5.69	38.44	41.64	24.44	49.45	59.67	8.67	75.36
	E13	mean	18.65	3.07	0.07	0.67	0.53	6.27	7.19	0.16
		CV	6.46	49.6	103.01	23.95	24.91	29.87	12.35	134.23
	E14	mean	18.34	0.73	0.05	0.39	0.23	6.49	6.72	0.01
		CV	4.13	15.05	34.01	42.18	18.2	18.95	11.36	54.31
	E15	mean	17.22	3.17	0.22	0.54	0.18	5.94	6.79	0.37
		CV	4.93	20.22	24.45	25.36	33.85	34.93	9.76	50.13
	E16	mean	17.9	1.03	0.15	0.42	0.33	5.66	6.65	0.09
		CV	6.72	127.76	43.18	28.77	51.06	32.39	10.53	194.76
	E17	mean	17.99	0.62	0.14	0.33	0.18	5.23	6.56	0.02
		CV	6.57	37.14	174.22	36.32	33.86	42.58	9.21	117.08
	E18	mean	17.39	1.35	0.06	0.37	0.71	4.31	6.49	0.03
		CV	8.09	129.59	38.68	69.16	93.68	61.94	8.78	154.23

Table 5.6: Results of principal component analysis and Spearman's rank correlation coefficients between stream variables and principal components for all collections (n=432)

Variable	Stream sites			Principal components				
	Min	Max	Mean ± SE	PC-1	PC-2	PC-3	PC-4	PC-5
Elevation (m)	159.00	1,813.00	891.40 ± 30.1	−0.751**	0.268**	0.522**	0.224**	0.049
Temperature (°C)	14.40	28.40	25.30 ± 0.17	0.636**	−0.230**	−0.622**	−0.285**	−0.059
Width (m)	0.12	7.90	2.95 ± 0.11	0.692**	0.322**	−0.132**	0.044	0.037
Depth (m)	0.02	0.85	0.54 ± 0.005	0.616**	0.171	0.34	0.034	0.124
Velocity (m/s)	0.16	1.03	0.42 ± 0.009	0.416**	0.544**	−0.217**	0.115	0.360**
Conductivity (mS/cm)	0.01	0.15	3.48 ± 0.001	−0.297**	0.162	−0.327**	0.001	0.328**
Dissolved oxygen (mg/l)	1.27	17.20	15.77 ± 0.14	0.148	0.336**	−0.051	0.363**	−0.576**
pH	4.32	8.53	5.20 ± 0.04	0.036	−0.080	−0.337**	0.739**	−0.155
Discharge (m^3/s)	0.002	1.56	2.23 ± 0.01	0.786**	0.376**	−0.208**	0.049	0.137
Canopy cover	open	complete	2‡	0.213**	−0.718**	0.175	0.315**	0.306**
Riparian	open	forest	2‡	0.400**	−0.763**	0.174	0.122	−0.015
Streambed	sand	bedrock	3‡	0.449**	0.176	−0.503**	0.274**	0.366**
% Variance explained in PCA								
Proportion				24.8	15.1	13.8	9.3	8.4
Cumulative				24.8	39.7	53.6	62.8	71.2

** $P < 0.001$

‡Median values given for riparian vegetation (1=open, 2= brush and 3=forest), streambed-particle size (min; 1=mud/silt and max; 6=bedrock), and canopy cover. (1=open, 2=partial and 3=complete). Rankings followed McCreadie *et al*. (2006).

Regression analysis for four black fly species is presented in Table 5.7. Forward logistic regression analyses were conducted for eight species, which were found in more than 10% (FO) of total collections. All regression models of species distribution except *S. decuplum*, *S. izuae*, *S. brevipar* and *S. bishopi*, were significant at $P<0.001$ with correct classification varying from 73.4 to 85.3%. *Simulium whartoni* was positively associated with PC-1 and PC-3. *Simulium* sp. (nr. *feuerborni*) was positively associated with PC-3, *S. tani* was positively associated with PC-1, PC-2 and PC-4. *Simulium angulistylum* was positively associated with PC-1 but negatively associated with PC-3 and PC-4. PC-5 was not related to any species.

An ordination diagram for 18 fixed-stream sites and species are presented in Figure 5.6 and Figure 5.7, respectively. CCA indicated that temperature, stream size and discharge were the most important factors in differentiating streams from different elevations. Therefore, these factors are good predictors for black fly species assemblages. The relationship between species and stream variable conditions was high (> 0.569) for the first three canonical axes, indicating that the variables used in this study were strongly related to black fly species assemblage. Temperature was the most important factor on the CCA axis 1. Species that associated with normal stream temperature were *S. cheongi* and *S. trangense*. The bottom left panel of the biplot is characterized by streams with wider and higher discharge. These sites were predominated by *S. tani*, *S. vanluni* and *S. jeffreyi*. The upper right side of the biplot is composed of sites with lower discharge and smaller streams. Black fly species found predominantly at these sites were *S. bishopi*, *S. izuae* and *S. longitruncum*. The bottom right panel of the biplot is characterized by low water temperature, which is characteristic of high elevation streams. Black flies predominating at these sites were *S. asakoae*, *S. caudisclerum* and *S.* sp. (nr. *feuerborni*) (Figure 5.7).

Table 5.7: Regression analyses for the distribution of preimaginal black fly species at 18 fixed-streams along an elevational gradient in Peninsular Malaysia

Species	Regression coefficient						P	% Correct
	K	PC-1	PC-2	PC-3	PC-4	PC-5		
Simulium whartoni	−1.048	0.403	–	0.741	–	–	< 0.001	73.4
Simulium sp.(nr. *feuerborni*)	−1.449	–	–	1.214	–	–	< 0.001	78.1
Simulium tani	−0.461	1.837	0.59	–	0.393	–	0.001	79.3
Simulium angulistylum	−2.463	0.805	–	−2.012	−0.444	–	< 0.001	85.3

Only species that were present at >10% of the sampling sites were analyzed. K = the intercept

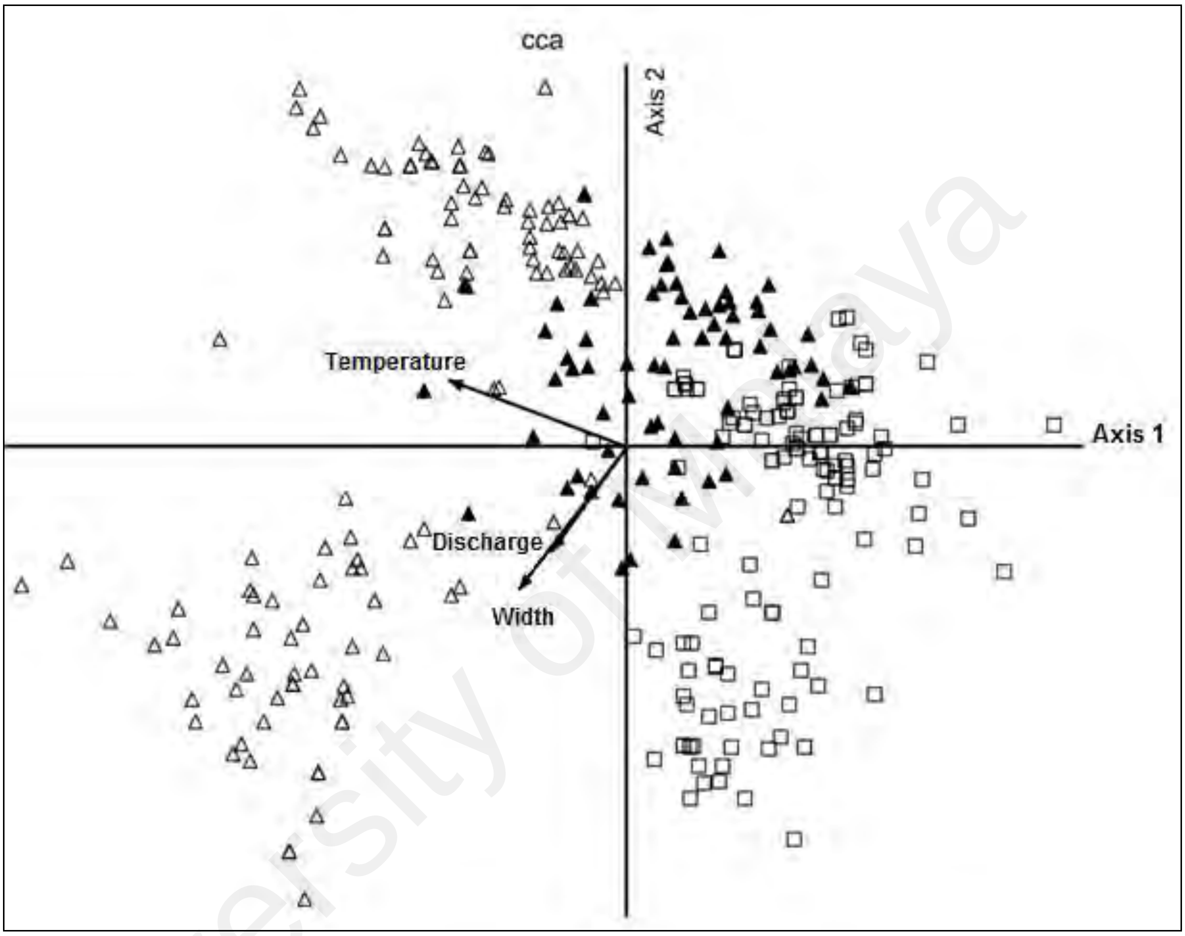

Figure 5.6: Ordination diagram of the first two axes of canonical correspondence analysis (CCA) of 432 sampling collections (*open triangles represent low-elevation sites; closed triangles represent middle-elevation sites; and open square represent high-elevation sites*). Arrows denote environmental variables with strength of the environmental condition indicated by arrow length of closeness to the CCA axis.

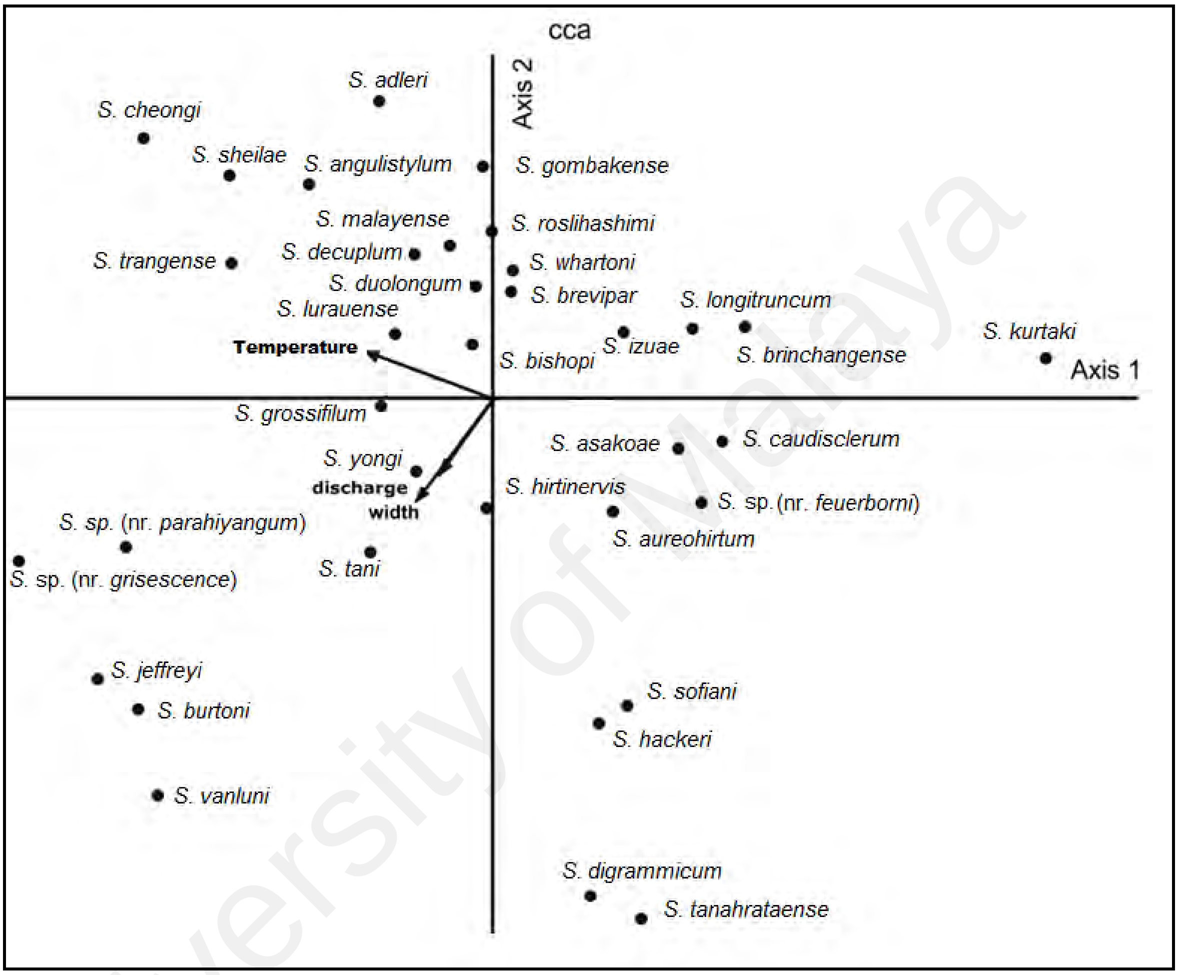

Figure 5.7: Ordination diagram of the first two axes of canonical correspondence analysis (CCA) of the 35 black fly species of the genus *Simulium* in Peninsular Malaysia.

5.4 Discussion and Conclusion

This in-depth survey on the vertical distribution of black flies was conducted for the first time in Peninsular Malaysia and yielded 35 species, representing 42.7% of total simuliids in Malaysia (82 species) (Adler & Crosskey, 2016). *Simulium digrammicum* from Cameron Highland, an earlier described species and previously considered as locally extinct, was discovered in this study. These findings indicated that all sampled streams are the natural breeding habitats for black flies. As far as medico-veterinary importance of black flies is concerned, we collected *S.* (*G.*) *asakoae*, one of the well-known species that have been reported to be infected with filarial parasites in Thailand (Fukuda *et al*., 2003). The host biting habits and vectorial capacity of Malaysian black flies, however, remain unknown.

Average number of species per total collections in this study was 3.2, slightly higher than previously reported (Hamada *et al*., 2002; McCreadie *et al*., 2005; Pramual & Kuvangkadilok, 2009; Pramual & Wongpakam, 2010). This study also revealed 74.3% of the sampled black flies as rare species (FO<10%). This pattern was consistent with the nationwide study in Peninsular Malaysia (Chapter 3) and other geographical regions (Hamada *et al*., 2002; McCreadie & Adler, 2008; Couceiro *et. al*., 2015). In contrast, 22.8% (eight species) of total species had higher frequency of occurrence (10.6 to 31.7%), stream occurrence (44.4 to 77.8%) and total specimens collected, confirming the previous observations where the species were relatively abundant, on average and also widely distributed (Gotelli & Simberloff, 1987; Gaston & Lawton, 1988). The capability of these species to adapt over a broad range of stream physicochemical conditions has allowed them to occur almost in all places and become generalist.

In contrast, rare species require a more specialised habitat, which consequently limits their distribution to certain streams conditions and defines them as specialist. This situation also corroborated with the prediction on taxa distribution (Hutchinson, 1957) and neutral theory (Hubbell, 2005).

This study showed that the five principal components had eigenvalues >1.0 and accounted for 71.2% of total intersite variance of the stream condition variables. Streams at low elevation, with normal water temperature (23°C–25°C), wider, deeper and faster, low conductivity, higher discharge, more canopy covers and riparian vegetations and with larger stream-bed particles, accommodate more preimaginal black fly species. Some of these core factors were consistent with previous studies (Grillet & Barrera, 1997; Scheder & Waringer, 2002; Hamada *et al*., 2002; Pramual & Kuvangkadilok, 2009; Pramual & Wongpakam, 2010; Srisuka *et al*., 2015).

The first two CCA axes indicated the differentiation of low, middle and high elevation streams (Figure 5.5). As expected, temperature, stream size and discharge are varied as elevation increases. The general conditions of low elevation streams were warmer, wider and higher discharge with average mean 23.8°C, 2.9m, and 0.2m^3/s respectively. High elevation sites were cooler (17.8°C) than middle elevation sites (20.6°C). However, the average mean for stream size and discharge observed both at middle (1.0m; 0.09m^3/s) and high (1.5m; 0.1m^3/s) elevations were less different. In fact, stream sites at these elevations are smaller and slower compared to low elevation sites. The present results showed gradual decrease of water temperature values as elevation increased. Dudgeon (2008) suggested low water temperature as the most characteristic feature of high elevation streams. This finding is consistent with previous studies in other geographical regions (Tomanova *et al*., 2007; Henriques-Oliveira & Nessimian, 2010). Besides, Tomanova *et al.* (2007) reported stream depth as another factor that negatively related to elevation.

Regarding difference in elevations, Srisuka *et al*. (2015) indicated that certain Thai simuliids occurred exclusively in a single zone while others were found in almost all gradients. This result was consistent with a previous study on other aquatic macroinvertebrates (Henriques-Oliveira & Nessimian, 2010). The present study corroborates this trend, species such as *Simulium* sp. (nr. *feuerborni*) of the subgenus *Nevermannia* and *S. asakoae* of the subgenus *Gomphostilbia* were found to be restricted to middle and high elevations. The Thai *S. asakoae*, however, was distributed from low to high elevations (500–2,100 m) with predominance at low elevation (Srisuka *et al*., 2015). Based on these distinct ecological conditions between Thai populations and Malaysian populations, coupled with the previous genetic evidence (Low *et al*., 2015), we suggest the presence of cryptic species in *S. asakoae*. Three species in each of the subgenus *Gomphostilbia* (*S. duolongum*, *S. cheongi* and *S. adleri*) and the subgenus *Simulium* (*S. vanluni*, *S. jeffreyi* and *Simulium* sp. nr. *grisescens*) were distributed at low elevation streams in this study (e.g. 159–423 m), further supporting previous published ecological data (Takaoka & Davies, 1995; Jitklang & Kuvangkadilok, 2007). Nearly half of total species collected (45.7% or 16 species) were euryzonal or showing wide vertical distributions. These species, for example, *S. brevipar*, *S. whartoni* and *S. bishopi* were found occupying more than 80% (16 streams) of total surveyed streams varying from low to higher elevations. *Simulium tani* and *S. angulistylum* were found at 12 and ten elevations, respectively (Table 5.4). A similar trend observed in Thai simuliid species such as *S. yuphae* was found distributed from 650–2,534 m (Srisuka *et al*., 2015). This wide vertical distribution pattern was also observed in other aquatic macroinvertebrates in temperate streams (Dudgeon, 2008). Black fly species that are widely distributed and adaptable in various physicochemical conditions are likely to be a species complex (Adler & McCreadie, 1997). This situation has been highlighted in previous studies where the Thai *S. tani* and *S. angulistylum* were found to be a cytological species complexes (Tangkawanit *et al*., 2009; Pramual & Kuvangkadilok, 2012). The present results revealed that some of the widespread species (i.e. *S. tani*, *S. angulistylum*, *S. bishopi*) along this elevational gradient were also commonly found in other locations in Peninsular Malaysia (Chapter 3). Cryptic diversity might be found in Malaysian samples and further cytogenetic and molecular studies would help to clarify this hypothesis.

The observed spatial distributions of preimaginal black flies in this study were predictable on the basis of stream-site characters. These results revealed that distributions of four common species were related to elevation, temperature, stream size, velocity, streambed particle and discharge. Most of these factors are consistent with the patterns observed in tropical streams in the Oriental region and other geographical regions (Hamada *et al*., 2002; McCreadie *et al*., 2005; Pramual & Kuvangkadilok, 2009). Temperature is a well-known variable that reversely correlated with elevation and has been widely associated with black fly distribution (Hamada *et al*., 2002; McCreadie *et al*., 2005; Pramual & Kuvangkadilok, 2009; Pramual & Wongpakam, 2010; Srisuka *et al*., 2015). Moreover, Henriques-Oliveira and Nessimian (2010) indicated that both temperature and stream size were the influencing factors in other aquatic insect distribution and composition in Southeastern Brazil. Based on the present observation, species such as *Simulium* sp. (nr. *feuerborni*) was largely found in cooler streams (E11-E18) with temperature ranging between 14°C and 19°C and the mean between 17°C and 19°C. In a broader context, this species was reported as a species complex, which comprised cytoforms A and B in Thailand (Pramual & Wongpakam, 2013), cytoform C in Malaysia and cytoform D in Indonesia (Pramual *et al*., 2015). In particular, high frequency of B chromosome was detected in the *S. feuerborni* cytoform C, a unique character for temperate and arctic species (Pramual *et al*., 2015). Similarly, *S. caudisclerum*, S. *hackeri*, *S. digrammicum* and *S. tanahrataense* were inhabitants of cooler streams (Takaoka & Davies, 1995; Takaoka *et al*., 2014). The populations of these high-elevation specialists are fragmented and isolated at high elevation probably as a result of a glacial period, and thus, considered more vulnerable to extinction (Finn & Adler, 2006; Adler *et al*., 2010). In contrast, species such as *S. cheongi* was a common inhabitant at normal stream temperatures (23.9°C–25°C) (Takaoka & Davies, 1995). Based on this observation, the availability of larger streams with higher discharge rates gradually decreases with increasing elevation. Species such as *S. jeffreyi* and *S. vanluni*, were largely associated with these stream characters and abundantly found at low elevation. In fact, stream velocity has been emphasized as one of the important factors determining the distribution of black fly larvae (Zhang *et al*., 1998; Palmer & Craig, 2000; Pramual & Kuvangkadilok, 2009; Hamada *et al*., 2002; McCreadie *et al*., 2005; Pangjanda & Pramual, 2015).

Species richness and composition are strongly interconnected with their habitat characteristics (Hamada *et al*., 2002). Species richness could increase with elevation but decline at above 1,500 m (Lawton *et al*., 1987; Wolda, 1987; McCoy, 1990; McCain & Grytnes, 2010; Srisuka *et al*., 2015). The current study corroborated these trends, where species richness started to decline at 1,405 m. Similar results found with other macroinvertebrates where taxa richness starts to decrease as elevation increases (Jacobsen *et al*., 1997; Huamantinco, 2004; Jacobsen, 2004; Henriques-Oliveira & Nessimian, 2010). Regarding species composition observed in cluster analysis, there was a marked separation of streams at similar elevation (1,405 m).

This reflects the remarkable change of abiotic factors particularly in the observed water temperature and thus creates a boundary for most of the species with narrow ecological tolerance except several generalists and highly specialised taxa or high-elevation specialists. A similar pattern was reported in other macroinvertebrate studies along elevational gradients (Palmer *et al*., 1994; Huamantinco & Nessimian, 2004).

Regards to species co-occurrence pattern, the assemblages observed in this study were not random and most of the samplings recorded the co-existence of species (76.6% or 331), implying high stream environmental heterogeneity (e.g. larger stream with larger streambed particles) (Pramual & Kuvangkadilok, 2009). However, species co-existence could also be found in homogenous streams with respect to the availability of microhabitats (Figueirò *et al*., 2012). A group of species in this particular stream would require similar microhabitat preference (Figueiro *et al*., 2012; Pramual *et al*., 2012). A recent study indicated that habitat filtering is a major factor that shaping community structure of black flies in tropical streams (Pangjanda & Pramual, 2015). Co-existence species usually show similar morphological traits that associate with stream conditions. The results revealed that most of the co-occurring species possess similar labral fan morphologies, the food-filtering organ that is strongly associated with stream conditions (Zhang & Malmqvist, 1996). These species for example, *S. angulistylum* and *S. trangense* were found co-existing in 50% of total samplings, while other species such as *S. tani*, *S. jeffreyi* and *Simulium* sp (nr. *parahiyangum*) were found co-occurring in 46% of total samplings. Therefore, patterns of species assemblage of the black flies in tropical streams in Malaysia mirror previous findings in Thailand and suggest that ecological conditions of the larval habitat play a significant role in determining black fly species assemblage.

In conclusion, this comprehensive survey on the vertical distribution of black flies was conducted for the first time in Peninsular Malaysia and yielded 35 species, representing 42.7% of total simuliids in Malaysia. The current study has provided new insight into the distribution patterns of preimaginal black fly along an elevational gradient in Peninsular Malaysia. The results indicated that physicochemical characteristics of the stream habitats that are associated with black fly distribution (e.g. stream size, velocity and temperature) varied along an elevational gradient. Thus, species diversity and assemblages varied accordingly. Certain black fly species are habitat specialists, whereas some are habitat generalists and distributed in wide range of ecological conditions. These species are likely to contain cryptic taxa and further taxonomic study using cytogenetic and molecular methods are required to support this hypothesis. Moreover, this study could deepen the knowledge on the ecology and biology of the specialised taxa in response to environmental changes.

CHAPTER 6: GENERAL DISCUSSION AND RECOMMENDATIONS

As the second most significant group of medically important insects, black flies have been totally neglected in the Southeast Asia region (Currie & Adler, 2008) and as a consequence, nothing is known about their ecology, biology and other related information. Hence, the present study aims to report detailed identification keys and taxonomic descriptions of new species from Peninsular Malaysia. This first comprehensive black fly exploration in the Peninsular Malaysia encompassing northern (Perlis, Perak, Kedah, Pulau Pinang), Eastern (Kelantan, Terengganu and Pahang), Western (Selangor) and southern (Negeri Sembilan and Johor) regions has successfully discovered and described five new species based on mature larva, pupa, male and female namely *Simulium* (*Simulium*) *vanluni*, *Simulium* (*Nevermannia*) *ledangense*, *Simulium* (*Nevermannia*) *pairoti* and *Simulium* (*Gomphostilbia*) *azhari* and *Simulium* (*Gomphostilbia*) *johorense*.

In this study, *S. ledangense*, *S. azhari*, and *S. johorense* were described based solely on morphotaxonomical approach, whereas *S. vanluni* and *S. pairoti* were described with the initial evidence from chromosomal or molecular data. *Simulium vanluni* in Peninsular Malaysia is indeed a true cryptic species with no recognizable morphological variation among the three genetically distinct lineages reported by Low *et al*. (2016). *Simulium pairoti*, on the other hand, is a pseudocryptic species where the morphological differences were only detected after its unique lineage has been revealed from the chromosomal and molecular approaches (Pramual *et al*., 2015). To document both cryptic/pseudocryptic species as the valid species, their formal descriptions and naming were carried out in the present study. The five new species generated in this study have significantly increased the number of Peninsular Malaysian species from 56 to 61.

Immature stages of black flies (i.e. larva and pupa) can reach a density of a million per m^2 in some rivers (Currie & Adler, 2008), and participate in ecosystem processes (e.g., nutrient cycling), thus, they are useful taxa for testing ecological hypotheses and for assessing patterns of biodiversity (Grillet & Barrera, 1997; Hamada & McCreadie, 1999; Hamada & Grillet, 2001; Hamada *et al*., 2002). Black fly assemblage is a colony of different species occurring in similar ecological or habitat requirements. Defining this assemblage is of paramount importance in evaluating the comparative richness of populations, and the effect of isolation or fragmentation (Dufrene & Legendre, 1997). In Malaysia, as well as other countries in Southeast Asia (except Thailand), there is still a lack of information in many aspects of black fly studies including, preimaginal black fly species distribution pattern, co-occurrence pattern, preferred microhabitat of most species, breeding preference of specific species at different geographical scales. Therefore, the present study was conducted for the first time in Peninsular Malaysia, aiming to elucidate preimaginal black fly distribution pattern at a nationwide scale (Chapter 4) and along an elevational gradient scale (Chapter 5).

These extensive surveys revealed that more than half of species were rare species (found only once of total samplings). At nationwide scale, species such as *S. adleri*, *S. aziruni*, *S. azhari*, and *S. kisapense* were only collected once. Meanwhile, at elevational gradient scale, several species were rarely obtained, for example, *S. hackeri*, *S. digrammicum* and *S. caudisclerum.* High numbers of rare species have also been recorded in most of the black fly studies in other geographic regions (Hamada *et al*., 2002; McCreadie & Adler, 2008; Couceiro *et al*., 2014). These species are important as part of organisms influencing the function of ecosystem (Lyons *et al*., 2005). The species occurrence pattern which largely influenced by species dispersal ability and the stream ecological conditions (Adler *et al*., 2004; Pramual & Wongpakam, 2010; Couceiro *et al*., 2014), are the important factors determining whether or not the species are rare. These species require a more specialized habitat, which consequently limits their distribution to certain stream conditions and defines them as specialists. Further investigations on the ecology and biology of these rare species are needed to reveal the possible relationship between the morphological traits and habitat preference as well as to uncover the factors that influence their occurrences.

In contrast, the present study also revealed four common species (e.g. *S. vanluni*, *S. tani*, *S. trangense*, *S. whartoni*), which were found almost in all states in Peninsular Malaysia (Chapter 4). Meanwhile, of total 35 species discovered from 432 samplings conducted along an elevational gradient, 45.7% (Chapter 5) were euryzonal or showing wide vertical distributions varying from low to high elevations. These species were *S. brevipar*, *S. whartoni*, *S. bishopi*, *S. tani* and *S. angulistylum*. The capability of these species to adapt over a broad range of stream physicochemical conditions has allowed them to occur almost in all places and become generalist. Black fly species that are widely distributed and adaptable in various physicochemical conditions are likely to be a species complex (Adler & McCreadie, 1997). This situation has been highlighted in previous studies where the widespread species, *S. tani* and *S. angulistylum* in Thailand were in fact consisted of several complex taxa (Tangkawanit *et al*., 2009; Pramual & Kuvangkadilok, 2012). Therefore, additional cryptic diversity might be found in Malaysian specimens and further chromosomal and molecular studies would help to clarify this hypothesis.

On the other hand, the elevational study (Chapter 5) was successfully discovered specialist species, a group of species that require a more specialized habitat, which limits their distribution to certain stream conditions. This situation corroborates the species distribution theory that strongly interconnected with the habitat characteristics (Hamada *et al*., 2002; McCreadie *et al*., 2005; Pramual & Kuvangkadilok, 2009). Three stream parameters: temperature, stream width and discharge were revealed as the most important ecological variables differentiating the stream at different elevation and were the important limitation factors for species distribution (Chapter 5). *Simulium* sp. (nr. *feuerborni*) of the subgenus *Nevermannia* and *S. asakoae* of the subgenus *Gomphostilbia* were found to be restricted to middle and high elevations (e.g. 1,000 to 1,813 m). At this elevational range, the observed mean stream temperature, width and discharge were 18°C, 1.3 m and 0.08 m^3/s respectively. Species such as *S. hackeri*, *S. digrammicum*, *S. caudisclerum* and *tanahrataense* were distributed at elevation more than 1,300 m asl. The observed mean of associated stream temperature, width and discharge were 17°C, 1.6 m and 0.1 m^3/s respectively. Three species in each of the subgenus *Gomphostilbia* (*S. duolongum*, *S. cheongi* and *S. adleri*) and the subgenus *Simulium* (*S. vanluni*, *S. jeffreyi* and *S.* sp. nr. *grisescens*) were distributed at low elevation streams (e.g., 159–423 m) with the observed mean for associated streams temperature, width and discharge were 24°C, 3.0 m and 0.23 m^3/s respectively. These findings were further supported by the previous published ecological data (Takaoka & Davies, 1995; Jitklang & Kuvangkadilok, 2007). Based on the current findings, water temperature plays as the most obvious stream factor, limiting the species diversification particularly those with small ecological niche tolerance. Therefore, an in-depth investigation should be targeted on these specialists (especially the high-specialized taxa), for deepening the knowledge on their population dynamic and survival strategy in response to the environmental change.

Stream characters such as at low elevation, normal water temperature (23°C–25°C), wider, deeper and faster, low conductivity, higher discharge, more canopy covers and riparian vegetations and larger stream-bed particles tend to accommodate more preimaginal black fly species. These stream ecological characters provide a variety of niches that support various species with different ecological tolerance (Pramual & Kuvangkadilok, 2009). These results are largely consistent with the pattern of species richness found in previous studies in other geographical regions (Grillet & Barrera, 1997; Hamada *et al*., 2002; Scheder & Waringer, 2002; McCreadie *et al*., 2005; Pramual & Kuvangkadilok, 2009; Pramual & Wongpakam, 2010). In addition to the mentioned stream conditions, other associated factors related to black fly ecology have also been pointed out, for example, water chemistry (Townsend *et al*., 1983; Jenkins *et al*., 1984; Erman & Erman, 1995; McCreadie *et al*., 2005), habitat disturbance (Erman & Erman, 1995; Palmer *et al*., 1995; Pramual & Kuvangkadilok, 2009; Couceiro *et al*., 2014), elevation (Tate & Heiny, 1995; Srisuka *et al*., 2015), intense riparian vegetation (Lautenschläger & Kiel, 2005) as well as the labral fan of black fly larvae (Pangjanda & Pramual, 2015).

The findings on the most preferred stream habitat of preimaginal black fly species would be greatly useful as the target areas for future vector control program. It has been suggested that epidemiology and vector-borne disease are affected directly by inter-annual and inter-decade climate changes (Figueiro & Gil-Azevedo, 2010) and it has been expected that there will be an increasing case of vector-borne diseases by the year 2100, as a result of global temperature risen (1.0°C−3.5°C) (Githeko *et al*., 2000). Previous study in Florida (Cilex & Schaediger, 2004) have linked the severe infestation of *Simulium slossonae* Dyar and Shannon with the increased precipitation in the winter, which caused by the El Nino phenomenon. The effects of global climate change in black flies, however, has been little studied in the Southeast Asian region.

Although the current study has significant outputs for the black flies in Peninsular Malaysia, there are couples of limitations need to be addressed. Breeding habitat characters, particularly the biotic factors such as competition and predation, as well as food availability and trophic relationship have not been examined in the current study. Because the current study was a preimaginal-based (i.e. pupa and larva) survey, host-related information such as biting habits, latent period of parasite in the flies, survival rate per gonotrophic cycle and the amount of flies that release all infective larvae at blood meal are not known. Other habitat characteristics in relation to female ovipositional selection behaviour which may influence the larval density and distribution also have not been investigated in this study.

With regards to the limitations outlined herein, several recommendations for future black fly studies are proposed. All species discovered from this study should be subjected for DNA barcoding, phylogenetic and genome studies, a modern biology, aiming to identify the relationship between species and to reveal the cryptic species in Malaysia. An extensive preimaginal black fly survey needs to be conducted in east Malaysia (Sabah and Sarawak) to discover potential new species, species diversity and distribution pattern in relation to the associated environmental data.

The bionomic, population ecology and population dynamic of black fly species, which are all have been widely neglected in the literature, should be carried out accordingly in the Oriental region. Specifically, attention should be given on the species dynamic in response to their associated biotic factors (i.e., competition and predation). In addition, study on the ecological interaction such as symbiosis between trichomycete fungi and black fly larvae could be an interesting topic since this interaction can increase their survival rates under stress conditions (Crosskey, 1990; McCreadie *et al*., 2005). Furthermore, in the context of biological control, a considerable attention can be given on the mermithid parasitism pattern because this endoparasitic nematode known to cause intersexuality development and behaviour modification in black flies (Fredeen, 1970; Sharp & Hunter, 2008).

Identification of the human-biting or/and animal-biting species and detection of their associated pathogens will be beneficial in understanding the disease transmission cycle which will therefore contribute to the technical know-how of implementing effective vector control programs.

CHAPTER 7: CONCLUSION

1. Five new species namely *Simulium* (*Simulium*) *vanluni* sp. nov., *Simulium* (*Nevermannia*) *pairoti* sp. nov., *Simulium* (*Nevermannia*) *ledangense* sp. nov., *Simulium* (*Gomphostilbia*) *azhari* sp. nov. and *Simulium* (*Gomphostilbia*) *johorense* sp. nov. were successfully discovered and described from Peninsular Malaysia.

2. Forty-seven species were discovered from 180 stream points encompassing four geographical regions and 10 states in Peninsular Malaysia.

3. The most abundant species were *S. trangense* (36.7%) and *S. angulistylum* (33.3%), *S. cheongi* (29.4%), *S. tani* (25.6%), *S. vanluni* (16.2%), *S. sheilae* (14.5%) and *S. bishopi* (10.6%).

4. *Simulium vanluni*, *S. trangense*, *S. tani*, *S. sheilae*, *S. angulistylum*, *S. cheongi* and *S. whartoni* were the most widely distributed species in Peninsular Malaysia.

5. Species richness was positively and significantly associated with streams characterized by larger, deeper, faster, higher discharge, larger streambed particles, more riparian vegetation and high pH.

6. *Simulium bishopi* prefers streams located at higher elevation with low water temperature and conductivity, high dissolved oxygen, densely covered and more riparian vegetations.

7. *Simulium vanluni* (previously treated as *S. nobile* Edwards) was likely to breed in streams characterized by at low elevation, wider, deeper, higher discharge and acidity (pH), bigger streambed particles and with a lot of riparian vegetations.

8. *Simulium tani* prefers streams with similar characters with those preferred by *S. vanluni* and *S. bishopi.*

9. *Simulium sheilae* was likely to be an inhabitant for the streams located at low elevation, normal water temperature, high conductivity, low dissolved oxygen, less covered and little riparian vegetation.

10. Thirty-five species were discovered along an elevational gradient, and *S. tani, S. whartoni, S.* sp. (nr. *feuerborni*), *S. decuplum*, *S. angulistylum*, *S. bishopi* and *S. izuae* were the frequently collected species.

11. Species diversity and richness increased with elevation and declined at 1,600 m and above.

12. Species were not randomly distributed and high co-existence were observed (76.6%) in the community along an elevational gradient.

13. Temperature, stream size and discharge were the most important factors in differentiating streams from different elevations.

14. Five patterns of species distribution were observed: (1) six species distributed at low elevation (159–423 m), (2) three species distributed from low to middle elevations (159–1,000 m), (3) two species distributed from middle to high elevations (711–1,813 m) (4) eight species distributed at high elevation (1,405–1,813 m) and (5) 16 species distributed from low to high elevations (generalist species).

15. *Simulium* sp. (nr. *feuerborni*) and *S. asakoae* were found to be restricted to middle and high elevations.

16. *Simulium duolongum*, *S. cheongi* and *S. adleri S. vanluni*, *S. jeffreyi* and *S.* sp. (nr. *grisescens*) were only found at low elevation streams and identified as low-elevation specialist species.

17. *Simulium caudisclerum*, *S. hackeri*, *S. digrammicum* and *S. tanahrataense* were only collected at high elevation streams and designated as high-elevation specialist species.

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PRESENTATIONS

Oral Presentations

1. **Ya'cob**, **Z.**, Takaoka, H., & Sofian-Azirun, M. (2015). The black flies of Johor and taxonomic note on two new species, *Simulium* (*Nevermannia*) *ledangense* sp. nov. and *Simulium* (*Gomphostilbia*) *johorense* sp. nov. The International conference on Biodiversity, 16−17 November 2015, University Tun Hussien Onn, Johor, Malaysia.

2. **Ya'cob**, **Z.**, Takaoka, H., Pramual, P., Low, V. L., & Sofian-Azirun, M. (2016). Breeding habitat preference of preimaginal black flies in tropical streams of Peninsular Malaysia. The Japan Society of Medical Entomology and Zoology, 15−17 April 2016, Tochigi, Japan.

Poster Presentations

1. **Ya'cob, Z.**, Takaoka, H., Sofian-Azirun, M., & Chen, C. D. (2014). Fauna of black flies (Diptera: Simuliidae) along elevational gradients in Malaysia with taxonomic notes on four new species and three new records. International Symposium on Insects, 1−3 November 2014, Bayview Hotel, Malacca, Malaysia.

2. **Ya'cob, Z.**, Takaoka, H., Pramual, P., Low, V. L., & Sofian-Azirun, M. (2016). Distribution pattern of black flies (Diptera: Simuliidae) assemblage along an altitudinal gradient in Peninsular Malaysia. First Asian Simuliidae Symposium, 24−26 July 2016, Mahasarakham University, Maha Sarakham, Thailand.

LIST OF PUBLICATIONS AND PAPERS PRESENTED

Research articles

1. **Ya'cob, Z**., Takaoka, H., Low, V. L., & Sofian-Azirun, M. (2017). First description of a new cryptic species, *Simulium vanluni* from Peninsular Malaysia: An integrated morpho-taxonomical and genetic approach for naming cryptic species in the family Simuliidae. *Acta Tropica*, *167*, 31−39. (**ISI-Cited Publication**)

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8. **Ya'cob**, Z., Takaoka, H., Low, V. L., & Sofian-Azirun, M. Uncovering the mask of the *Simulium feuerborni* complex (Diptera: Simuliidae): description of *Simulium pairoti* sp. nov. from Malaysia based on integrative taxonomic evidence.

Research articles offshoot the current study

1. Takaoka, H., Sofian-Azirun, M., **Ya'cob**, **Z**., Chen, C. D., Low, V. L. & Harmonis (2016). A new species of *Simulium* from Kalimantan, Indonesia with keys to identify 19 Bornean species of the subgenus *Gomphostilbia*. *Journal of Medical Entomology*, *53*, 798−806. (**ISI-Cited Publication**)

2. Takaoka, H., Low, V. L., Sofian-Azirun, M., Otsuka, Y., **Ya'cob**, **Z**., Chen, C. D., Lau, K. W., & Lardizabal, M. L. (2016). Dimorphic male scutal patterns and upper-eye facets of *Simulium mirum* n. sp. (Diptera: Simuliidae) from Malaysia. *Parasites & Vectors*, *9*, 136. (**ISI-Cited Publication**)

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8. Takaoka H, Sofian-Azirun, M., **Ya'cob, Z**, Hashim, R., & Otsuka, Y. (2014). A New Species of *Simulium* (*Gomphostilbia*) (Diptera: Simuliidae) From Malaysia, with keys to 32 species of the *Simulium ceylonicum* species-group. *Journal of Medical Entomology*, *51*, 517−528. (**ISI-Cited Publication**)

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Highlands, Peninsular Malaysia, with keys to 21 species of the *Simulium asakoae* species-group. *Zootaxa*, *3765*, 54–68. (**ISI-Cited Publication**)

12. Takaoka, H., Sofian-Azirun, M., & **Ya'cob**, **Z**. (2014). Two new species of the *Simulium batoense* species-group of *Simulium* (*Gomphostilbia*) (Diptera: Simuliidae) from Peninsular Malaysia. *Zootaxa*, *3774*, 473–480. (**ISI-Cited Publication**)

13. Takaoka, H., Sofian-Azirun, M., & **Ya'cob**, **Z**., Chen, C. D., Lau, K. W., & Pham, X. A. (2014). Three new species of *Simulium* (*Nevermannia*) (Diptera: Simuliidae) from Vietnam. *Zootaxa*, *3866*, 555–571. (**ISI-Cited Publication**)

14. Low, V. L., Takaoka, H., Pramual, P., Adler, P. H., **Ya'cob**, **Z**., Chen, C. D., Yotopranoto, S., Zaid, A., Hadi, U. K., Lardizabal, M. L., Nasruddin-Roshidi, A., & Sofian-Azirun, M. (2016). Three taxa in one: Cryptic diversity in black fly *Simulium nobile* (Diptera: Simuliidae) in Southeast Asia. *Journal of Medical Entomology*, *53*, 972−976. (**ISI-Cited Publication**)

15. Low, V. L., Takaoka, H., Adler, P. H., **Ya'cob, Z**., Norma-Rashid, Y., Chen, C. D., & Sofian-Azirun, M. (2015). A multi-locus approach resolves the phylogenetic relationships of the *Simulium asakoae* and *Simulium ceylonicum* species groups in Malaysia: evidence for distinct evolutionary lineages. *Medical and Veterinary Entomology*, *29*, 330−337. (**ISI-Cited Publication**)

16. Pramual, P., Thaijarern, J., Sofian-Azirun, M., **Ya'cob**, **Z**., Hadi, U. K. & Takaoka, H. (2015). Cytogenetic and molecular evidence of additional cryptic

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17. Low, V. L., Adler, P. H., Takaoka, H., **Ya'cob**, **Z**., Lim, P. E., Tan, T. K., Lim, Y. A. L., Chen, C. D., Norma-Rashid, Y., & Sofian-Azirun, M. (2014). Mitochondrial DNA markers reveal high genetic diversity but low genetic differentiation in the black fly *Simulium tani* Takaoka & Davies along an elevational gradient in Malaysia. *PLoS ONE*, *9*, e100512 (**ISI-Cited Publication**)

Proceedings

1. **Ya'cob**, **Z.**, Takaoka, H., Pramual, P., Low, V. L, & Sofian-Azirun, M. (2016). Breeding habitat preference of preimaginal black flies in tropical streams of Peninsular Malaysia. *Medical Entomology and Zoology*, *67*(Supplement), 94. (Non-ISI/Non-SCOPUS Cited Publication)

2. Chen, C. D., Lau, K. W., Takaoka, H., Tan, P. R., Chin, A. C., Low, V. L., Norma-Rashid, Y., **Ya'cob**, **Z**., & Sofian-Azirun M. (2016). Insecticide susceptibility status of black fly, *Simulium nobile* (Diptera: Simuliidae) against DDT, dieldrin, propoxur, malathion and permethrin in Malaysia. *Medical Entomology and Zoology*, *67*(Supplement), 95. (Non-ISI/Non-SCOPUS Cited Publication).

3. Low, V. L., Takaoka, H., Adler, P. H., **Ya'cob**, **Z**., Norma-Rashid, Y., Chen, C. D., & Sofian-Azirun, M. (2015). Phylogenetic relationships of the *Simulium asakoae* and *Simulium ceylonicum* species groups (Diptera: Simuliidae) in Malaysia. *Medical Entomology and Zoology*, *67*(Supplement), 95. (Non-ISI/Non-SCOPUS Cited Publication).

APPENDICES

Appendix A: Location details and associated ecological data of 180 sampled points across Peninsular Malaysia.

State	Stream Code	GPS	Province	Location	altitude (m)	temp (°C)
Pahang	KG1	N03°55.237′ E102°33.251′	Jerantut	Kota Gelanggi	94	26.0
Pahang	KG2	N03°55.824′ E102°33.417′	Jerantut	Kota Gelanggi	91	26.0
Pahang	KG3	N03°54.028′ E102°31.944′	Jerantut	Kota Gelanggi	52	24.0
Pahang	KG4	N03°01.323′ E102°36.909′	Jerantut	Kota Gelanggi	136	23.0
Pahang	KG5	N03°59.892′ E102°35.626′	Jerantut	Kota Gelanggi	97	24.0
Pahang	KG6	N04°00.817′ E102°38.157′	Jerantut	Kota Gelanggi	118	24.0
Pahang	KG7	N04°00.751′ E102°39.150′	Jerantut	Kota Gelanggi	126	24.0
Pahang	KG8	N04°01.163′ E102°36.694′	Jerantut	Kota Gelanggi	66	23.0
Pahang	KG9	N04°05.880′ E102°37.306′	Jerantut	Kota Gelanggi	184	23.0
Pahang	KG10	N04°06.685′ E102°37.109′	Jerantut	Kota Gelanggi	287	26.0
Pahang	KG11	N04°06.919′ E102°36.801′	Jerantut	Kota Gelanggi	387	23.0
Pahang	LT1	N04°06.959′ E102°36.618′	Jerantut	Lepar/Tekam	405	24.0
Pahang	LT3	N04°06.694′ E102°37.126′	Jerantut	Lepar/Tekam	265	24.0
Pahang	LT4	N04°05.857′ E102°37.315′	Jerantut	Lepar/Tekam	162	24.0
Pahang	LT5	N04°05.023′ E102°37.285′	Jerantut	Lepar/Tekam	123	24.0
Pahang	LT6	N04°01.807′ E102°29.046′	Jerantut	Lepar/Tekam	247	23.0
Pahang	LT7	N04°03.180′ E102°29.393′	Jerantut	Lepar/Tekam	394	24.0
Pahang	LT8	N03°57.433′ E102°44.161′	Jerantut	Lepar/Tekam	119	24.0
Pahang	LT9	N03°57.475′ E102°44.427′	Jerantut	Lepar/Tekam	137	25.0
Pahang	LT10	N03°59.545′ E102°45.943′	Jerantut	Lepar/Tekam	233	24.0
Pahang	LT11	N03°56.075′ E102°26.797′	Jerantut	Lepar/Tekam	62	24.0
Pahang	JT1	N03°45.176′ E102°39.058′	Jerantut	Jerantut	71	26.0
Pahang	JT2	N03°38.996′ E102°41.759′	Jerantut	Jerantut	65	27.0
Pahang	JT3	N03°51.456′ E102°17.705′	Jerantut	Jerantut	70	24.0
Pahang	JT4	N03°51.659′ E102°17.838′	Jerantut	Jerantut	91	24.0
Pahang	JT6	N03°51.788′ E102°17.680′	Jerantut	Jerantut	106	25.0
Pahang	JT7	N03°59.923′ E102°07.277′	Jerantut	Jerantut	93	26.0
Pahang	MT1	N04°38.784′ E102°08.235′	Lipis-Merapoh	Mount Tahan	388	25.0
Pahang	MT2	N04°38.610′ E102°08.207′	Lipis-Merapoh	Mount Tahan	239	25.0
Pahang	MT6	N04°39.172′ E102°10.643′	Lipis-Merapoh	Mount Tahan	685	21.2
Pahang	MT7	N04°39.245′ E102°11.114′	Lipis-Merapoh	Mount Tahan	769	21.1
Pahang	MT8	N04°38.300′ E102°18.966′	Lipis-Merapoh	Mount Tahan	346	22.6
Pahang	MT9	N04°33.282′ E102°18.995′	Lipis-Merapoh	Mount Tahan	212	22.7
Pahang	MT10	N04°33.195′ E102°19.106′	Lipis-Merapoh	Mount Tahan	212	22.7
Pahang	MT11	N04°32.994′ E102°19.254′	Lipis-Merapoh	Mount Tahan	218	23.0
Pahang	MT12	N04°32.843′ E102°19.249′	Lipis-Merapoh	Mount Tahan	225	22.8
Pahang	MT13	N04°32.644′ E102°19.253′	Lipis-Merapoh	Mount Tahan	236	23.4
Pahang	MT14	N04°32.424′ E102°19.327′	Lipis-Merapoh	Mount Tahan	227	23.9
Pahang	MT15	N04°32.420′ E102°19.554′	Lipis-Merapoh	Mount Tahan	226	23.4
Pahang	MT16	N04°32.349′ E102°19.755′	Lipis-Merapoh	Mount Tahan	224	23.6
Pahang	MT17	N04°32.310′ E102°19.856′	Lipis-Merapoh	Mount Tahan	222	23.2
Pahang	MT18	N04°27.453′ E102°23.084′	Lipis-Merapoh	Mount Tahan	165	23.7

Pahang	MT19	N04°27.454′ E102°23.083′	Lipis-Merapoh	Mount Tahan	116	23.2
Pahang	MT20	N04°27.456′ E102°23.089′	Lipis-Merapoh	Mount Tahan	115	23.0
Pahang	MT21	N04°24.013′ E102°24.184′	Lipis-Merapoh	Mount Tahan	110	24.0
Pahang	MT22	N04°24.064′ E102°24.174′	Lipis-Merapoh	Mount Tahan	96	24.1
Pahang	GA1	N04°04.910′ E102°53.161′	Jerantut	Gunung Aiss	272	25.5
Pahang	GA2	N04°08.075′ E102°51.029′	Jerantut	Gunung Aiss	312	25.0
Pahang	GA3	N04°05.978′ E102°49.826′	Jerantut	Gunung Aiss	186	25.0
Pahang	FH1	N03°43.464′ E101°42.864′	Raub	Fraser Hill	956	20.0
Pahang	FH2	N03°43.118′ E101°42.963′	Raub	Fraser Hill	1002	20.0
Pahang	FH3	N03°43.497′ E101°42.861′	Raub	Fraser Hill	950	20.0
Pahang	CHS14	N04º34.760' E101º20.507'	Cameron	Cameron Highland	1602	18.8
Pahang	CHS11	N04º24.112' E101º22.356'	Cameron	Cameron Highland	1405	17.0
Pahang	CHS15	N04º28.738' E101º22.979'	Cameron	Cameron Highland	1813	15.3
Pahang	RBS17	N04º23.715' E101º36.443'	Raub	Raub	423	24.2
Terengganu	PR1	N04°35.125′ E102°57.355′	Dungun	Pasir Raja	210	24.0
Terengganu	PR2	N04°34.365′ E102°57.960′	Dungun	Pasir Raja	109	25.0
Terengganu	PR3	N04°34.079′ E102°57.605′	Dungun	Pasir Raja	82	24.0
Terengganu	PR4	N04°33.985′ E102°57.429′	Dungun	Pasir Raja	95	24.0
Terengganu	PR5	N04°33.251′ E102°57.074′	Dungun	Pasir Raja	238	25.0
Terengganu	PR6	N04°35.372′ E102°56.871′	Dungun	Pasir Raja	191	24.0
Terengganu	PR7	N04°35.488′ E102°56.464′	Dungun	Pasir Raja	243	23.0
Terengganu	TK1	N04°35.372′ E102°56.871′	Hulu Terengganu	Tasik Kenyir	159	24.0
Terengganu	TK2	N04°32.300′ E102°57.784′	Hulu Terengganu	Tasik Kenyir	164	24.0
Terengganu	TK3	N04°46.582′ E102°45.283′	Hulu Terengganu	Tasik Kenyir	165	25.0
Terengganu	TK4	N04°46.714′ E102°45.106′	Hulu Terengganu	Tasik Kenyir	165	25.0
Terengganu	TK5	N04°57.907′ E102°50.465′	Hulu Terengganu	Tasik Kenyir	415	24.0
Kelantan	GS1	N05°22.111′ E101°56.016′	Gua Musang	Gunung Stong	400	23.0
Kelantan	GS2	N05°22.156′ E101°55.111′	Gua Musang	Gunung Stong	251	25.0
Kelantan	JP3	N05°44.059'E102°22.039'	Pasir Puteh	Jeram Pasu	150	25.0
Kelantan	LR5	N05°18.067'E102°17.097'	Kuala Krai	Lata Rek	144	26.0
Kelantan	L01	N04°40.677′ E101°31.481′	Gua Musang	Lojing	497	23.4
Kelantan	L02	N04°40.731′ E101°31.795′	Gua Musang	Lojing	436	22.8
Kelantan	L03	N04°41.106′ E101°36.261′	Gua Musang	Lojing	413	23.0
Kelantan	L04	N04°43.435′ E101°40.201′	Gua Musang	Lojing	354	30.9
Kelantan	L05	N04°43.682′ E101°40.770′	Gua Musang	Lojing	338	29.3
Kelantan	BG1	N05°07.699′ E101°31.511′	Tanah Merah	Batu Gajah	156	22.9
Kelantan	BG2	N05°07.699′ E101°31.511′	Tanah Merah	Batu Gajah	140	24.0
Kelantan	BG3	N05°07.699′ E101°31.511′	Tanah Merah	Batu Gajah	90	24.0
Kelantan	BG4	N05°07.699′ E101°31.511′	Tanah Merah	Batu Gajah	115	21.0
Kelantan	BG5	N05°07.699′ E101°31.511′	Tanah Merah	Batu Gajah	124	22.5
Kelantan	JL6	N05°07.699′ E101°31.511′	Jeli	Jeli	225	24.7
Kelantan	JL7	N05°07.699′ E101°31.511′	Jeli	Jeli	130	24.0
Kelantan	JL8	N05°07.699′ E101°31.511′	Jeli	Jeli	210	24.0
Kelantan	JL9	N05°07.699′ E101°31.511′	Jeli	Jeli	112	25.0

Kelantan	BG10	N05°07.699′ E101°31.511′	Tanah Merah	Batu Gajah	251	23.0
Kelantan	BG11	N05°07.699′ E101°31.511′	Tanah Merah	Batu Gajah	253	23.7
Kelantan	BG12	N05°07.699′ E101°31.511′	Tanah Merah	Batu Gajah	350	23.0
Kedah	LK1	N06°22.334′ E099°51.924′	Langkawi	Langkawi	42	24.0
Kedah	LK2	N06°24.078′ E099°49.251′	Langkawi	Langkawi	142	23.0
Kedah	LK3	N06°21.635′ E099°41.776′	Langkawi	Langkawi	32	25.0
Kedah	LK4	N06°22.270′ E099°41.047′	Langkawi	Langkawi	10	25.0
Kedah	LK5	N06°23.597′ E099°42.204′	Langkawi	Langkawi	22	24.0
Kedah	LK6	N06°23.887′ E099°42.167′	Langkawi	Langkawi	102	25.5
Kedah	LK7	N06°24.319′ E099°45.391′	Langkawi	Langkawi	32	25.6
Kedah	LK8	N06°23.815′ E099°44.592′	Langkawi	Langkawi	45	26.0
Kedah	LK9	N06°23.532′ E099°42.843′	Langkawi	Langkawi	29	25.0
Kedah	LK10	N06°23.875′ E099°40.368′	Langkawi	Langkawi	100	25.0
Kedah	LK11	N06°23.140′ E099°47.409′	Langkawi	Langkawi	176	24.0
Kedah	LK12	N06°23.359′ E099°47.575′	Langkawi	Langkawi	227	24.0
Kedah	LK13	N06°23.103′ E099°48.151′	Langkawi	Langkawi	491	23.0
Kedah	LK14	N06°23.189′ E099°48.495′	Langkawi	Langkawi	609	23.0
Kedah	LK15	N06°25.035′ E099°40.479′	Langkawi	Langkawi	179	24.0
Kedah	LK16	N06°23.036′ E099°40.482′	Langkawi	Langkawi	182	24.0
Kedah	LK17	N06°22.287′ E099°40.461′	Langkawi	Langkawi	33	25.0
Kedah	SS2	N05°24.753′ E100°46.762′	Kulim	Sungai Sedim	81	24.0
Kedah	SS3	N05°24.495′ E100°47.138′	Kulim	Sungai Sedim	196	23.0
Kedah	SS5	N05°24.251′ E100°46.988′	Kulim	Sungai Sedim	144	24.0
Kedah	SS7	N05°24.118′ E101°46.833′	Kulim	Sungai Sedim	141	24.0
Kedah	SS8	N05°24.761′ E100°46.865′	Kulim	Sungai Sedim	115	23.0
Kedah	GJ15	N05°48.780′ E100°26.461′	Kuala Muda	Gunung Jerai	757	22.0
Kedah	GJ16	N05°24.749′ E100°26.413′	Kuala Muda	Gunung Jerai	779	22.0
Kedah	YB18	N05°48.403′ E100°26.636′	Yan	Yan Besar	31	25.0
Kedah	PJ1	N06°22.391′ E100°33.488′	Padang Terap	Puncak Janing	111	24.0
Kedah	WG2	N05°59.277′ E100°53.752′	Baling	Weng	281	24.0
Kedah	LB3	N05°43.018′ E100°48.889′	Baling	Lata Bayu	62	21.0
Kedah	BH4	N05°43.013′ E100°48.893′	Baling	Bukit Hijau	110	23.0
Kedah	SS5	N05°24.251′ E100°46.988′	Kulim	Sungai Sedim	196	23.0
Perak	CD19	N04°21.282′ E101°14.181′	Batang Padang	Chenderiang	129	24.0
Perak	TP2	N04°14.203' E101°18.354'	Batang Padang	Tapah	159	23.0
Perak	TP3	N04°16.316' E101°19.022'	Batang Padang	Tapah	235	22.0
Perak	TP4	N04°16.522' E101°18.996'	Batang Padang	Tapah	224	22.0
Perak	PP1	N04°14.392′ E100°34.184′	Larut Matang	Pulau Pangkor	28	25.0
Perak	PP2	N04°14.833′ E100°33.625′	Larut Matang	Pulau Pangkor	53	26.0
Perak	BL3	N04°52.013′ E100°45.748′	Manjung	Bukit Larut	117	24.0
Perak	BL6	N04°51.776′ E100°45.700′	Manjung	Bukit Larut	70	28.0
Perak	LG7	N05°10.910′ E100°59.989′	Hulu Perak	Lenggong	102	23.0
Perak	LG8	N05°10.632′ E100°56.036′	Hulu Perak	Lenggong	468	22.7
Perak	LG9	N05°10.418′ E100°55.789′	Hulu Perak	Lenggong	533	22.4
Perak	LG10	N05°10.418′ E100°55.789′	Hulu Perak	Lenggong	514	23.1
Perak	BH11	N04°49.583′ E100°51.183′	Kuala Kangsar	Batu Hampar	110	23.4
Perak	BH12	N04°49.577′ E100°51.151′	Kuala Kangsar	Batu Hampar	117	23.9
Perak	JG13	N04°38.178′ E100°51.962′	Kuala Kangsar	Jeliang	118	25.0

Perak	UC14	N04°42.344′ E100°04.482′	Kinta	Ulu Chepor	115	24.0
Perak	UC15	N04°42.344′ E100°04.482′	Kinta	Ulu Chepor	94	25.0
Perak	UC16	N04°42.344′ E100°04.482′	Kinta	Ulu Chepor	26	25.0
Perak	GP17	N04°27.397′ E100°13.369′	Kinta	Gopeng	138	23.0
Perak	GP18	N04°27.385′ E101°12.265′	Kinta	Gopeng	132	25.0
Perak	SP1	N04º34.760' E101º20.507'	Kinta	Simpang Pulai	1345	19.0
Perlis	BM1	N06°32.800′ E100°10.021′	Mata Ayer	Bukit Mata Ayer	69	24.0
Perlis	GK2	N03°59.923′ E102°07.277′	Kaki Bukit	Gua Kelam	50	24.0
Perlis	SP3	N06°42.075′ E100°11.937′	Kaki Bukit	State Park	155	23.1
Perlis	SP4	N06°41.963′ E100°11.580′	Kaki Bukit	State Park	158	24.0
Perlis	WK5	N06°40.495′ E100°11.287′	Kaki Bukit	Wang Kelian	121	24.5
Pulau Pinang	YP1	N 05°25.974' E100°17.825	Balik Pulau	Youth Park	132	25.0
Pulau Pinang	BG2	N05°25.999' E100°16.001'	Balik Pulau	Botanical Garden	251	24.0
Pulau Pinang	SA3	N05°19.909' E100°15.979'	Balik Pulau	Sungai Ara	150	24.2
Pulau Pinang	BP4	N05°21.002' E100°14.097'	Balik Pulau	Balik Pulau	140	24.4
Johor	GL2	N02°19.262′ E102°37.033′	Ledang	Gunung Ledang	67	27.8
Johor	GL3	N02°19.706′ E103°37.041′	Ledang	Gunung Ledang	79	28.5
Johor	GL4	N02°20.485′ E102°36.944′	Ledang	Gunung Ledang	119	24.6
Johor	GL5	N02°20.490′ E102°37.039′	Ledang	Gunung Ledang	143	24.0
Johor	GL6	N02°22.116′ E102°36.518′	Ledang	Gunung Ledang	1113	18.0
Johor	GL7	N02°22.076′ E102°36.615′	Ledang	Gunung Ledang	1011	16.3
Johor	GL8	N02°22.076′ E102°36.616′	Ledang	Gunung Ledang	1001	18.0
Johor	GL9	N02°21.857′ E102°36.542′	Ledang	Gunung Ledang	973	19.8
Johor	GL10	N02°03.915′ E103°31.576′	Ledang	Gunung Ledang	78	23.9
Johor	KT11	N01°22.076′ E102°36.615′	Kota Tinggi	Kota Tinggi	27	27.9
Johor	KT12	N01°49.786′ E103°50.038′	Kota Tinggi	Kota Tinggi	50	25.5
Johor	GP13	N01°48.666′ E103°52.475′	Kota Tinggi	Gunung Panti	53	24.6
Johor	GP14	N01°35.423′ E103°31.018′	Pontian	Gunung Pulai1	67	26.3
Johor	GP15	N01°34.336′ E103°30.856′	Pontian	Gunung Pulai2	65	24.0
Johor	GP16	N01°48.666′ E103°52.475′	Pontian	Gunung Pulai2	65	24.0
Johor	MR17	N01°48.666′ E103°52.475′	Mersing	Mersing	115	25.0
Negeri Sembilan	JB1	N03°01.269′ E102°02.053′	Jelebu	Jelebu	184	23.7
Negeri Sembilan	JB2	N02°49.951′ E102°02.563′	Jelebu	Jelebu	952	21.3
Negeri Sembilan	JB3	N03°01.269′ E102°02.053′	Jelebu	Jelebu	150	25.0
Selangor	HL9	N03°04.751′ E101°47.098′	Cheras	Hulu Langat	141	24.4
Selangor	HL7	N03°04.751′ E101°47.098′	Cheras	Hulu Langat	140	24.4
Selangor	HL8	N03°04.751′ E101°47.098′	Cheras	Hulu Langat	140	24.4
Selangor	HL5	N03°04.751′ E101°47.098′	Cheras	Hulu Langat	145	25.0
Selangor	UY1	N03°19.882′ E101°42.120′	Hulu Selangor	Ulu yam	226	23.7
Selangor	UY2	N03°21.103′ E101°41.874′	Hulu Selangor	Ulu yam	365	23.1
Selangor	UY3	N03°21.791′ E101°41.643′	Hulu Selangor	Ulu yam	251	24.0
Selangor	SD5	N03°24.353′ E101°41.010′	Hulu Selangor	Sungai Sendat	140	24.4
Selangor	SM6	N03°26.145′ E101°44.302′	Hulu Selangor	Semangkok	315	24.0
Selangor	SM7	N03°36.435′ E101°41.485′	Hulu Selangor	Semangkok	333	24.7
Selangor	SM8	N03°37.292′ E101°44.504′	Hulu Selangor	Semangkok	408	23.0
Selangor	SM9	N03°38.665′ E101°44.435′	Hulu Selangor	Semangkok	645	22.0