

**BIO-ECOLOGICAL STUDIES OF MALAYSIAN
ODONATES AND AN INTEGRATED TAXONOMIC STUDY
ON THE GENUS *Rhinocypha***

NOORHIDAYAH BINTI MAMAT

**FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
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ODONATES AND AN INTEGRATED TAXONOMIC
STUDY ON THE GENUS *Rhinocypha***

NOORHIDAYAH BINTI MAMAT

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**BIO-ECOLOGICAL STUDIES OF MALAYSIAN ODONATES AND AN
INTEGRATED TAXONOMIC STUDY ON THE GENUS *Rhinocypha***

ABSTRACT

Studies on Odonata have gained worldwide attention as well as here, locally in Malaysia. Although there is a wealth of data available to be utilized for solving taxonomic problems but ecological and behavioural research areas are more favoured in contrast to taxonomy and systematics. Thus, there are existing confusions for correct identifications in closely related and sympatric species, especially in female odonates. One such example is in the genus *Rhinocypha*, in which one of the objectives of this study is to fill in this gap. Consequently, the research aims and study techniques were; to illuminate the nationwide distribution and diversity of Odonata from Peninsular Malaysia forest reserves and relate these to the environmental parameters. Secondly, applying the molecular technique to elucidate the phylogeographic pattern of *Rhinocypha fenestrella* which a predominant species in this study. Thirdly, a focused taxonomic work was conducted on *Rhinocypha*, employing multi-approaches, in the form of morphological procedures (Field Emission Scanning Electron Microscope, FESEM and geometric morphometric analysis); bio-material property investigations by using Laser Scanning Confocal Microscopy (LSCM), Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM). Overall, 1193 individuals from 70 species were collected from the 22 sampling localities. Chlorocyphidae was the dominant family and *R. fenestrella*, *R. biforata*, and *Euphaea ochracea* were the most abundant species. The PCA analysis confirmed that higher species richness was associated with the lower water chemical characteristics (compositions of sulphate, ammonia, iron and nitrite). The genetic diversity, expressed by CO1 and 16S rRNA genes for *R. fenestrella* was high, with 26 and 10 unique haplotypes, while 33

haplotypes were recovered by both combined datasets. The TCS analysis revealed the common ancestor of *R. fenestrella* was from the state of Negeri Sembilan. For the taxonomic study, 17 morphological characteristics were created to differentiate between the females of *Rhinocypha* spp. The FESEM on the female's ovipositor was done to focus on the anal appendages and sheathing valve (V3). Also, the phylogenetic patterns and canonical variate analysis for the wing geomorphometry revealed three clusters that supported the distinction of the *Rhinocypha* group. Exploration on bio-material, illustrate a general widespread distribution of resilin patches and cuticular spikes along the longitudinal veins of the wings. A novel technique applied in this study, was on nanoindentation (AFM) clarified the presence of varying size of nanostructures for all sample sections (membranes, mobile and immobile joints), and the elasticity values differed between sections. In summary, this study had effectively developed an integrated approach of classic morphological and trendy molecular as well as biomaterial studies, combined with different microscopy techniques, LSCM, SEM and AFM which provided corroborative evidence in resolving taxonomic uncertainties.

Keywords: Atomic force microscopy, dragonflies, laser scanning electron microscopy, nanoindentation, phylogeography

**KAJIAN BIO-EKOLOGI PEPATUNG MALAYSIA DAN KAJIAN INTEGRASI
TAKSONOMI KE ATAS GENUS *Rhinocypha***

ABSTRAK

Kajian mengenai Odonata telah mendapat perhatian di seluruh dunia sama seperti mana di sini, di Malaysia. Walaupun terdapat banyak data yang tersedia untuk digunakan dalam menyelesaikan masalah taksonomi, tetapi bidang penyelidikan ekologi dan tingkah laku lebih disukai dan berbeza dengan bidang taksonomi dan sistematik. Oleh itu, terdapat permasalahan dalam mengenal pasti spesies-spesies yang rapat dan spesies yang simpatrik, terutamanya bagi pepatung betina. Salah satu contohnya adalah dalam genus *Rhinocypha*, di mana salah satu objektif kajian ini adalah untuk mengisi jurang yang terdapat di sini. Oleh yang demikian, objektif serta teknik penyelidikan utama bagi kajian ini adalah; untuk melaporkan corak taburan dan kepelbagaian pepatung di seluruh negara dari hutan rizab di Semenanjung Malaysia dan mengaitkannya dengan parameter persekitaran. Kedua, dengan menggunakan teknik molekular untuk menjelaskan corak filogeografik bagi spesies *Rhinocypha fenestrella* yang merupakan spesies yang dominan dalam kajian ini. Ketiga, kajian taksonomi difokuskan ke atas kumpulan *Rhinocypha* dengan menggunakan pelbagai teknik, bagi kaedah morfologi (Mikroskop Elektron Imbasan Pancaran Medan, FESEM dan analisis morfometrik geometri) dan bagi analisis sifat bio-material, dengan menggunakan pengimbasan mikroskop confokal laser (LSCM), pengimbasan mikroskop elektron (SEM) dan mikroskop daya atom (AFM). Secara keseluruhannya, 1193 individu daripada 70 spesies telah direkodkan dari 22 kawasan penyempelan. Keluarga Chlorocyphidae adalah yang dominan dan spesies *R. fenestrella*, *R. biforata*, dan *Euphaea ochracea* adalah spesies paling banyak direkodkan. Analisis PCA mengesahkan bahawa kekayaan spesies yang lebih tinggi dikaitkan dengan kandungan sifat kimia air yang rendah

(komposisi sulfat, ammonia, besi dan nitrit). Bagi kepelbagaian genetik spesies *R. fenestrella* daripada gen CO1 dan 16S rRNA, adalah tinggi dengan 26 dan 10 haplotip unik, manakala 33 haplotip diperolehi daripada gabungan kedua-dua dataset. Analisis TCS mendedahkan bahawa keturunan spesies *R. fenestrella* adalah berasal daripada Negeri Sembilan. Untuk kajian taksonomi, 17 ciri morfologi telah dicipta untuk membezakan spesies betina bagi kumpulan *Rhinocypha*. FESEM pada ovipositor betina dijalankan untuk memberi tumpuan kepada bahagian anal dan injap pelapis (V3). Selain itu, corak filogenetik dan analisis variasi kanonik untuk kajian geomorfometrik sayap menunjukkan tiga kelompok yang berbeza yang menyokong pembezaan di antara kumpulan *Rhinocypha*. Eksplorasi mengenai sifat bio-material sayap mendedahkan bahawa terdapat taburan yang umum bagi tompokan resilin dan duri di sepanjang urat membujur sayap. Kaedah baru yang digunakan dalam kajian ini iaitu nanoindentasi (AFM), menjelaskan kehadiran pelbagai saiz struktur nano untuk semua bahagian sampel (membran, sendi bergerak dan tidak bergerak), dan nilai keanjalannya berbeza-beza di antara bahagian. Secara ringkasnya, kajian ini secara efektif telah membangunkan pendekatan bersepadu bagi kaedah klasik morfologi dan kaedah molekular terkini bersama-sama dengan kajian bio-material, yang digabungkan dengan teknik mikroskopi yang berbeza iaitu LSCM, SEM dan AFM, dapat memberikan keterangan dan bukti yang kuat untuk menyelesaikan masalah taksonomi.

Kata kunci: Filogeografik, mikroskop daya atom, nanoindentasi, pengimbasan mikroskop confokal laser, pepatan

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	degree Celsius
=	equal to
δ	indentation depth
<	less than
\leq	less than and equal to
μl	microliter
μm	micrometer
μS	microsiemens
'	minute
>	more than
%	percent
\pm	plus or minus
ν	Poisson's ratio
16S rRNA	16S ribosomal RNA
ANOVA	analysis of variance
Ap	anal appendages
As	articulated setae
B	bootstrap
bp	basepair
Bs	basiconic sensilla
cm	centimeter
COI	cytochrome c oxidase subunit I
COII	cytochrome c oxidase subunit II
CVA	canonical variate analysis
DA	dalton

<i>df</i>	degree of freedom
DNA	deoxyribonucleic acid
DO	dissolved oxygen
E	east
<i>E</i>	elastic modulus
e.g.	exempli gratia ("for example")
<i>et al.</i>	et alia ("and others")
F	regression analysis value
<i>F</i>	force
Fe	Iron
FO	frequency of occurrence
g/l	gram per liter
GPS	global positioning system
Gs	group of sensilla
h	hour
Hd	haplotype diversity
IQR	Interquartile
ITS1	ribosomal internal transcribed spacer 1
ITS2	ribosomal internal transcribed spacer 2
kHz	kilohertz
km	kilometer
kV	kilovolt
Lam	lamina valvarum
LO	local occurrence
m	meter
m/s	meter per second
max	maximum

mg/l	milligram per liter
min	minimum
mm	millimeter
mM	millimolar
mS	millisiemens
ms ⁻¹	meter per second
MWCO	molecular weight cut-off
N	north
n	number of sample size (“total”)
N/m	newton per meter
NaH ₂ PO ₄	sodium dihydrogen phosphate
ND1	NADH dehydrogenase subunit 1
Nitrate	NO ₃ ⁻ -N
Nitrite	NO ₂ ⁻ - N
Sulfate	SO ₄ ²⁻
Phosphate	PO ₄ ³⁻
Ammonia	NH ₃ -N
NJ	neighbour-joining
nm	nanometer
nmol	nanomoles
<i>P</i>	possibility value
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
PCA	principle components analysis

PCR	polymerase chain reaction
PCs	principle components (“plural”)
pH	potential of Hydrogen
Pi	nucleotide diversity
pmol	picomole
ppm	parts per million
ppt	parts per thousand
<i>r</i>	correlation coefficient
<i>Rc</i>	radius of tip curvature
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
S. l.	sensu lato (“in the broad sense”)
S10	10 th segment
S8	8 th segment
S9	9 th segment
SD	standard deviation
SE	standard error
sec	second
SO	stream occurrence
sp.	species (“singular”)
spp.	species (“plural”)
St	stylus
TDS	total dissolved solids
tRNA	transfer ribonucleic acid
UV	ultraviolet
V3	valvulae 3
vs	versus

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CHAPTER 1: INTRODUCTION

1.1 GENERAL INTRODUCTION

Odonata is taxonomically isolated and very ancient (Norman, 1997) and are also very primitive (Orr *et al.*, 2004). Today two suborders are known, Anisoptera (unequal wings) or true dragonflies, and Zygoptera (equal wings) often referred to as damselflies. In Malaysia, Anisoptera and Zygoptera are about equally represented (Orr *et al.*, 2004), but there remains substantial scopes for new discoveries, especially in Borneo.

The odonates possess anatomical features relating to feeding, flight and reproduction, which are unique among insects. Dragonflies are conspicuous inhabitants of many types of country: they are large, active predators which hunt by day. Odonata contained 5956 described species (39 families, 659 genera), of which 2942 belong to the suborder Zygoptera (309 genera, 27 families), 3012 to the Anisoptera (348 genera, 11 families) and 2 to the Anisozygoptera (1 family, 1 genus) which was described in 2012 (Suhling *et al.*, 2015). With approximately 6000 species currently described, the taxonomy of the Odonata has been largely considered as well established, and it has been estimated that 95% of all extant species will be described by 2030 (Dijkstra *et al.*, 2013).

Odonata are among the most recognizable of insects and have been used in a wide array of studies dealing with functional morphology, behaviour, ecology, and evolution (Corbet, 1999). They are well suited to biotope characterization and environmental monitoring, because they occupy a wide spectrum of aquatic habitats. All of them are predators either as larvae or adults. They are mostly relatively large insects occurring at only moderate densities by being near the top of the food chain, besides are

of little economic significance. However, perhaps most significantly, odonates are interesting in their behaviour, ecology and zoogeography (Orr *et al.*, 2004).

Dragonflies and damselflies are known as a flagship group, and an important component of aquatic ecosystems, in which they can often be top predators (Balzan, 2012) and their sensitivity to environmental conditions makes them as excellent biological indicators of environmental conditions (Brown, 1991; Clark & Samways, 1996; Samways *et al.*, 2010). As the odonates inhabit both aquatic and terrestrial environments, they may better reflect environmental variation at different scales, ranging from the characteristics of the aquatic habitat that includes the characterizing of vegetation that plays an important role throughout the lifecycle of the odonates, to the surrounding terrestrial landscape which offer resources and conditions required for the persistence of the adult stages (Balzan, 2012).

According to Corbet (1999), the odonates play an important role in ecological food webs, feeding on smaller insects, including fingerlings and young tadpoles, or serving as prey for adult fishes. Moreover, they are also resourceful indicators of water quality (Pires *et al.*, 2013) due to the preference of both larval and adult stages for certain environmental conditions for their establishment (Corbet, 1999, Clausnitzer *et al.*, 2009). They are a key component of many freshwater ecosystems that are generally perceived to be good fliers, potentially capable of wide dispersal and possibly less sensitive to habitat fragmentation than other freshwater taxa (Watts *et al.*, 2006).

On the other hand, the odonates also known to be good indicators of forest structure and landscape composition (Clausnitzer, 2003) besides their assemblages are highly visible and sensitive indicators of long-term environmental conditions of the water environment (Stewart & Samways, 1998). The presence of dragonflies is an important indicator of ecological balance. By way of reproduction, these insects lay

their eggs in or near only freshwater (Corbet, 1999) and thus, their high abundance in an area is a good indication of the quality of freshwater. Odonata are used as bioindicators for wetland quality in Europe, Japan, the USA, Australia (Clausnitzer & Jodicke, 2004) and in South Africa (Clark & Samways, 1996; Stewart & Samways, 1998). The greatest numbers of species are found at sites that offer a wide variety of microhabitats (Ameilia *et al.*, 2006).

The use of odonates as indicators offers several advantages; they are widespread and represent one of the historically most studied insect groups, and so there is a good knowledge of the ecological requirement of a large number of species and distribution. Besides, they are relatively easy to observe and identify, and finally they are well dependent on the ecological conditions of the environment (Corbet, 2004). Odonata assemblages are associated with different habitat types (Bried & Ervin, 2005). Consequently, increased habitat heterogeneity can lead to increased Odonata diversity at a particular site (Dolny & Harabis, 2012; Clausnitzer *et al.*, 2012) and many ecological factors affect the distribution of the nymphs. The acidity of water, the amount and type of aquatic vegetation, the temperature, and whether the water is stationary or flowing affect the distribution of odonate nymphs (Acquah *et al.*, 2013).

Odonates are appreciated for their aesthetic value (Norma-Rashid, 2000), besides having wide application in the area of conservation (Samways, 1992), biomonitoring (Schmidt, 1958; Steffens & Smith, 1999), biocontrol (Corbet, 1999) and more recently, in the design of surveillance tools fondly named 'Dragon Spies' which mimic the form and habit of the dragonfly.

The association of the odonates with their habitats, together with other characteristics of this taxon that include their functional importance within ecosystems, and their association with other species and resources, therefore, make the surveys of this insect group communities an important tool for characterizing and assessing the land-water interface through their function as indicators of ecosystem quality. The adults of odonates are conspicuous, easy to record, taxonomically well studied, and susceptible to habitat changes induced by human activities, all characteristics desirable of bioindicator groups (Brown, 1991). On the other hand, the odonate larvae may also occur in artificial water bodies such as reservoirs, nonetheless their diversity in such environments is little studied (Williams *et al.*, 2008).

Although there is a wealth of data available to be utilized for solving taxonomic problems, but ecological and behavioral research areas are more favored in contrast to taxonomy and systematics. Thus, there exist confusions for correct identifications in closely related and sympatric species, especially in female odonates. One such example is in the genus *Rhinocypha*, in which one of the objectives of this study is to fill in this gap.

The odonates are now receiving worldwide attention as objects of research, besides their phylogenetic position makes them being importance in comparative studies on the evolution of genomic innovations. Surprisingly, despite there are many odonate studies, few are taxonomic in nature, especially in Malaysia. In this study, for the first time, a nationwide survey of odonate diversity related to environmental factors is conducted in Peninsular Malaysia and distinguishes female of sympatric species of *Rhinocypha* group by using a cohesive approach of morphology, molecular and bio-materials of the wings. The present study will offer new insights into odonate research with the utilization of combined classic as well as modern tools and methods. These

findings will hopefully prompt more investigations on the potentially vast aspects in such study to promote greater interest on odonates.

1.2 RESEARCH QUESTIONS

Peninsular Malaysia is made up of a rugged geographical topology and thus provided a wide variety of natural habitat structures suitable for specific communities of organisms including odonates. With the presence of odonate communities within the diversity of habitats there are sympatric species which are congeneric; some of these species can be a challenging task for taxonomy work. General research questions relevant to this study:

- 1) Would the population of odonates be evenly or explicitly distributed across the available habitat structures? and if distinct, what are the determining parameters that affect them?
- 2) Among the diversity found are there iconic species worthy of conservation and can be important bioindicators? and thus needing a focussed study as in the genus *Rhinocypha*.
- 3) Sympatric species of close taxonomic levels can be a challenge in identification, especially for indistinguishable females and so can the 3 combined approaches of molecular techniques, morphology and mechanical studies on wing resilin be able to resolve the problematic taxonomy?

1.3 AIMS AND OBJECTIVES

The aim of this research is an integrated approach which is to reveal the bio-ecological studies of Malaysian odonates in Peninsular Malaysia, together with the taxonomic study on a particular genus, *Rhinocypha*. In light of this, the present study was performed to address the following specific objectives:

1.3.1 To illuminate the nationwide distribution and diversity of Odonata from the Reserve Forest in Peninsular Malaysia.

- i. To document the population and assemblage patterns of the odonates.
- ii. To elucidate the distribution patterns of odonates throughout their habitat in Peninsular Malaysia

1.3.2 To reveal the environmental parameters related to odonate diversity and distribution.

- i. To correlate the relationship between species richness and associated environmental factors.
- ii. To reveal the suitable habitat for the odonates species.
- iii. To relate the function of odonates as the bio-indicator of the environment.

1.3.3 To reveal the phylogeographic pattern of the common species for the focussed genus, *Rhinocypha fenestrella* inferred from COI and 16S rRNA gene sequences.

- i. To investigate the intraspecific genetic diversity of *Rhinocypha fenestrella*.
- ii. To reveal the phylogeographic pattern of the specific species.
- iii. To illuminate the ancestral haplotype of *Rhinocypha fenestrella* in Malaysia.

1.3.4 To resolve the taxonomic problems within the females of *Rhinocypha* by utilizing 4 contrasting tools:

- i. To describe the morphological diagnostics and produce a simplified dichotomous key for the genus group
- ii. To define the female's ovipositor of the three species of *Rhinocypha* using Field Emission Scanning Electron Microscope (FESEM)
- iii. To quantify and analyze wing morphological features in the female of *Rhinocypha* spp. using landmark-based geometric morphometric method
- iv. To reveal phylogenetic patterns using COI and 16S rRNA genes.

1.3.5 To demonstrate the morphology and characteristics of the *Rhinocypha*'s wing.

- i. To examine the distribution of resilin vein-joint types and cuticular spikes on the wing of *Rhinocypha* spp.
- ii. To reveal the wing elasticity of the particular genus.
- iii. To study the amino acid components of the resilin in the wing of *Rhinocypha* spp.

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1.4 SPECIFIC INTRODUCTION CORRESPONDING TO THE STATED OBJECTIVES

1.4.1 Odonates Diversity and Distribution in Peninsular Malaysia

Dragonflies, including the smaller damselflies, belong to an order of insects, Odonata, are charismatic, culturally important, play important functional roles in ecosystems as both predators and prey (Luke *et al.*, 2017), and have the potential to provide valuable pest-control services to agricultural systems (Corbet, 1999). They are taxonomically isolated and very ancient (Norman, 1997), and they possess anatomical features relating to feeding, flight and reproduction which are unique among insects. On the other hand, dragonflies and damselflies are extant representatives of the first ancient winged insects (Misof *et al.*, 2014).

Today odonates are conspicuous inhabitants of many types of country: they are large, active predators which hunt by day. Odonata is the second largest insect order with an obligatory aquatic stage in the life history after the Trichoptera and only very rarely are the larvae secondarily terrestrial (Orr *et al.*, 2004). However, the diversity of odonates is not well known in certain regions of the world (Pires *et al.*, 2013). According to Kalkman *et al.* (2008) about 1,000 to 1,500 species are yet to be described. If true, the actual number of extant species will lie between 7000 and 7500 (Dijkstra *et al.*, 2013).

According to Luke *et al.* (2017), the Oriental region, which including southeast Asia from southern China to Java, Sundaland, and South Asia south of the Himalayas, has a highly diverse dragonfly fauna, with some of the highest numbers of described species and genera for all biogeographical regions. Several families are largely confined

to the region (e.g. Chlorogomphidae, Euphaeidae, Devadattidae, Philosinidae and Pseudolestidae) and several others are mostly found there (e.g. Chlorocyphidae and Platystictidae) resulting in very high levels of endemism (Kalkman *et al.*, 2008).

In Malaysia, previous studies found that odonate fauna comprises 342 named species, include 161 species of Zygoptera in 10 families, and 181 species of Anisoptera in 5 families as presented in Table 1.1 below. Overall 239 species are known from Sabah, Sarawak and Brunei, and 226 from Peninsular Malaysia (including Singapore). From the total number, 123 species or 36% are shared between Peninsular Malaysia and Sabah–Sarawak. As expected the family compositions of the two regions are very similar, but much higher levels of endemism are found in Borneo (40%) than the peninsular (11%) which shares much of its non- Bornean fauna with either Sumatra or Thailand (Orr *et al.*, 2004).

The highest levels of endemism within the peninsular species occurred in the family of Platystictidae; with nine members of the widespread genera *Protosticta* and *Drepanosticta* are all endemic. No other family exhibits an endemism level of more than 33% and in seven families there are no endemic species at all. Nevertheless the genera *Sundacypha* (Chlorocyphidae), *Podolestes* (Megapodagrionidae), *Pericnemis* (Coenagrionidae), *Brachygonia*, *Chalybeothemis* and *Pornothemis* (Libellulidae) all occur in both regions of Malaysia but are elsewhere restricted to Sundaland (which also includes southern Thailand, Sumatra, Java, Bali and smaller adjacent islands).

Table 1.1: Species richness and endemism of odonate families in Peninsular Malaysia and Sabah–Sarawak. Significantly high proportions of endemics, 40% and above (*), adopted from Orr *et al.* (2004).

Taxon	Peninsular Malaysia		Sabah and Sarawak		Total species	% common to Peninsular - Borneo
	No. species	% endemic	No. species	% endemic		
Zygoptera	88	16	105	66	161	20
Amphipterygidae	1	0	1	100*	2	0
Calopterygidae	6	0	9	78*	13	15
Chlorocyphidae	10	0	18	67*	23	22
Euphaeidae	3	0	8	75*	9	22
Lestidae	5	0	3	33	6	33
Megapodagrionidae	4	25	5	80*	8	13
Caenagrionidae	33	9	28	43	47	30
Platycnemididae	10	10	11	82*	19	11
Platystictidae	9	100*	11	100*	20	0
Protoneuridae	7	14	11	55*	14	31
Anisoptera	138	8	134	19	181	50
Gomphidae	32	19	24	46*	46	21
Chlorogomphidae	3	33	1	0	3	33
Aeshnidae	15	13	27	15	30	43
Corduliidae	14	14	13	46*	18	47
Libellulidae	74	0	69	7	84	69
Total	226	11	239	40*	342	3

Interestingly, Sabah and Sarawak have endemism rates exceeding 70% for the Zygopteran families (Amphipterygidae, Chlorocyphidae, Euphaeidae, Calopterygidae, Megapodagrionidae, Platystictidae and Platycnemididae). An astonishing 66% of all Zygoptera are endemic while for the Anisoptera is only 19%, although rates of 46% occur in the Gomphidae and Corduliidae, still considerably higher than in Peninsular Malaysia. Endemic genera include *Rhinoneura* (Chlorocyphidae), *Matronoides* (Calopterygidae), *Bornargiolestes* (Megapodagrionidae), *Linaeshna* (Aeshnidae) and *Pseudagrionoptera* (Libellulidae) (Orr *et al.*, 2004).

In general, the distribution and composition of aquatic insects and such as Odonata may undergo changes due to adaption to environmental changes (Lenat, 1993; Che Salmah *et al.* 1998; Ameilia *et al.*, 2006). Peninsular Malaysia is made up of a rugged geographical topology and thus provided a wide variety of natural habitat structures including for odonates. However, the diversity of odonates of Peninsular Malaysia was poorly known until relatively recently, and based solely on scattered records and descriptions (Norma-Rashid, 2010; Farizawati *et al.*, 2014; Choong, 2014; Dow *et al.*, 2016). Thus, the aim of this first study was to illuminate the nationwide distribution and diversity of Odonata from the Reserve Forest in Peninsular Malaysia and to document the population, assemblage and distribution patterns of the dragonflies in Peninsular Malaysia.

1.4.2 Correlations of Physical and Chemical Parameters with Odonate Diversity and Distribution

As frequently stated, odonates are a key component of many freshwater ecosystems that are generally perceived to be good fliers and potentially capable of wide dispersal, besides having the possibility of being less sensitive to habitat fragmentation compared to other freshwater taxa (Watts *et al.*, 2006). From the conservation point of view, the order Odonata is among the most thoroughly studied insect groups. This is because the odonates are widely acknowledged as biological indicators (Foote & Rice 2005; Rouquette & Thompson 2005; Clark & Samways, 1996; Samways *et al.*, 2010), they are widespread, and so there is a good knowledge of the ecological requirements of a large number of species and their distribution.

In addition, the use of odonates as indicators offers several advantages; they are relatively easy to observe and identify, and they are well dependent on the ecological conditions of the environment (Corbet, 2004). Odonata assemblages are associated with different habitat types (Bried & Ervin, 2005). Consequently, increased habitat heterogeneity can lead to increased Odonata diversity at a particular site (Dolny & Harabis, 2012; Clausnitzer *et al.*, 2012), and many ecological factors affect the distribution of the nymphs. The acidity of water, the amount and type of aquatic vegetation, the temperature, and whether the water is stationary or flowing all affect the distribution of odonate nymphs (Acquah *et al.*, 2013), hence, many Odonata (and Lepidoptera) are flagship species for conservation in some countries (Samways, 1999).

According to Merritt and Cummings (1996), the odonate larvae occupy a variety of running and standing freshwater environments such as rivers, lakes, ponds, wetlands and, to a lesser extent, phytotelmata, brackish waters and riparian areas of rivers. In these habitats, they are known to be effective bio-indicators of both aquatic and terrestrial

habitats including the agricultural land and farmland ponds (Clark & Samways, 1996; Briers & Biggs, 2003) and they have played an important role in ecological food webs, feeding on smaller insects, including fingerlings and young tadpoles, or serving as prey for adult fishes (Corbet, 1999). Additionally, previous studies showed that they are also efficient indicators of water quality (Pires *et al.*, 2013) due to the preference of both larval and adult stages for certain environmental conditions for their establishment (Corbet, 1999; Clausnitzer *et al.*, 2009). Odonate larvae may also occur in artificial water bodies such as reservoirs, but their diversity in such environments has been little studied (Williams *et al.*, 2008).

Furthermore, Odonata have a bipartite life-cycle. According to Corbet (1999), at a local scale, the odonates depend on healthy waterbodies for growth and emergence during their larval stages, and egg deposition during their adult stages, and on the other hand, at a larger scale, adult of odonates depend on the quality of the terrestrial landscape for dispersal, feeding and roosting. Therefore, the presence of odonates at ponds or certain habitat not only reflects the habitat quality, but also reflects the quality of the surrounding landscape and a failure to consider aquatic habitats at the landscape-scale has aggravated odonate declines (Declerk *et al.*, 2006; Thompson & Watts, 2006). Besides to the habitat loss (Fahrig, 2003), landscape fragmentation causes the decline or local extinction of dragonfly populations (Watts *et al.*, 2004, 2006)

Odonates are known as insects that are highly specialized for a specific wetland habitat. Daily changes in environmental variables may affect the distribution of aquatic insects (Fulan *et al.*, 2011), such as human activities that contributed to the changes in the aquatic environment as well the quality of water. According to previous studies, rapid industrial development, population growth, agriculture, mining and logging activities affected the watercourses and drastically reduced vegetation in the habitats (Abu Bakar, 1985; Allan & Johnson, 1997; Ameilia *et al.*, 2006).

Leaching of fertilizers and pesticides used in agricultural areas would alter chemical properties of water and the human residents living by the rivers undeniably contributed anthropogenic wastes in portions of the rivers that passed the areas (Ameilia *et al.*, 2006). The physical and chemical characteristics of the water body, mainly determined the fauna colonizing the area (Townsend *et al.*, 1983; Wright *et al.*, 1984; Dissanayake & Chandrasekara, 2014), even though within a certain range of values, they showed no relationship with the abundance of certain odonate species (Che Salmah *et al.*, 1998).

Moreover, differences in river orders and habitat can influence the composition of odonate larval communities (Hawking & New, 1999) because of the habitat is necessarily the location where an organism develop from young to adult and thus, a suitable habitat must fulfil the ecological needs for all life stages. According to Corbet (1999), the terrestrial landscape is probably as important as the aquatic habitat due to it provides several conditions and resources that are required by the odonate phase and the habitat characteristics on Odonata assemblages have been identified particular dragonfly associations to specific habitat types (Clark & Samways, 1996; Samways, 1996; Schindler *et al.*, 2003).

Monitoring changes in biodiversity subsequent from forest modification and destruction require the study of a wide range of taxa, embracing species with very different ecologies and life histories (Lawton *et al.*, 1998). As the odonates can be a good indicator of forest structure and landscape composition (Clausnitzer, 2003), their assemblages are highly visible and sensitive indicators of long-term environmental conditions of the water environment (Stewart & Samways, 1998).

According to Clausnitzer *et al.* (2009), threatened species are have been clustered in tropical areas, especially in the Indo-Malayan region. Up to now, regional

or local faunistic surveys of dragonflies in Borneo have been very limited, especially those that relate the occurrence to habitat (Orr, 2006; Dolny *et al.*, 2011). Besides, according to some researchers (Thompson & van Tol, 1993; Orr, 2001, 2006), the northern part of Borneo is certainly the best-researched part of the island, especially the sultanate of Brunei, and to a slighter extent Sarawak (Dow, 2008; Dow & Reels, 2008, 2010) and Sabah (Yagi & Kitagawa, 2001) that includes the highest mountain in Borneo, Mount Kinabalu (Laidlaw, 1934; Hämäläinen, 1994). However, the Peninsular Malaysia is no exception. Very little is known about the environmental parameters related to the odonate diversity and distribution.

Knowing the sensitivity of odonates to conditions and environments across both terrestrial and freshwater habitats means that the odonates can be good indicators of habitat disturbance, by means of changes in assemblages and abundance, giving information about the habitat quality, and, potentially the status of a range of other taxes that are more difficult to sample (Chovanec & Waringer, 2001; Opperl, 2005; Simaika & Samways, 2009; Kutcher & Bried, 2014; Golfieri *et al.*, 2016). Thus, this second study will correlate the relationship between species richness of the odonates and associated environmental factors, to reveal the suitable habitat for the odonates species, and to relate the function of odonates as the bio-indicator of the environment.

1.4.3 Molecular Phylogeography of *Rhinocypha fenestrella* Based on Analyses of Mitochondrial COI and 16s rRNA Genes

Rhinocypha (Rambur, 1842), from the family of Chlorocyphidae is the most characteristic genus of odonates of tropical Asia (Lieftinck, 1945). Rhinocyphae are of a very particular nature of their habitat (Lieftinck, 1945) and certain species are more sensitive to habitat disturbance and primarily found in undisturbed areas (Jumawan *et al.*, 2012). This characteristic makes them a good indicator of environmental quality.

Rhinocypha fenestrella Rambur, syn, *Aristocypha fenestrella*, also known as a peacock jewel, is the most widespread species in the genus, and one of the most active species of the family, which occurs in Peninsular Malaysia, Thailand, Burma, Laos, Vietnam and southern China (Sharma, 2010). It is one of several common mountain stream damselflies and is usually found in the primary forests (Lieftinck, 1945).

Though Malaysia is known to be one of the three mega-biodiversity countries in South East Asia, the phylogeographic pattern of odonates has been relatively scarce, especially for this particular species, little has been published. Studies of phylogeographic and biogeographic patterns between insects are frequently limited to species with the limited dispersal ability (Vogler & DeSalle, 1993; Butlin *et al.*, 1998; Lunt *et al.*, 1998) or to the taxa originate on the island archipelagoes (Fleischer *et al.*, 1998; Polhemus & Polhemus, 1998; Roderick & Gillespie, 1998). Instead, the species with a widespread distribution that includes the dragonflies normally will have complexes multiple lineages or variation in the genetic diversity in the geographic region (Angulo & Icochea, 2010; Damm *et al.*, 2010a; Low *et al.*, 2015a, b, 2016a). With a view of zoogeography, the Rhinocyphae are substantial important, for instance, in the Malay Archipelago, each large island, mostly has its own group of endemic species (Lieftinck, 1945).

With the advances of molecular techniques, mitochondrial DNA has been identified as an excellent genetic marker of gene flow in matrilineal inheritance (Jisha & Sebastian, 2015) and it is the most widely used markers to study the molecular ecology in animal taxa (Simon *et al.*, 1994; Norris, 2002; Pramual *et al.*, 2005). Particularly, cytochrome C oxidase subunit I (COI) and 16S ribosomal RNA are known to be the reliable genetic markers and the most commonly applied markers in Odonata (Dijkstra *et al.*, 2014; Artiss *et al.*, 2001; Yong *et al.*, 2014; Kim *et al.*, 2014).

Additionally, these markers also provide well resolved and supported trees from species to family level (Hasegawa & Kasuya, 2006; Ballare & Ware, 2011). Given the high resolution of mitochondria-encoded 16S rRNA and COI genes reported in odonates, this third study attempts to characterize the intraspecific genetic diversity and population genetic structure of *R. fenestrella*, for the first time across its range in Malaysia.

1.4.4 Taxonomic Studies within the Females of *Rhinocypha* by Utilizing 4 Contrasting Tools

In suborder Zygoptera, *Rhinocypha* spp. was the most abundant species found in the forest reserve (Wahizatul Afzan *et al.*, 2006) and the most abundant damselflies in Selangor (Noorhidayah, 2013). Mapi-ot *et al.* (2013) found that this species can adapt and tolerate to the disturbed habitats, while Villanueva (2012) observed that this species can be found even in areas with significant human activity and it can tolerate streams that have agricultural and domestic runoffs.

However, *Rhinocypha* spp. can be a challenge to the studies on dragonflies. Besides the scarce study of phylogeographic patterns of this genus, the females are more cryptic at species level, and identifying the females is challenging. They are difficult to differentiate with other females of the same genus even though the male of *Rhinocypha* are conspicuous and easy to identify with its distinct blue thoracic marks.

While the phylogeny of the Anisoptera has been reasonably well studied and its classification is fairly settled (Ware *et al.*, 2007; Fleck *et al.*, 2008a), recent studies of Zygoptera rely on rather incomplete molecular data sets (Bybee *et al.*, 2008; Carle *et al.*, 2008; Dumont *et al.*, 2010). Besides the morphological studies, mitochondrial gene region cytochrome c oxidase subunit 1 (COI) and 16S ribosomal RNA (16S rRNA) can be used for species confirmation of the Malaysian taxon and preliminary interspecific phylogeny of the *Rhinocypha* group.

Female-limited colour polymorphism in damselflies is a counter-adaptation to male mating harassment; therefore, it is expected to alter population dynamics through relaxing sexual conflict (Takahashi *et al.*, 2014). Such female-limited colour polymorphisms are widespread among damselflies. Typically, females have two or

more morphs, where one ‘andromorph’ showing a male-like colour pattern and one or two ‘gynomorph(s)’ expressing colour patterns which are different from the males.

Additionally, according to Bechly *et al.* (2001), in this group of insects (Odonata), the endophytic oviposition is expected to be a plesiomorphic feature. The odonate females deposit their eggs within plant tissues as a result of a well-developed ovipositor composed of the genitals appendages of the 8th and 9th abdominal segments (Matushkina, 2011).

Throughout the last 20 years, extensive work has been done on the comparative and functional morphology of the plesiomorphic well-developed ovipositor in Odonata. For instance, previously specific studies have been focused on the skeleton and musculature (Klass, 2008; Matushkina, 2004, 2008a, 2008b; Matushkina & Gorb, 1997; Matushkina & Klass, 2011; Matushkina & Lambret, 2011), cuticular microstructures (Matushkina, 2008b; Matushkina & Lambret, 2011; Matushkina & Klass, 2011), and functional aspects of the endophytic ovipositor (Matushkina & Gorb, 2002, 2007; Matushkina & Lambret, 2011; Matushkina & Klass, 2011).

Besides, it is being understood that the majority of phylogenetic reconstructions of higher-level relationships in Odonata suffer from the absence of a common morphological character system apart from the wing venation (Pritykina, 1980; Bechly, 1996; Lohmann, 1996; Trueman, 1996). This highlights the importance of a search for new phylogenetically informative characters, and according to Matushkina (2005), the ovipositor is expected to provide such characters. The three species of *Rhinocypha* as well as all Zygoptera and aeshnid Anisoptera have a cutting ovipositor, used for egg deposition within plant tissues (St. Quentin, 1962).

In addition, insect wings have been the subject of geometric morphometric analysis in the past many years (Rohlf & Slice, 1990; Baylac & Daufresne, 1996). They are especially attractive because they can be treated with biological realism in only two dimensions. Morphometric is the study of variation and covariation of biological form (Bookstein, 1991; Dryden & Mardia, 1998; Adams *et al.*, 2004). According to Rohlf and Marcus (1993), the morphometric methods are important for description and statistical analysis of the shape of an organism, while the term 'geometric morphometric' was introduced to distinguish it from the measurement-based techniques of 'traditional' morphometric.

The geometric morphometric bring up to the approach in morphometry where shapes are expressed as geometric coordinates and the representation and comparison of these shapes are subject to mathematical and statistical techniques (Zelditch *et al.*, 2004). This method will allow visualization of shape independent of size (Rohlf & Marcus, 1993; Adams *et al.*, 2004) and also evidences useful in phylogenetic investigation (Monteiro, 1999; Pierce *et al.*, 2008). Moreover, the geometric morphometric method is a relatively innovative technique that has generated valuable results in many fields of classic morphometry. A major advantage of the geometric framework is a complete use of information about the shape that available from a set of landmarks (Bookstein, 1996).

In consequence, wing morphometrics can help to characterize populations within a species, as shown by the previous studied such as the analysis of geographic variation in populations of *Drosophila lummei* (Haas & Tolley, 1998), *Drosophila serrata* (Hoffman & Shirrifs, 2002) and *Scythris obscurella* (Lepidoptera) (Roggero & d'Entrèves, 2005). Besides, wings also showed useful to study complexes of species, for example, in Diptera (De La Riva *et al.*, 2001), or examine the effects of hybridization, such as in *Apis mellifera* subspecies (Smith *et al.*, 1997).

Traditionally, taxonomy is based on phenotypic analyses; although several researchers found that in many taxa this approach is impossible due to the lack of sufficient morphological characters (Wilkerson *et al.*, 1993; Chilton *et al.*, 1995; Floyd *et al.*, 2002). For several aquatic insect orders such as Ephemeroptera (Ball *et al.*, 2005; Williams *et al.*, 2006; Alexander *et al.*, 2009), Diptera (Pfenninger *et al.*, 2007), Coleoptera (Balke *et al.*, 2007; Dutton & Angus, 2007) and Trichoptera (Pauls *et al.*, 2010), morphological characters only do not allow reliable distinction. Henceforth, the molecular genetic techniques have become widespread in taxonomic studies. Though there are increasing number of studies combining DNA sequences and morphology, relatively few studies have been focused on odonates (Pilgrim *et al.*, 2002; Stoks *et al.*, 2005; Pilgrim & von Dohlen, 2007). Expectedly, many debated regarding the taxonomic connections still remain in this order (Schmidt, 2001; Dijkstra, 2003; Dijkstra & Lewington, 2006).

Hereafter, in this present forth study, the morphological diagnostics, ovipositor characteristics, geometric morphometric of the wings and phylogenetic patterns of adult females of three congeneric damselfly species, *R. biforata*, *R. fenestrella*, and *R. perforata*, were studied to discover the problems in differentiating with other females of the same genus.

1.4.5 Morphology and Characteristics Properties of the *Rhinocypha* Wings

Insects were among the first animals that have been recognized to have unique structures of elastic proteins that assist in movements (Neff *et al.*, 2000). Insect wings, including those of dragonflies are complex mechanical structures. Both of dragonflies and damselflies have two pairs of elongated membranous wings with strong cross veins and many small veins that criss-cross the wings and add strength and flexibility to the wings (Talucdher, 2013). Remarkably, the venous system of dragonfly wings is composed of both stiff and flexible materials. The veins at the trailing edge and the wing tip where the loads are small are simple bilayer tubular structures with both an inner and an outer layer, while at the basal and leading edges where the loads are higher, the veins are thick and display multi-layered structures (Zhao *et al.*, 2011).

In addition, Zhao *et al.* (2011) also reported a venous system for dragonfly wings, mainly composed of veins and membranes, possessing both stiff and flexible materials. The veins and membrane are formed by sun drying of blood vessels and muscles during the metamorphosis, transformation from larva to fly (Talucdher, 2013). Wang *et al.* (2008), further reported the wing veins of the complex sandwich structure of chitinous shells and a protein layer containing fibrils, that further enhance the capability to absorb mechanical energy (Meyers *et al.*, 2008). This hierarchical composite structure is said to be common in biological materials (Ji & Gao, 2004; Alam, 2014).

The main focus of this fifth study is on the flexible element that has been found on the wings of damselflies, called resilin. Resilin is a member of a family of elastic proteins that includes elastin, as well as gluten, gliadin, abductin and spider silks (Elvin *et al.*, 2005). Resilin is the rubber-like protein found in specialized regions of the cuticle

of most insects that gives low stiffness, high strain and efficient energy storage (Andersen, 1964; Gosline *et al.*, 2002; van Eldijk *et al.*, 2012) that functions in insect flight (Weis-Fogh, 1960; Gorb, 1999). Weis-Fogh (1960) first described resilin from the flight systems of locusts and dragonflies, it was described to be similar to swollen isotropic rubber, but its elastic behaviour is unlike any other natural or synthetic polymer; resilin was shown to have remarkable mechanical properties.

Additionally, numerous authors found that irrespective of its small size, the resilin-filled joints played a role in bending shapes and for the whole flexibility of the wing (Donoughe *et al.*, 2011; Mountcastle & Combes, 2014). On the other hand, Gorb (1999) found that automatic performances of passive wing movements of odonates are the responsibility borne by the distribution pattern of resilin. The presence of resilin in some vein joints of odonate provided flexibility, besides functioning as a damper and stretchable component (Gorb, 1999; Jakle, 2003) and is believed to be involved in the mechanical control of wing torsion and in the storage of elastic energy (Fauziyah *et al.*, 2014).

A number of findings have discussed about the structure and mechanical properties of the membranes, alongside with the venations of the insect wings (Wootton, 1992; Wootton *et al.*, 1998; Combes & Daniel, 2003; Ganguli *et al.*, 2010) as well as on flight aerodynamics (Azuma *et al.*, 1985; Ho *et al.*, 2003; Wang, 2005; Tamai, 2007; Shyy *et al.*, 2008, 2010; Floreano *et al.*, 2009). Although the current understanding increased on the role of wing structural elements and mechanical properties, however, the precise function of the individual elements and their components are still vague. Over the past 16 years, scientific notion of insect flight has been substantively transformed by the presence of new experimental techniques for measuring from the

aerodynamics of flight, to the movement of actin and myosin proteins in the muscle of insect flight and the responses of flight control flying animals (Hedrick *et al.*, 2015).

Nanotechnology has become a major field in scientific discipline and engineering with many products ranging from cosmetics, auto parts, and electronics using nano-scale factor (Hoffman, 2010). Indentation testing is a simple and convenient way to measure the properties of a material. The hardness and elastic modulus are the two most common properties measured by indentation testing. Despite the fact that its primary function is to image the surface of a sample, the atomic force microscopy (AFM) has been employed as a nanoindenter (Bhushan & Konikar, 1994; Tranchida *et al.*, 2006; Bassani *et al.*, 2006). Most of the AFM methods have used the same principles as traditional nanoindentation where to gain accurate results, several properties of the AFM cantilever must be known, particularly the spring constant, sensitivity, and tip radius. These properties can be hard to discover; however, a new technique has been proposed that can estimate these properties by executing tests on reference samples (Tang *et al.*, 2008).

Thus, the work reported here would be the first comprehensive investigation of the microjoint wing properties in the suborder Zygoptera that shows the potential of combining techniques of laser scanning confocal microscopy (LSCM), scanning electron microscopy (SEM), and atomic force microscopy (AFM) to investigate resilin elasticity and analyze the protein components.

1.5 IMPORTANCE OF THE RESEARCH

- i. Outcomes of the bio-ecological studies will gauge the standards of human impacts on habitats and community populations, here odonates as a case study.
- ii. The morphology and characteristics of the *Rhinocypha*'s wings will offer many inspiring clues to improve the biomimetic designs for high-performance technology development, whereas the mechanical properties of dragonfly wings is a need to be understood in order to perform simulated models based on the structure and the function of biomechanical of dragonfly wing possibly.
- iii. The taxonomic focus on *Rhinocypha* using tools of classic morphological features and the trendy molecular analysis will contribute to the taxonomic clarifications and possible revisions of the generic group.
- iv. Contribute in developing The Dragonfly Biotic Index in Malaysia – a compound index based on geographical distribution, conservation status, and ecological sensitivity – (which currently applied in tropical Africa and elsewhere, Simaika & Samways (2009)).

CHAPTER 2: LITERATURE REVIEW

2.1 BIOLOGY AND TAXONOMY OF ODONATES

The Odonata are important models for many studies, such as in the field of ecology, behavior, evolutionary biology and biogeography (Grimaldi & Engel, 2005; Thomas *et al.*, 2013). Their phylogenetic position makes this insect group of central importance to comparative studies on the evolution of genomic innovations involved in the origins of physiological processes, for example, in flight, color vision, and metabolism, and of life history strategies, for instance in predation, mating, dispersal, and complex life cycles. Besides, they are known as a key component of many freshwater ecosystems that are in general perceived to be good fliers, theoretically capable of wide dispersal and possibly less sensitive to habitat destruction than other freshwater taxa.

Modern odonates have an exceptionally well documented behaviour and natural history (Cordoba-Aguilar, 2008). The Holarctic regions have the best described odonate faunas, however, the most understudied faunas are found in tropical areas, even though it has greatest species diversity (Bybee *et al.*, 2016), especially in Malaysia (Norma-Rashid, 2010; Farizawati *et al.*, 2014; Choong, 2014; Dow *et al.*, 2016). Keys and field guides for adult odonates are available for most areas of the world (Dijkstra & Lewington, 2006; Garrison *et al.*, 2010; Dijkstra & Kalkman, 2012), but, surprisingly, in Malaysia despite there are many odonate studies, few are taxonomic in nature. Characteristics such as their relatively large body size and conspicuous behaviour make them an ideal insect group to study components of adult fitness in natural populations (Fincke & Hadrys, 2001; Thompson *et al.*, 2011).

According to Pires *et al.* (2013), the richness and especially the abundance of the odonates were higher in farm ponds than in streams, and the higher diversities of species in lentic habitats have also been reported in the northern hemisphere, in Europe and North America. Conversely with the earlier study by Kalkman *et al.* (2008), where they found that the highest diversity of odonate is found in flowing waters in rainforests of the tropics, and the Oriental and Neotropical regions being the most spacious.

The differences between the lentic environments in relation to lotic have been associated with the characteristics such as higher colonization rates at lentic sites (Hof *et al.*, 2006, Niba & Samways, 2006, Stevens & Bailowitz, 2009). According to Ribera *et al.* (2003), the lentic environments tend to be geologically less predictable through time than lotic. This geological characteristic in lentic habitats presses the species that adapted to the environment to colonize them faster in order to be able to disperse and then persist (Hof *et al.*, 2006).

Factors and aspects that influenced the distribution of odonates diversity can be divided into historical (geological) and ecological factors. Both of the factors determine the current species diversity, whereas the composition at family and genus level is mainly determined by the first (Kalkman *et al.*, 2008). These days' patterns of odonate diversity correspond mainly with the present of the climatological zones (Suhling *et al.*, 2015). Overall, the diversity of the odonate increases with temperature and precipitation, with most species occurring in a tropical rainforest (Kalkman *et al.*, 2008; Clausnitzer *et al.*, 2012).

Over the last few decades, several damselfly species have modified their distributions and abundances in response to rising global temperatures (Hickling *et al.*, 2006; Hassall *et al.*, 2007; Sanchez-Guillen *et al.*, 2013). Long-term distributional data of adults demonstrate that the odonates are amongst the taxa that showing the strongest

poleward range expansions (Hickling *et al.*, 2006; Hassall *et al.*, 2007), making them outstanding study organisms for unravelling the still poorly documented rapid micro evolutionary changes, related to range expansions (Merila & Hendry, 2014). This study could be fixed in the several well-documented cases of latitudinal adaptation among the odonates.

In addition, tropical regions possess the greatest number of odonate species, and it suggested that the high diversity can be determined by the aquatic habitat abundance in the tropical forest (Orr, 2006). Furthermore, tropical mountains offer a diversity of niches and regional refugia (Kalkman *et al.*, 2008). The limited seasonality of tropical habitats raised the opportunities for specialist lifestyle, thus it supports the highest diversity of tropical odonates and other taxa.

The highest levels of endemism and species richness of odonates occur in North Borneo in the middle of forest stream dwellers in montane and mixed dipterocarp forest. However, Java, Sumatra and the Peninsular Malaysia, all host distinctive faunas (Kalkman *et al.*, 2008). According to the World Conservation Monitoring Centre (WCMC) (1992), the destruction and fragmentation of habitat are major causes of biodiversity loss. Organisms that sensitive to the effects of habitat fragmentation are probable to have a combination of low natural abundance or high area requirement, large population fluctuations, low intrinsic growth rate, specialized habitat requirements and/or poor dispersal capability (Henle *et al.*, 2004).

2.1.1 *Rhinocypha* spp. Rambur

The genus *Rhinocypha* Rambur, 1842, is a genus of damselflies in the family Chlorocyphidae. The *Rhinocypha*, synonym as *Aristocypha*, which presently is ranked either as a full genus (Bridges, 1994; Orr, 2005; van Tol, 2006) or a subgenus (Tsuda, 2000) of *Rhinocypha* group.

This genus is the most characteristic genus of dragonflies of Tropical Asia (Lieftinck, 1945) which having great beauty and brilliance of its members. Not only do their wings display an inimitable play of scintillating colors, ranging through flashing blues, greens, purples, bronzes to gorgeous fiery coppery red, but the bodies in most cases are also gaily decorated with red and blue or yellow in many shades. The development of the small clear area or “windows” in such a wing as that of fenestrata only serves to heighten the effect of these radiant rainbow hues. They are easily recognized insects on account of their unusually short bodies and projecting "nose", whence the name.

The *Rhinocyphae* are very particular as to the nature of their habitat, all of them being rigidly confined to well-aerated shady streams of forest-books in which they breed (Lieftinck, 1945). Some species are recognized more sensitive to habitat disturbance and primarily found in undisturbed areas (Jumawan *et al.*, 2012). They usually found perched on rocks in midstream, couples of males circling round one another may be observed above the water.

Within the group with fenestrate wings, males, which are, the more plastic and progressive sex, are found to differ constantly in certain characters from different islands, while the females, which are the more conservative sex, are often indistinguishable throughout the whole area. They have unmarked wings and are dull-

colored, sitting quietly on gravel and rocks in the stream bed, or congregating on the bare twigs of neighboring trees or bushes. *Rhinocyphae* are very rarely seen pairing, but the females may occasionally be observed ovipositing, unattended by the male on a piece of dead twig or rotten wood sticking between boulders in midstream.

In the world species list, they are 62 species (Schorr *et al.*, 2003); in South & Southeast Asia they have 27 species, while in East Asia they have 10 species. According to Hamalainen (2005), in Peninsular Malaysia they have three species of *Rhinocypha*; *R. fenestrella* (Rambur, 1842), *R. biforata* (Selys, 1859) and *R. perforata* (Percheron, 1835). No attempt has yet been made to establish mutual relationships, but the arrangement proposed above reflects the broadest possible view of a “species”. Hardly anything is yet known concerning the *Rhinocypha* fauna of the numerous smaller islands of Malaysia, the only regional islands where these stream-dwelling insects (only subspecies) had been found to be Nias, the Anambas, and Palawan. The classifications of *Rhinocypha* group are described as below:

Phylum: Arthropoda

Class: Insecta

Order: Odonata

Suborder: Zygoptera

Family: Chlorocyphidae

Genus: *Rhinocypha*

(Rambur, 1842)

2.2 ECOLOGICAL AND ENVIRONMENTAL FACTORS

Odonata exists in almost all types of freshwater habitats; even a few of the species are terrestrial or occur in quite saline habitats. Though, many odonate species are restricted to a certain habitat, for example, forest streams (where this study takes place), acidic waters, or in the tree holes. However, according to Suhling *et al.* (2015), the most important habitat conditions are the presence or absence of the predators.

Many odonate species have small distributional ranges, and they are habitat specialists, including inhabitants of alpine mountain bogs, seepage areas in tropical rain forests, and waterfalls (Kalkman *et al.*, 2008). They are often used as bioindicators for environmental health and conservation management regarding the quality of the ecosystem.

Many species and some families of odonate have their own specific environmental requirements (Orr *et al.*, 2004; Dijkstra, 2006), needed for their larval development, including open spaces for finding mates, suitable perches, open aspect, roosting sites, suitable plant species for ovipositing and suitable water quality. For examples, some of the odonate species prefers flowing waters, while others prefer standing water, such as Gomphidae lives in running water, while the Libellulidae live in still water. Besides, some species are found in temporary water pools and are capable of tolerating changes in water level, desiccation, and the resulting variations in temperature, while some genera such as *Sympetrum* (darters) have eggs and larvae that can resist drought and are stimulated to grow rapidly in warm, shallow pools, also often benefiting from the absence of predators there.

Moreover, the chemical properties of the water, including its trophic status, the degree of enrichment with nutrients, and pH can influence the habitat of dragonflies (Dissanayake & Chandrasekara, 2014) due to requirements of different species. Most species of odonates required moderate conditions, which is not too eutrophic and not too acid. A few species such as *Sympetrum danae* and *Libellula quadrimaculata* prefer acidic waters while other species such as *Libellula fulva* need slow-moving, eutrophic waters with reeds or similar waterside plants (Dijkstra, 2006).

In addition, the species of *Enallagma cyathigerum* for example, can occur at high densities in acid waters where fish are absent; such as in bog pools, where *Ischnura pumilio* in contrast, requires base-rich habitats and water with a slow flow-rate. It is found in ditches, quarries, seeps, flushes, marshes and pools and tolerates high levels of zinc and copper in the sediment, but requires suitable emergent plants for egg-laying without the water being choked with plants (Allen, 2009).

Aeshna viridis Eversmann, 1836, in Europe, is one example of a species that occupies a highly restricted habitat, where this species requires the leaves of water soldier *Stratiotes aloides* (Linnaeus, 1758), for oviposition and as a larval shelter (Suhonen *et al.*, 2013). Nevertheless, in contrast to the species, many other odonate species reproduce in a wide variety of habitats, for instance, the species of *Crocothemis erythraea*, is the most common African odonate expanding its range northward through Europe. Such opportunistic species are commonly widespread and can be found in almost all species assemblages of their distribution range such as, *Ischnura elegans* in the Palearctic and *Ischnura senegalensis* in the Paleotropics (Suhling *et al.*, 2015).

Besides of many odonate species are restricted to a certain habitat, several researchers also identified that the importance of macrophytes in influencing Odonata assemblages for several ecosystems (Clark & Samways, 1996; Samways, 1996; Stewart

& Samways, 1998; Painter, 1999; Schindler *et al.*, 2003; Samways & Taylor, 2004; Carchini *et al.*, 2005; Hofmann & Mason, 2005). Correlations investigating the influence of plant species on Odonata assemblages by Balzan (2012) appear to confirm the hypothesis made by Corbet (1999) that the structure and appearance of plant communities, rather than individual plant species, are probable to be the cues for habitat recognition.

Generally, different odonate groups were associated with different vegetation life-forms and height categories. Additionally, according to Samways & Taylor (2004), the odonate species have been reported to be predominantly threatened by invasive alien plant species along watercourses, which alter sunlight versus shade regimes along aquatic systems (Samways, 2005). Besides, Moore (1991) found that, in a 27-year study of dragonfly communities of small ponds, there are declines in the population of adults that coincided with the extensive growth of reed.

Furthermore, daily changes in environmental variables such as human activities which contributed to the changes in the aquatic environment, as well as the quality of water also affect the distribution of aquatic insects (Fulan *et al.*, 2011). Leaching of fertilizers and pesticides applied in the plantations would alter chemical properties of water (Ameilia *et al.*, 2006), and recently, Dissanayake and Chandrasekara (2014) found that the physical and chemical characteristics of the water body, mainly determined the fauna that colonizes the area. Damselflies' dependence on freshwater habitats makes them very vulnerable to damage to wetlands through drainage for agriculture or urban growth (Corbet, 1980).

2.3 MOLECULAR AND PHYLOGENETIC STUDIES OF ODONATES BASED ON MITOCHONDRIAL DNA

Molecular studies have in particular resulted in major changes in odonate taxonomy in recent years (Dijkstra & Kalkman, 2012). Over the past 20 years until recently, there are many studies progressing towards reconstructing the odonates phylogeny (Ware *et al.*, 2007; Bybee *et al.*, 2008; Blanke *et al.*, 2013; Dijkstra *et al.*, 2014; Misof *et al.*, 2014; Carle *et al.*, 2015; Bybee *et al.*, 2016). The mitochondrial DNA of the organisms functions as an excellent genetic marker with the gene flow in its matrilineal inheritance. It serves as maternal haploid genome and gathers nucleotide substitution five to ten times more rapidly than nuclear DNA and this makes the mitochondrial DNA suitable for examining population and sub population structures among related taxa (Weisser & Siemann, 2004).

In recent years, the molecular genetic studies have led to novel understandings into the spatial genetic structure of aquatic insects (Kelly *et al.*, 2002; Hughes *et al.*, 2003; Wilcock *et al.*, 2003, Sadeghi *et al.*, 2010). In various cases, the results of molecular have supported classical morphological work; however, there are several cases where it shows unexpected novelty findings (Parkes *et al.*, 2009).

The DNA barcoding studies have mostly focused also on distance-based methods to classify and delimited species (Hebert *et al.*, 2003). Nevertheless, this method can show difficulty for various reasons, such as the substitution rates of mitochondrial DNA differ between the different groups of species, thus resulting in a broad overlap of intra and interspecific distances (Will & Rubinoff, 2004; Hickerson *et al.*, 2006). But yet, several researchers have proven that the recent DNA barcoding can present as a fast identification method for assessing biodiversity of known species, and

had created excitement about a new, powerful tool for taxonomy (Hebert & Gregory, 2005; Vences *et al.*, 2005; Clare *et al.*, 2007; Pfenninger *et al.*, 2007)

Additionally, it is supported that a species identification system which based on the DNA sequences can provide a rapid, reliable and consistent method that is important, especially in crisis fields like conservation biology and biodiversity research (Goldstein & DeSalle, 2000; Janzen, 2004; Wheeler *et al.*, 2004; DeSalle *et al.*, 2005; Vogler & Monaghan, 2007; Waugh, 2007). In the taxonomic studies, the DNA sequence data now offer a powerful tool by greatly expanding the number of characters that can be used to distinguish species. The addition of such data, together with the more traditional morphological variables, can solve the problem of subjectivity in current species descriptions (Cracraft, 1992; Dayrat, 2005; Rubinoff, 2006; Vogler, 2006; Cardoso *et al.*, 2009). Besides, the taxonomic circle introduced by DeSalle *et al.* (2005) demonstrated a way in which different data sets can interact to discover new species.

Additionally, recent advances in molecular techniques have been proved as powerful tools for studies of intra and interspecific phylogeographic patterns. Though, according to several researchers, the studies of phylogeographic differentiation and biogeographic patterns among insects are frequently limited to species with the limited dispersal ability (Vogler & DeSalle, 1993; Butlin *et al.*, 1998; Lunt *et al.*, 1998) or to taxa found on island archipelagoes (Fleischer *et al.*, 1998; Polhemus & Polhemus, 1998; Roderick & Gillespie, 1998).

Artiss (2004) concluded that, to make the phylogeographic patterns to easy detectable, there are at least two significant characteristics of taxa which are distributed over large land. The first characteristic is the species whose distributions bridge geographic features that may performance as potential barriers to the gene flow,

possibly will become reproductively isolated, hence, over the time, these populations might become genetically differentiated. The second characteristic is the phylogeographic patterns may be expected in species whose historical patterns of dispersal are known or inferred, and principally if some populations have been historically isolated.

All animal mitochondrial genomes contain the same 37 genes: two for rRNAs, 13 for proteins and 22 for tRNAs (Boore, 1999). Among the mitochondrial DNA, cytochrome oxidase subunit I (COI) gene is one of the most important protein encoding genes by reason of lack of introns, simple alignment, limited exposure to recombination and availability of robust primer sites (Bernard *et al.*, 2011). Besides, 16S rRNA also has been most popular marker in the molecular studies of insects (Shouche & Patole, 2000; Yong *et al.*, 2014; Dijkstra *et al.*, 2014).

The COI gene alone was widely used by many researchers in the studies of molecular phylogeography, genetic variations and biogeography of dragonflies. For example the study of demographic history, population structure and intraspecific diversity of *Pantala flavescens* by Low *et al.* (2017), the clarification of taxonomic status of *Aciagrion occidentale* (Jisha & Sebastian, 2015), intraspecific patterns of molecular variation among geographically isolated populations of *Libellula quadrimaculata* (Artiss, 2004), and the genetic diversity of the tiny dragonfly *Nannophya pygmaea* in Asia (Low *et al.*, 2016b).

The combination of genes also been used in various molecular studies on odonates. Based on COI and 16S rRNA genes, the molecular phylogenetic reconstruction of Zygoptera had confirmed that the zygopteran were monophyletic (Dijkstra *et al.*, 2014), while Bernard *et al.* (2011) used the COII and 16S rRNA-ND1-genes to reveal the phylogeographic structures of taiga species, *Nehalennia speciosa*

and found the low genetic diversity between the populations in Eurasia. Besides, the COI and COII genes had been used to elucidate the phylogenetic of *Enallagma* damselflies (Turgeon & McPeck, 2002) and *Orthetrum* dragonflies (Yong *et al.*, 2014) using ITS 1 & 2, COI, COII and 16S rRNA genes.

Despite a variety of phylogenetic and population genetic studies, to date, species discovery has been based solely on classical taxonomic descriptions and no cryptic odonate species have been discovered (Misof *et al.*, 2000; Weekers *et al.*, 2001; Stoks *et al.*, 2005; Hadrys *et al.*, 2006; Hasegawa & Kasuya, 2006). This case indicates how important it is to combine different fields to determine species boundaries in modern taxonomy, as have molecular studies (Bybee *et al.*, 2008; Carle *et al.*, 2008; Fleck *et al.*, 2008b; Dumont *et al.*, 2010). A modern taxonomic system can be derived from both quantitative information and expert judgment. Combination of datasets from different disciplines into one character based matrix eventually allows the species discovery and species assignment in a more objective way.

2.4 MORPHOLOGICAL CHARACTERISTICS

Earlier, the main guide for classifying Odonata was wing venation; but as similar characters evolved multiple times, this is often not a reliable indication of close relationships (Suhling *et al.*, 2015). Studies incorporating other morphological features, including those of larvae, have helped to overcome this (von Ellenrieder, 2002; Rehn, 2003; Fleck *et al.*, 2008a), such as the structure of the male sex organs, the distance between the compound eyes, form and development of rear appendages, of other structures on the thorax and abdomen, and presence of an ovipositor.

The odonates are made out by their long and thin abdomen, with their large globular eyes that often making up a big part of the head, short antennae and long wings, which bear a conspicuous nodus and usually a pterostigma and also the legs that facilitate catching prey during flight (Hoell *et al.*, 1998). The zygopterans or damselflies have similar fore and hind wings, a broad head with widely spaced eyes. Most of the species rest with the wings at the closed position. Their larvae are slender and rely principally on two or three caudal gills for respiration (Kalkman *et al.*, 2008). Most of the odonate groups are relatively large insects; nonetheless the wingspans of odonates are range from 17 mm for some *Agriocnemis* damselflies, to 191 mm in helicopter damselfly, *Megaloprepus coerulatus*, and the largest dragonflies have a wingspan up to 160 mm (Suhling *et al.*, 2015).

Imagines of Odonata are divided into a head, thorax, and abdomen, and they have two pairs of wings formed by a network of stiffer veins and a flexible. The abdomen of odonates is long, with 10 visible segments, and terminates in clasping organs in both sexes. Females of all damselflies and several dragonflies' families carry a prominent ovipositor under abdominal segments S9-S10; while in males always possess secondary genitalia on the underside of abdominal segments S2-S3. In this study, for

Zygoptera identification we follow the taxonomy proposed by Dijkstra *et al.* (2014) and modified from Gunther (2009). The external features of the damselflies are shown in the figure below (Figure 2.1).

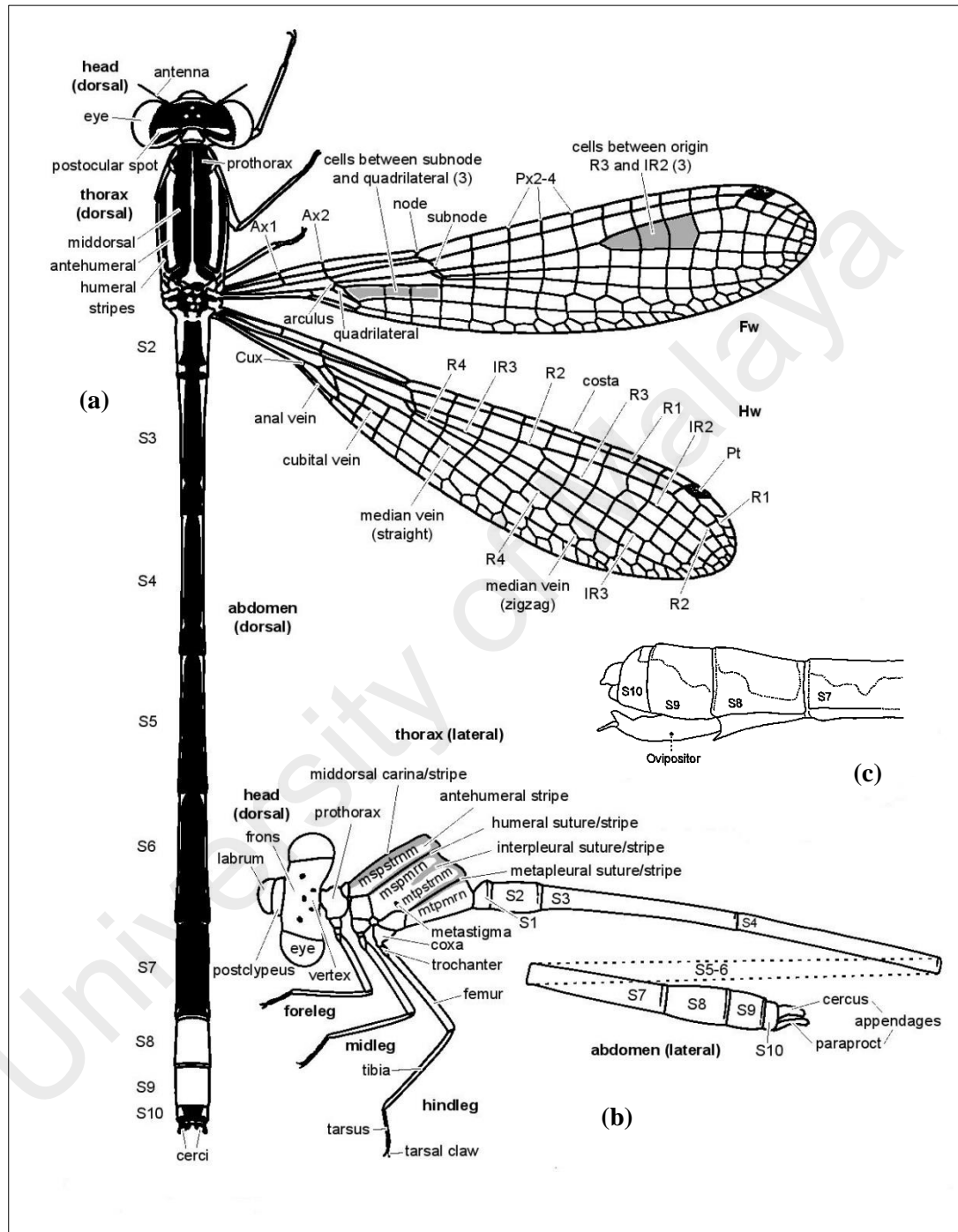


Figure 2.1: External features of damselfly imagines. (a) Dorsal view of a Zygoptera; (b) lateral view of thorax and abdomen; (c) last segments of female Zygoptera abdomen in lateral view showing the ovipositor. (Modified from Suhling *et al.*, 2015 and Dijkstra, 2016).

In scientific fields, taxonomy is very important and a correct identification of organisms establishes an essential infrastructure for other research areas (Dijkstra *et al.*, 2013). The numerous high-throughput technologies currently available allow for the characterization of the genome, transcriptome, proteome and even the morphology of an organism, for instance CT scans, (Busse *et al.*, 2015). The application of such technologies to taxonomic research in dragonflies and damselflies could increase the quality and quantity of data that can be applied, not only to the description of new species, but also to provide new perspectives for the correct identification of specimens (Raupach *et al.*, 2015).

However, according to Jisha and Sebastian (2015), the identification using traditional taxonomy is problematic due to the external changes in the organisms that caused by seasonal and geographical variations. Numerous of the organisms modify themselves physiologically and morphologically by reason of the unfavorable conditions in the environment. Therefore, the implementation of manual taxonomy frequently leads to a wrong identification of the species. Thus, this problem has influenced the development of the molecular taxonomic studies for the conformation and the improvement in the identification of species.

In 2005, the taxonomic circle has been suggested the components of a modern taxonomic system which are hypothesis testing, corroboration, reciprocal illumination and revision (DeSalle *et al.*, 2005). In this scheme more than one of the five components of the circle such as DNA (as discussed above), morphology, reproduction, ecology and geography has supported the hypothesis of a new species. The DNA based identification will provide an initial decision while non-DNA data can complement the database. Accordingly, the DNA based information can be associated with biological information

to include together the evolutionary and taxonomically background (Vogler & Monaghan, 2007).

For examples, the identification of the odonates species has been done using the morphometric studies (Bookstein, 1991; Dryden & Mardia, 1998; Adams *et al.*, 2004), geometric morphometric of the wing shape (Rohlf & Marcus, 1993; Adams *et al.*, 2004), the combination of the DNA sequences and morphology (Pilgrim *et al.*, 2002; Stoks *et al.*, 2005; Pilgrim & von Dohlen, 2007), and on the ovipositor in odonates such as on the skeleton and musculature, cuticular microstructures and functional aspects of the endophytic ovipositor (Matushkina & Lambret, 2011; Matushkina & Klass, 2011).

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2.4.1 Females Ovipositors

There are many characters and elements in describing the adult of odonates, and one of the elements that should be included in any description of an adult of odonates was the genitalia of both sexes, where the ovipositor of the female should be shown in ventral and lateral view, and the illustration of the anal appendages of the female would be very helpful (Heckman, 2008). Genitalia are conspicuously variable, even in closely related taxa that are morphologically very similar. Explaining genital diversity is a longstanding problem that is attracting renewed interest from evolutionary biologists (Hosken & Stockley, 2004).

The genitalia is a complex structure that the basis for species discrimination in most families and also in family identification (Powell, 2009). The uniqueness of the genitalia of a species led to the use of the morphological study of genitalia as one of the most important keys in taxonomic identification of taxa below family level, and with the advent of DNA analysis, the study of the genitalia has now become just one of the techniques used in taxonomy.

In most females, the ovipositor is the most obvious structure at the apex of the abdomen (Heckman, 2008). The ovipositor of odonates can be divided into two, which are exo- and endophytic. The exophytic oviposition (Carle *et al.*, 2008) was practiced by Anisoptera with reduced appendicular ovipositors, for examples for the family of Gomphidae, Corduliidae, Libellulidae, Chlorogomphidae, and Neopetaliidae (Carle & Louton, 1994; St. Quentin, 1962) which included 43% of recent dragonflies (Bridges, 1993).

Otherwise, all zygopteran and aeshnid Anisoptera have a cutting ovipositor that used in egg deposition within the plant tissues (St. Quentin, 1962). The egg-laying behaviour is very important for the continuation of almost all insect species (Matushkina & Lambret, 2011) and there are about 54% species of recent odonates demonstrate endophytic egg-laying including all the Zygoptera group, Anisozygoptera and anisopteran Aeshnidae (Bridges, 1993).

The ovipositor of Zygoptera and Aeshnidae has evolved to facilitate oviposition into substrates such as plants. A pair of cutting valves, covered by outer valves when at rest, makes a hole in the plant tissue by alternating movements, and the eggs are inserted by muscles contracting in S9 (Matushkina & Lambret, 2011). In other Anisoptera there is a gradual reduction of ovipositor complexity, ending with a simple genital plate in Corduliidae and Libellulidae.

2.4.2 Wings Structures of the Odonates

The dragonfly wing is made up of a three-dimensional skeletal network of relatively hard longitudinal veins as described by Suhling *et al.* (2015). They are interconnected through thin membranous areas, known as cells. Former studies on the functional morphology of odonate wings have revealed that the camber and angle of attack are automatically maintained under aerodynamic load by a set of internal mechanisms. These are included of vein pattern, vein curvature, and type of joints between cross- and longitudinal veins.

There are more than six different kinds of crossvein junctions of longitudinal veins are known, however, these junctions can be divided into two main types of joints, which are immobile and mobile. The mobile vein joints are known to contain resilin, a

rubberlike protein that is responsible for elastic energy storage during deformations which is an important component in insect flight (Gorb, 1999). Apparently, such joints are essential to the highly maneuverable flight of odonates, especially under conditions where unstable wind pulses act on the flying insect.

Insect flight has fascinated many scientists in the fields of biology and engineering. Over the past 14 years, the scientific understanding of insect flight has been extensively transformed by the appearance of new experimental techniques for measuring everything from the aerodynamics of flight, to the movement of actin and myosin proteins within insect flight muscle and also the flight control responses of freely flying animals. Many insects establish impressive flight abilities, for instance hovering (Liu & Kawachi, 1998, Aono *et al.*, 2008), taking off (Dickinson *et al.*, 1999), backward (Ellington, 1999) and sideward flights (Ristroph *et al.*, 2009). Moreover, in the last decade, several efforts have been made to explore the effects and correlations of morphology and material properties on the biomechanics of insect wings such as by Young *et al.* (2009), Appel and Gorb, (2011, 2014), Dirks *et al.* (2013), Mountcastle and Combes (2013), and Rajabi *et al.* (2015).

Both dragonflies and damselflies exhibit very different flight behaviors. While most of the dragonflies are fast fliers with broad-based wings and posterior-distally curved longitudinal veins (Ruppell & Hilfert, 1993), damselflies with their narrow-based and nearly identical fore- and hindwings, in contrast, are known by their slow flight, low flapping frequency and large wing twisting (Ruppell, 1989; Wootton & Kukalova-Peck, 2000). The damselflies are classified as perchers; use both up- and downstrokes for an aerodynamic force generation (Wootton & Kukalova-Peck, 2000). Their wings may undergo extremely large deformations, and are not only due to

aerodynamic and inertial forces in flight (Wootton, 1981, 1990, 1991, 1992), but also because of the interactions between wings and solid obstacles.

In 1981, Wootton was the first person who created attention to the importance of passive deformability of insect wings (Wootton, 1981). He studied the effects of the wing architecture, including venation pattern, relief, thickened areas, and flexion lines on its local deformations, while Newman (1982) was the first to describe the morphology of the connections between the longitudinal veins and some of the cross veins in dragonfly wings. He categorized these vein joints into two main groups of flexible and fused joints and also proposed that they may contribute to the passive deformation of insect wings. In addition, according to Appel & Gorb (2014), the wings of Odonata are dominated by combinations of one or two specific vein joint types considering the distribution of the vein joints within the wings of 22 species of Odonata, there are five groups of wings with different vein joint patterns distinguished.

Even though several parameters influence the deformation of the wings in Odonata, considering their distribution over the wing, vein microjoints have been recognized as one of the most effective elements, in contrast to the others (Rajabi *et al.*, 2015, 2016). The dragonfly wings are attractive biological composite structures consisting of an ultra-thin membrane reinforced with a network of hollow veins. Insect wing veins are known as biological composites of chitin and protein that's arranged in a complex lamellar configuration.

According to Donoughe *et al.* (2011), insect wings differ from vertebrates where their lack of internal musculature extending into the aerodynamic surface of the wing. Hence, while birds and bats can actively modulate the form and flexibility of their wings, insects have little active control over wing properties, and most deformations are a product of the passive mechanical properties of the wing (Wootton, 1992) interacting

with the inertial and aerodynamic forces it generates while flapping (Daniel & Combes, 2002). The flexibility in insect wings may enhance aerodynamic performance and may also help to protect against permanent wing damage.

Newman and Wootton (1986) who the first suggested that dragonfly wings appear to be adapted for reversible failure in response to excess loads, allowing them to avoid permanent structural damage and their flight performance has been the subject of various aerodynamic, kinematic and morphological studies (Wootton, 1981, 1991; Newman, 1982; Ruppell, 1989; Vargas *et al.*, 2008; Wootton & Newman, 2008; Kim *et al.*, 2009; Jongerius & Lentink, 2010).

Understanding of wing's structure and morphology is imperative to mimic a synthetic flying system. Formerly several researchers had been exploring the morphological features of dragonfly wings, but not the damselfly. For instance, Wang *et al.* (2008) showed microstructural features of dragonfly wing veins cross-section and spanwise plan view of a dragonfly wing near the proximal and also the microstructure of tubular Costa. On the other hand, the surface of the veins was studied by Zhao *et al.* (2010), where they showed veins surface had wave microstructure for example huge surface roughness, while other researchers, Song *et al.* (2007) showed that the membrane cross-section features which consists of three layers, dorsal surface, middle layer and ventral surface along the direction of thickness. One of the main functions of the veins is evidence to offer the structural framework of the wing (Wootton, 1992; Smith *et al.*, 2000); indeed, they are highly specialized elements which enable insects to withstand large aerodynamic forces in flight (Wu & Sun, 2004; Nakata & Liu, 2012).

A rubber-like protein, resilin was originally discovered and analyzed in detail by Weis-Fogh (1960, 1961a, b), and later confirmed by Andersen and Weis-Fogh (1964). This elastomeric protein stands out for its long-range deformability, coupled with an

almost complete elastic recovery (97%; Andersen & Weis-Fogh, 1964; Andersen, 2010). They are found to give low stiffness, high strain and efficient energy storage (Andersen, 1964; Gosline *et al.*, 2002; van Eldijk *et al.*, 2012) and that functions in insect flight (Weis-Fogh, 1960; Gorb, 1999). Additionally, numerous authors found that irrespective of its small size, the resilin-filled joints played a role in bending shapes and for the whole flexibility of the wing (Donoughe *et al.*, 2011; Mountcastle & Combes 2014).

The presence of resilin in some vein joints of odonate provided flexibility, as well as serving as a damper and stretchable component (Gorb, 1999; Jakle, 2003). Therefore, a study on the flexible element that has been found on the wings of damselflies is important in order to response the key of successfulness of these small damselflies, in contrast to hydrated resilin, which has a Young's modulus of about 0.1–3.0MPa, sclerotized cuticle exhibits a relatively high stiffness, with a Young's modulus of up to 20 GPa (Vincent & Wegst, 2004; Elvin *et al.*, 2005; Peisker *et al.*, 2013).

CHAPTER 3: METHODOLOGY

3.1 ODONATES DIVERSITY AND DISTRIBUTION IN PENINSULAR MALAYSIA

3.1.1 Study Sites

Two specific sites from the 11 states in Peninsular Malaysia were selected as sampling sites for the study. A total of 22 localities (Figure 3.1) were covered which comprised of forest reserves or forested parks. The samplings were conducted from the June 2014 to October 2015. Details of samplings are as in Table 3.1.

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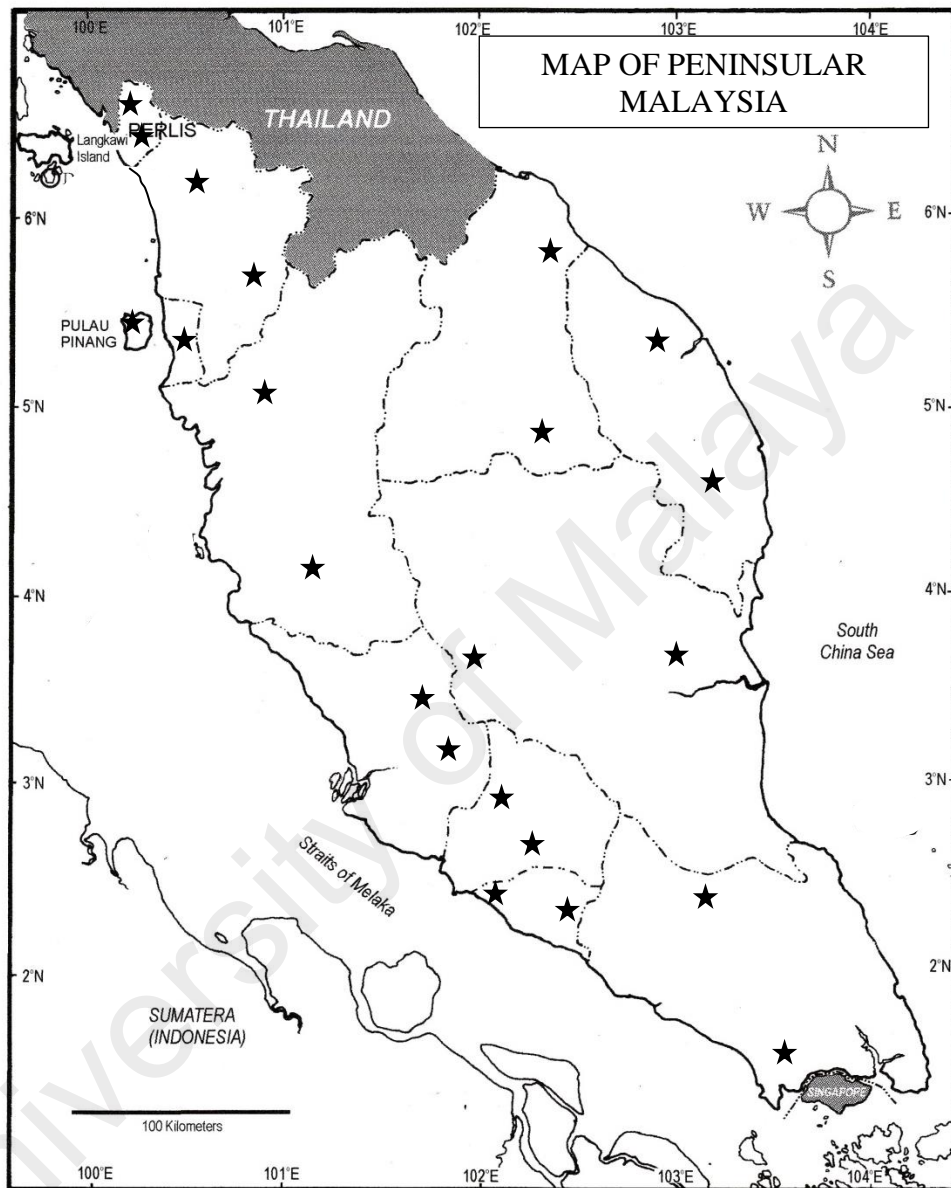


Figure 3.1: Map showing the 22 sampling sites (★) in Peninsular Malaysia, two localities for all 11 state boundaries.

Table 3.1: Details of sampling sites of odonates in Peninsular Malaysia

Region	GPS coordinates	Collecting date
Northern		
<i>Kedah</i>		
Bukit Wang Recreational Forest	N 06 18' 11.30" E 100 28' 50.55"	September, 2014
Lata Bayu Recreational Forest	N 05 43' 02.9" E 100 48' 50.9"	September, 2014
<i>Perak</i>		
Lata Kekabu Recreational Forest	N 05 03' 01.1" E 100 56' 32.5"	November, 2014
Lata Iskandar Waterfalls	N 04 19' 27.18" E 101 19' 31.36"	June, 2014
<i>Perlis</i>		
Kelam Cave	N 06 38' 40.65" E 100 12' 11.6"	August, 2015
Sungai Jernih Recreation Park	N 06 32' 46.6" E 100 16' 17.3"	August, 2015
<i>Pulau Pinang</i>		
Teluk Bahang Forest Park	N 05 26' 47.2" E 100 13' 01.8"	September, 2015
Taman Rimba Recreational Forest	N 05 21' 33.7" E 100 29' 36.8"	September, 2015
Southern		
<i>Johor</i>		
Sungai Bantang Recreational Forest	N 02 20' 46.7" E 103 09' 23.9"	August, 2014
Gunung Pulai Recreational Forest	N 01 25' 25.8" E 103 31' 01.2"	August, 2014
<i>Melaka</i>		
Sungai Linggi Recreational Forest	N 02 23' 39.7" E 101 59' 18.4"	June, 2014
Ayer Keroh Recreational Forest	N 02 17' 09.7" E 102 17' 50.0"	March, 2015
<i>Negeri Sembilan</i>		
Ulu Bendul Recreational Forest	N 02 43' 38.9" E 102 04' 33.8"	October, 2015
Jeram Toi Recreational Forest	N 02 51' 52.7" E 102 00' 52.0"	June, 2014
Central		
<i>Selangor</i>		
Sungai Sendat Recreational Forest	N 03 24' 14.4" E 101 41' 00.5"	April, 2015
Sungai Gabai Waterfalls	N 03 09' 56.5" E 101 54' 28.6"	Februari, 2015
East Coast		
<i>Kelantan</i>		
Jeram Linang	N 05 44' 33.4" E 102 22' 26.2"	October, 2014
Jeram Pelangi	N 04 56' 24.0" E 102 02' 55.1"	October, 2014
<i>Pahang</i>		
Teladas Waterfalls	N 03 35' 28.4" E 102 45' 56.9"	March, 2015
Sungai Chamang Waterfalls	N 03 30' 34.2" E 101 51' 36.3"	March, 2015
<i>Terengganu</i>		
Sekayu Recreational Forest	N 04 57' 45.8" E 102 57' 11.9"	October, 2015
Lata Belatan Recreational Forest	N 05 38' 21.5" E 103 35' 16.6"	December, 2014

3.1.2 Sampling Methods, Preservation and Identification

Methods of sampling and preservation of Odonata were based on Orr (2003) and Borror and White (1970). Odonata were caught with a sweep net, which had been standardized during hot sunny days, 10:00 to 15:00, and same sampling efforts of two same collectors throughout the study areas. The odonates were collected along the river from the starting point within 2 kilometers up to the hill.

The Odonata were grasped by its body and stunned by pinching the thorax after it was removed from the net. Then, the specimens caught were placed in the triangle envelope with the wings folded together above the body. Data on collection and information such as locality, date, time and the collector's name were written on the outside of the envelopes. The microhabitats frequented by the odonates were recorded at every site where odonates were sampled. All specimens were identified to species level.

3.1.3 Data Analysis

The sample frequencies consist of the information regarding the number of species present at the sampling sites and the common species for their total abundance. Besides, the frequency of species occurrence (FO) and sampling sites occurrence (SO) was designated as percentages. The presence-absence data for the species were used and expressed as binary scales which are 0 = absent, while 1 = present (Hamada & McCreadie, 1999; McCreadie *et al.*, 2004; Pramual & Kuvangkadilok, 2009).

The sampling efficiency was obtained from the species richness estimators, and a rarefaction species accumulative curve, which was based on the number of individuals, in order to determine if the species in the site were adequately sampled (Brühl, 2001). The species diversity estimations were calculated as percentage of

observed versus expected species from the estimators. Incidence-based data was used to calculate the richness estimators, namely: Chao Present & Absent, Chao & Lee 2, 1st Order Jackknife, 2nd Order Jackknife, the Bootstrap and Michaelis-Menten. The detailed description of the mathematical background, see Appendix A. Moreover, a Chi-square test was done in order to confirm the status of no significant difference between the sites. This analysis was run using Species Diversity & Richness 4.1.2 software (Seaby & Henderson, 2007).

Additionally, a hierarchical cluster that is the most common method was used to group all the samples collected into a series of clusters producing taxonomic tree of groups and subgroups of the similar species. The binary data was subjected to Jaccard and the average-linkage-between-groups method, often aptly called UPGMA (unweighted pair-group method using arithmetic averages) that defines the distance between two clusters, as the average of the distances between all pairs of cases in which one member of the pair is from each of the clusters were applied. This analysis was calculated using IBM SPSS statistic 24 for Windows (SPSS Inc., Chicago).

3.2 CORRELATIONS OF PHYSICAL AND CHEMICAL PARAMETERS WITH ODONATE DIVERSITY AND DISTRIBUTION

3.2.1 Study Sites

Physical and chemical parameters were measured and collected at the time of each sampling time. The detailed information on sampling sites is given in the Section 3.1.1.

3.2.2 Physical and Chemical Measurement

For the descriptions of physical and chemical variables were measured for each site as follows (Table 3.2):

Table 3.2: The environmental variables measured for sampling sites.

	Variable	Unit of measurement (Transformation)
1	Altitude	m ($\log_{10} x$)
2	Flow rate	ms ⁻¹
3	Water temperature	°C
4	Ambient temperature	°C
5	Humidity	Percentage relative humidity (%)
6	Salinity	ppt
7	pH	pH
8	DO (Dissolved oxygen)	ppm
9	Conductivity	mS , uS
10	TDS (Total dissolved solids)	g/L
11	Nitrate	mg/L NO ₃ ⁻ N
12	Nitrite	mg/L NO ₂ ⁻ - N
13	Sulfate	mg/L SO ₄ ²⁻
14	Phosphate	mg/L PO ₄ ³⁻
15	Ammonia	mg/L NH ₃ -N
16	Iron	mg/L Fe
17	Depth of water body	cm/m
18	Width of water body	cm/m

The conductivity, salinity and total dissolved solids (TDS) were measured by a microprocessor based instrument, Jenco model 3010, while for the dissolved oxygen (DO) and water temperature were measured using the dissolved oxygen meter, Mi 605. PH meter Jenco model 6010N and Twirling Sling Psychrometer (Wet Dry Bulb Hygrometer) were used to measure water pH and humidity, and the relative humidity was determined by locating the intersection of the wet and dry-bulb temperatures on a psychrometric chart. For the ambient temperature, it was also measured by Twirling Sling Psychrometer, and for the water depth and width were measured using meter tape. For each site, three readings were taken and the average of the three readings was used.

Selected chemical parameters such as; Nitrate, Nitrite, Sulfate, Phosphate, Ammonia, and Iron were measured using DR/2400 Portable Spectrophotometer. The determination of all the chemical parameters were carried out immediately after the water sample was brought back to the base camp or hotel. For each chemical parameter, the water samples were measured for three times and the average of the three readings was used.

3.2.3 Data Analysis

All the data were tested for normality using the Shapiro-Wilk method (Table 3.3). For correlation analysis, both Pearson's and Spearman's correlation were used. From the outcome of the Shapiro-Wilk test, if the variables were normally distributed, the Pearson's correlation was used, in contrast, if the variables were not normally distributed or the relationship between the variables was not linear, the Spearman's rank correlation was used.

Table 3.3: Normality test using Shapiro-Wilk test of all variables measured across all sampling sites in Peninsular Malaysia. ($\rho < 0.05$, asterisk indicates the data normally distributed)

Parameters	Shapiro-Wilk		
	Statistic	df	Sig.
Elevation (m)	.784	22	.000*
Conductivity (ms)	.501	22	.000*
Humidity (°C)	.959	22	.465
DO (ppm)	.838	22	.002*
pH	.939	22	.188*
Salinity	.417	22	.000*
Water Temp (°C)	.837	22	.002*
Ambient Temp (°C)	.974	22	.805
Flow Rate (s/m)	.957	22	.427
Water Depth (m)	.649	22	.000*
Water Width (m)	.974	22	.808
TDS (g/L)	.546	22	.000*
Iron (mg/L Fe)	.716	22	.000*
Nitrate (mg/L NO ₃ ⁻ -N)	.806	22	.001*
Nitrite (mg/L NO ₂ ⁻ - N)	.915	22	.061
Ammonia (mg/L NH ₃ -N)	.609	22	.000*
Phosphorus (PO ₄ ³⁻)	.903	22	.034*
Sulphate (mg/L SO ₄ ²⁻)	.406	22	.000*

The Principal Components Analysis was used in order to reduce the variables into groups of independent components. All the principal components (PCs) that had the eigenvalues more than 1.0 were taken as variables. For this analysis, Rotation Method (Varimax with Kaiser Normalization) was used so that the clusters of the variables fall as closely as possible to them, and it also simplifies the expression of a particular subspace in terms of just a few major items each. However, the actual coordinate system is unchanged.

Besides, logistic regression that used binary data was performed to measure the relationship between the categorical dependent variable and one or more independent variables by estimating probabilities using a logistic function, which is the cumulative logistic distribution. In this study, it was used to test the relationship between species richness (i.e. number of species in each sampling site) and the variables of the sampling sites (i.e. PC scores). Moreover, a log-logistic regression was combined with bootstrap method. The bootstrap method (Efron & Tibshirani, 1993) is an effective tool for the internal validation of logistic regression models and performs multiple resampling with replacement within the data set. Bootstrap regression can be considered a compromise between the power of resampling and fitting an underlying distribution and was found to be especially useful for small data sets where a good model fit is achieved (Wheeler *et al.*, 2002).

All analysis and statistical calculations were performed using Species Diversity and Richness (SDR) 4.1.2 software (Seaby & Henderson, 2006), IBM SPSS statistic 24 for Windows (SPSS Inc., Chicago) and STATISTICA 8 (StatSoft, 2007).

3.3 MOLECULAR PHYLOGEOGRAPHY OF *Rhinocypha fenestrella* BASED ON ANALYSES OF MITOCHONDRIAL COI AND 16S RRNA GENES

3.3.1 Animal Material

Rhinocypha fenestrella syn. *Aristocypha fenestrella* (Rambur, 1842) was collected from eight populations in Peninsular Malaysia during 2014-2015 (Table 3.4). A total of 147 male specimens were used in the analysis.

Table 3.4: Sampling localities and geographic position of sampling sites of *Rhinocypha fenestrella*.

Abbreviation	State	District	Locality	Geographic position
SS1	Negeri Sembilan	Jelevu	Jeram Toi Waterfall	N 02 51' 52.7" E 102 00' 52.0"
SS2	Johor	Bekok	Sungai Bantang Waterfall	N 02 20' 46.7" E 103 09' 23.9"
SS3	Kedah	Banting	Lata Bayu Waterfall	N 05 43' 02.9" E 100 48' 50.9"
SS4	Perak	Tapah	Lata Iskandar Waterfall	N 04 19' 27.18" E 101 19' 31.36"
SS5	Kelantan	Machang	Jeram Linang Waterfall	N 05 44' 33.4" E 102 22' 26.2"
SS6	Terengganu	Kuala Berang	Sekayu Waterfall	N 04 57' 45.8" E 102 57' 11.9"
SS7	Pahang	Bentong	Chamang Waterfall	N 03 30' 34.2" E 101 51' 36.3"
SS8	Selangor	Hulu Selangor	Sungai Sendat Waterfall	N 03 24' 14.4" E 101 41' 00.5"

3.3.2 DNA Isolation and Amplification

Genomic DNA was extracted from four to six legs of each fresh specimen using the i-genomic CTB DNA Extraction Mini Kit (iNtRON Biotechnology Inc., Seongnam, South Korea) see Appendix B. The DNA amplifications of both COI and 16S rRNA genes were conducted using an Applied Biosystems Veriti 96-Well Thermal Cycler (Applied Biosystems Inc., Foster City, CA, USA) with the amplification protocol consisted of 300 sec at 94°C followed by 35 cycles of 50 sec at 94°C, 50 sec at 50°C and 50 sec at 72°C, and a final 7 min at 72°C.

Primers for the amplification of the mitochondrial-encoded COI gene, were adopted from Folmer *et al.* (1994) (forward primer: 5'- GGT CAA CAA ATC ATA AAG ATA TTG G – 3') and Barrett and Hebert (2005) (reverse primer: 5'- GGA TGG CCA AAA AAT CAA AAT AAA TG –3'). For 16S rRNA gene, ODO 12852 and ODO 13393 primer set (forward primer, 5'- AGA AAC CGA CCT GGC TTA AA -3'; reverse primer, 5'- CGC CTG TTT ATC AAA AAC AT -3') were adopted from Dijkstra *et al.* (2014). Each PCR amplification was performed in a reaction mixture containing 50–100 ng of genomic DNA, 25 µL of NEXpro e-PCR 2x Master Mix (Genes Labs Inc., Gyeonggi-do, South Korea), and 10 pmol of each forward and reverse primer.

3.3.3 DNA Purification and Sequencing

The amplified samples were then electrophoresed on 2% agarose gel pre-stained with SYBR Safe™ (Invitrogen Corp., Carlsbad, CA, U.S.A.) and the PCR products were sent to a commercial company for DNA sequencing in both forward and reverse directions. The samples were sequenced using the BigDyeH Terminator 3.1 Sequencing Kit.

3.3.4 DNA Sequence Alignment

All sequences were assembled and edited using Molecular Evolutionary Genetics Analysis (MEGA) software Version 6.0 (Tamura *et al.*, 2013) and BioEdit 7.0.9.0 (Hall, 1999) and preliminarily aligned using CLUSTALX (Thompson *et al.*, 1997).

3.3.5 DNA Data Analysis

The aligned COI and 16S rRNA sequences comprised 614 bp and 534 bp, respectively, while the sequences of both COI and 16S rRNA were concatenated to yield a total length of 1148 bp. The genetic diversity or haplotype networks of *R. fenestrella* were analyzed using TCS 1.13[®] (Clement *et al.*, 2000) to calculate the minimum number of mutational steps by which the sequences could be joined with >95% confidence. Of all the 147 sequences generated from this study, 26 were unique for COI and 10 for 16S rRNA genes, while 33 distinct haplotypes (AB1-AB33) were detected based on COI + 16S rRNA. The unique sequences generated in this study were deposited in the NCBI GenBank database under accession numbers KY678719-KY678744 for COI and KY678745-KY678754 for 16S rRNA.

To assess the haplotype diversity (H_d) and nucleotide diversity (π), sequences were analyzed using DnaSP 5.0 program (Librado & Rozas, 2009). Uncorrected (p) pairwise genetic distances were assessed using PAUP 4.0B10 (Swofford, 2002) to measure the genetic divergence of *R. fenestrella*. The analysis of DNA sequence variation and nucleotide composition was performed using Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 (Tamura *et al.*, 2013).

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3.4 TAXONOMIC STUDIES WITHIN THE FEMALES OF *Rhinocypha* BY UTILIZING 4 CONTRASTING TOOLS

3.4.1 Type Specimens

Adult females of *Rhinocypha fenestrella*, *Rhinocypha biforata* and *Rhinocypha perforata* collected from peninsular Malaysia were used.

3.4.2 Morphological Description of Female *Rhinocypha* spp.

Five females for each species of *Rhinocypha* were investigated and examined to create dichotomous keys. To ensure correct pairs of species, the female individuals were collected during pairings or matings.

Several characters were highlighted in order to create the key identification for females of *Rhinocypha* spp. using the morphological nomenclature by Dijkstra *et al.* (2014) and modified from Gunther (2009). There are 1) wings, to observe the wing venations, 2) pterostigma, 3) nodus, 4) thorax in dorsal view to see the metepimeron, metanepisternum, mesanepisternum, and etc, 5) abdominal segments (S1-10) length, and width and 6) length of the wing (Figure 3.2).

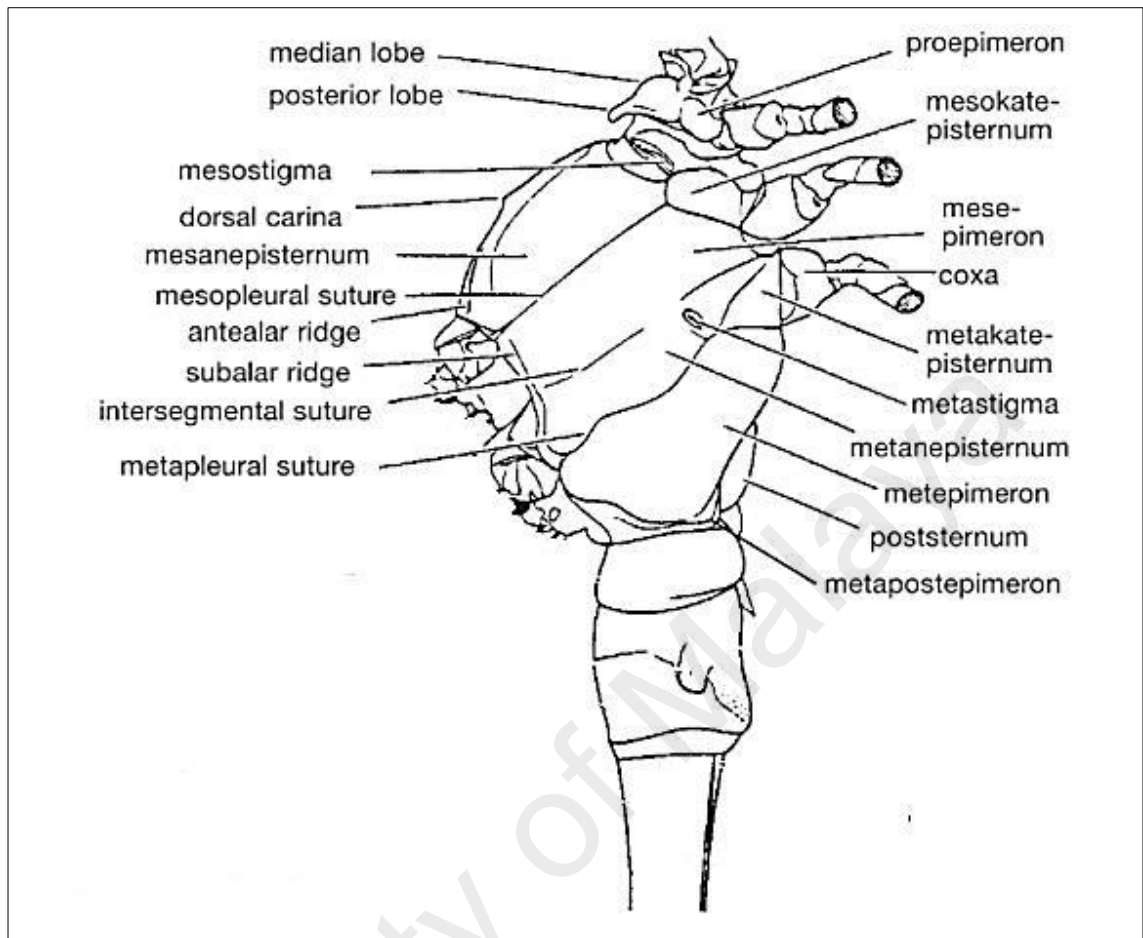


Figure 3.2: Lateral view of thorax and anterior abdomen. Characters used in order to create the key identification for females of *Rhinocypha* spp. using the morphological nomenclature by Djikstra *et al.* (2014) and modified from Gunther (2009).

3.4.3 Field Emission Scanning Electron Microscope (FESEM)

Three air-dried females of each species of *Rhiniocypha* were first examined with stereo microscope and then with a field emission scanning electron microscope. This study was focused on the ovipositor part of the adult females of *Rhinocypha* spp. For FESEM, the female's abdomen was cut at the S7 and was mounted using carbon tape on a stub. All the specimens were then examined with a FEI QUANTA 450 FEG field emission scanning electron microscope. A general description of the odonate endophytic ovipositor was provided by Matushkina (2008).

To record the morphometric data, eleven continuous characters were measured from the FESEM images captured. The mean and the standard deviation values of the characters were taken. These characters were: length of 8th, 9th and 10th segments, the length of the anal appendages, basal width of the anal appendages, length of the stylus, width of V3, peak of the tooth to the median base, the space between the tooth, the width of the distal tooth, and width of the stylus. For the details images of the measurements as shown in section 4.4.2.

3.4.4 Geometric Morphometric Analysis of the Wings

A total of 30 individuals of females of each *Rhinocypha* species were used in this analysis. The right wing of each individual was carefully removed from the specimen and placed on a white paper with the dorsal side of the wing facing upwards. A ruler with minimum scales of 1 mm was placed on the white paper to calibrate of the measurement and a digital image of each specimen was taken with a Dino-lite EDGE AM7115MZT attached with RK-10 Stand. Images were imported into tpsDig (Rohlf, 2005) for digitization of landmarks. 15 homologous landmarks were chosen in this study to quantify wing shape variation such as shown in the below figure (Figure 3.4), *Rhinocypha biforata* as an example.

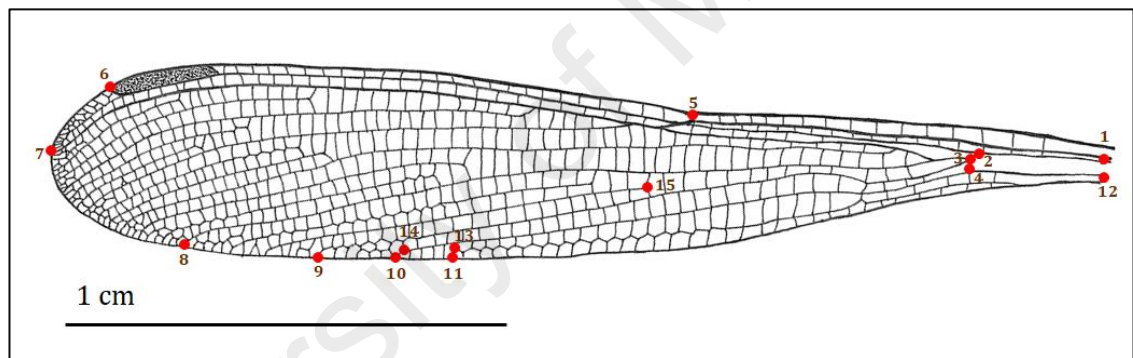


Figure 3.4: Landmark configuration of *Rhinocypha* spp.

Fifteen landmarks were used in geometric morphometric analysis. Landmarks represent: (1) costa – subcostal connection, (2, 3 & 4) distal angles of arculus, (5) the nodus, (6) posterior intersection of the pterostigma and radius 1 (R1), (7) distal tip of the wing, (8) posterior end of the radius 4 (R4), (9) posterior end of the anterior media (MA), (10) posterior end of the Cubital Vein (CuP), (11) posterior end of the Anal Vein 1 (A1), (12) proximal apex of anal triangle, (13) anterior end of the cubital vein supplementary (Cupspl); (14) anterior end of the anterior media supplementary (Mspl); and (15) anterior end of the radius 4 supplementary (R4spl).

The coordinates of all the samples were superimposed to remove the information of size, position and orientation to standardize each specimen according to centroid size. For analyzing wing shape variation within the females of the three species of *Rhinocypha*, principle component analysis (PCA) were conducted on the landmark coordinates data set, while to examine the amount of symmetric variation and shape dimorphism, Procrustes ANOVA were used. Thin plate spline deformation grids were generated and used to visualize shape variation along PC axes (Bookstein, 1991). On the other hand, canonical variate analyses (CVA), a multivariate statistical method was conducted to determine the shape characteristics that best distinguished the groups of specimens from each other by using these coordinates. All analyses were then run using MorphoJ software version 1.06d (Klingenberg, 2011).

3.4.5 Phylogeny Comparison

A total of five individuals for each *Rhinocypha* species were used to conduct a phylogenetic tree of this group. For DNA isolation and amplification, DNA purification and sequencing and DNA sequence alignment, the materials and methods were followed as in the previous section, Sections 3.2.2, 3.2.3 and 3.2.4.

For data analysis, the step was further analyzed to build a Neighbor-joining phylogenetic tree of *Rhinocypha* species based on combined COI + 16S rRNA sequences with the bootstrap replicate of $n = 1000$. The evolutionary distances were computed using the Maximum Composite Likelihood method. The evolutionary analyses were conducted and performed using Molecular Evolutionary Genetics Analysis (MEGA) version 6 (Tamura *et al.*, 2013).

3.5 MORPHOLOGY AND CHARACTERISTICS PROPERTIES OF THE *Rhinocypha* WINGS

3.5.1 Laser Scanning Confocal Microscopy (LSCM)

The forewing of the samples (*Rhinocypha fenestrella*, *Rhinocypha perforata* and *Rhinocypha biforata*) were dried, mounted between two coverslips and observed using a ZeissLSM 700 laser scanning confocal microscope (Figure 3.5) in wavelengths of UV Bands (excitation 405nm, emission 400-420nm). Resilin was known to have auto fluorescence at a narrow band of wavelengths ~420nm (Andersen & Weis-Fogh, 1964; Neff *et al.*, 2000; Burrows *et al.*, 2008; Donoughe *et al.*, 2011). The wing veins were examined for the presence of resilin, which appears as a deep blue colour under UV excitation on both sides, dorsal and ventral sides of the wing. All the images were captured with a specific digital camera AxioCam MRm up to a magnification of 40x, and a detailed joint-by-joint mapping of resilin on the both sides of the wings of the *Rhinocypha* spp. were conducted. A given longitudinal vein was scored as “present” of resilin that showed visible blue fluorescence and “absent” for versa. The veins were identified using homology system developed by (Riek & Kukalova-Peak, 1984). It should be mentioned here that there was a limitation in the mapping of resilin since there was a variation in the size (strong to weak) of resilin patches that were subjected fluorescence intensity. Thus, the presence and absence of the resilin at the specific veins (using the homology system) were more focused and thus scored here.

3.5.2 Scanning Electron Microscopy (SEM)

After imaging by laser scanning confocal microscopy (LSCM), the forewings of all the samples used were coated with gold-palladium and examined using a JOEL JCM-6000 NeoScope Benchtop scanning electron microscopy (SEM) (Figure 3.6) at an accelerating voltage of 5kV. The wings of the three species of damselflies were mounted using carbon tape and examined the presence of cuticular spikes in close proximity to a vein joint dorsally and ventrally. Subsequently, detailed joint-by-joint mappings of the spikes of the wings on both sides were done.

3.5.3 Atomic Force Microscopy (AFM)

For this purpose, the forewings of the three species of *Rhinocypha* were dissected into sections that comprised of (1) wing surface, (2) mobile joint and (3) immobile joints. These samples used were characterized using atomic force microscopy (AFM), where the wing parts were mounted onto a platform using a double-sided tape. All the samples were observed and the images were recorded by using a Scanning Probe Microscope (AFM5300E, Hitachi High-Tech Science Corporation, Japan) (Figure 3.7), at the room temperature using a silicon cantilever type probe (SI-DF3). To obtain accurate results, several properties of the AFM cantilever must be known, particularly the spring constant, sensitivity, and tip radius. The cantilever was set to a radius tip curvature of 10 nm, with a spring constant of 1.7 N/m and frequencies of 28 kHz. The samples were scanned using tapping mode AFM with size of $1\mu\text{m} \times 1\mu\text{m}$. Three samples were used for each species of *Rhinocypha* spp., and the force curves from 10 different points in each section (wing membrane, mobile and immobile joints) were recorded from each sample for further elasticity measurements. The average values and standard deviations from the 30 different points at each section of the three species were used as the

modulus values of each section of the wings. Prior to this, a force curve of a glass substrate was recorded as a reference.

3.5.4 Mechanical Properties – Elasticity Measurements with AFM

Elasticity or Young's Modulus values were generated by analyzing the force curves according to the Hertz Model using conical tip geometry (JPK Instrument, 2017). The loading force is defined as:

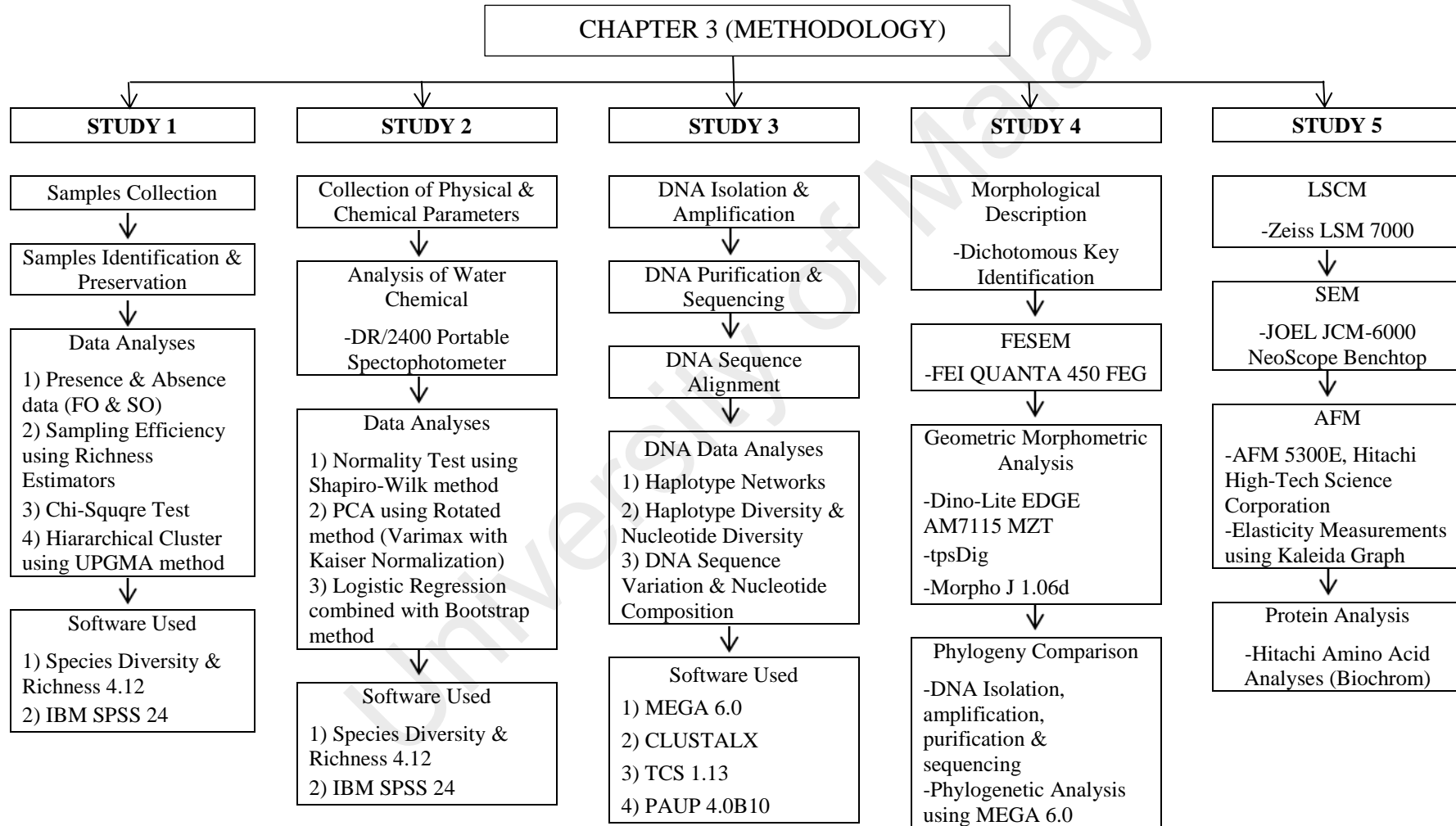
$$F = \frac{4\sqrt{Rc}}{3} \frac{E}{1-\nu^2} \delta^{3/2},$$

where F is the force, Rc is the radius of tip curvature, E is the elastic modulus, ν is the Poisson's ratio and δ is the indentation depth. Afterwards, E -value was derived by fitting the indentation curve using Kaleida graph software.

3.5.5 Protein Analysis of the Wing Samples

In this study, 16 dried wings were used from 4 individuals (fore- and hindwings) for each species of the *Rhinocypha*. The wing samples were dried and grind to a powder. Approximately 8 mg of wing samples were hydrolyzed in 8 M urea, 100 mM NaH₂PO₄, 10 mM Tris = pH 7.4, and stirred overnight. Then, the lysate was centrifuged (10000 rpm, 90 min, r.t.) to transfer the supernatant into a membrane filter (100 – 500 DA MWCO) for dialysis process for 5 days. Subsequently, the hydrolyzates were freeze-dried under a vacuum using an EYELA Rotary Vacuum Evaporator. The amino acid was analyzed by the Research Resources Centre of RIKEN Brain Science Institute using a Hitachi Amino Acid Analyzer (Biochrom) and about 0.7 mg/100 uL, 0.8 mg/100uL and 09 mg/100 uL of the filtrate was taken for amino acid analysis for *R. biforata*, *R. perforata* and *R. fenestrella* respectively.

3.6 SCHEMATIC FLOW DIAGRAM FOR THE RESEARCH METHODOLOGY



CHAPTER 4: RESULTS

4.1 ODONATES DIVERSITY AND DISTRIBUTION IN PENINSULAR MALAYSIA

4.1.1 Odonates Species Composition

Overall, 1193 individuals of 70 odonate species from 10 families were collected from the 22 localities representing the diversity of odonates in Peninsular Malaysia forest reserves. Chlorocyphidae was the dominant family with 40.3%, followed by Libellulidae (23.3%) and Platycnemididae (12.7%), while Megapodagrionidae was the least with 0.2% (Figure 4.1). The highest species number across all the sites was from the Sungai Chamang Waterfall, the state of Pahang with 20 species found, followed by Sungai Bantang Recreational Forest (Johor) and Lata Kekabu (Perak) with 17 species at each site. The most abundant among all sampling sites was from Sungai Chamang Waterfall, followed by Sungai Gabai Waterfall (Selangor) and Sungai Bantang Recreational Forest (Figure 4.2). The complete checklist of the odonates in all the sampling sites is given in Appendix C.

The abundance, frequency of occurrence (FO) and sampling sites occurrence (SO) is shown in Table 4.1. The most frequently collected species (FO) was *Rhinocypha fenestrella* with 20.1%, *Rhinocypha biforata*, and *Euphaea ochracea*, with 11.8% and 10.6% respectively. Relatively common species was *Rhinocypha perforata* (7.5%), *Prodasineura humeralis* (4.9%) and *Prodasineura laidlawii* (4.3%). Other species were collected at a frequency lower than 4% and considered as rare. In terms of sampling sites occurrence (SO), a species of *Vestalis gracilis* and *Rhinocypha biforata* were the widest distributed species (14 sites or 63.6% each), followed by *Zygonyx iris* (12 sites or

54.5%) and 11 sites or 50.0% of species for *Euphaea ochracea*, *Rhinocypha fenestrella* and *Neurobasis chinensis*.

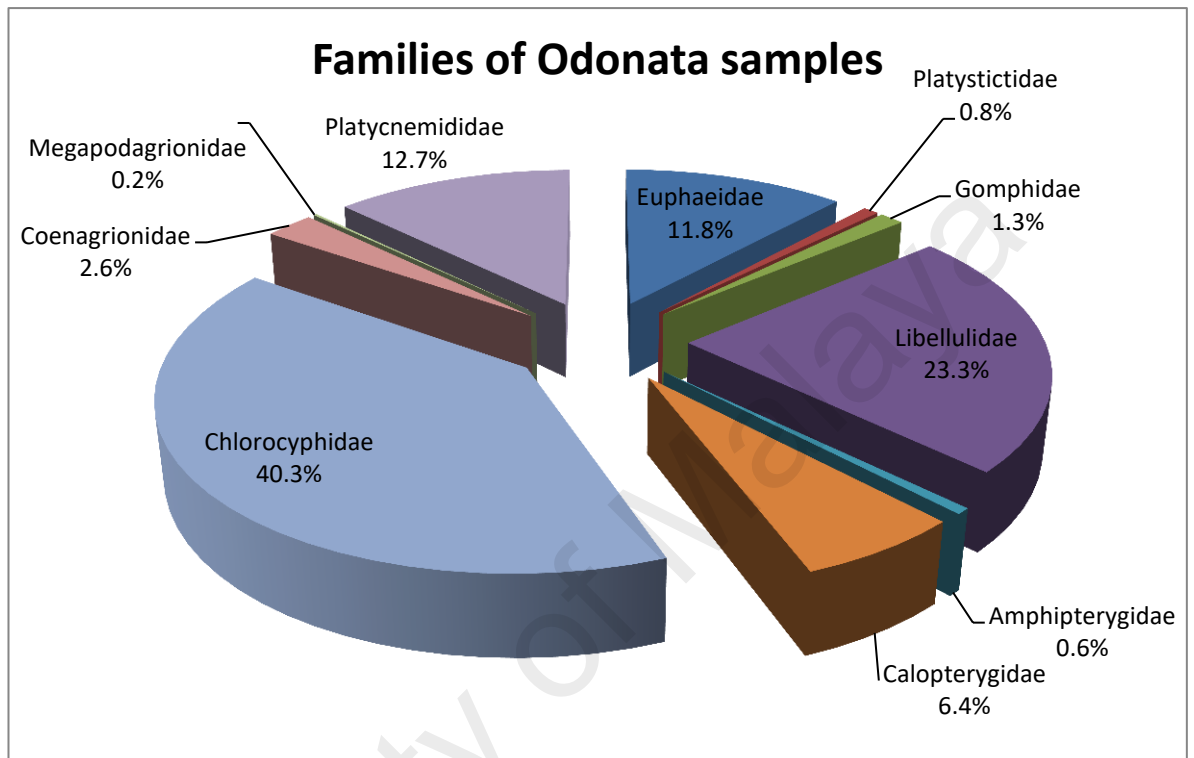


Figure 4.1: Percentage of family groups of Odonata surveyed in forest reserves in Peninsular Malaysia

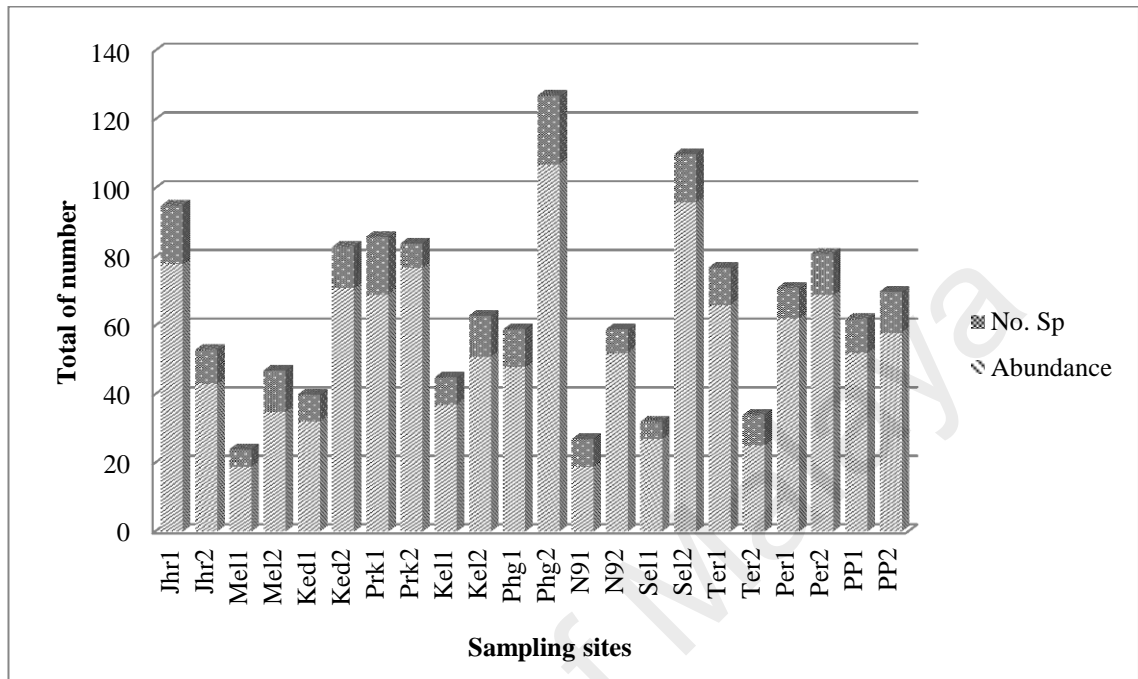


Figure 4.2: Odonate abundance and number of species surveyed in Peninsular Malaysia. Abbreviations; Jhr: Johor, Mel: Melaka, Ked: Kedah, Prk: Perak, Kel: Kelantan, Phg; Pahang, N9: Negeri Sembilan, Sel: Selangor, Ter: Terengganu, PP: Pulau Pinang.

Table 4.1: Abundance, frequency of occurrence (FO) and sampling sites occurrence (SO) of 70 odonates species in Peninsular Malaysia.

Species	Abundance	%	
		FO	SO
<i>Gomphidictinus perakensis</i> Laidlaw, 1902	2	0.2	9.1
<i>Heliaeshna crassa</i> Krüger, 1899	1	0.1	4.5
<i>Ictinogomphus acutus</i> Laidlaw, 1914	5	0.4	9.1
<i>Ictinogomphus decoratus</i> Selys, 1854	2	0.2	4.5
<i>Nepogomphus walli</i> Fraser, 1924	1	0.1	4.5
<i>Paragomphus capricornis</i> Förster, 1914	2	0.2	9.1
<i>Stylogomphus malayanus</i> Sasamoto, 2001	2	0.2	4.5
<i>Acisoma panorpoides</i> Rambur, 1842	16	1.3	9.1
<i>Aethriamanta gracilis</i> Brauer, 1878	7	0.6	4.5
<i>Agrionoptera sexlineata</i> Selys, 1879	1	0.1	4.5
<i>Brachydiplax chalybea</i> Brauer, 1868	1	0.1	4.5
<i>Cratilla lineata</i> Brauer, 1878	1	0.1	4.5
<i>Cratilla metallica</i> Brauer, 1878	8	0.7	13.6
<i>Crocothemis servilia</i> Drury, 1773	2	0.2	9.1
<i>Hydrobasileus croceus</i> Brauer, 1867	1	0.1	4.5
<i>Onychothemis coccinea</i> Lieftinck, 1953	1	0.1	4.5
<i>Orthetrum chrysis</i> Selys, 1891	31	2.6	18.2
<i>Orthetrum glaucum</i> Brauer, 1865	20	1.7	36.4
<i>Orthetrum pruinatum</i> Burmeister, 1839	2	0.2	9.1
<i>Orthetrum Sabina</i> Drury, 1773	10	0.8	9.1
<i>Orthetrum testaceum</i> Burmeister, 1839	22	1.8	27.3
<i>Orthetrum triangulare</i> Selys, 1878	1	0.1	4.5
<i>Nesoxenia lineata</i> Selys, 1879	1	0.1	4.5
<i>Neurothemis fluctuans</i> Fabricius, 1793	19	1.6	31.8
<i>Neurothemis fulvia</i> Drury, 1773	1	0.1	4.5
<i>Pantala flavescens</i> Fabricius, 1798	14	1.2	9.1
<i>Potamarcha congener</i> Rambur, 1842	1	0.1	4.5
<i>Pseudothemis jorina</i> Förster, 1904	1	0.1	4.5
<i>Rhyothemis Phyllis</i> Sulzer, 1776	4	0.3	9.1
<i>Tetrathemis platyptera</i> Selys, 1878	5	0.4	9.1
<i>Tramea transmarina</i> Brauer, 1867	4	0.3	9.1
<i>Trithemis aurora</i> Burmeister, 1839	36	3.0	36.4
<i>Trithemis festiva</i> Rambur, 1842	24	2.0	31.8
<i>Tyriobapta torrida</i> Kirby, 1889	4	0.3	13.6
<i>Urothemis signata</i> Rambur, 1842	4	0.3	4.5
<i>Zygonyx ida</i> Selys, 1869	1	0.1	4.5
<i>Zygonyx iris</i> Kirby, 1869	35	2.9	54.5
<i>Devadatta argyoides</i> Selys, 1859	7	0.6	13.6
<i>Neurobasis chinensis</i> Linnaeus, 1758	25	2.1	50.0
<i>Vestalis amoena</i> Hagen in Selys, 1853	6	0.5	9.1
<i>Vestalis amethystina</i> Lieftinck, 1965	15	1.3	31.8

Table 4.1, continued.

Species	Abundance	%	
		FO	SO
<i>Vestalis gracilis</i> Rambur, 1842	30	2.5	63.6
<i>Libellago aurantiaca</i> Selys, 1859	3	0.3	4.5
<i>Libellago lineata</i> Burmeister, 1839	6	0.5	18.2
<i>Rhinocypha biforata</i> Selys, 1859	141	11.8	63.6
<i>Rhinocypha fenestrella</i> Rambur, 1842	240	20.1	50.0
<i>Rhinocypha perforata</i> Percheron, 1835	90	7.5	36.4
<i>Sundacypha petiolata</i> Selys, 1859	1	0.1	4.5
<i>Agriocnemis femina</i> Brauer, 1868	1	0.1	4.5
<i>Agriocnemis rubescens</i> Selys, 1877	2	0.2	4.5
<i>Ceriagrion cerinorubellum</i> Brauer, 1865	1	0.1	4.5
<i>Ischnura senegalensis</i> Rambur, 1842	5	0.4	13.6
<i>Onychargia atrocyana</i> Selys, 1865	1	0.1	4.5
<i>Mortonagrion aborense</i> Laidlaw, 1914	1	0.1	4.5
<i>Pseudagrion pruinatum</i> Burmeister, 1839	10	0.8	9.1
<i>Pseudagrion rubriceps</i> Selys, 1876	9	0.8	18.2
<i>Teinobasis ruficollis</i> Selys, 1877	1	0.1	4.5
<i>Dysphaea dimidiata</i> Selys, 1853	7	0.6	18.2
<i>Euphaea impar</i> Selys, 1859	7	0.6	13.6
<i>Euphaea ochracea</i> Selys, 1859	127	10.6	50.0
<i>Podolestes buwaldai</i> Lieftinck, 1940	2	0.2	4.5
<i>Coeliccia albicauda</i> Förster in Laidlaw, 1907	1	0.1	4.5
<i>Coeliccia octogesima</i> Selys, 1863	1	0.1	4.5
<i>Copera marginipes</i> Rambur, 1842	21	1.8	36.4
<i>Copera vitata</i> Selys, 1863	2	0.2	9.1
<i>Indocnemis orang</i> Förster in Laidlaw, 1907	16	1.3	18.2
<i>Prodasineura humeralis</i> Selys, 1860	58	4.9	27.3
<i>Prodasineura interrupta</i> Selys, 1860	2	0.2	4.5
<i>Prodasineura laidlawii</i> Förster in Laidlaw, 1907	51	4.3	22.7
<i>Drepanosticta fontinalis</i> Lieftinck, 1937	10	0.8	4.5

In relation to dispersion model, out of 70 species surveyed, 41 species were aggregated with the highest dispersion statistic (20.5) and 430.50 chi value of the dispersion statistic for the species of *Prodasineura humeralis*. Instead, 29 species were found randomly disperse with the dispersion statistic >1.00 (Table 4.2).

In addition, the diversity indices are presented in Figure 4.3. Majority of sampling sites (14 localities or 63.6%) had diversity values ranged from 1.60 to 2.19. A total of 6 localities of forested areas (27.3%) showed highest diversity index ranging from 2.00 to 2.19. In contrast, dominance index was highest in 5 localities (or 22.7% each) where each sampling sites represented by 0.31-0.35 and 0.46-0.50 species.

Table 4.2: Dispersion model of 70 odonates species in Peninsular Malaysia.

Species	Dispersion (I)	Chi of I	Dispersion
<i>Prodasineura humeralis</i> Selys, 1860	20.50	430.50	Aggregated
<i>Rhinocypha fenestrella</i> Rambur, 1842	16.46	345.66	Aggregated
<i>Prodasineura laidlawii</i> Förster in Laidlaw, 1907	14.77	310.17	Aggregated
<i>Rhinocypha biforata</i> Selys, 1859	13.00	273.00	Aggregated
<i>Acisoma panorpoides</i> Rambur, 1842	12.33	258.93	Aggregated
<i>Rhinocypha perforata</i> Percheron, 1835	11.76	246.96	Aggregated
<i>Euphaea ochracea</i> Selys, 1859	11.05	232.05	Aggregated
<i>Drepanosticta fontinalis</i> Lieftinck, 1937	10.00	210.00	Aggregated
<i>Orthetrum chrysis</i> Selys, 1891	8.77	184.17	Aggregated
<i>Orthetrum Sabina</i> Drury, 1773	8.11	170.31	Aggregated
<i>Pseudagrion rubriceps</i> Selys, 1876	8.11	170.31	Aggregated
<i>Pantala flavescens</i> Fabricius, 1798	8.01	168.21	Aggregated
<i>Aethriamanta gracilis</i> Brauer, 1878	7.00	147.00	Aggregated
<i>Trithemis aurora</i> Burmeister, 1839	5.74	120.54	Aggregated
<i>Orthetrum testaceum</i> Burmeister, 1839	5.71	119.91	Aggregated
<i>Indocnemis orang</i> Förster in Laidlaw, 1907	4.34	91.14	Aggregated
<i>Vestalis amoena</i> Hagen in Selys, 1853	4.25	89.25	Aggregated
<i>Pseudothemis jorina</i> Förster, 1904	4.11	86.31	Aggregated
<i>Urothemis signata</i> Rambur, 1842	4.00	84.00	Aggregated
<i>Devadatta argyoides</i> Selys, 1859	3.71	77.91	Aggregated
<i>Cratilla metallica</i> Brauer, 1878	3.55	74.55	Aggregated
<i>Trithemis festiva</i> Rambur, 1842	3.48	73.08	Aggregated
<i>Ictinogomphus acutus</i> Laidlaw, 1914	3.32	69.72	Aggregated
<i>Neurothemis fluctuans</i> Fabricius, 1793	3.01	63.21	Aggregated
<i>Libellago aurantiaca</i> Selys, 1859	3.00	63.00	Aggregated
<i>Orthetrum glaucum</i> Brauer, 1865	2.61	54.81	Aggregated
<i>Tetrathemis platyptera</i> Selys, 1878	2.49	52.29	Aggregated
<i>Copera marginipes</i> Rambur, 1842	2.44	51.24	Aggregated
<i>Rhyothemis Phyllis</i> Sulzer, 1776	2.43	51.03	Aggregated
<i>Tramea transmarina</i> Brauer, 1867	2.43	51.03	Aggregated
<i>Zygonyx iris</i> Kirby, 1869	2.43	51.03	Aggregated
<i>Euphaea impar</i> Selys, 1859	2.21	46.41	Aggregated
<i>Ischnura senegalensis</i> Rambur, 1842	2.07	43.47	Aggregated
<i>Argiocnemis rubescens</i> Selys, 1877	2.00	42.00	Aggregated

Table 4.2, continued.

Species	Dispersion (I)	Chi of I	Dispersion
<i>Ictinogomphus decoratus</i> Selys, 1854	2.00	42.00	Aggregated
<i>Podolestes buwaldai</i> Lieftinck, 1940	2.00	42.00	Aggregated
<i>Prodasineura interrupta</i> Selys, 1860	2.00	42.00	Aggregated
<i>Stylogomphus malayanus</i> Sasamoto, 2001	2.00	42.00	Aggregated
<i>Dysphaea dimidiata</i> Selys, 1853	1.91	40.11	Aggregated
<i>Vestalis amethystina</i> Lieftinck, 1965	1.87	39.27	Aggregated
<i>Neurobasis chinensis</i> Linnaeus, 1758	1.79	37.59	Aggregated
<i>Libellago lineata</i> Burmeister, 1839	1.46	30.66	Random
<i>Tyriobapta torrida</i> Kirby, 1889	1.38	28.98	Random
<i>Vestalis gracilis</i> Rambur, 1842	1.23	25.83	Random
<i>Agriocnemis femina</i> Brauer, 1868	1.00	21.00	Random
<i>Agrionoptera sexlineata</i> Selys, 1879	1.00	21.00	Random
<i>Brachydiplax chalybea</i> Brauer, 1868	1.00	21.00	Random
<i>Ceriagrion cerinorubellum</i> Brauer, 1865	1.00	21.00	Random
<i>Coelliccia albicauda</i> Förster in Laidlaw, 1907	1.00	21.00	Random
<i>Coelliccia octogesima</i> Selys, 1863	1.00	21.00	Random
<i>Cratilla lineata</i> Brauer, 1878	1.00	21.00	Random
<i>Heliaeshna crassa</i> Krüger, 1899	1.00	21.00	Random
<i>Hydrobasileus croceus</i> Brauer, 1867	1.00	21.00	Random
<i>Mortonagrion aborense</i> Laidlaw, 1914	1.00	21.00	Random
<i>Nepogomphus walli</i> Fraser, 1924	1.00	21.00	Random
<i>Nesoxenia lineata</i> Selys, 1879	1.00	21.00	Random
<i>Neurothemis fulvia</i> Drury, 1773	1.00	21.00	Random
<i>Onychargia atrocyana</i> Selys, 1865	1.00	21.00	Random
<i>Onychothemis coccinea</i> Lieftinck, 1953	1.00	21.00	Random
<i>Orthetrum triangulare</i> Selys, 1878	1.00	21.00	Random
<i>Potamarcha congener</i> Rambur, 1842	1.00	21.00	Random
<i>Pseudagrion pruinosum</i> Burmeister, 1839	1.00	21.00	Random
<i>Sundacypha petiolata</i> Selys, 1859	1.00	21.00	Random
<i>Teinobasis ruficollis</i> Selys, 1877	1.00	21.00	Random
<i>Zygonyx ida</i> Selys, 1869	1.00	21.00	Random
<i>Copera vitata</i> Selys, 1863	0.95	19.95	Random
<i>Crocothemis servilia</i> Drury, 1773	0.95	19.95	Random
<i>Gomphidictinus perakensis</i> Laidlaw, 1902	0.95	19.95	Random
<i>Orthetrum pruinosum</i> Burmeister, 1839	0.95	19.95	Random
<i>Paragomphus capricornis</i> Förster, 1914	0.95	19.95	Random

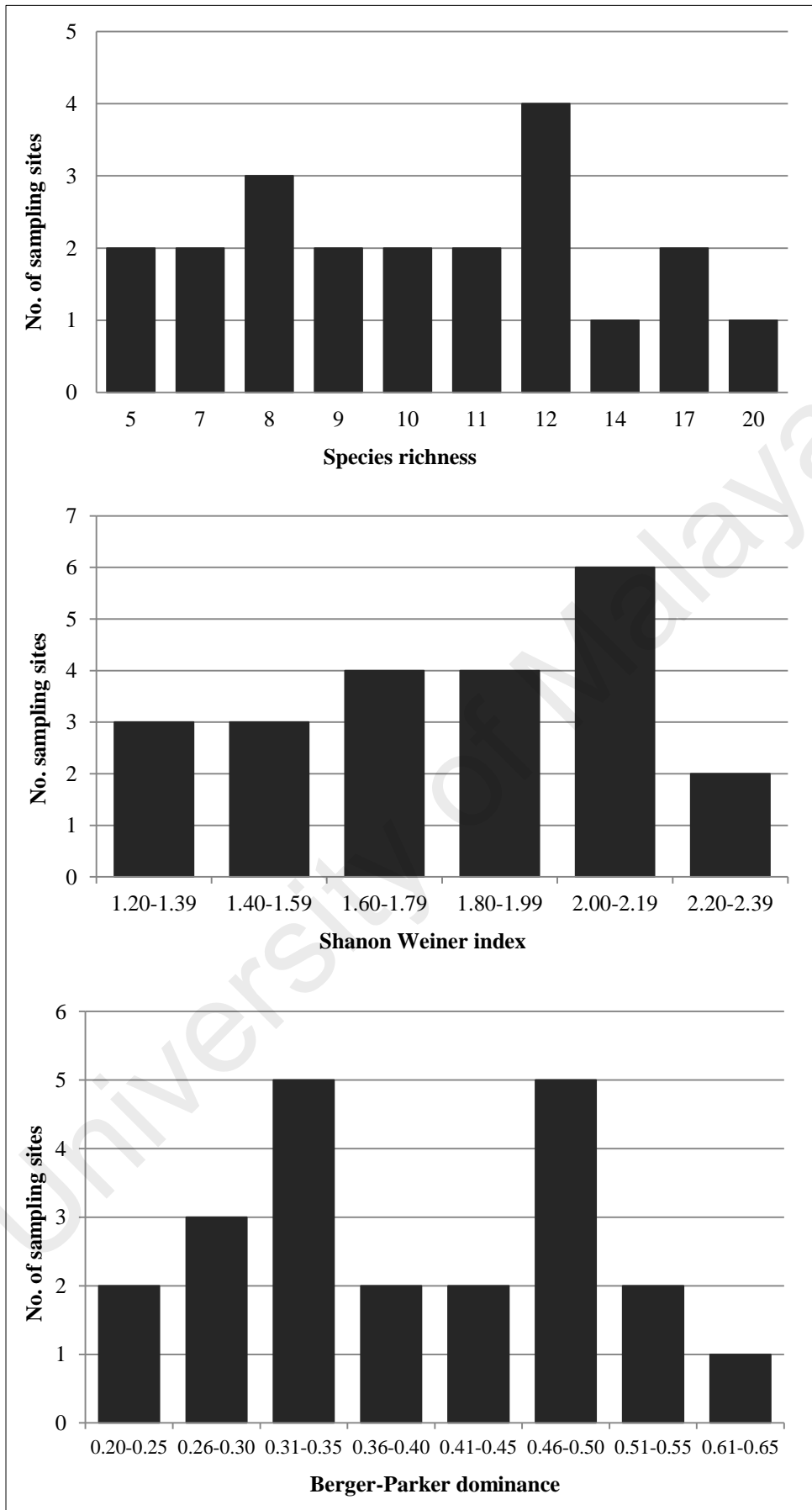


Figure 4.3: Diversity indices of odonates species in Peninsular Malaysia

4.1.2 Sampling Efficiency

The species richness (local and regional) and estimated richness are presented in Table 4.3 and Table 4.4 respectively. Sampling efficiency was varying greatly between sites and estimators. The total estimated species richness ranged between 71 to 113 species, which yielded 61.5% - 98.2% of sampling efficiency.

Moreover, the species accumulation curve is shown in Figure 4.4. It showed that the accumulation curve had a curve nearing an asymptote. However, the curve slightly increased towards the end. On the other hand, heterogeneity test as done as extrapolation to estimate the total species complement for the habitat, is only possible if the species accumulation curve is derived from a homogeneous (stable) community. From the Figure 4.5, it showed that the Coleman curve is above the observed species acquisition curve, which suggesting that some sample heterogeneity.

Table 4.3: Local and regional richness of odonates in Peninsular Malaysia

Region	Total collection	Richness	Mean Richness ± SE
Northern			
<i>Kedah</i>			
Bukit Wang Recreational Forest	32	8	0.11±0.32
Lata Bayu Recreational Forest	71	12	0.17±0.38
<i>Perak</i>			
Lata Kekabu Recreational Forest	69	17	0.24±0.43
Lata Iskandar Waterfalls	77	7	0.10±0.30
<i>Perlis</i>			
Kelam Cave	62	9	0.13±0.34
Sungai Jernih Recreation Park	69	12	0.17±0.38
<i>Pulau Pinang</i>			
Teluk Bahang Forest Park	52	10	0.14±0.35
Taman Rimba Recreational Forest	58	12	0.17±0.38
Total	490	87	0.16±0.36
Southern			
<i>Johor</i>			
Sungai Bantang Recreational Forest	78	17	0.24±0.43
Gunung Pulai Recreational Forest	43	10	0.14±0.35
<i>Melaka</i>			
Sungai Linggi Recreational Forest	19	5	0.07±0.26
Ayer Keroh Recreational Forest	35	12	0.17±0.38
<i>Negeri Sembilan</i>			
Ulu Bendul Recreational Forest	19	8	0.11±0.32
Jeram Toi Recreational Forest	52	7	0.10±0.30
Total	246	40	0.14±0.35
Central			
<i>Selangor</i>			
Sungai Sendat Recreational Forest	27	5	0.07±0.26
Sungai Gabai Waterfalls	96	14	0.20±0.40
Total	123	19	0.14±0.34
East Coast			
<i>Kelantan</i>			
Jeram Linang	37	8	0.11±0.32
Jeram Pelangi	51	12	0.17±0.38
<i>Pahang</i>			
Teladas Waterfalls	48	11	0.16±0.37
Sungai Chamang Waterfalls	107	20	0.29±0.46
<i>Terengganu</i>			
Sekayu Recreational Forest	66	11	0.16±0.37
Lata Belatan Recreational Forest	25	9	0.13±0.34
Total	334	71	0.17±0.38

Table 4.4: Actual species number and sampling efficiency percentages (given in brackets) of the different species estimators of odonates in Peninsular Malaysia.

Estimators	Actual species collected	Estimated richness (Sampling efficiency %)
Choa Present & Absent	70	102.1 (68.6)
Choa & Lee 2	70	71.3 (98.2)
1st Order Jackknife	70	98.6 (71.00)
2nd Order Jackknife	70	113.8 (61.5)
Bootstrap	70	82.7 (84.6)
Michaelis-Menten	70	87.5 (80.0)

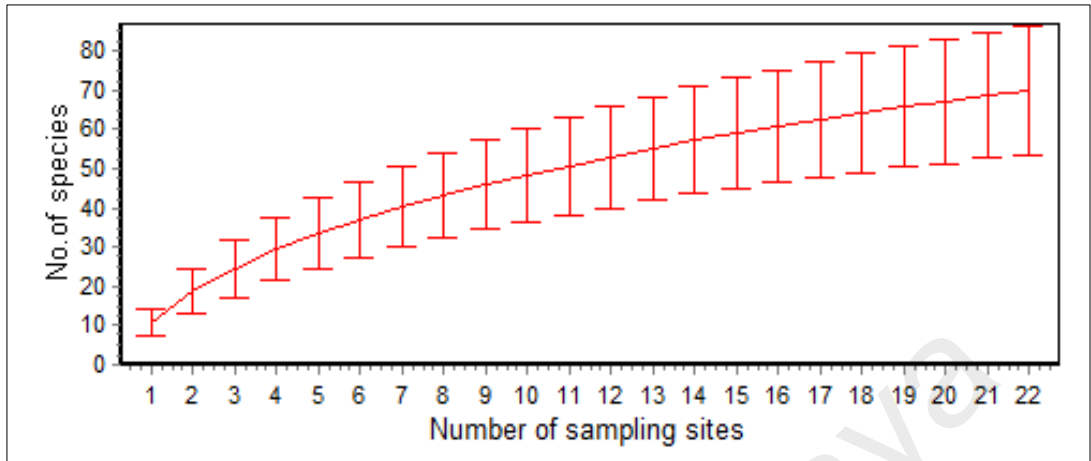


Figure 4.4: Accumulation curve with error bars for all sampling sites in Peninsular Malaysia.

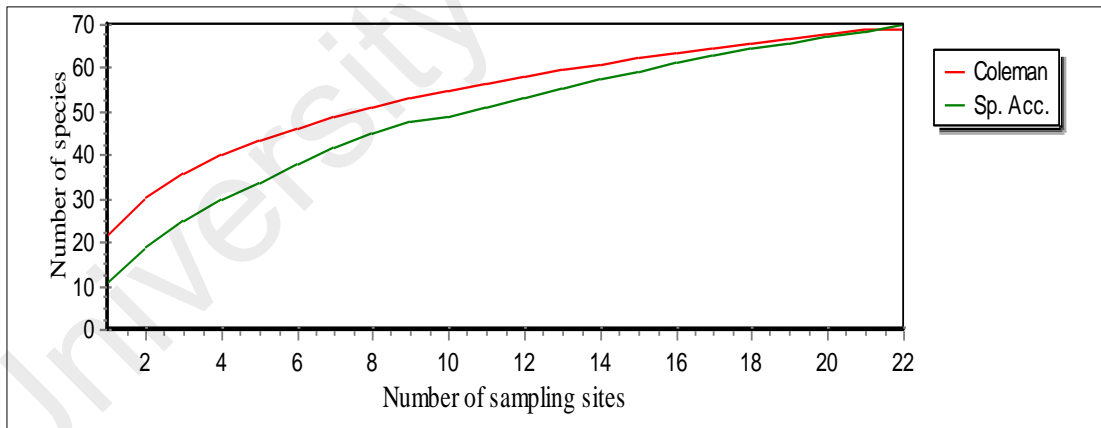


Figure 4.5: Coleman curve using heterogeneity test of all species across all the sampling sites in Peninsular Malaysia.

4.1.3 Hierarchical Cluster Analysis

The Jaccard index was calculated and a hierarchical cluster analysis was performed and UPGMA was used as clustering algorithms (Figure 4.6). The UPGMA dendrogram displayed four distinct clusters from all the species sampled. The lower distance between the species showed they shared high similarities of diversity, while more distance between the species, it showed they are more diverse between each other.

From the results showed that there were several species that are closely related to each other with only one distance; (1) *Nepogomphus walli*, *Zygonyx ida*, *Onychothemis coccinea*, (2) *Agrionoptera sexlineata*, *Potamarcha congener*, *Agriocnemis femina*, (3) *Drepanosticta fontinalis*, *Hydrobasileus croceus*, *Coeliccia octogesima*, (4) *Ictinogomphus decoratus*, *Urothemis signata*, *Aethriamanta gracilis*, *Brachydiplax chalybea*, *Ceriagrion cerinorubellum*, and (5) *Libellago aurantiaca*, *Sundacypha petiolata*, *Heliaeshna crassa*.

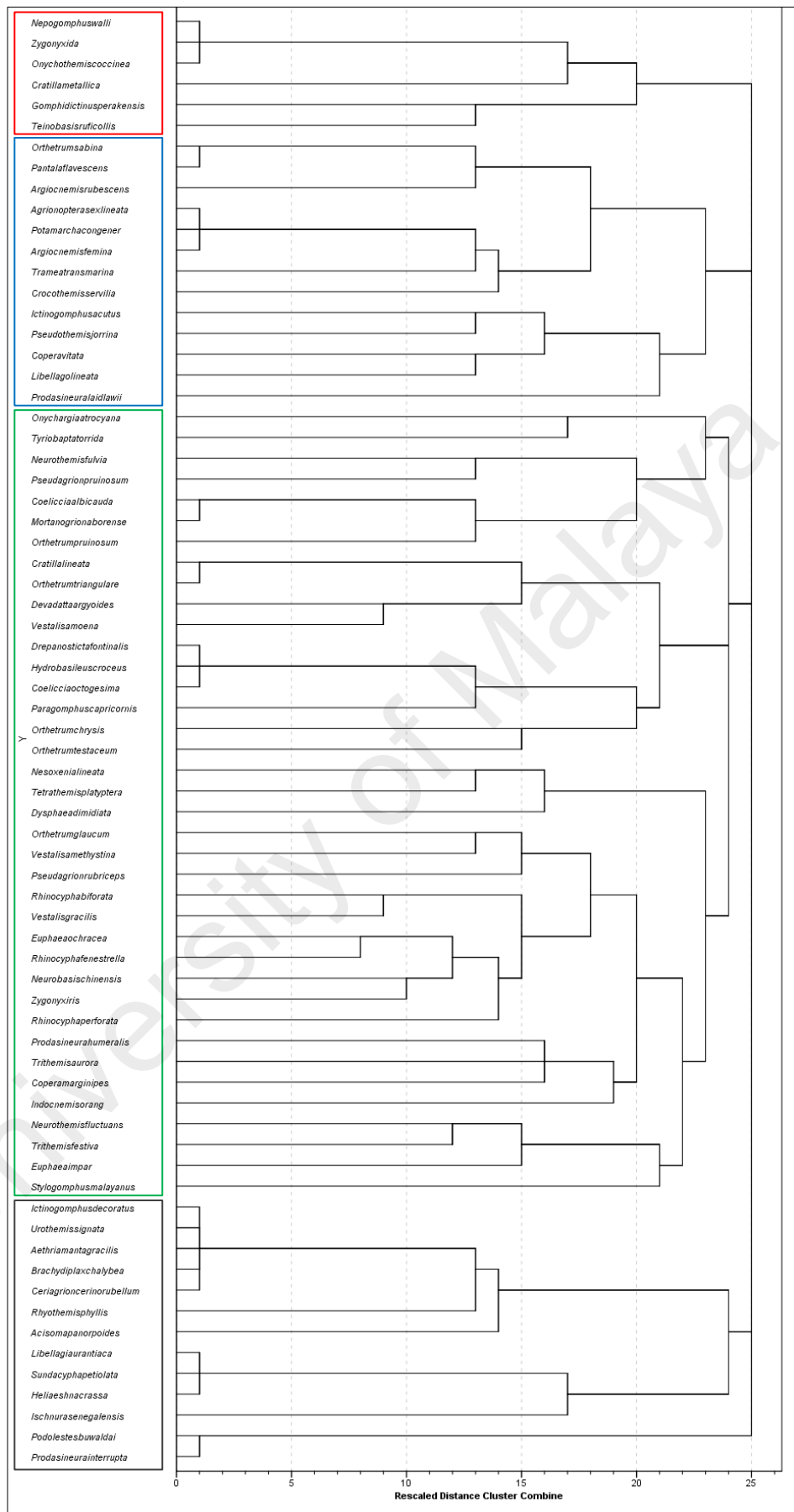


Figure 4.6: UPGMA dendrogram using Jaccard method as distance measurements. (Coloured boxes displayed distinct cluster from all the species sampled).

4.2 CORRELATIONS OF PHYSICAL AND CHEMICAL PARAMETERS WITH ODONATE DIVERSITY AND DISTRIBUTION

4.2.1 Correlation with Environmental Parameters

Eighteen environmental parameters variables were collected in the study sites throughout two years of sampling in 2014 and 2015 (Appendix D). Shapiro-Wilk test showed that there are five variables out of 18 were not significant, which are normally distributed; pH, ambient temperature, flow rate, water width and one of the chemical components of the water which is Nitrite. These variables were used Pearson correlation, while the rest of the variables, which were significantly different and not normally distributed were used Spearman correlation (elevation, conductivity, humidity, dissolved oxygen, salinity, water temperature, water depth, total dissolved solids, iron, nitrate, ammonia, phosphorus and sulfate).

Data in Table 4.5 provides the correlation matrix, Pearson and Spearman rank order correlations of the environmental parameters which including the water quality parameters obtained from the principal component analysis (PCA). From the results it showed that there are very strong positive correlations between TDS and conductivity with 0.944. Besides, strong correlations similarly can be observed between conductivity and water temperature, salinity and TDS, and water temperature and TDS, with 0.699, 0.611, and 0.692 respectively.

On the other hand, a very strong negative correlation can also be observed between the humidity and ambient temperature with -0.836. In addition, strong negative correlations also found between ammonia and water width, conductivity and elevation, and nitrate and salinity, with -0.506, -0.465, and -0.443 respectively.

Table 4.5: Correlation matrix using Pearson and Spearman rank order correlations of all the variables obtained from the principal component analysis. (# indicates Pearson correlations). * Correlation is significant at the 0.05 level (2-tailed).

	Elevation	Conductivity	Humidity	DO	pH	Salinity	Water Temp	Ambient Temp	Flow Rate	Water Depth	Water Width	TDS	Iron	Nitrate	Nitrite	Ammonia	Phosphate	Sulphate
Elevation	1																	
Conductivity	-0.465*	1																
Humidity	-0.216	-0.154	1															
DO	-0.315	0.169	0.248	1														
pH	0.032	0.011	-0.072#	-0.330	1													
Salinity	-0.330	0.596	0.050	-0.193	0.227	1												
Water Temp	-0.432*	0.699	-0.396	0.003	0.226	0.493*	1											
Ambient Temp	-0.013	0.330	-0.836#	-0.213	0.185#	0.048	0.559	1										
Flow Rate	-0.228	0.231	-0.003#	0.420	-0.393#	-0.315	0.061	-0.007#	1									
Water Depth	-0.132	0.012	-0.183	-0.218	-0.062	0.082	0.120	0.327	-0.041	1								
Water Width	0.017	-0.247	0.261#	0.199	-0.231	-0.245	-0.191	-0.178#	0.153#	0.442*	1							
TDS	-0.432*	0.944	-0.211	0.183	0.010	0.611	0.692	0.384	0.078	0.124	-0.176	1						
Iron	-0.171	0.493*	-0.184	0.082	-0.233	0.438*	0.499*	0.134	0.029	0.147	-0.081	0.495*	1					
Nitrate	-0.156	0.047	-0.141	0.233	-0.395	-0.443*	-0.031	0.069	0.353	0.158	0.284	-0.030	0.003	1				
Nitrite	-0.190	0.346	-0.079#	-0.023	0.204#	0.256	0.441*	0.105#	0.152#	-0.163	-0.346#	0.183	0.529*	-0.059	1			
Ammonia	-0.220	0.498*	-0.032	-0.102	0.435*	0.517*	0.502*	0.313	-0.127	-0.019	-0.506*	0.516*	0.167	-0.320	0.203	1		
Phosphorus	0.173	0.143	-0.311	-0.348	-0.053	0.185	0.029	0.472*	0.102	0.216	-0.346	0.049	0.029	-0.180	0.168	0.236	1	
Sulfate	-0.074	0.574	-0.004	0.315	0.067	0.208	0.328	0.135	0.212	-0.399	-0.380	0.496*	0.001	0.200	0.128	0.579	0.038	1

4.2.2 Habitat Preference of Odonata

In PCA, eigenvalues are normally used to determine the number of principal components (PCs) that can be retained for further study. A scree plot for the eigenvalues obtained in this study shows a pronounced change of slope after the five eigenvalue (Figure 4.7). The scree plot is a simple line segment plot that shows the fraction of the total variance in the data as explained or represented by each PC. The PCs are ordered, and by decreasing order of contribution to the total variance.

Cattell and Jaspers (1976) and Vega *et al.* (1998) suggested using all of the PCs up to and including the first one after the break. Therefore, the first five PCs with the eigenvalues of 4.705, 3.306, 2.565, 2.223 and 1.262 were used for further analysis from all collections which have eigenvalues >1.0 accounted for 78.12% of total intersite variance of the physical and chemical conditions.

As can be seen in the Table 4.6, PC-1 to PC-5 were highly influenced (negatively or positively) by most of the variables, thus hindering the interpretation regarding which parameters are more important than the others in influencing water quality variations within a given season. Data in Table 4.6 shows the rotated component matrix that revealed the sites with higher PC-1, which explained 20.01% of the total variance, were mostly influenced by the chemical components of the water. The level of water chemical at PC-1 were increased if the level of sulphate, ammonia, iron, nitrite and DO are higher, while the level of water chemical will decrease with the increase of water width level.

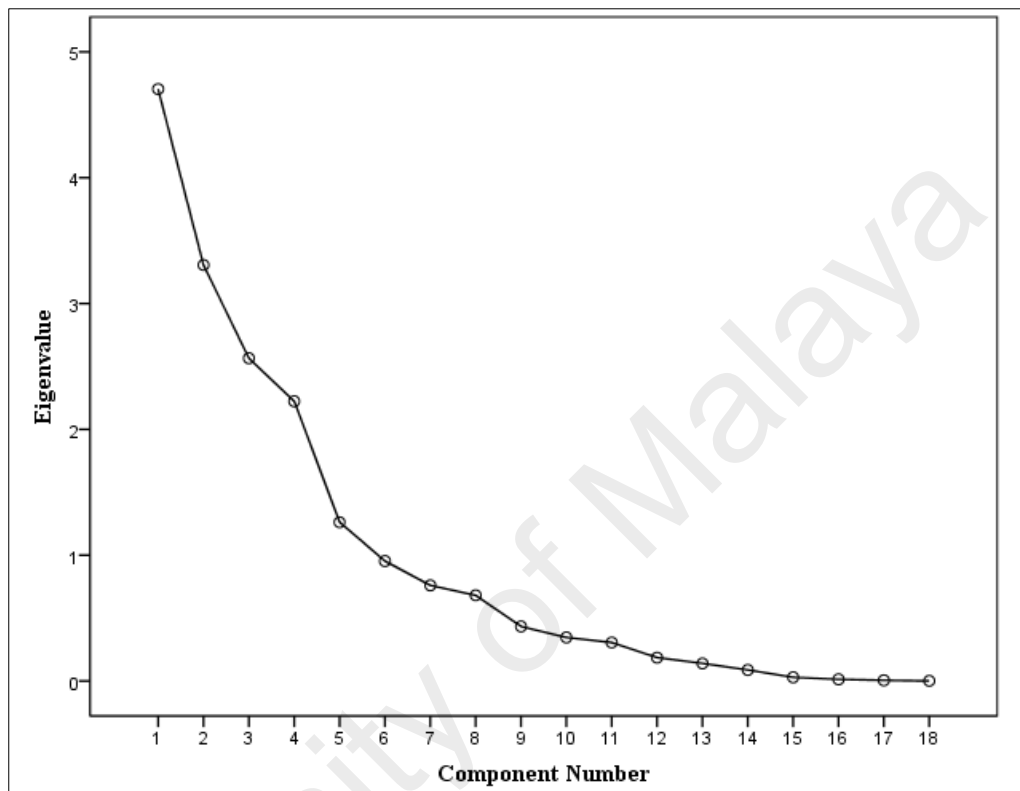


Figure 4.7: Scree plot of the eigenvalues of principles components from all the sampling sites in Peninsular Malaysia.

Table 4.6: Rotated component matrix between samplings sites variables and principal components (PCs).

Variables	Sampling sites			Principle components				
	Min	Max	Mean \pm SE	PC-1	PC-2	PC-3	PC-4	PC-5
Sulphate	-2.000	25.000	1.5 \pm 1.15	0.941*	-0.015	-0.091	0.017	0.016
Ammonia	-0.020	0.450	0.06 \pm 0.02	0.932*	0.144	-0.030	-0.166	0.055
Iron	0.004	1.010	0.20 \pm 0.06	0.715*	0.265	0.089	0.038	0.188
Nitrite	0.000	0.011	0.004 \pm 0.0006	0.649*	0.259	0.112	-0.044	-0.033
DO	2.950	10.700	6.77 \pm 0.31	0.597*	-0.551	-0.062	0.190	0.210
Water Width	2.130	14.300	8.59 \pm 0.68	-0.502*	0.040	-0.089	0.420	0.367
Conductivity	16.100	512.000	73.03 \pm 25.56	0.160	0.952*	0.118	-0.126	0.047
TDS	0.010	0.330	0.05 \pm 0.02	0.130	0.904*	0.333	-0.010	0.145
Salinity	0.000	0.200	0.02 \pm 0.13	0.281	0.895*	-0.049	-0.205	0.033
Ambient Temp	24.000	33.500	28.66 \pm 0.46	0.017	0.088	0.937*	-0.107	-0.186
Humidity	60.000	96.000	75.91 \pm 2.66	-0.053	0.022	-0.824*	0.069	0.398
Water Temp	24.400	39.100	28.44 \pm 0.66	0.200	0.367	0.775*	0.137	0.342
Water Depth	0.210	1.800	0.51 \pm 0.07	-0.221	0.165	0.681*	0.424	0.254
pH	5.460	10.520	7.74 \pm 0.32	0.122	0.000	0.202	-0.818*	0.261
Flow Rate	0.800	3.200	1.78 \pm 0.15	0.149	-0.268	0.089	0.711*	-0.035
Nitrate	0.000	0.180	0.05 \pm 0.01	-0.127	-0.161	0.247	0.709*	0.342
Phosphate	0.040	0.250	0.11 \pm 0.01	0.014	0.073	0.292	0.064	-0.781*
Elevation	20.000	440.000	119.68 \pm 19.71	-0.206	-0.199	-0.113	-0.019	-0.739*
Eigenvalue				4.71	3.31	2.57	2.22	1.26
% total variance				20.01	18.34	16.65	12.21	10.90
Cumulative %				20.01	38.35	55.01	67.22	78.12

* $p < 0.05$

In addition, PC-2 accounted for 18.34% of the total variance. Conductivity, total dissolved solids and salinity were found variables that positively contributed to the components or sites. For PC-3 explained 16.65% of the total variance. Sites with higher of this component score were having higher ambient and water temperature, deeper level of streams and lower degree of humidity.

Moreover, from the table showed that PC-4 accounted for 12.21% of total variance. The flow rate and level of nitrate were found positively contributed to the component and negatively contributed by the pH level. Lastly, PC-5 explained with 10.90% of total variance. Sites with higher PC-5 were significantly related to lower level of phosphate and had lower elevation.

Figure 4.8 represented the rotated component plot using varimax normalized method extracted from the principal components. It showed the component loading (Component 1 vs Component 2 vs Component 3) of all the variables among the study sites.

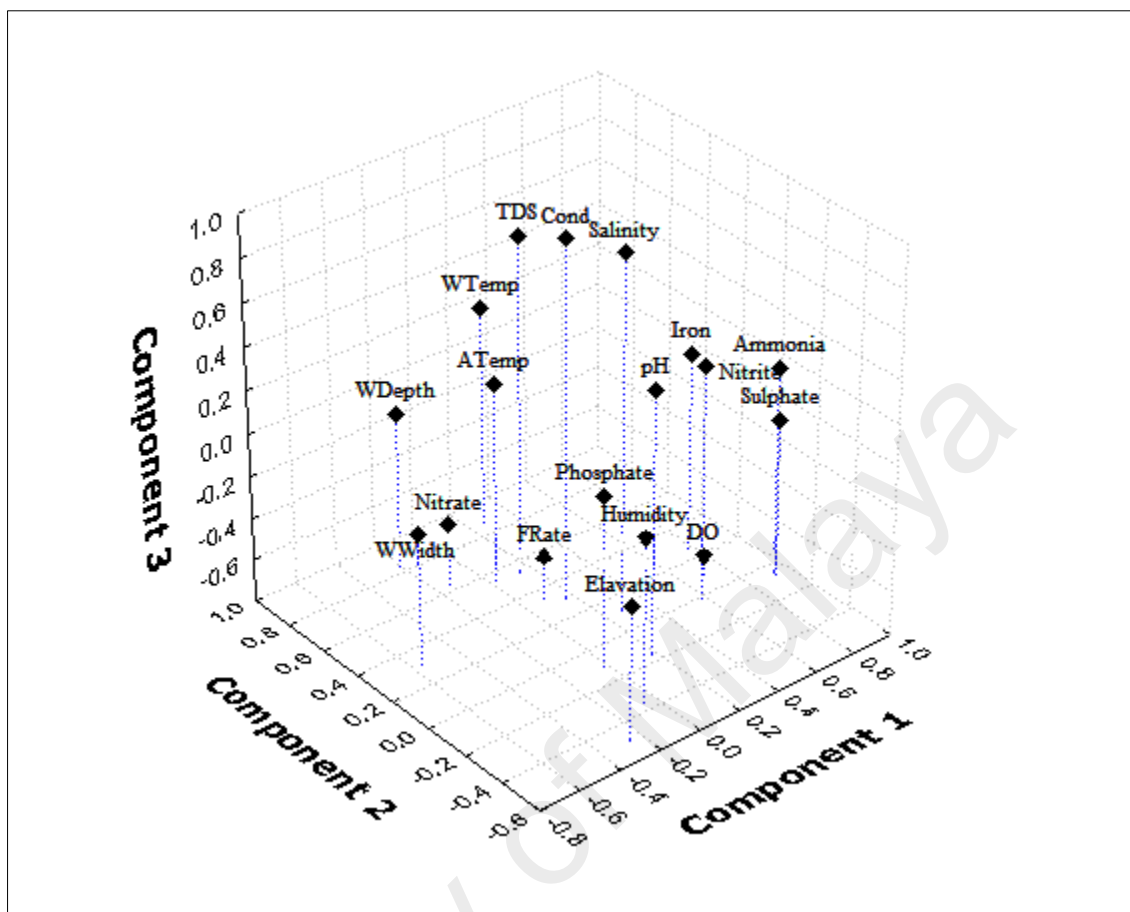


Figure 4.8: Rotated component plot using varimax normalized method extracted from the principal components for all variables among the study sites.

2.3 Logistic Regression Analysis

Logistic regression analysis of 11 odonate species is presented in Table 4.7 using 500 bootstrap replicates of the species richness estimate ($B = 500$) to show how the components (PCs) affected the presence/absence of the odonates. The logistic regression analysis was conducted for 21 species of odonates which were found in more than 20% of the sampling sites. All the regression models of species distribution were significant at $p < 0.05$.

PC-1 which was related to the chemical properties of the water was significantly correlated with the occurrence of seven species (*Dysphaea dimidiata*, *Eupahea ochracea*, *Orthetrum chrysis*, *Neurothemis fluctuans*, *Rhinocypha biforata*, *Rhinocypha fenestrella* and *Zygonyx iris*). However, if the water chemical level was increased, the species of *D. dimidiata*, *E. ochracea*, *R. biforata*, *R. fenestrella* and *Z. iris* will decrease or disappeared, in contrast with another two species (*O. chrysis*, *N. fluctuans*); they were adapted with the higher level of chemical properties and shallow water as in PC-1.

The occurrence of *Libellago lineata* and *Prodasineura humeralis* was correlated with PC-2, which associated with the highest level of conductivity, TDS and salinity. Moreover, in PC-3, four species of odonate were correlated with the site (PC-3). The occurrence of *E. ochracea* and *R. biforata* will decrease or disappeared with the increased of ambient and water temperature and water depth, and decreased level of humidity, but oppositely goes to the species *O. chrysis* and *N. fluctuans*.

Additionally, the occurrences of *P. humeralis* was positively associated with PC-4 which are the pH, flow rate and nitrate, while *Prodasineura laidlawii* was negatively correlated with the site. On the other hand, five species out of six species (*E. ochracea*, *Orthetrum testaceum*, *P. humeralis*, *R. fenestrella* and *Z. iris*) were found negatively correlated, which will decreased or disappeared with the lower level of phosphate and at lower elevation, but for the species of *R. biforata*, it was positively correlated with PC-5 which will increase the abundance or population with lower levels of phosphate and at lower elevation.

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Table 4.7: Logistic regression analysis for the distribution of odonate species in Peninsular Malaysia. (B = 500).

Species	Regression coefficient						p
	PC-1	PC-2	PC-3	PC-4	PC-5	K	
<i>Dysphaea dimidiata</i> Selys (1853)	-3.469*	-2.793	1.014	-.888	.847	-3.372	
<i>Euphaea ochracea</i> Selys (1859)	-2.696*	-1.162	-1.388*	.003	-1.180*	-.701	<0.05
<i>Libellago lineata</i> Burmeister (1839)	.166	1.477*	-.019	-.326	.091	-1.783	<0.05
<i>Orthetrum chrysis</i> Selys (1891)	1.274*	-2.692	2.030*	-1.038	-.457	-3.234	<0.05
<i>Orthetrum testaceum</i> Burmeister (1839)	.889	-.852	.736	-1.156	-1.585*	-2.134	<0.05
<i>Neurothemis fluctuans</i> Fabricius (1793)	1.229*	-1.262	2.536*	.654	1.187	-1.261	<0.05
<i>Prodasineura humeralis</i> Selys (1860)	-.018	2.473*	-.610	1.601*	-.808*	-1.548	<0.05
<i>Prodasineura laidlawii</i> Förster in Laidlaw (1907)	.009	-.294	1.386	-3.498*	-.692	-3.348	<0.05
<i>Rhinocypha biforata</i> Selys (1859)	-1.288*	-.735	-1.965*	-.423	2.167*	1.273	<0.05
<i>Rhinocypha fenestrella</i> Rambur (1842)	-3.637*	-.658	.425	.381	-2.586*	-.045	<0.05
<i>Zygonyx iris</i> Kirby (1869)	-6.819*	.622	-.782	.857	-1.927*	-.518	<0.05

4.3 MOLECULAR PHYLOGEOGRAPHY OF *Rhinocypha fenestrella* BASED ON ANALYSES OF MITOCHONDRIAL COI AND 16S RRNA GENES

4.3.1 Haplotype Network Construction

A statistical parsimony network of 147 taxa revealed 26 haplotypes from 614 characters of the COI gene and 10 haplotypes from 534 characters of the 16S rRNA gene. For concatenated sequences, 33 haplotypes were revealed from a total of 1148 characters of both COI and 16S rRNA genes (Figure 4.9). Haplotype AB1 was the common ancestor and the most widespread haplotype based on its prevalence in Peninsular Malaysia. Notably, the A2, A3, A4 and A5 haplotypes from COI gene, and the AB2, AB3 AB4 and AB5 from combining COI and 16S rRNA dataset, mainly originated from the same population, Johor state, formed a single cluster, shown in Figure 4.9a and 4.9c. Consequently, from this study, it revealed that a most recent common ancestor of the *R. fenestrella* odonate may exist from the Negeri Sembilan state as discovered from the COI, 16S rRNA and COI + 16S rRNA genes haplotype network analysis.

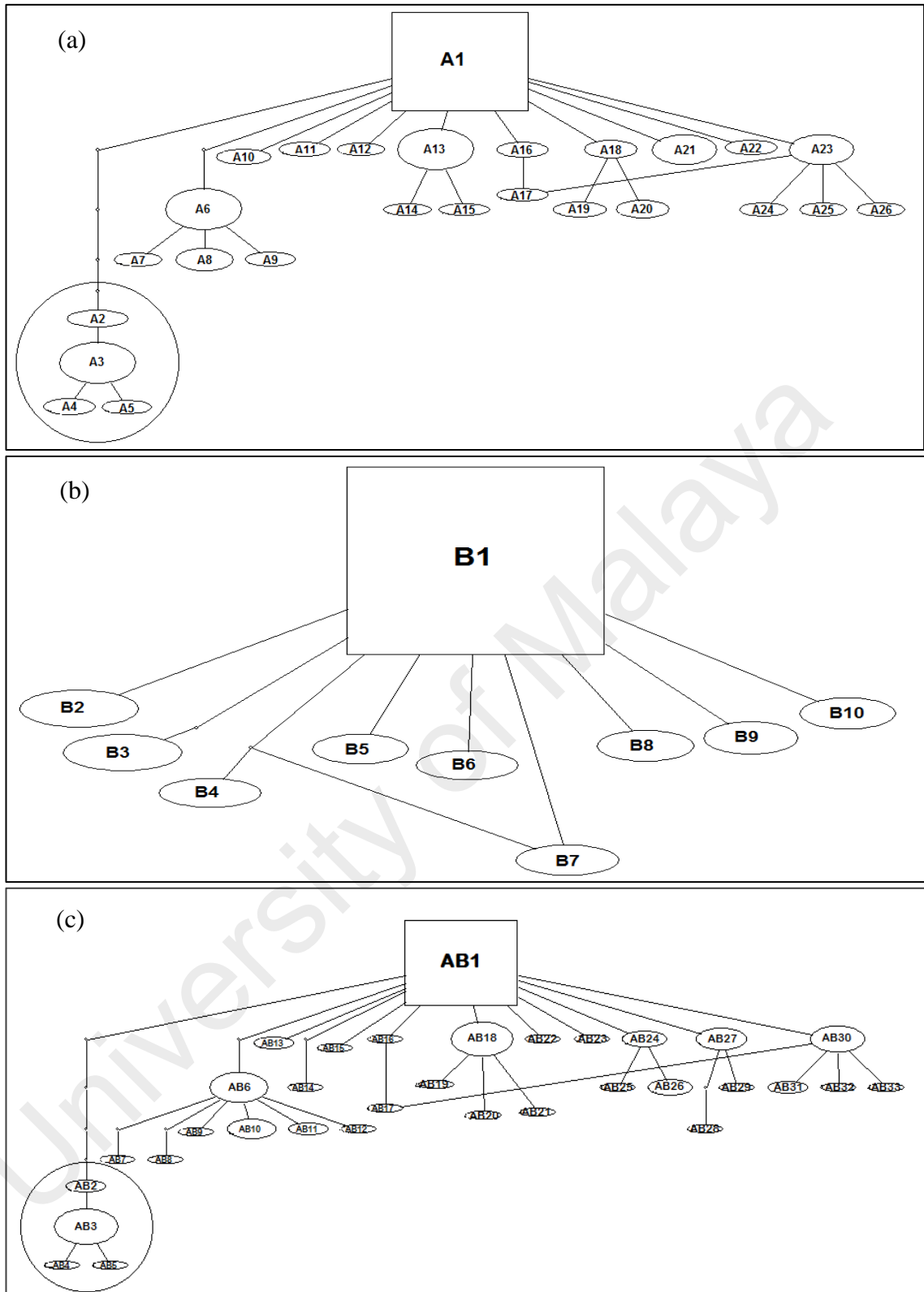


Figure 4.9: Statistical parsimony network for *Rhinocypha fenestrella* in Malaysia. a) COI gene, b) 16S rRNA gene and c) COI + 16S rRNA genes haplotypes for *R. fenestrella* in Malaysia. Lines represent parsimonious connections between haplotypes; probabilities of > 95% with one mutational step representing each line. The small circles indicate missing haplotypes and the relative sizes of ovals and squares indicating the haplotype frequency. A1, B1 and AB1 in the networks were inferred as hypothetical ancestral haplotypes.

4.3.2 Haplotype and Nucleotide Analysis

The combined COI + 16S rRNA dataset and COI gene alone shared greater haplotype diversity with 0.889 than 16S rRNA gene with 0.205. The haplotype diversity in a population, for COI gene, ranged from 0.195 in Kedah state to 0.889 in Pahang state, nonetheless, for 16S rRNA gene, it ranged from 0.000 in most of the population (Johor, Perak, Kelantan, Pahang and Selangor) to 0.205 in Terengganu state. However, for the combine COI + 16S rRNA dataset, the haplotype diversity ranged from 0.284 in Kedah state to 0.889 in Pahang state. In addition, COI gene showed greater nucleotide diversity (0.00401) than the combined COI + 16S rRNA (0.00040) dataset or 16S rRNA (0.00228) alone (Table 4.8).

Table 4.8: Sample size (n), number of haplotype (h), haplotype diversity (Hd), and nucleotide diversity (Pi) based on COI, 16S rRNA and COI + 16S rRNA of *Rhinocypha fenestrella* in Malaysia.

Site	n	COI			16S rRNA			COI + 16S rRNA		
		h	Hd	Pi	h	Hd	Pi	h	Hd	Pi
Negeri Sembilan	19	3	0.433	0.00074	2	0.105	0.00020	4	0.509	0.00049
Johor	20	4	0.432	0.00076	1	0.000	0.00000	4	0.432	0.00041
Kedah	20	3	0.195	0.00033	2	0.100	0.00019	4	0.284	0.00026
Perak	20	5	0.558	0.00129	1	0.000	0.00000	5	0.558	0.00069
Kelantan	20	4	0.726	0.00328	1	0.000	0.00000	8	0.821	0.00228
Terengganu	19	6	0.778	0.00282	3	0.205	0.00040	6	0.778	0.00169
Pahang	18	9	0.889	0.00401	1	0.000	0.00000	9	0.889	0.00215
Selangor	11	3	0.564	0.00130	1	0.000	0.00000	3	0.564	0.00070
Total	147	26	0.885	0.00505	10	0.106	0.00026	33	0.894	0.00282

4.3.3 Genetic Divergence

Uncorrected sequence divergences for both intra and inter populations recorded ranged from 0.16 – 1.63 % of the representative COI haplotype of *R. fenestrella* (Table 4.9). Oppositely, the genetic distance of the representative 16S rRNA haplotype ranged from 0 – 0.75%, where it showed there is no genetic distance between haplotype H1 and H9 (Table 4.10).

4.3.4 Sequence Characteristics

The aligned 16S rRNA sequences which consist of 534 characters, of these, 524 sites were highly conserved (98.1%), 8 were variable (1.5%) and none were parsimony informative. In contrast, a slightly higher variation was observed in COI sequences with out of 614 sequences, 586 were conserved (95.4%), 28 were variable (4.6%) and 18 were parsimony informative (3%). Tables 4.11 and 4.12 presented the variable sites of *Rhinocypha fenestrella* haplotypes based on COI and 16S rRNA sequences. The base composition of the COI and 16S rRNA segment showed a significant A + T nucleotide bias (A = 30.6%; 29.1%, C = 17.7%; 11.5%, G = 19.4%; 18.6%, T = 32.3%; 40.8%), respectively.

Table 4.9: Percentage (%) of uncorrected “p” distance matrix among the 26 representative COI haplotypes of *Rhinocypha fenestrella* in Malaysia.

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22	H23	H24	H25	H26
H1	-																									
H2	0.16	-																								
H3	0.16	0.33	-																							
H4	1.14	0.98	1.30	-																						
H5	1.30	1.14	1.47	0.16	-																					
H6	0.98	0.81	1.14	0.16	0.33	-																				
H7	1.30	1.14	1.47	0.16	0.33	0.33	-																			
H8	0.33	0.16	0.49	1.14	1.30	0.98	1.30	-																		
H9	0.33	0.16	0.49	1.14	1.30	0.98	1.30	0.33	-																	
H10	0.49	0.33	0.65	1.30	1.47	1.14	1.47	0.49	0.49	-																
H11	0.49	0.33	0.65	1.30	1.47	1.14	1.47	0.49	0.49	0.65	-															
H12	0.33	0.16	0.49	1.14	1.30	0.98	1.30	0.33	0.33	0.16	0.49	-														
H13	0.33	0.16	0.49	1.14	1.30	0.98	1.30	0.33	0.33	0.49	0.49	0.33	-													
H14	0.49	0.33	0.65	1.30	1.47	1.14	1.47	0.49	0.49	0.65	0.65	0.49	0.49	-												
H15	0.65	0.49	0.81	1.47	1.63	1.30	1.63	0.65	0.65	0.81	0.81	0.65	0.65	0.16	-											
H16	0.33	0.16	0.49	1.14	1.30	0.98	1.30	0.33	0.33	0.49	0.49	0.33	0.33	0.49	0.65	-										
H17	0.65	0.49	0.81	1.47	1.63	1.30	1.63	0.65	0.65	0.81	0.81	0.65	0.65	0.16	0.33	0.65	-									
H18	0.33	0.16	0.49	1.14	1.30	0.98	1.30	0.33	0.33	0.49	0.49	0.33	0.33	0.49	0.65	0.33	0.65	-								
H19	0.65	0.49	0.81	1.47	1.63	1.30	1.63	0.65	0.65	0.81	0.81	0.65	0.65	0.16	0.33	0.65	0.33	0.65	-							
H20	0.16	0.33	0.33	1.30	1.47	1.14	1.47	0.49	0.49	0.65	0.65	0.49	0.49	0.65	0.81	0.49	0.81	0.49	0.81	-						
H21	0.33	0.16	0.49	1.14	1.30	0.98	1.30	0.33	0.33	0.49	0.16	0.33	0.33	0.49	0.65	0.33	0.65	0.33	0.65	0.49	-					
H22	0.33	0.16	0.49	1.14	1.30	0.98	1.30	0.33	0.33	0.49	0.49	0.33	0.33	0.49	0.65	0.33	0.65	0.33	0.65	0.49	0.33	-				
H23	0.49	0.33	0.65	1.30	1.47	1.14	1.47	0.16	0.49	0.65	0.33	0.49	0.49	0.65	0.81	0.49	0.81	0.49	0.81	0.65	0.16	0.49	-			
H24	0.49	0.33	0.65	1.30	1.47	1.14	1.47	0.49	0.49	0.33	0.65	0.16	0.49	0.65	0.81	0.49	0.81	0.49	0.81	0.65	0.49	0.49	0.65	-		
H25	0.49	0.33	0.65	1.30	1.47	1.14	1.47	0.49	0.49	0.65	0.33	0.49	0.49	0.65	0.81	0.49	0.81	0.49	0.81	0.65	0.16	0.49	0.33	0.33	-	
H26	0.49	0.33	0.65	1.30	1.47	1.14	1.47	0.49	0.49	0.65	0.33	0.49	0.49	0.65	0.81	0.49	0.81	0.49	0.81	0.65	0.16	0.49	0.33	0.65	0.33	-

Table 4.10: Percentage (%) of uncorrected “p” distance matrix among the 10 representative 16S rRNA haplotypes of *Rhinocypha fenestrella* in Malaysia.

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
H1	-									
H2	0.19	-								
H3	0.19	0.38	-							
H4	0.19	0.38	0.38	-						
H5	0.38	0.56	0.56	0.38	-					
H6	0.19	0.38	0.38	0.38	0.56	-				
H7	0.38	0.56	0.56	0.56	0.75	0.56	-			
H8	0.19	0.38	0.38	0.38	0.56	0.38	0.56	-		
H9	0.00	0.19	0.19	0.19	0.38	0.19	0.39	0.19	-	
H10	0.19	0.38	0.38	0.38	0.56	0.38	0.56	0.38	0.19	-

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Table 4.11: Unique sequences of *Rhinocypha fenestrella* haplotypes (H1-H26) based on COI sequences in Malaysia.

Haplotype	Variation sites in COI sequences																											
	9	10	16	30	55	103	108	145	156	175	205	217	234	241	256	295	307	322	334	358	379	409	448	453	505	535	558	
H1	A	A	G	C	G	G	G	G	A	A	G	C	C	C	A	C	A	C	T	C	A	G	A	G	G	A	G	
H2	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
H3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	
H4	-	-	A	-	A	-	-	-	-	G	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	G	A
H5	-	-	A	-	A	-	A	-	-	G	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	G	A
H6	-	-	A	-	A	-	-	-	-	G	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	A
H7	-	-	A	-	A	-	-	-	-	G	-	-	-	T	-	T	-	-	-	-	-	-	-	-	-	-	G	A
H8	-	-	-	-	-	-	-	-	-	G	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	-	G	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H10	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	T	G	-	-	-	-	-	-
H11	-	-	-	-	-	-	-	-	G	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-
H12	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-
H13	T	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H14	-	-	-	-	-	-	-	-	-	G	-	-	-	-	G	-	-	T	-	-	-	-	-	-	-	-	-	-
H15	-	-	-	-	-	-	-	A	-	G	-	-	-	-	G	-	-	T	-	-	-	-	-	-	-	-	-	-
H16	-	-	-	-	-	-	-	-	-	G	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H17	-	-	-	-	-	-	-	-	-	G	-	-	-	-	G	-	-	T	-	-	-	-	A	-	-	-	-	-
H18	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-
H19	-	-	-	-	-	-	-	-	-	G	A	-	-	-	G	-	-	T	-	-	-	-	-	-	-	-	-	-
H20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-
H21	-	-	-	-	-	-	-	-	G	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H22	-	-	-	-	-	A	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H23	-	-	-	-	-	-	-	-	G	G	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H24	-	-	-	G	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-
H25	-	-	-	G	-	-	-	-	-	G	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H26	-	-	-	-	-	-	-	-	G	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-

Table 4.12: Unique sequences of *Rhinocypha fenestrella* haplotypes (H1-H10) based on 16S rRNA sequences in Malaysia.

Haplotype	Variation sites in 16S RNA sequences									
	242	264	298	355	370	442	493	496	513	527
H1	G	T	A	-	G	G	T	G	A	G
H2	-	-	G	-	-	-	-	-	-	-
H3	-	-	-	-	A	-	-	-	-	-
H4	-	-	-	-	-	-	G	-	-	-
H5	-	-	-	-	-	-	C	C	-	-
H6	-	-	-	-	-	-	-	-	G	-
H7	-	-	-	-	-	T	-	-	-	T
H8	-	C	-	-	-	-	-	-	-	-
H9	-	-	-	T	-	-	-	-	-	-
H10	T	-	-	-	-	-	-	-	-	-

4.4 TAXONOMIC STUDIES WITHIN THE FEMALES OF *Rhinocypha* BY UTILIZING 4 CONTRASTING TOOLS

4.4.1 Morphological Description of Female *Rhinocypha* spp.

The male of *Rhinocypha* is easy to identify with its distinct blue thoracic marks on their thorax or abdomen. However, identifying the females is challenging and difficult to differentiate with other females of the same genus (Hamalainen & Divasiri, 1997; Willem, 2007). The *Rhinocypha* spp. can be a challenge to the studies on odonates, although the males are conspicuous with established key identification (Lahiri & Sinha, 1985; Orr, 2002; Hamalainen *et al.*, 2009), but females are more cryptic at species level.

Generally, the female species of *Rhinocypha* group can be identified within each species among other features is the pterostigma. The coloration of the pterostigma was distinct from each species, although it looks very similar with the naked eyes (Figure 4.10) besides the obvious brown marking at the tip of the *R. biforata* wing. In addition, the markings on thorax seen in lateral view (Figure 4.11) suggested that they had different yellow marking and some sort of blue marking in *R. biforata* species at the thorax. The details of the dichotomous key identification for females of *Rhinocypha* spp. as described below:

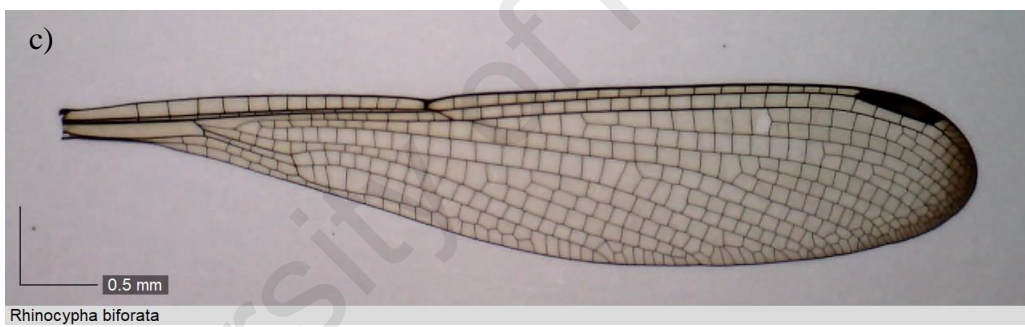
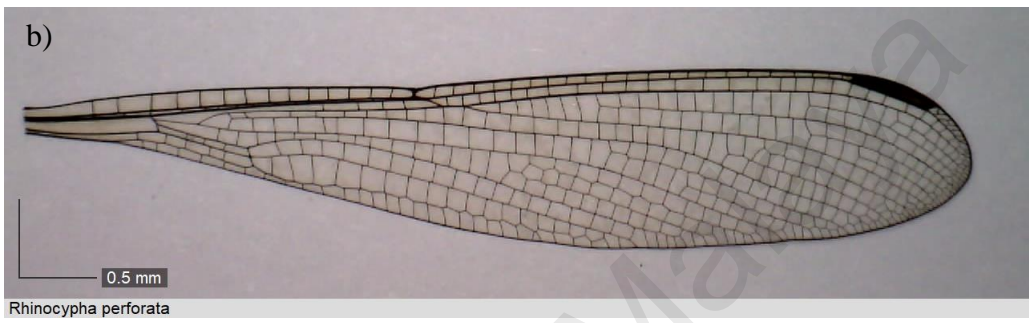
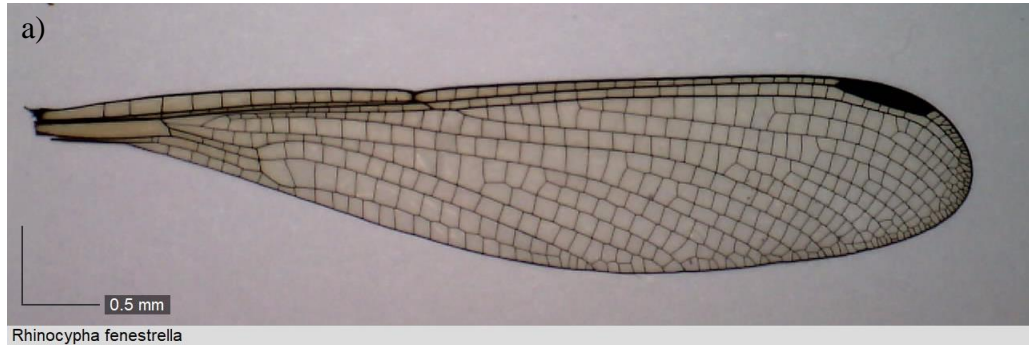


Figure 4.10: Wing of *Rhinocypha* spp. (a) *Rhinocypha fenestrella*, (b) *Rhinocypha perforata*, and (c) *Rhinocypha biforata*.



Figure 4.11: Thorax of *Rhinocypha* spp. (a) *R. fenestrella*, (b) *R. perforata*, and (c)

Key to species for the genus *Rhinocypha* Rambur (Females)

1. Discoidal cell is a simple quadrilateral, sometimes traversed by crossveins, occasionally open at base.....Suborder Zygoptera 2
2. Numerous antenodal crossvein in both the costal and subcostal spaces.....Chlorocyphidae 3
3. Abdomen shorter than wings, pterostigma present.....*Rhinocypha* 4
4. Yellow at the centre of the black pterostigma and wing enfumed; front of the synthorax almost dark black; median part of synthorax yellow with yellow stripe; yellow patch on metepimeron covering approximately most of it length and width with broadened to the tip5
- 5a. Tiny yellow stripe at below of mesopleural suture6a
- 5b. Widened yellow stripe at below of mesopleural suture6b
- 6a. Yellow marking on metanepisternum, occupying approximately half of width with extend to mesepimeron at anterior part7a
- 6b. Much more extensive of yellow marking on metanepisternum, occupying more to two third of width with extend to mesepimeron at anterior part7b
- 7a. Tiny yellow stripe at the tip of intersegmental suture, tiny yellow stripe at upper part of mesanepisternum which form a circle to near of mesostigma segment9a
- 7b. Bellow intersegmental suture, widened yellow mark at the tip.....8
- 8a Tiny of yellow stripe at upper part of mesanepisternum segment with yellow stripe extending down in front of mesopleural suture9b

8b Long yellow stripe at the upper part of mesanepisternum which form a yellow lobe to near of mesostigma segment and broadened at the tip9c

9a. Metakatepisternum dark-brown with pale yellow at below segment; no marking at the wing; yellow at the centre of the pterostigma distinct, Wing length : Wing width (24-26 mm : 5 mm), Abdomen length 16.6-18.8 mm.....*fenestrella* Rambur

9b. Metakatepisternum dark-brown and surrounded by yellow pale; brown marking at the tip of the wing; pterostigma generally markedly paler of black colour, Wing length : Wing width (23-24½ mm : 4 mm), Abdomen length 14.8-17.7 mm..... *biforata* Seysl

9c. Metakatepisternum dark-brown with pale yellow at upper segment; no marking at the wing; pterostigma with brownish color, Wing length : Wing width (24-25 mm : 4½ mm), Abdomen length 15.6-17.8 mm.....*perforata* Percheron

University of Malaysia

4.4.2 Description of the Female's Ovipositor of the Three Species of *Rhinocypha* using Field Emission Scanning Electron Microscope (FESEM)

Figure 4.12 showed the lateral view of the external morphology of the ovipositor of *Rhinocypha* spp. generated from the field emission scanning electron micrograph. Basal plate of the ovipositor (Lam) was connecting the first valves with sternite of 8th segment (S8) and tergite of 9th segment (S9). The sheathing valves (V3) showed ensheathing cutting valves laterally in resting position. Besides, the anal appendages (Ap) were connected with the 10th segment (S10), and the stylus (St) and distal tooth (Dt) were connected at the end of the V3.

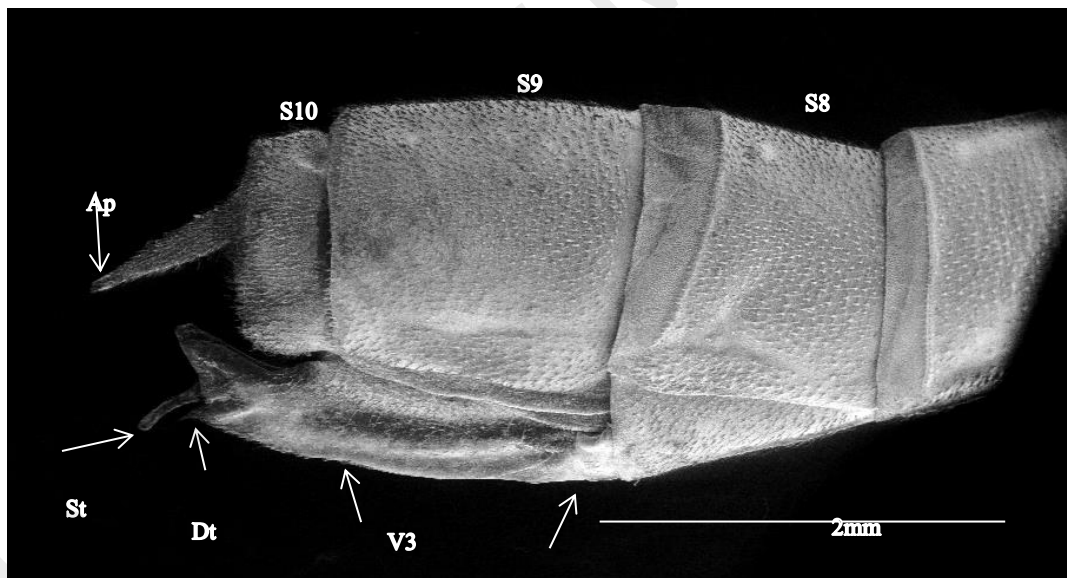


Figure 4.12: Lateral view of the external morphology of the ovipositor of *Rhinocypha* spp. Ap: anal appendages; St: stylus; Dt: distal tooth; V3: third valves of ovipositor (valvulae 3); Lam: basal plate of ovipositor (lamina valvarum).

Below the figure, Figure 4.13 showed the images of 8th, 9th and 10th segments, together with ovipositor parts representatives of each species of the *Rhinocypha* group, *Rhinocypha biforata*, *Rhinocypha fenestrella* and *Rhinocypha perforata* generated by FESEM in lateral view. Besides, it showed the morphometric measurements taken for each part of each individual of the samples.

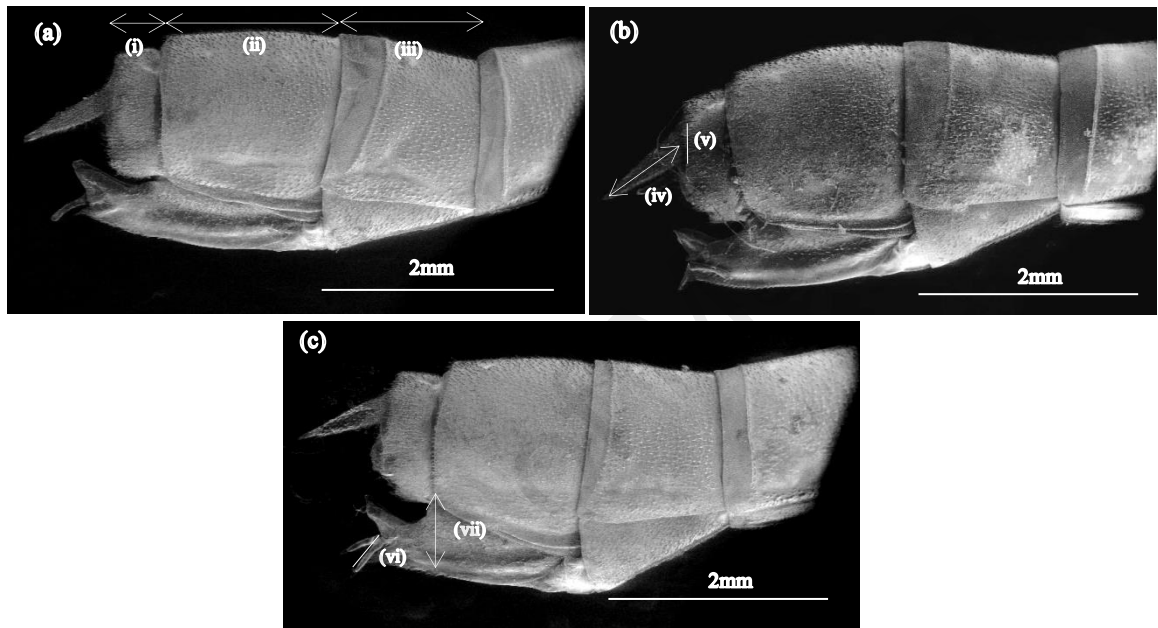


Figure 4.13: Scanning electron micrographs of the morphometric measurements of the ovipositor for the females of *Rhinocypha* spp. (lateral view) - (a) *Rhinocypha biforata*, (b) *Rhinocypha fenestrella*, (c) *Rhinocypha perforata*. (i, ii & iii) length of each segment; (iv) length of anal appendages; (v) basal width of anal appendages; (vi) length of stylus; (vii) width of the V3.

Based on high-resolution images generated in this study, the ovipositor of females *Rhinocypha* species were categorized in the following morphological types:

- 1) The sensilla and the setae of the anal appendages
- 2) The characteristics of the sheathing valve (V3) and distal tooth
- 3) The hair sensilla of the stylus

Anal appendages. After visualization of the anal appendages for each species of *Rhinocypha*, it showed three primary characteristics (Figure 4.14). For the species of *R. biforata*, they had few long articulated setae (Figure 4.14a), compared to species of *R. fenestrella* (Figure 4.14b). However, for the species of *R. perforata*, they had short articulated setae (Figure 4.14c). On the other hand, the distribution of the basiconic sensilla in *R. biforata*, they had a compact of the basiconic sensilla in their anal appendages, whereas in *R. fenestrella* anal appendages, they had more space between the basiconic sensilla, and as the articulated setae, the *R. perforata* had a compact of short basiconic sensilla. Instead, *R. fenestrella* had a lot of coeloconica-like sensilla compared to the other two species.

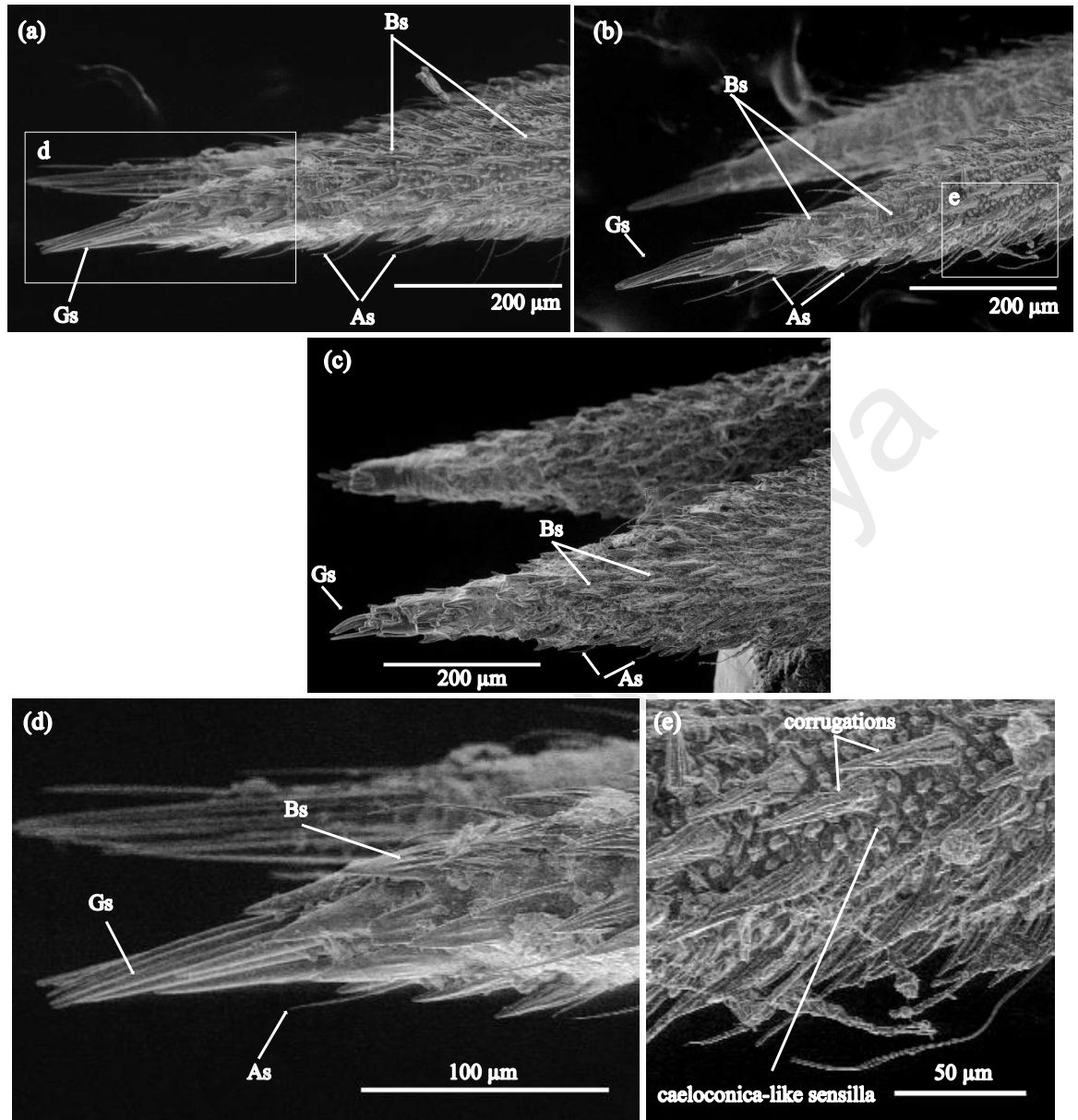


Figure 4.14: Scanning electron micrographs of anal appendages of *Rhinocypha* spp. – (a) *Rhinocypha biforata* – inset indicates the group of sensilla; (b) *Rhinocypha fenestrella* – inset shows the caeloconica-like sensilla; (c) *Rhinocypha perforata*; (d) group of sensilla on the tip of anal appendages; (e) caeloconica-like sensilla on the surface of the anal appendages. Gs: group of sensilla; Bs: basiconic sensilla; As: articulated setae.

Sheathing valves (V3). Internal view of the apical part of carina showed that the three species of *Rhinocypha* had a different shape between each other (Figure 4.15). For the species of *R. biforata*, they had sharply pointed of the carina and they are more diagonal in projection (Figure 4.15a), while for the species of *R. fenestrella*, they had evenly sharply pointed of the carina and they are more vertical in projection (Figure 4.15b). Contrast in *R. perforata* species, they had blunt and rounded tip of carina in the sheathing valves (V3) (Figure 4.15c). In addition, the three species had no differences of the distal tooth (Figure 4.15e, f, g).

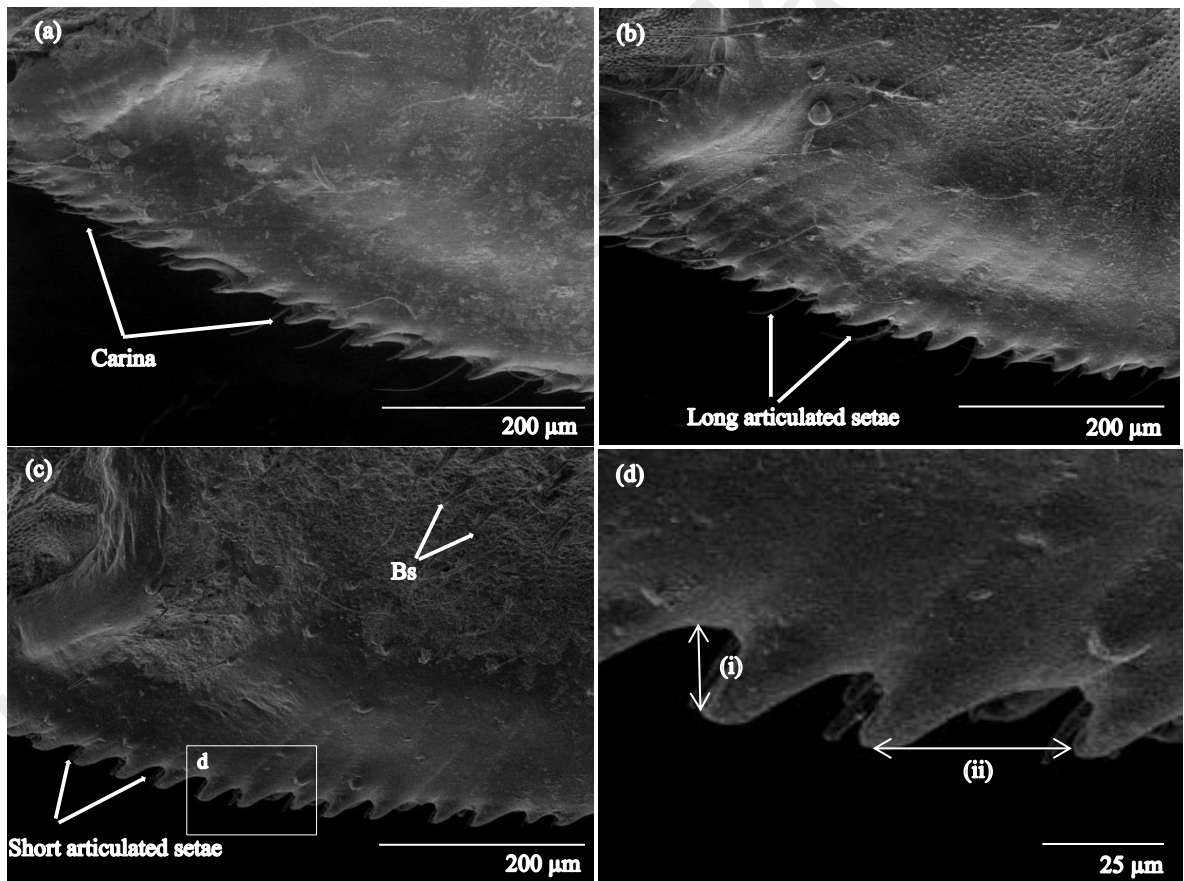


Figure 4.15: Scanning electron micrographs of sheathing valve (V3) and distal tooth of *Rhinocypha* spp. (a) *Rhinocypha biforata*, (b) *Rhinocypha fenestrella*, (c) *Rhinocypha perforata* – inset shows the carina, (d) measurement of (i) the peak of the tooth to the median base (ii) the space between the tooth. (e, f & g) shows the scanning electron micrographs of distal tooth of *Rhinocypha* spp.: (e) *Rhinocypha biforata* - (iii) indicates the width of the distal tooth, (f) *Rhinocypha fenestrella* – inset shows the campaniform sensilla, (g) *Rhinocypha perforata*, (h) campaniform sensilla at the distal tooth surface.

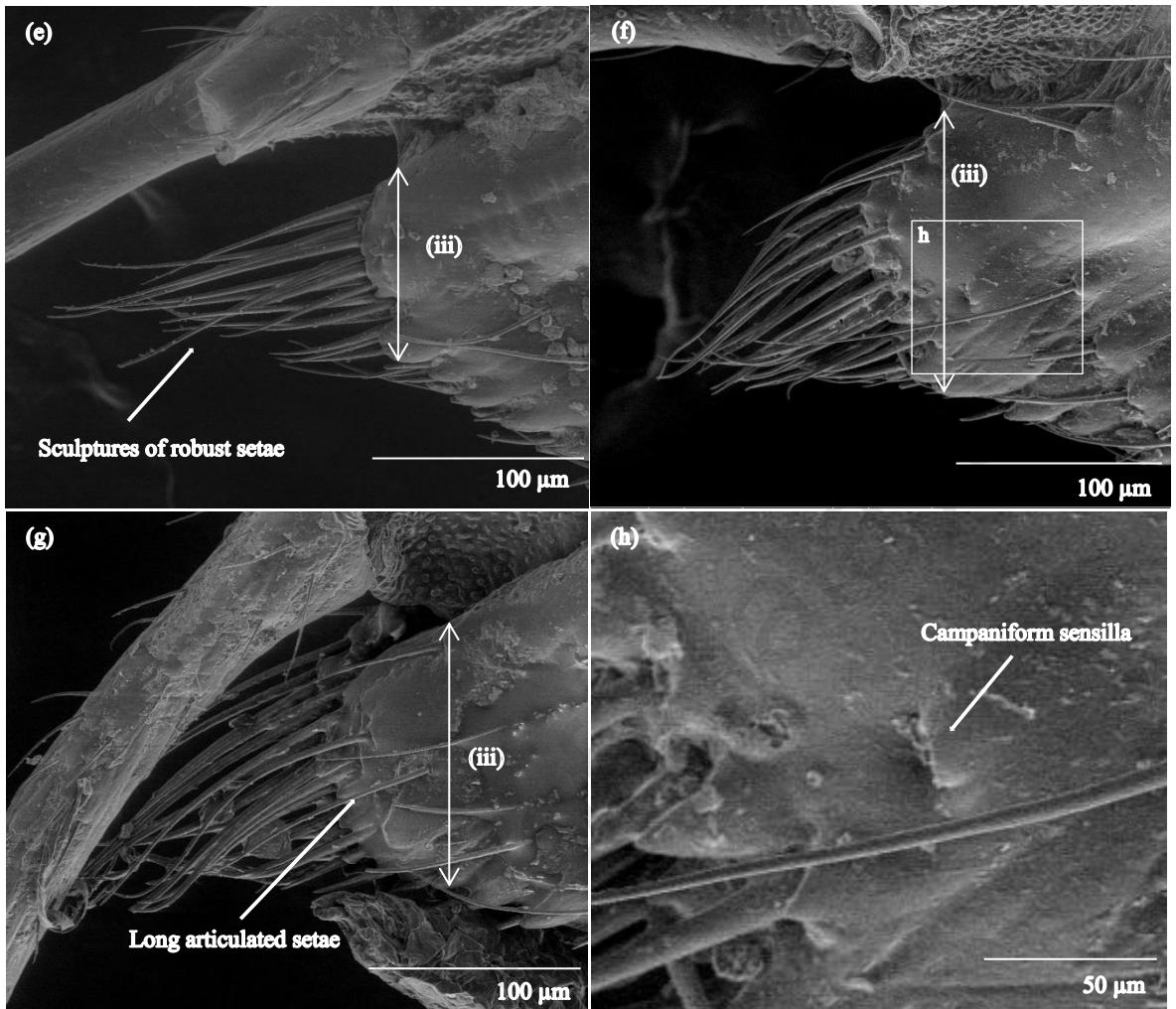


Figure 4.15, continued.

Stylus. Examining the apex of stylus showed they had differences shape and distribution of sensilla and knobble (Figure 4.16). For the species of *R. biforata*, the sensilla were gathered at the tip of the stylus, while *R. fenestrella* species, the sensilla scattered throughout the stylus. Besides, for *R. perforata* species, the sensilla was scattered at the tip of the stylus. On the other hand, Figure 4.16 d, e and f showed the base of the stylus of the three studies species where the knobbls of *R. biforata* were rounded and not compact as in the species of *R. fenestrella* that had rounded, more compact of the knobbls and evenly distributed at the base of stylus. Conversely, the base of stylus of the species of *R. perforata* showed they had flat and scattered knobbls.

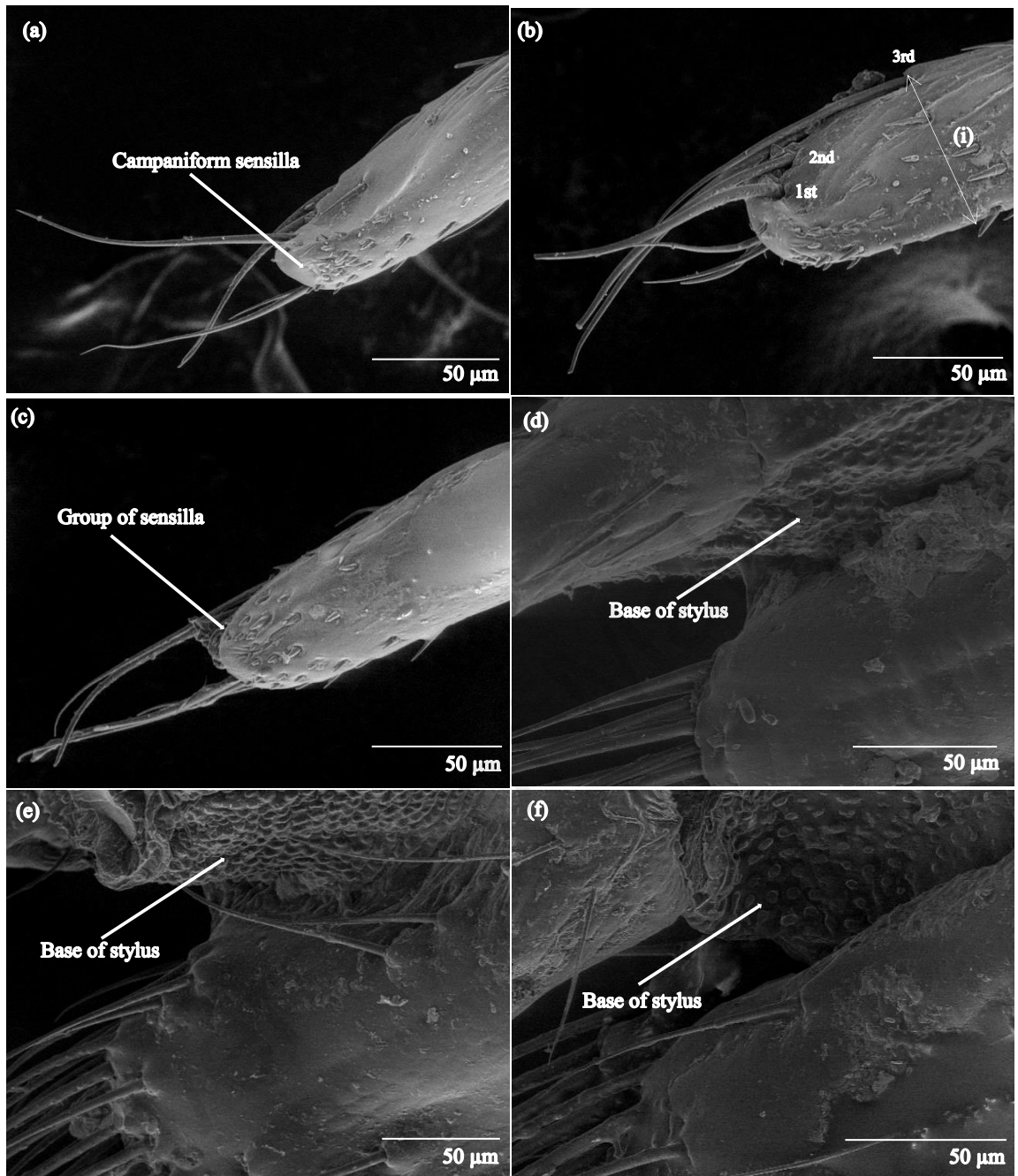


Figure 4.16: Scanning electron micrographs of the stylus and the base of stylus of *Rhinocypha* spp. (a) *Rhinocypha biforata*, (b) *Rhinocypha fenestrella* – (i) width of the stylus from the third of hair sensilla, (c) *Rhinocypha perforata*. (d, e & f) scanning electron micrographs of the base of stylus. (d) *Rhinocypha biforata*, (e) *Rhinocypha fenestrella*, (f) *Rhinocypha perforata*.

On top of that, from the micrographs generated by using the FESEM, the morphometric measurements were taken in several parts of the female ovipositor (Table 4.13). The table below shows that species of *R. fenestrella* had longer of 8th and 9th segment compared to the two species, *R. biforata* and *R. perforata*, but had the shortest 10th segment. Besides, *R. biforata* species had the longest anal appendages, while *R. perforata* had the widest base of the anal appendages and stylus, and the widest stylus compared to the other two species.

Moreover, in terms of V3, *R. biforata* had the widest of V3, and also showed they had more space between the distal tooth. As well, consistent with the micrograph taken from the FESEM, the distal tooth of *R. fenestrella* was wider and more in upright protrusion likened the species of *R. biforata* and *R. perforata*.

Table 4.13: Morphometric measurements calculated from the ovipositor of the females' of *Rhinocypha* spp. The values showed the mean \pm standard deviations of each characteristic.

Species	Length of 8th segment	Length of 9th segment	Length of 10th segment	Length of anal appendages	Basal width of anal appendages
<i>R. biforata</i>	1.205 \pm 0.088	1.512 \pm 0.044	0.375 \pm 0.062	0.877 \pm 0.094	0.221 \pm 0.018
<i>R. fenestrella</i>	1.383 \pm 0.002	1.563 \pm 0.023	0.318 \pm 0.023	0.789 \pm 0.103	0.241 \pm 0.062
<i>R. perforata</i>	1.215 \pm 0.122	1.446 \pm 0.160	0.359 \pm 0.076	0.846 \pm 0.067	0.256 \pm 0.062

Table 4.13, continued.

Species	Width of V3	Peak of tooth to the median base	Space between the tooth	Width of the distal tooth	Length of stylus	Width of the stylus
<i>R. biforata</i>	0.625 \pm 0.093	0.016 \pm 0.002	0.042 \pm 0.019	0.119 \pm 0.023	0.215 \pm 0.082	0.040 \pm 0.007
<i>R. fenestrella</i>	0.620 \pm 0.038	0.017 \pm 0.003	0.040 \pm 0.016	0.160 \pm 0.008	0.158 \pm 0.009	0.043 \pm 0.008
<i>R. perforata</i>	0.620 \pm 0.039	0.016 \pm 0.002	0.032 \pm 0.008	0.141 \pm 0.005	0.287 \pm 0.024	0.050 \pm 0.005

4.4.3 Geometric Morphometric Analysis of the Wings

The landmark configuration of the Procrustes superimposed coordinates for the wings are presented in Figure 4.17. Overall, the landmarks 6, 7 and 15 of the forewings of *Rhinocypha* spp. are more variable than the other landmarks. Between the three *Rhinocypha* damselflies, the landmarks of the *R. biforata* demonstrated more shape variation than the other species, suggested by the percentage of variance of the principal component analysis.

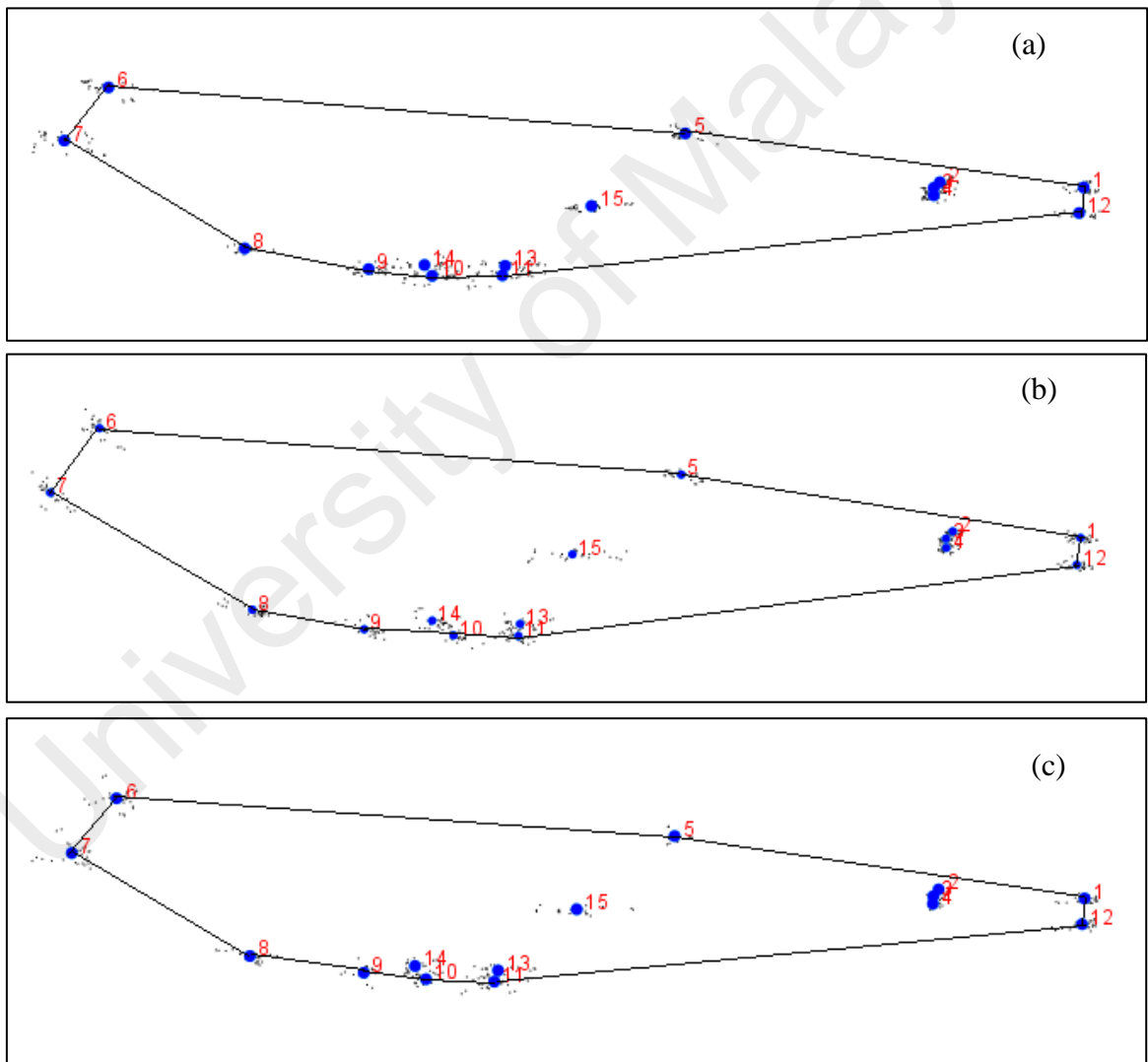


Figure 4.17: Scatterplot of all 15 landmarks configurations after Procrustes superimposition. (a) *Rhinocypha biforata*, (b) *Rhinocypha fenestrella*, (c) *Rhinocypha perforata*.

The wireframe in Figure 4.18 visualize the shape variation on the axes. PC1 of *R. biforata* species accounted for 72.92% of the total variance, besides for *R. fenestrella* accounted for 40.53% and 52.29% for *R. perforata*. The species with high scores on PC1, *R. biforata*, have a shorter wing length compared to the other species; the species with lowest PC1 scores have a longer wing length.

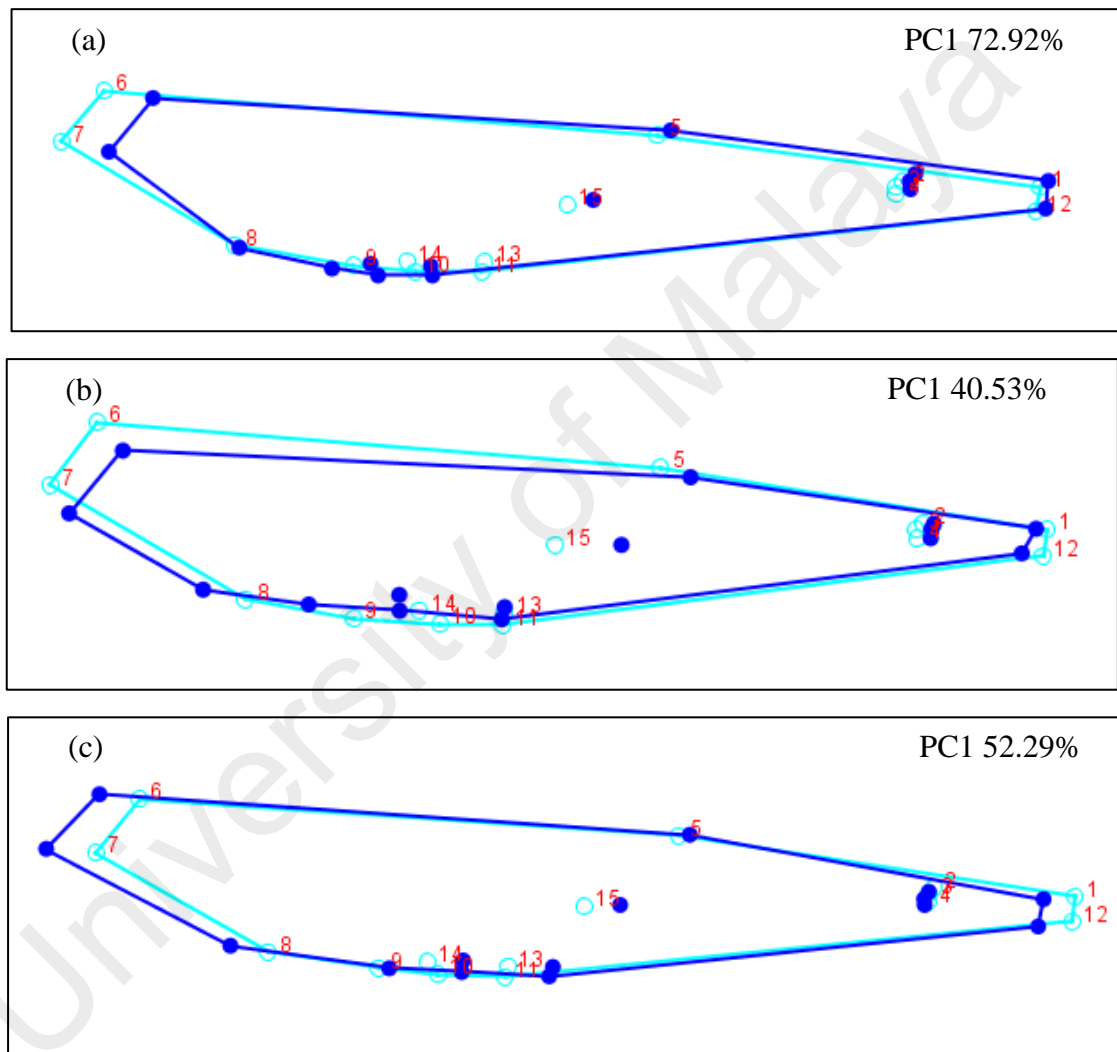


Figure 4.18: Wireframe visualization of shape variation along the principal components one from geometric morphometric analysis. (a) *Rhinocypha biforata*, (b) *Rhinocypha fenestrella*, (c) *Rhinocypha perforata*. Light blue landmarks represent the configuration of average specimen; dark blue landmarks represent one approximate extreme of the variation on that axis. Percentages indicate the proportion of total variance explained by each axis.

The PCA plot graph (Figure 4.19), showed considerable dispersion across morphospace among species. The first five principal components explaining 88.68% of total variation accounted for 48.64%, 17.45%, 9.96%, 8.33% and 4.30% respectively. A total up to six axes were required to cover more than 90% of the shape variation.

Accordingly, the PCA analysis of the three species explained 66.09% of shape variation within samples by the two first PCA axes extracted from the variance-covariance matrix (PC1 explains 48.64% and PC2, 17.45%). A plot of PC1 and PC2 demonstrated overlapping of wing shapes between the three species of *Rhinocypha*.

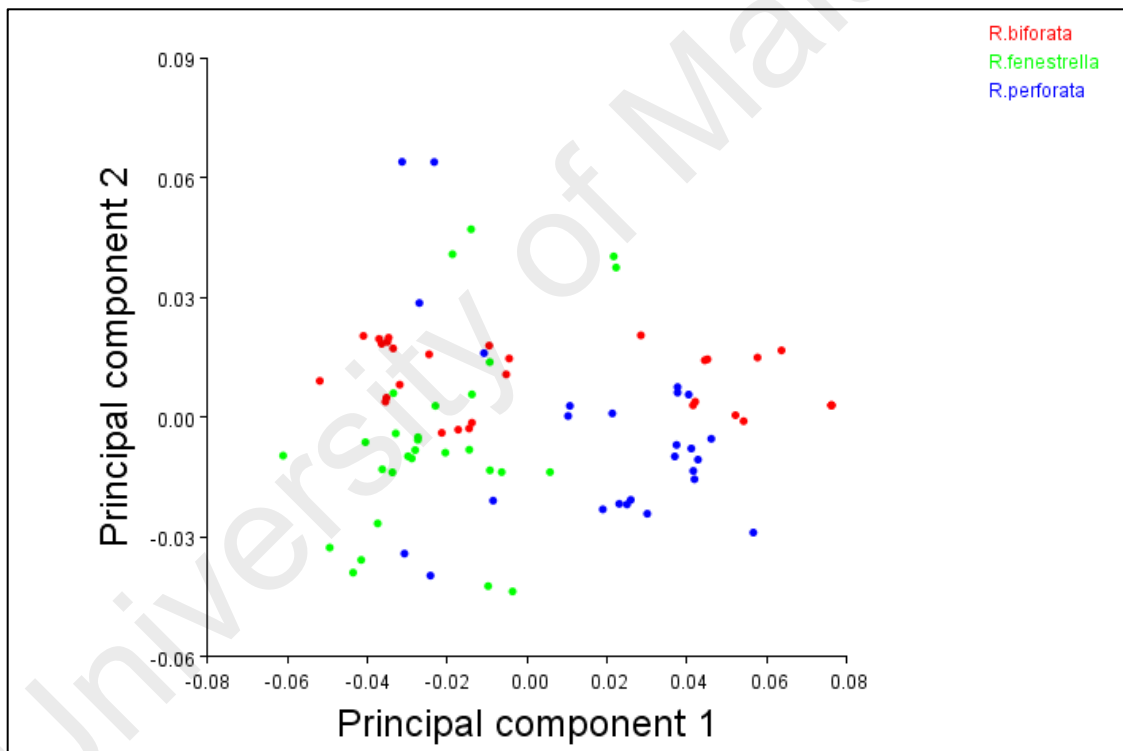


Figure 4.19: Results of principal components analysis of all specimens. PC1= 48.64%, PC2= 17.45%, accounting for 66.09% of the total variation.

Differences in shape among species were described in terms of thin-plate deformation grids and the coordinates of landmarks were used for estimating the overall size of the wing known as centroid size, an isometric estimator defined as the square root of the sum of the squared distances of all landmarks from their centroid. Figure 4.20 showed the thin-plate spline deformation grids of wing shape variation and the species-specific differentiation was evident in the forewing in the three species of *Rhinocypha*.

From the thin-plate deformation grids, *Rhinocypha biforata* presented narrower wings, whereas *Rhinocypha fenestrella* had broader wings. On the other hand, the species of *Rhinocypha perforata* had a broader elongated apex.

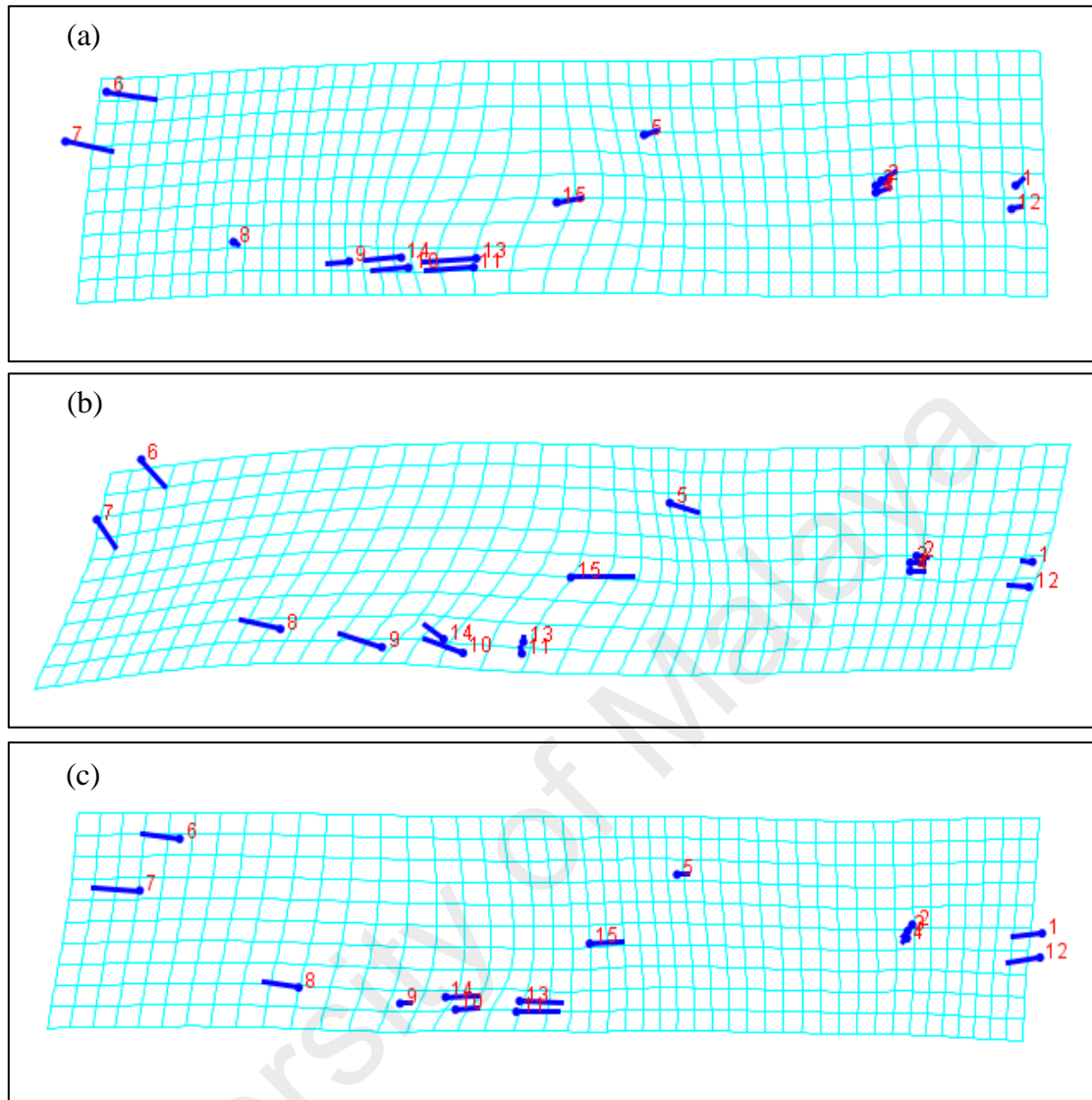


Figure 4.20: Thin-plate spline deformation grids of wing shape variation in *Rhinocypha* spp. (a) *Rhinocypha biforata*, (b) *Rhinocypha fenestrella*, (c) *Rhinocypha perforata*, demonstrating the directions (arrows).

In contrast to PCA, the differences between species well illustrated by a canonical variate analysis (CVA) plot. The CVA was applied to the Procrustes coordinates extracted from the fore wings of all the samples. A scatter plot of CV1 (eigenvalue 8.887) vs. CV2 (eigenvalue 2.150) showed that the wing shapes of the three species of *Rhinocypha* were not overlapping each other and well clustered according to species (Figure 4.21). This suggested that the geometric morphometric of the wing shapes successfully differentiate between the species of *Rhinocypha* group.

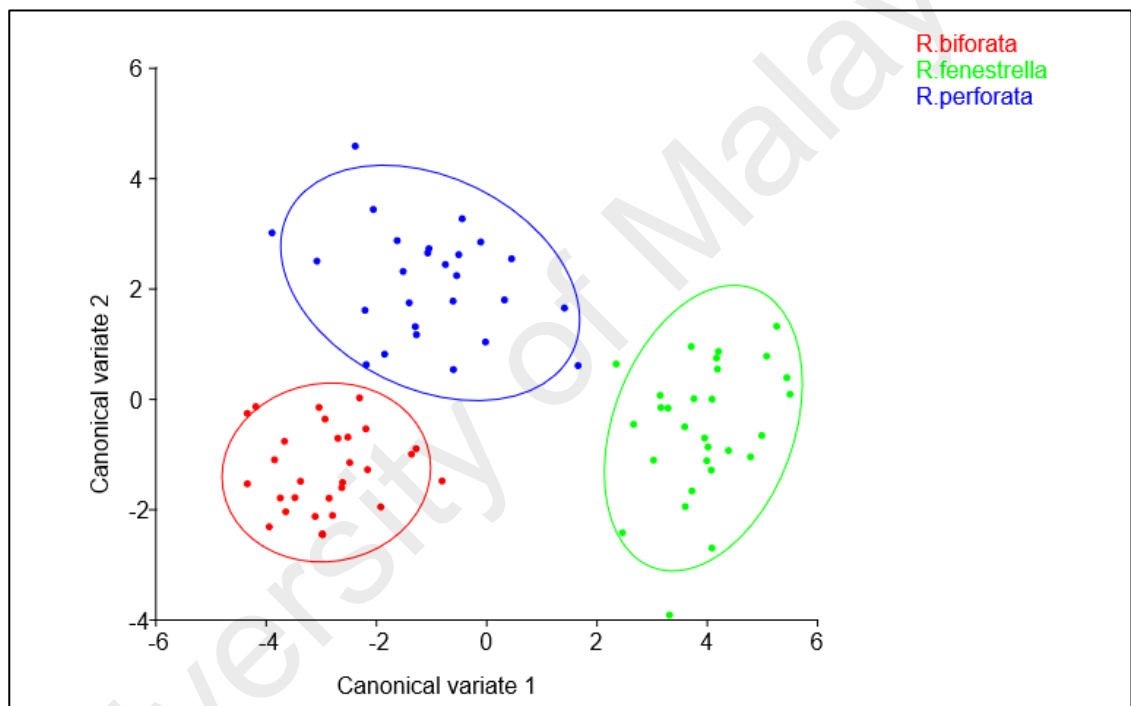


Figure 4.21: Canonical Variate analysis (CVA) plot. CV1 (eigenvalue 8.887) vs CV2 (eigenvalue 2.150). 90% confidence ellipses of CVA scores. Colour of ellipses corresponds to the species written alongside.

4.4.4 Phylogeny Comparison

The phylogenetic relationships of the investigated damselflies were recovered using two different DNA regions; COI and 16S rRNA (Figure 4.22). The Neighbor-joining (NJ) analysis revealed that the phylogeny of all the samples was separated into three clades. The species of *R. perforata* formed separate monophyletic clade with very high bootstrap support for both regions, 100. *R. biforata* and *R. fenestrella* together formed a monophyletic group clearly separated from the investigated *R. perforata* species.

Additionally, *R. biforata* species were recovered as sister taxon to the species of *R. fenestrella*. On the whole, the phylogenetic relationships support the generic status of *Rhinocypha* where the three species clustered well into their own specific cluster.

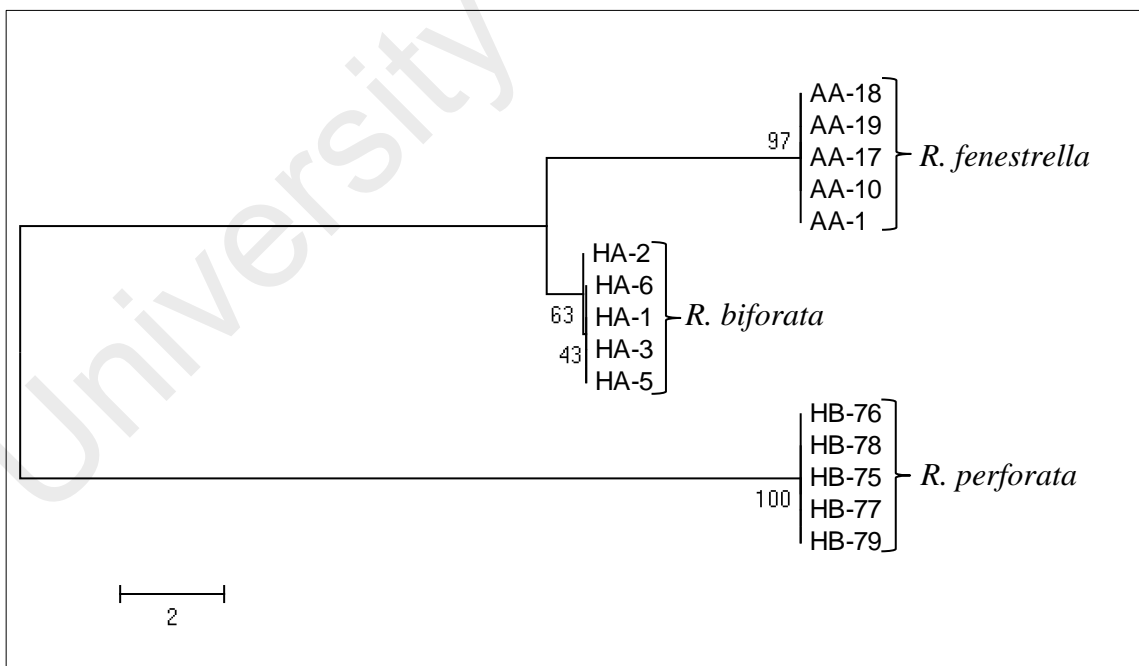


Figure 4.22: Neighbor-joining phylogenetic tree of *Rhinocypha* spp. based on combined COI + 16S rRNA sequences. Bootstrap values are shown on the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and the bar indicates substitutions per site.

4.5 MORPHOLOGY AND CHARACTERISTICS PROPERTIES OF THE *Rhinocypha* WINGS

4.5.1 Wing Joint Morphology

Initial scanning electron microscopy (SEM) on the wings of *Rhinocypha* group; *R. fenestrella*, *R. biforata* and *R. perforata* confirmed that there are two main types of joints which are i) mobile and ii) immobile (Figure 4.23). The mobile joints (Figures 4.23a, 4.23c) were disjoined to the longitudinal veins, oppositely with the immobile joints (Figures 4.23b, 4.23c), where they were resolutely connected to the longitudinal veins.

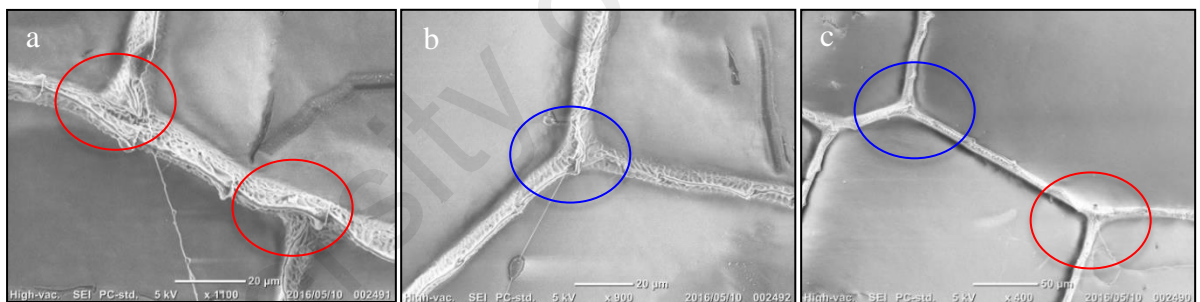


Figure 4.23: Scanning electron microscope (SEM) of the two types of vein joints. Mobile joint (red circle) and immobile joint (blue circle). (a): Mobile joint at the cross vein attached with the longitudinal vein. (b): Immobile joint at the trailing edge of the wing. (c): Two types of the vein joint in the region of the MP(-).

On the other hand, confocal laser scanning microscopy (LSCM) in the UV band revealed the presence of the blue-fluorescing material in the vein-joints which is known as resilin, and it was confirmed by the resilin-specific custom filter set (Figures 4.24, 4.25 and 4.26). The resilin was found mostly in the mobile joints where the cross veins meet the longitudinal veins (Figures 4.24d, 4.25e, and 4.26e); whereas strong patches of resilin were found within the nodus for both surfaces of the wings, dorsally and ventrally surfaces (Figures 4.24c, 4.25c, and 4.26c).

Interestingly, the presence of resilin varied at the dorsal and ventral sides of specific joints. From the study it revealed a variation of 4 resilin distributions occurred which were; (1) contained resilin only dorsally (e.g., Figure 4.26e), (2) only ventrally (e.g., Figure 4.25d), (3) both surfaces dorsally and ventrally at the joints (e.g., Figures 4.24d, 4.25e and 4.26d) or (4) neither side of the joint contained resilin (e.g., Figure 4.24e).

Overall, Figure 4.27a present a summarized of resilin mapping for the *Rhynocypha* group investigated in this current work. From the results, it is postulated that 3 trends of resilin mapping existed: (1) nodus is constantly enriched with resilin for both dorsal and ventral surfaces (2) the dorsal longitudinal vein RA always have resilin present (3) both dorsal and ventral surfaces with the longitudinal veins RP2, RP3/4 and trailing edge vein MP have resilin present.

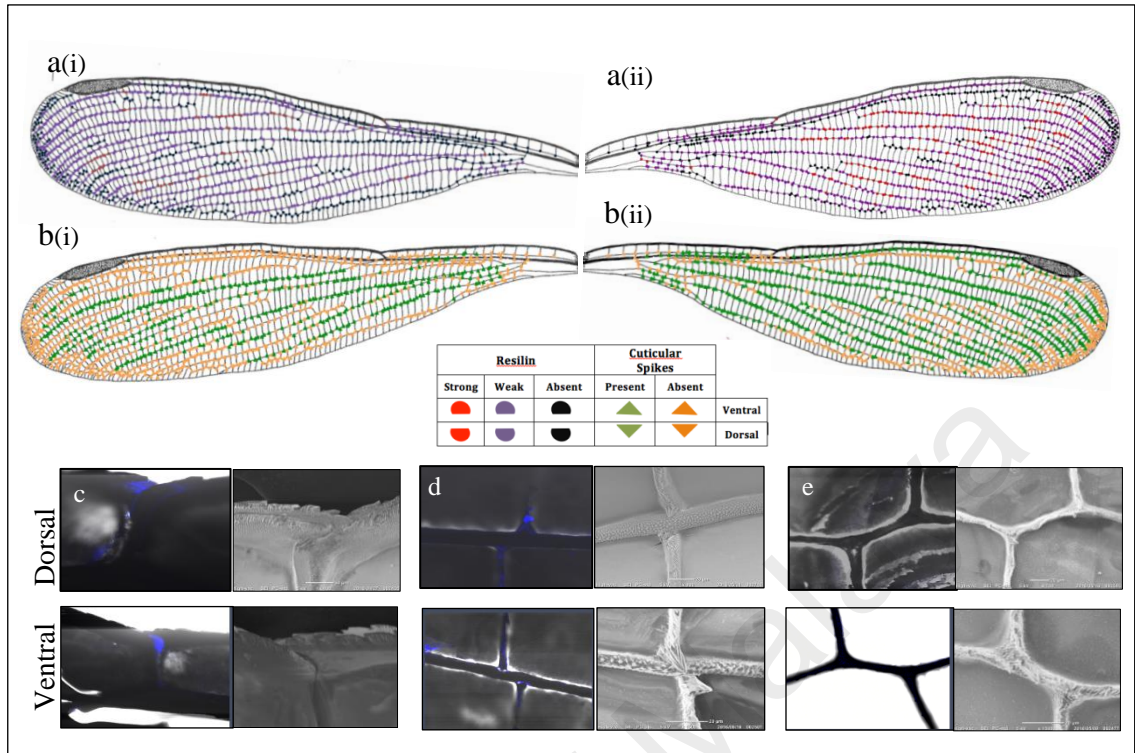


Figure 4.24: Distribution of resilin and spikes in the vein joints of *Rhinocypha fenestrella* wing. Dorsal (a(i), b(i)) and ventral (a(ii), b(ii)) side of the forewing. c-d: LSCM (right) and SEM (left) images at the selected joint for dorsal (upper) and ventral (below) sides. (c): Large resilin patch in both dorsal and ventral side. (d): Resilin patches at the mobile joint. (e): No resilin patch at the immobile joint.

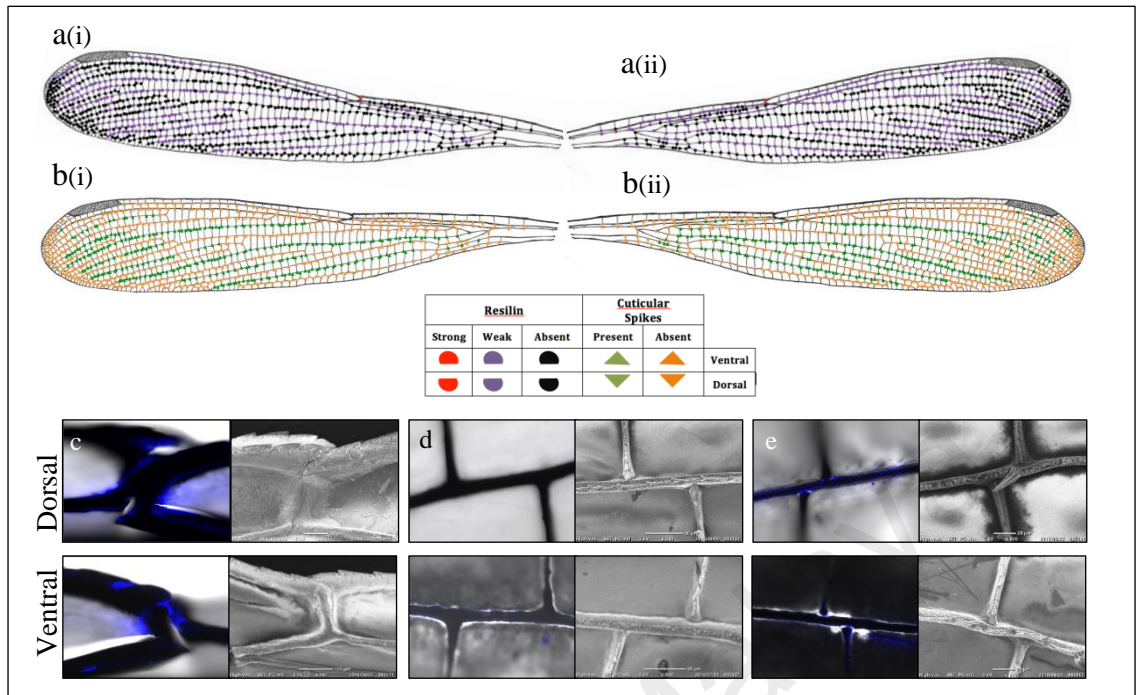


Figure 4.25: Distribution of resilin and spikes in the vein joints of *Rhinocypha perforata* wing. Dorsal (a(i), B(i)) and ventral (a(ii), b(ii)) side of the forewing. c-d: LSCM (right) and SEM (left) images at the selected joint for dorsal (upper) and ventral (below) sides. (c): Large resilin patch in both dorsal and ventral side. (d): No resilin patches at the ventral side. (e): Resilin patches at the mobile joint for both dorsal and ventral side.

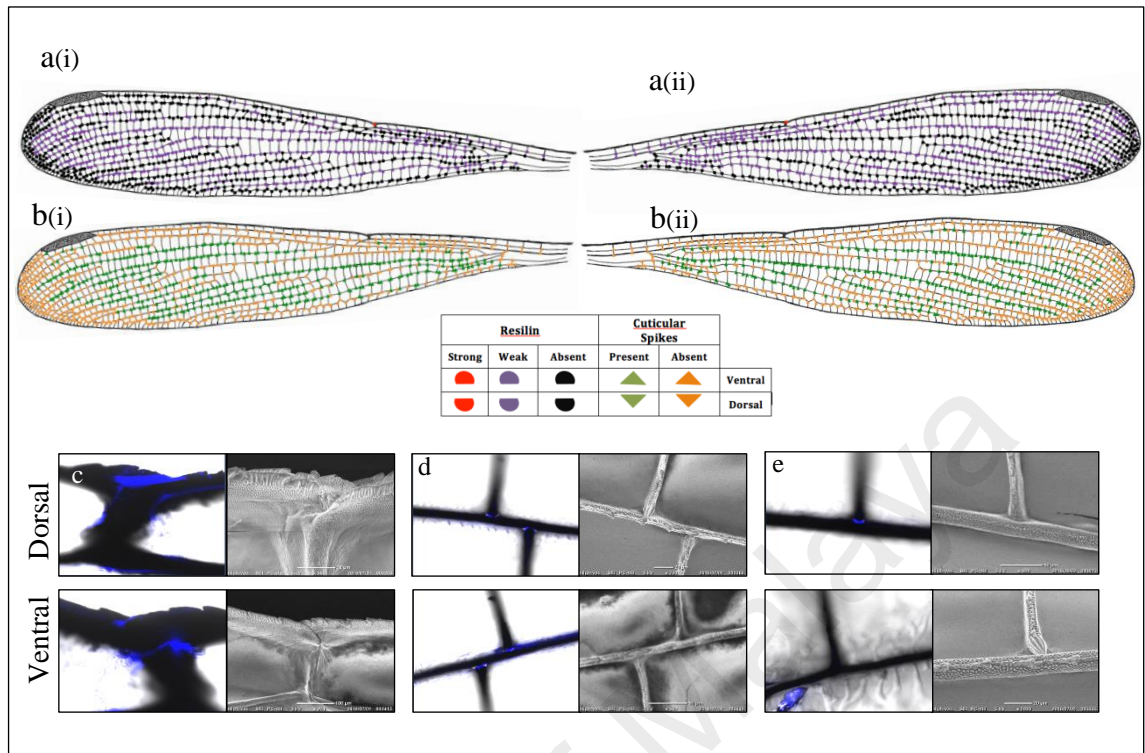


Figure 4.26: Distribution of resilin and spikes in the vein joints of *Rhinocypha biforata* wing. Dorsal (a(i), b(i)) and ventral (a(ii), b(ii)) side of the forewing. c-d: LSCM (right) and SEM (left) images at the selected joint for dorsal (upper) and ventral (below) sides. (c): Large resilin patch in both dorsal and ventral side. (d): Resilin patches at both dorsal and ventral side. (e): Only resilin patch at ventral side.

In the context of scanning electron micrographs (SEM), it showed the position of spikes in relation to the longitudinal veins. Generally, there were several positions of spikes observed from SEM; i) spikes were either upright protrusions pointing directly away from the wing surface (Figures 4.28a, 4.28c), ii) placed adjacent to the longitudinal veins (Figures 4.28b, 4.28c), iii) or orientated towards the longitudinal veins which impacted the wing veins (Figure 4.28d) by restricting the free movements of the joints.

The presence of spikes at the wing vein joints were equivalent to resilin, ie: only dorsally (e.g., Figures 4.25d, 4.26d), ventrally only (e.g., Figure 4.26e), or on both dorsal and ventral sides (e.g., Figure 4.24d). Figure 4.27a illustrated the patterns of spike distributions true for all species except category (4): (1) longitudinal vein RA, absence of spikes at the dorsal side, (2) longitudinal vein RP2 presence of spikes on the dorsal surface, (3) spikes found on the trailing edge MP ventrally and (4) specific for *R. fenestrella*, longitudinal veins IR2 and MA have spikes on both dorsal and ventral surfaces. In general, the results showed that spikes are widespread throughout the wing surfaces.

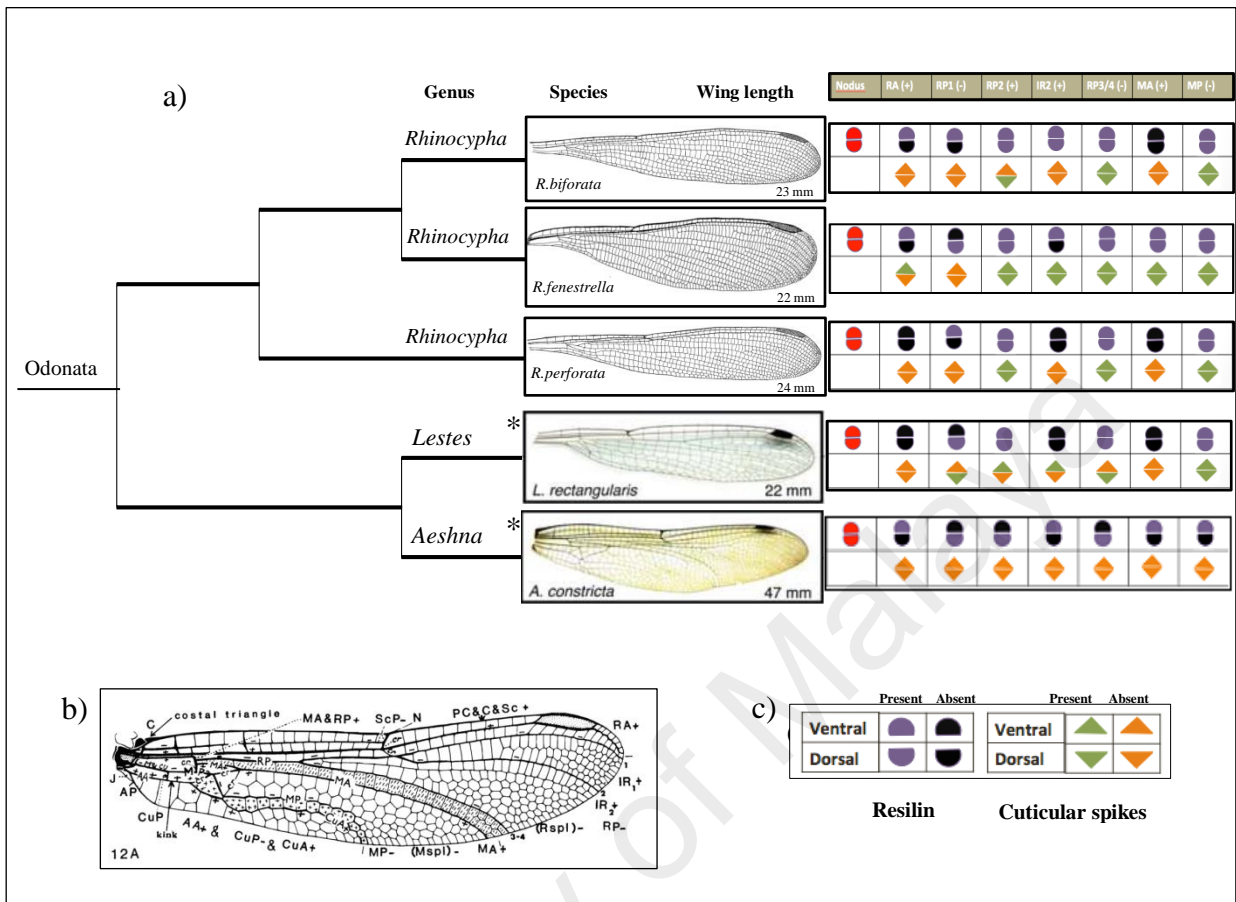


Figure 4.27: Summary of the distribution of resilin and spikes in the vein joints of the *Rhinocypha* spp. (a) Phylogenetic comparison of resilin patches and spikes in damselflies. Branches show the relationship of taxa based on molecular, COI gene. (b) Nomenclature of wing structure according to Riek & Kukalova-Peck (1984). (c) Symbols used for resilin and spikes mapping. “Present” and “Absent” are marked at the vein that showed blue fluorescence and versa, and at the vicinity of vein joint for spikes. *based on Donoughe *et al.* (2011).

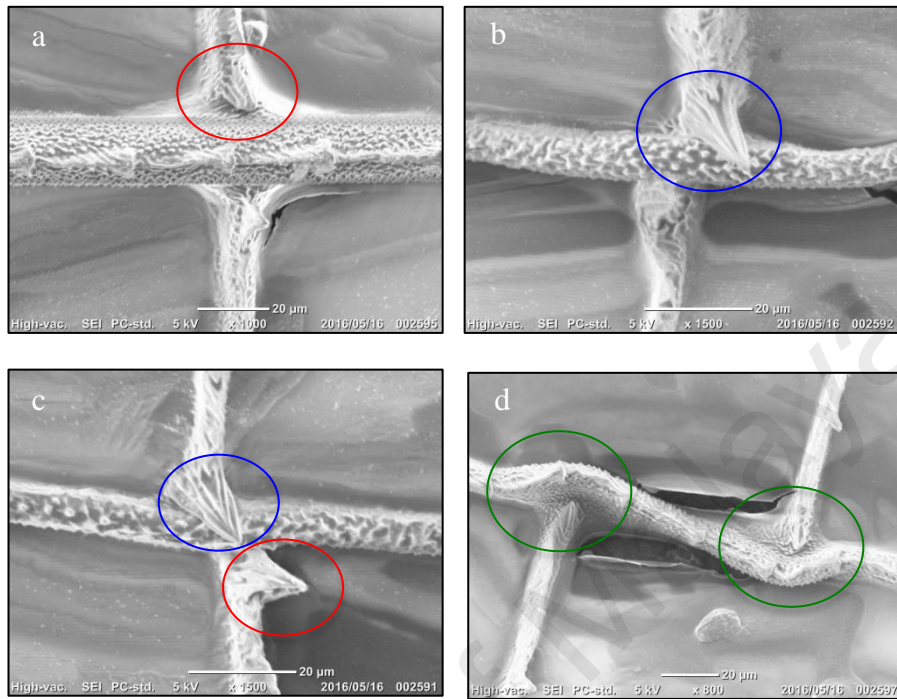


Figure 4.28: Relation of cuticular spikes with longitudinal vein by SEM imaging. (a): A spike upright protrusions pointing directly away from the wing surface. (b): A spike placed adjacent to the longitudinal vein. (c): Two types of spikes between cross vein and longitudinal vein. (d): Spikes orientated towards the longitudinal vein which impacted the wing veins.

4.5.2 Mechanical Properties of the Wing

This study investigated 3 sections of the wings: (1) membrane of the wing (2) mobile joint; and (3) immobile joint using the scanning electron microscopy (SEM) together with atomic force microscopy (AFM) to obtain the topography images and to determine the roughness of each sample.

As the results, Figure 4.29 shows the representative images of the surface for each section of the wings of *Rhinocypha* spp. using the SEM, whereas Figure 4.30 shows the representative topography images of the surface for each section of each sample that used the AFM in non-contact mode. Imaging obtained from the scanning electron microscopy (Figure 4.29) revealed interesting morphologies on the surfaces of the wings. The surfaces of the *Rhinocypha* spp. shows in the form of wavelike patterns (Figure 4.29c (i)), and additionally, there were globular nanostructures (e.g., Figures 4.29a (ii), 4.29b (ii)) observed on the surface of the wings.

Force curves of the sample surfaces were recorded using a parabolic tip, and then it was analyzed according to the Hertz theory to give the quantitative data on sample elasticity (Young's modulus). The Kruskal Wallis test was applied due to non-normal distribution of sample data as a nonparametric test to compare the Young's modulus values for the membrane of the wing, mobile joint and immobile joint among the three species. The results indicated that there were significant difference among species for the membrane of the wing (KW=35.433, $p < 0.001$), mobile joint (KW=7.5367, $p = 0.024$) and immobile joint (KW=23.458, $p < 0.001$).

Table 4.14 shows Young's modulus values for the wing membranes, mobile and immobile joint sections for each of the three species of *Rhinocypha* and the Kruskal Wallis test pairwise comparison. The modulus values for the membrane of the wing,

mobile joint and immobile joint of *R. fenestrella* were 0.04 ± 0.04 GPa, 2.0 ± 1.8 GPa and 6.0 ± 5.6 GPa (mean \pm standard deviations), respectively. Whereas the elasticity of the membrane of the wing, mobile joint and immobile joint of *R. perforata* was 0.04 ± 0.03 GPa, 1.8 ± 1.9 GPa and 2.1 ± 2.8 GPa, as well 0.16 ± 0.17 GPa, and the values; 1.1 ± 1.6 GPa and 1.8 ± 1.8 GPa for *R. biforata*.

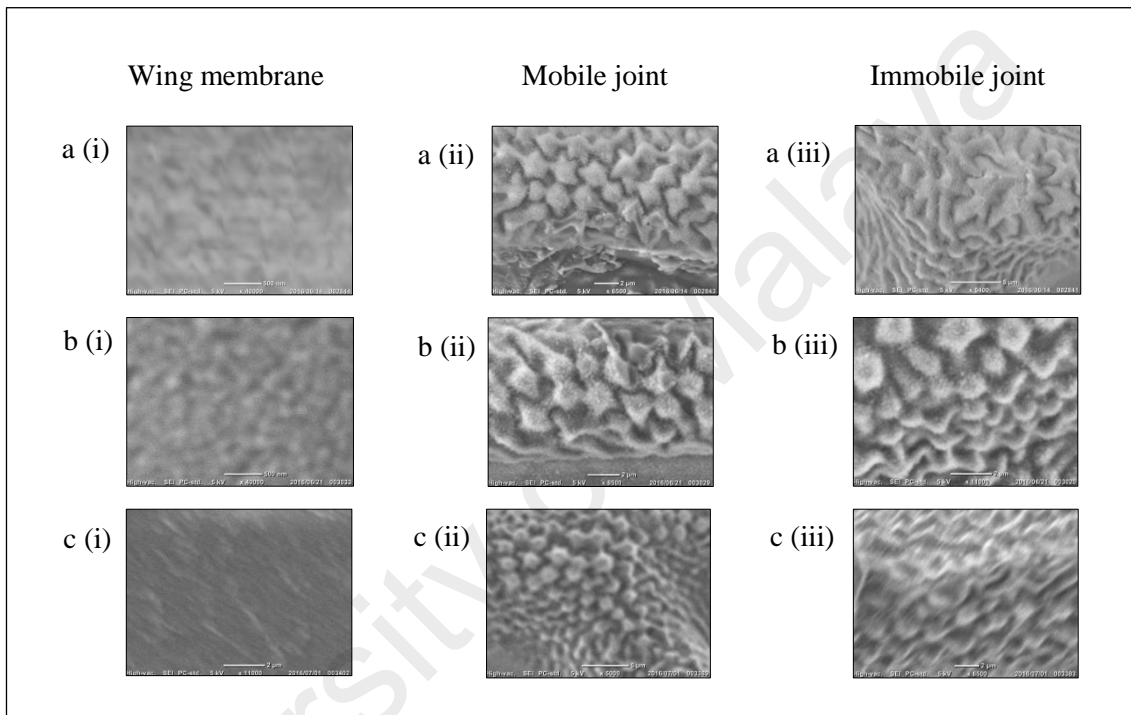


Figure 4.29: Surface of the wing images by SEM. (a) *Rhinocypha fenestrella*, (b) *Rhinocypha perforata*, and (c) *Rhinocypha biforata*.

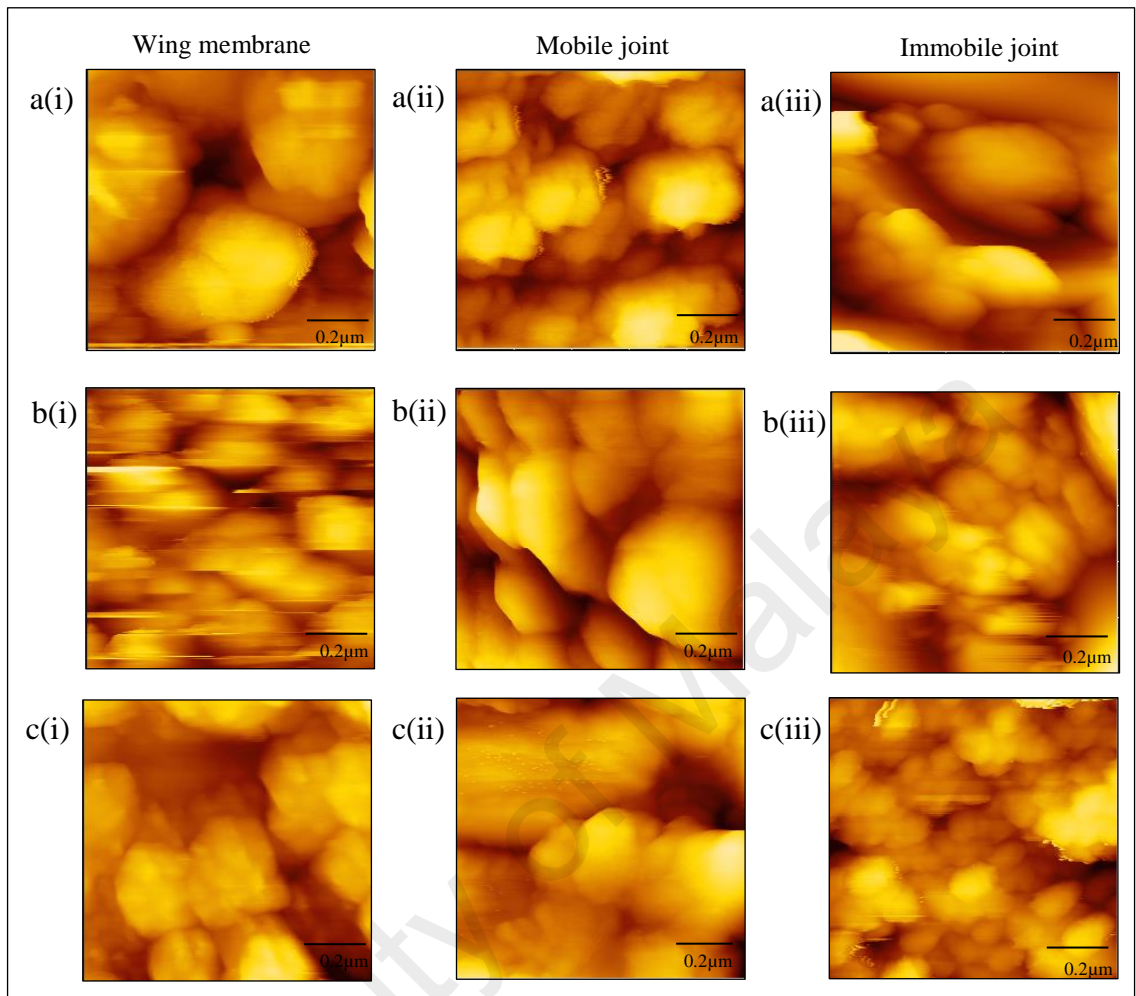


Figure 4.30: Morphologies of *Rhinocypha* spp. wings as observed under AFM. (a) *Rhinocypha fenestrella*, (b) *Rhinocypha perforata*, and (c) *Rhinocypha biforata*.

Furthermore, Figure 4.31 shows a typical force-displacement curve with glass samples as a standard reference of the three sections for each species of *Rhinocypha*. For all the three species, the immobile joint had greater hard substance compared to both mobile joint and membrane of the wing. For the force-indentation curve (Figure 4.32) for the three sections of the sample reinforced these results further, revealing descending order of stiffness; immobile joint > mobile joint > membrane of the wings for all the three species of *Rhinocypha*. Thus, it shows these results were consistent with Young's modulus value where within all the species, immobile joint always have bigger elasticity value compared to mobile joint and the membrane of the wings as shown in the table below (Table 4.14).

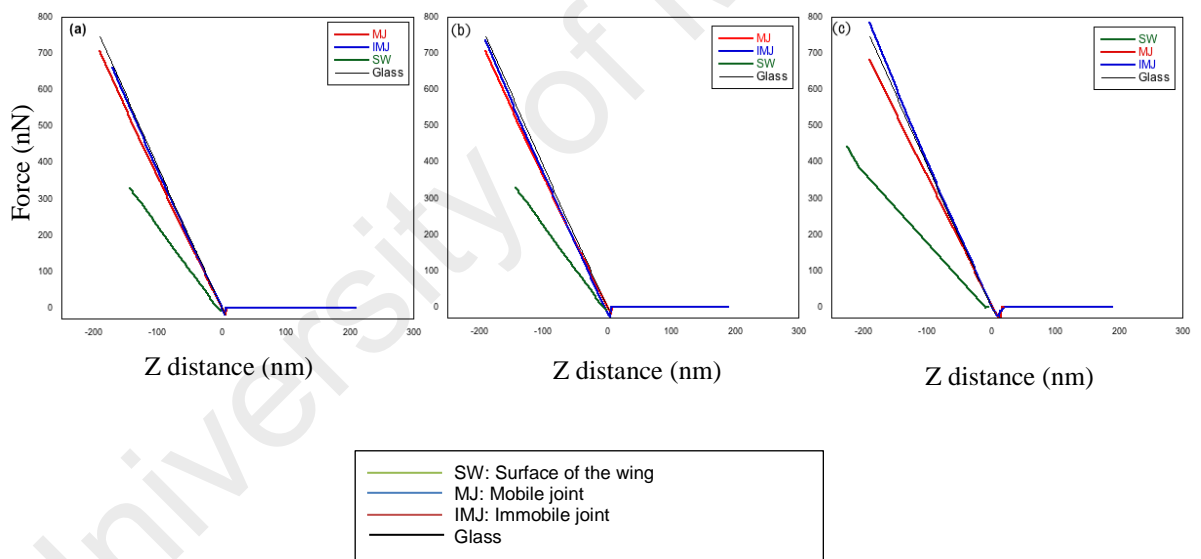


Figure 4.31: Force-displacement curve of the three sections of *Rhinocypha* spp. (a) *Rhinocypha fenestrella*, (b) *Rhinocypha perforata*, and (c) *Rhinocypha biforata*

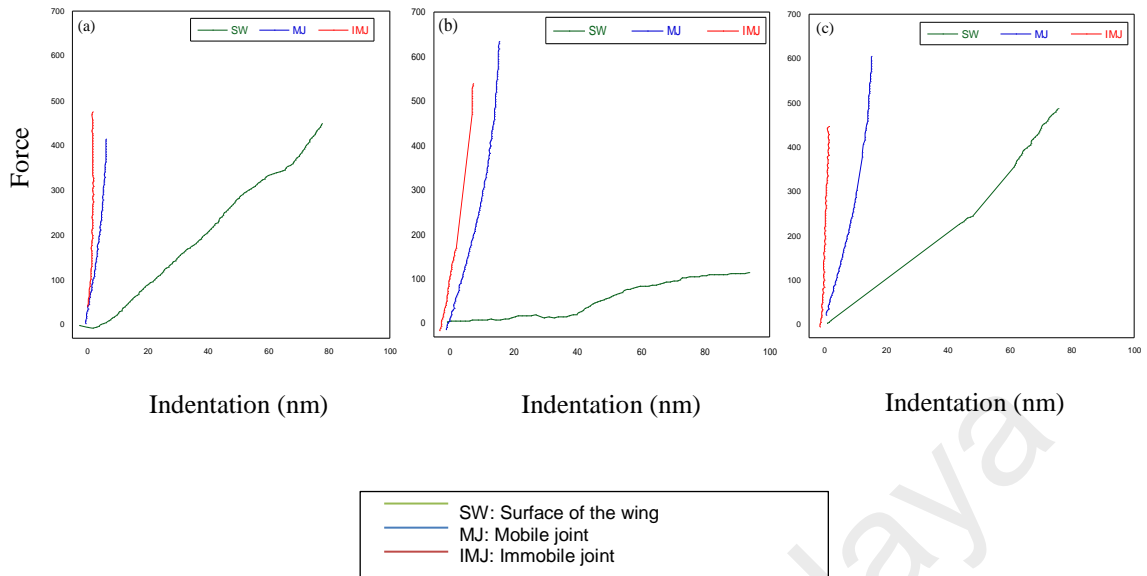


Figure 4.32: Force-indentation curve of the three sections of *Rhinocypha* spp. (a) *Rhinocypha fenestrella*, (b) *Rhinocypha perforata*, and (c) *Rhinocypha biforata*.

Table 4.14: Calculated values using the Young's Modulus formula for the 3 section wing samples from the 3 species, genus *Rhinocypha*.

Species	Membrane of Wing			Mobile Joint		Immobile Joint	
	n	Mean±SD	Median (IQR)	Mean±SD	Median (IQR)	Mean±SD	Median (IQR)
<i>R. fenestrella</i>	30	0.043±0.039 ^b	0.03 (0.03)	2.107±1.829 ^{ab}	2.0 (3.90)	5.960±5.609 ^a	4.0 (5.20)
<i>R. perforata</i>	30	0.036±0.038 ^b	0.02 (0.03)	1.800±1.896 ^a	1.0 (1.89)	2.073±2.783 ^b	1.0 (1.65)
<i>R. biforata</i>	30	0.162±0.172 ^a	0.10 (0.12)	1.054±1.609 ^b	0.3 (0.83)	1.780±1.791 ^b	1.0 (10.3)

Means with same letter are not statistically significant at 0.05

4.5.3 Amino Acid Composition of the Wing

In the context of amino acid, the amino acid sequences play the main role in defining the mechanical properties at a microscopic level. For this study, fifteen amino acids were found in our samples, with glycine, glutamic acid and alanine (except for *R. fenestrella*, alanine was replaced by serine) were found in higher concentrations (Figure 4.33). Glycine content ranged from 0.38 to 1.48 nmol; with 25.9% for *R. perforata*, 22.9% and 21.3% for *R. fenestrella* and *R. biforata* respectively.

For aspartic acid, they were found in varying concentrations from all the samples investigated; *R. fenestrella* (8.8%), *R. biforata* (9.0%) and least in *R. perforata* (1.8%), oppositely for threonine, they share the same value for all the samples. For the rest of the amino acids which were arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine and valine, they were low and accounted for less than 7% component in all species of *Rhinocypha*.

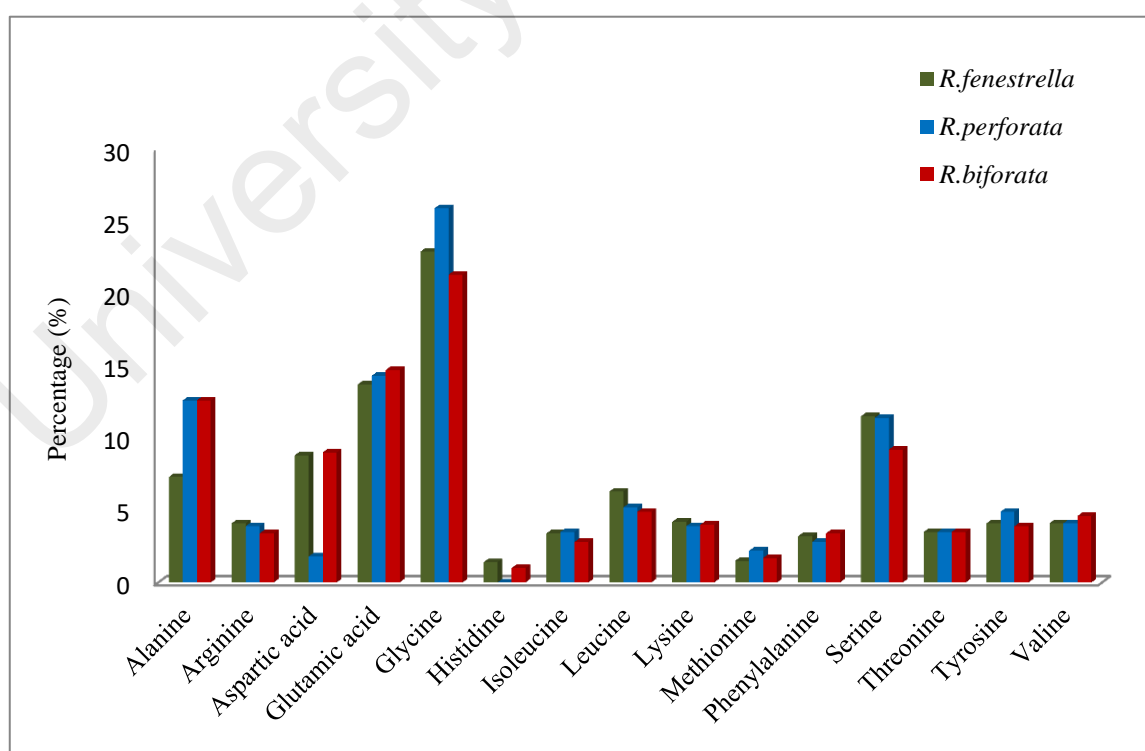


Figure 4.33: Amino acid composition in the *Rhinocypha* spp. wings (*Rhinocypha fenestrella*, *Rhinocypha biforata* and *Rhinocypha perforata*).

CHAPTER 5: DISCUSSION

5.1 ODNATES DIVERSITY AND DISTRIBUTION IN PENINSULAR MALAYSIA

Fairly rich communities of odonates were recorded from the 22 localities of forest reserve in Peninsular Malaysia. Overall, 1193 individuals were collected and a total of 70 species belonging to 10 family groups were identified. Among these, it showed that Chlorocyphidae was the dominant family found in the reserve forest in Peninsular Malaysia, followed by the family of Libellulidae. Chlorocyphidae was the species native to the Old World tropics, where they are known to occur along the forest streams, besides; they are most diverse in Southeast Asia (Hamalainen & Karube, 2001a).

This work found Libellulidae to be the other dominant group in support to previous work which reported the common occurrence of Libellulidae, for example, in a study conducted in the southern lowlands of New Guinea, Indonesia by Keize and Kalkman (2011), reported that the two largest families were Libellulidae and Coenagrionidae. This is not surprising as Libellulidae has frequently been reported to be the predominant family in odonate diversity studies (Lim & Furtado, 1975; Hamalainen, 1994; Gupta *et al.*, 1995; Norma-Rashid, 1995a, 1995b, 1998, 1999; Norma-Rashid *et al.*, 2001; Subramanian *et al.*, 2008; Noorhidayah, 2013). In contrast, family of Megapodagrionidae was the less dominant family found. This small megapodagrionid fauna is largely endemic, sharing little in common with the extensive argiolestine fauna to the east (Orr *et al.*, 2004).

Known patterns of environmental preferences concerning lentic and lotic conditions in the study areas were significant, at both the family and genus levels (Pires *et al.*, 2013). It was found in this work; Calopterygidae and Gomphidae predominated in lotic waters, Coenagrionidae, Libellulidae, and Megapodagrionidae were found in both lentic and lotic environments, as previously found in other countries (Merritt & Cummings, 1996; Muzón *et al.*, 2008; Ellenrieder, 2009).

However, Hawking and New (1999) reported that the different distribution of odonates among rivers could be affected by other elements such as the physical-chemical characteristics of the rivers or availability of food sources (Furse *et al.*, 1984; Askew 1998; Ameilia *et al.*, 2006), with higher species diversity when the abundances were more balanced. The highest species number across all the study sites was from the Sungai Chamang Waterfall, the state of Pahang, Recreational Forest Sungai Bantang (Johor) and Lata Kekabu (Perak). On the other hand, the most abundance individuals from all sampling sites were found from Sungai Chamang Waterfall, followed by Sungai Gabai Waterfall (Selangor) and Recreational Forest Sungai Bantang. Generally, the more species-rich families have larger distributions, and families with a small distribution mainly inhabit running water (Suhling *et al.*, 2015). Moreover, MacArthur (1965) suggested that high species abundance is related to ecosystem diversity; it could be due to the presence of rivers and forest streams that provide many different microhabitats for the species to thrive and divide themselves spatially.

Additionally, the frequency of occurrence can be used as a good indication of how common a species is, instead of using number of individuals (Chung & Maryati, 1996). From the study, the most frequently collected species (FO) were *Rhinocypha fenestrella*, *Rhinocypha biforata*, and *Euphaea ochracea*. Relatively, common species were *Rhinocypha perforata*, *Prodasineura humeralis* and *Prodasineura laidlawii*. Other

species were collected at a frequency lower than 4% and can be considered as rare. Shelton and Edward (1983) explained that common species have more individuals compared to the rare species due to their ability to survive in existing environmental conditions, while some other species may rarely visit a stream except for oviposition, or be very inconspicuous and well hidden. Rare species are important as part of organism influencing the function of the ecosystem (Lyons *et al.*, 2005), whereas 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.

Moreover, from the species listed in this study, there were some species that are interesting. In terms of sampling sites occurrence (SO), *Neurobasis chinensis*, belonged to the Calopterygidae family were recorded for 50% of sampling sites. This species, like other members of the Calopterygidae, occur in pristine habitats such as clear forest streams and swamp forest with good water quality (Orr, 2003). *Neurobasis chinensis* has the potential to act as bioindicators of rivers with diverse substrates and fast flowing undisturbed water (Wahidatul Afza, 2004).

However, contrasting with the presence of *Trithemis* spp., they may indicate habitat disturbances (Samways, 2008; Damm *et al.*, 2010b) and also can be found in all kinds of freshwater habitat (Damm *et al.*, 2010b; Dijkstra & Clausnitzer, 2015). In general, the *Trithemis* spp. has large and presumably good dispersal capability (Damm *et al.*, 2010b). The openness of the habitat as a result of vegetation architecture and flow rate of the water bodies play a more important role on the biotope of this genus (Damm *et al.*, 2010b). In brief, most *Trithemis* species, especially the red species are associated with disturbed environment (Adu *et al.*, 2016).

Interestingly, within all the species listed, there was one species listed as near threatened by the IUCN. *Drepanosticta fontinalis* is known only from Peninsular Malaysia, with published records from Pahang (Lieftinck, 1937, Kalkman, 2004), Bukit Mertajam in Penang (Fraser, 1942) as collected from this study, and Bukit Larut (formerly Maxwell Hill) in Perak (Tsuda & Kitagawa, 1987). Kalkman's record from Pahang is from Tioman Island, 50 km from the mainland and Choong (2007) has recorded this species at Gunung Jerai in Kedah, Panti Forest Reserve in Johor and, most recently, Angsi Forest Reserve in Negeri Sembilan.

According to Orr (2005), *D. fontinalis* is the “commonest member of genus” in Peninsular Malaysia, it certainly appears to be widespread, and it certainly appears to be common in some locations, for instance Panti Forest Reserve in Johor (Choong, 2007). It is likely to be under-recorded. The habitat loss through the clear-cutting of forests is a recognized threat that is likely to have a negative impact on this species (Dow, 2009).

Movement and dispersal play a fundamental role in the ecology and evolution of species. From the study, it showed that the dispersal patterns of 41 species out of 70 were aggregated. From the number, the species of *Prodasineura humeralis* had the highest dispersion value, followed by *Rhinocypha fenestrella* and *Prodasineura laidlawii*, while 29 other species were found to be randomly dispersed.

The dispersal ability of most insect groups is very limited; comprehensive understanding of dispersal ability is important for effective management of endangered species (Bohonak & Jenkins, 2003) and an effort to understand the ecology and conservation requirements of the species (Rouquette & Thompson, 2007). The dispersal ability of dragonflies reflects species habitat specificity (Harabis & Dolny, 2011), but is also influenced by local environmental conditions, as is true for other major groups of flying insects (Benard & McCauley, 2008; Dolny *et al.*, 2014). Most studies of dispersal

in insects have concentrated on Lepidoptera, with relatively few studies for Odonata. It is clear that carefully conducted studies of movement and dispersal are key components in guiding invertebrate conservation strategies, whereas understanding movement and dispersal is becoming increasingly important as landscapes become ever more fragmented and species numbers continue to decline (Baguette *et al.*, 2000; Petersen *et al.*, 2004).

The diversity indices revealed that the majority of sampling sites (14 localities) had diversity values ranged from 1.60 to 2.19. A total of 6 localities had highest diversity index, ranging from 2.00 to 2.19. In contrast, dominance index was highest in 5 localities where each sampling site represented by 0.31-0.35 and 0.46-0.50 species. Most standard indexes of diversity are based on two components, the number of species present, or species richness, and the relative abundance of the species (Peet, 1974, Cook, 2008). Both variables were taken into account by standard diversity indexes calculated from species abundance data (Pielou, 1975; Magurran, 1988; Southwood & Henderson, 2000). Instead, Simpson's index (Simpson, 1949) is a method measuring diversity similar to the better known Shannon index, and like it, is widely used in ecology (Magurran, 1988; Southwood & Henderson, 2000). According to Dash (2003), Javaid and Ashok (2013) mature and stable communities will have high Simpson dominance index value (0.6 to 0.9), while the communities under stress conditions will exhibit low diversity index which is usually close to zero values (Dash, 2003).

Conceivably the location of sampling sites played a significant factor in determining the diversity and richness of odonate communities in the forest reserve in Peninsular Malaysia. All the sampling sites were located in forest reserves, which in general were more suitable for tolerant species. Thus the richness of the community was restricted to those groups of species due to suitability and adaptability to the chosen

habitats. When sampling program is designed to cover all microhabitats available in the whole stretch of a river, the diversity of insect communities is likely to be richer (Hawking & New, 1999).

Generally, sampling efficiency, which was observed and estimated species vary greatly between the estimators. The lowest estimated richness was 61.5%, while the highest was 98.2% from Choa & Lee 1 and 2 estimators. However, according to the species accumulation curve, it showed that the accumulation curve had a curve nearing an asymptote and the curve was slightly increased towards the end. To improve this problem, sampling effort need to increase. Nevertheless, the spatial scale is also important for assessing the odonate diversity in the region which was restricted to forest reserves in Peninsular Malaysia. Differences in river classification orders too, can influence the composition of odonate larval communities (Hawking & New 1999, Pires *et al.*, 2013).

The total 230 species of Odonata in Peninsular Malaysia (including Singapore) was listed by Orr (2003), meanwhile, this study had listed 70 species from 22 localities in forested areas of Peninsular Malaysia and, it's approximately 31% of the odonates fauna documented in Orr (2005). Therefore, more sampling efforts are needed to be done in the future. Additional surveys of exuviae would also be highly desirable to determine whether the species are successfully completing their life cycle at these sites (Cordoba-Aguiler, 2008; Raebel *et al.*, 2010), but it is recognized that exuviae can be challenging to survey, and so can often under-estimate richness (Cordoba-Aguiler, 2008; Bried *et al.*, 2012, Luke *et al.*, 2017).

The diversity patterns shown in the study by the suborders Anisoptera and Zygoptera largely coincide with that of Odonata in general. Odonates occurred in all types of freshwater habitats, and hence a broad array of habitats needs to be sampled to get a proper overview of an area's fauna. It is necessary to spread the sampling not only across a variety of habitats, but also across the season and the day. In addition, the presence of odonates is generally perceived to indicate a healthy ecosystem (Carle, 1979; Moore, 1984; Schmidt, 1985; Castella, 1987; Clark & Samways, 1996; Corbet, 1999), and this group has already been identified as a 'flagship' species within the field of conservation biology (Sahlén & Katarina, 2001). Thus, this study should provide useful information to document the population and assemblage patterns of the dragonflies, besides can elucidate the distribution patterns of odonates throughout their habitat in Peninsular Malaysia especially in forested areas.

5.2 CORRELATION OF PHYSICAL AND CHEMICAL PARAMETERS WITH ODONATE DIVERSITY AND DISTRIBUTION

The odonate community within an area were affected by the habitat structure (Hawking & New, 1999), and other factors such as the physical-chemical characteristics of the rivers or availability of food sources have an impact on the distribution of odonates (Furse *et al.*, 1984; Askew, 1998), where the species diversity is higher if their abundances are more balanced. According to Corbet (1999) and McPeck (2008), many odonate taxa require specific habitat features, but most of the species recorded during adult counts within the study area were found in both lotic and lentic water-bodies.

The correlations of the environmental parameters, including the water quality parameters obtained from the principal component analysis (PCA) in this study showed that there are very strong positive correlations between TDS and conductivity as well as between conductivity and water temperature, salinity and TDS, and water temperature and TDS. Contrastingly, a very strong negative correlation was seen between the humidity and ambient temperature, while strong negative correlations found between ammonia and water width, conductivity and elevation, and nitrate and salinity.

APHA (1992) defined the total dissolved solids (TDS) as materials in the water that will pass through a filter with a pore size of 2 μm or smaller. Most of the dissolved matter in freshwaters consists of inorganic salts, small amounts of organic matter, and dissolved gasses (Sawyer *et al.*, 2003). Waters that have high concentrations of TDS may have objectionable tastes or cause adverse physiological effects when consumed by humans and livestock. The TDS value of the study sites were found to vary between 0.010 to 0.330 g/L a higher reading when compared to North Selangor Peat swamp Forest Reserve (NSPSF) as reported by Yule and Gomez (2009). The differences are

probably associated with environmental factors such as catchments geology and vegetation cover, climate and runoff quality. The influence of land use also varied from one area to another.

Conductivity is a measure of water's ability to conduct an electric current and is directly related to the total dissolved salts (ions) in the water. Some pollution discharges and polluted runoff into waters can cause changes in conductivity, especially if the pollutants include inorganic dissolved solids such as ions, for examples bicarbonate, sulphate, chloride, calcium, magnesium, sodium, potassium, and phosphate. Moreover, according to Carrino-Kyker and Swanson (2007), the conductivity showed positive correlation with agriculture following the application of fertilizer and pesticide, and this was parallel with the findings in this study where the TDS had a strong positive correlation with the conductivity. The increased of conductivity level could result from low precipitation, higher atmospheric temperatures resulting in higher evapotranspiration rates and higher total ionic concentration.

From the Principal Component Analysis (PCA), the first five PCs with the eigenvalues >1.0 were used for further analysis according to Cattell and Jaspers (1976) and Vega *et al.* (1998). They suggested using all of the PCs up to and including the first one after the brake. As for the results, PC-1 to PC-5 were highly influenced (negatively or positively) by most of the variables, thus hindering the interpretation regarding which parameters are more important than the others in influencing water quality variations within a given season. PC-1, were mostly influenced by the chemical components of the water, which could be seen that the level of water chemical at PC-1 increased when the level of sulphate, ammonia, iron, nitrite and DO were higher, while the level of water chemical would decrease with the increase of water width level.

Conductivity, total dissolved solids and salinity positively contributed to the components or sites PC-2, while PC-3 had higher ambient and water temperature, deeper level of streams and lower degree of humidity. Moreover, the flow rate and level of nitrate had positively contributed to the PC-4 and negatively contributed by the pH level, and lastly, PC-5 was significantly related to lower level of phosphate and had lower elevation.

Abiotic factors were known to be important in determining the species composition of Odonata assemblages (Corbet, 1999), and in this study dissolved oxygen (DO) was positively correlated with the species number in Peninsular Malaysia. DO levels in natural and waste waters depended on the physical (temperature and pressure), chemical (concentrations of various ions) and biochemical activities in the water body (Hutchinson, 1957; APHA, 1992). In general, the concentrations of the dissolved oxygen resulted from the biological activity and DO would be the key test for water pollution and waste treatment process control.

The concentrations of unpolluted fresh water would be close to 10 mg/l, and when the DO reading dropped to levels below 5 mg/L, mobile aquatic fauna prefer to move to areas with sufficient DO. According to Behar (1996), the level of dissolved oxygen between 0-2 mg/L would be insufficient to support life, 2-4 mg/L, only a few fish and aquatic insects could survive, 4-7 mg/L, good for many aquatic animals and low for cold water fish, while 7-11 mg/L, very good for most stream fish. From this study, the DO levels were between 2.95 to 10.70 mg/L which showed that the odonates were able to withstand low oxygen levels and more tolerant for organic matter enrichment.

Dissolved oxygen is an important parameter for all the aquatic organisms since exposure to low oxygen levels may result in the slowing of growth rates, reproductive difficulties, stress, susceptibility to disease, and, in severe cases of depletion, premature death. Other studies by Voshell and Simmons (1978), Corbet (1999) and Fulan *et al.* (2008) had shown that variations in oxygen availability in lacustrine (low oxygenation) and lotic (high oxygenation) environments determined the diversity of odonate species. In aquatic ecosystems, oxygen availability is one of the limiting factors for the survival of insect larvae, such as Odonata (Gaufin *et al.*, 1974; Corbet, 1999; Hoback & Stanley, 2001; Apodaca & Chapman, 2004).

Besides oxygen, the surface water temperature also influenced the distribution of Odonata larvae (Lutz, 1974; Ward, 1992; Corbet, 1999). According to Corbet (1999), four orders of aquatic insects (Odonata, Hemiptera, Diptera, and Coleoptera) were resistant to significant changes in water temperature. According to Schott & Brusven (1980), larvae of dragonflies were able to tolerate changes in water temperature through an enzymatic mechanism that promoted a fast physiological response. Results from this current work showed that the water temperatures were varied between the sampling sites from 24.40 to 39.10 °C. Several factors that affected water temperature were: weather, removal of bank vegetation, discharge of cooling water, and runoff water. Previous study by LeBlanc *et al.* (1997) discovered that shade of riparian vegetation, groundwater discharge, and stream width had the greatest influence on the stream temperature. The importance of abiotic factors like water dissolved oxygen and temperature in the presence of Odonata larvae near macrophytes was also shown for a lake lateral to Paranapanema River, Brazil (Fulan & Henry, 2006).

Other environmental factors, such as electrical conductivity, pH, and depth also had an effect on the abundance of Odonata larvae (Fulan *et al.*, 2011). Ammonia was found to positively contribute to the PC-1. NH₃ is called Unionized Ammonia since it has no charge and with higher concentrations could be toxic to aquatic organisms and estimated from the proportions of NH₄⁺ dependent on the dissociation dynamic, which were governed by pH and temperature (Trussell, 1972; Emerson *et al.*, 1975). Natural unpolluted waters had range of values: 0 to 3 mg/L and from all the sampling sites within the Peninsular Malaysia showed the ammonia values were between -0.020 to 0.450 mg/L, thus concluded to be non-toxic to aquatic organisms.

Besides ammonia, nitrite was also found to be one of the chemical components of the water that contributed to the habitat preference for odonates. According to Wetzel (1983), nitrification defines as the "biological conversion of organic and inorganic nitrogenous compounds from a reduced state to a more oxidised state". An increase of NO₂⁻ concentration in deeper waters indicated anaerobic decomposition of submerged plants to be higher in waters at the bottom as compared to the surface (Zakaria-Ismail & Sabariah, 1995).

Logistic regression analysis of 11 odonate species using 500 bootstrap replicates for the species richness estimate (B = 500) showed the components (PCs) affected the presence/absence of the odonates. The logistic regression analysis was conducted for 21 species of odonates which were found in more than 20% of the sampling sites.

PC-1 represented the chemical properties of the water was significantly correlated with the occurrence of seven species (*Dysphaea dimidiata*, *Eupahea ochracea*, *Orthetrum chrysis*, *Neurothemis fluctuans*, *Rhinocypha biforata*, *Rhinocypha fenestrella* and *Zygonyx iris*). The distributions of those species were related to the concentration levels of sulphate, ammonia, iron, nitrite and DO and also the width of the

stream. Previous study by Fulan *et al.* (2011) revealed that dragonflies were most affected by dissolved oxygen and temperature among other abiotic factors.

For example, the abundance of a species of *Telebasis* was positively influenced by the level dissolved oxygen (Fulan & Henry, 2006) due to in aquatic ecosystems, oxygen availability is one of the limiting factors for the survival of insect nymphs, such as Odonata (Gaufin *et al.*, 1974; Corbet, 1999; Hoback & Stanley, 2001; Apodaca & Chapman, 2004), and the amount of dissolved oxygen in water could affect the behavior, metabolism, and survival of Odonata nymphs (Corbet, 1999; Hofmann & Mason, 2005).

Moreover, this study found that the occurrence of species *Libellago lineata* and *Prodasineura humeralis* was correlated with PC-2, associated with the highest level of conductivity, TDS and salinity. Four species of odonate were correlated with PC-3 (*E. ochracea*, *R. biforata*, *O. chrysis* and *N. fluctuans*) that were related to ambient and water temperatures, humidity and depth of the stream, while the species of *P. humeralis* and *P. laidlawii* were correlated with PC-4 and related to the level of pH, flow rate and nitrate. Six other species (*E. ochracea*, *Orthetrum testaceum*, *P. humeralis*, *R. fenestrella*, *R. biforata* and *Z. iris*) were found to be correlated with PC-5 with increased abundance in lower levels of phosphate and at lower elevation.

Associated factors that influenced odonate (*Telebasis* and *Erythemis*) ecology were reported to be electrical conductivity and depth (Fulan *et al.*, 2011), pH on the abundances of *Acanthagrion* and *Tauriphila* (Corbet, 1999), whereas the abundance of molluscs was correlated with increased in dissolved oxygen, salinity, pH and decomposed organic matter (Bath *et al.*, 1999).

The identification of odonate-habitat associations provided an essential tool for characterizing the response of dragonflies to changes in the environment (Balzan, 2012). Besides, highlighting which rivers to conserve can be accomplished through using indices involving the odonates (Kietzka *et al.*, 2017). This study successfully identified several variables across different physiochemical characteristics that influenced the occurrence and abundance of Odonata in aquatic habitats. Habitat-odonate interactions identified in this study provided important implications for odonate monitoring, and thus the beneficial use of Odonata as bioindicators.

University of Malaya

5.3 MOLECULAR PHYLOGEOGRAPHY OF *Rhinocypha fenestrella* BASED ON ANALYSES OF MITOCHONDRIAL COI AND 16S rRNA GENES

Throughout the last 5 decades, the understanding on the ecology and evolution of odonates had increased dramatically (Cordoba-Aguiler, 2008) plus recent advances in molecular techniques had inspired several phylogeographical studies on Odonata. This particular study successfully defined the intraspecific genetic diversity, phylogeographical patterns and mitochondrial variations for *Rhinocypha fenestrella* using COI and 16S rRNA genes for the first time across eight populations within Peninsular Malaysia. Among the populations studied, haplotype A1 in COI and 16S rRNA genes and AB1 in COI + 16S rRNA datasets were the common ancestor of *R. fenestrella* that evolved over the time and diversified into numerous haplotypes enabled them to adapt to the climatic changes that had affected their habitat, and dispersed them across the region.

Moreover, the genetic diversity of this peacock jewel can be considered as high when compared to other species of the same suborder, *Nehalennia speciosa* (present in Germany, Poland, Lithuania, Russia, Japan) by Bernard *et al.* (2011), *Hawaiian megalagrion* (i.e. Hawaiian islands) by Jordan *et al.* (2005), where there were 26 haplotypes revealed by COI and 10 haplotypes by 16S rRNA, and 33 haplotypes revealed by the combination of COI + 16S rRNA genes. A greater genetic structure was found in the other suborder, the migratory dragonfly *Libellula quadrimaculata* (Artiss, 2004) and *Pantala flavensens* (Low *et al.*, 2017). Maintaining the genetic diversity would directly contribute to population viability (Saccheri *et al.*, 1998; Madsen *et al.*, 1999; Spielman *et al.*, 2004), also for the transformation potential of a species to react to the environmental change (Frankham *et al.*, 2002; Reed & Frankham, 2003; Schmitt & Hewitt, 2004).

Zygoterans were typically weak fliers not diffusing outside several kilometers (Conrad *et al.*, 1999; Geenen *et al.*, 2000; Purse *et al.*, 2003; Watts *et al.*, 2004), but it is somewhat surprising that genetic differentiation happened in this particular species, *R. fenestrella*, where their population separated by more than 500 km. Similar findings on genetic differentiation on damselflies were reported by Andres *et al.* (2000, 2002), where they found in their work among the damselflies population for the species *Ischnura graellsii* and *Ceriagrion tenellum* which were separated up to 100 km, and also separated not in short distance for the species of *Lestes viridis* (Geenen *et al.* 2000; Wong *et al.* 2003; but cf. Watts *et al.* 2004).

In the dataset from the results, the genetic distance based on COI gene ranged from 0.16 – 1.63%, while 16S rRNA gene ranged from 0 – 0.75%. It shows a slightly higher genetic distance between Malaysian *R. fenestrella* from all the populations contrasted with the same genus, *R. taiwana*, *R. uenoi* and *R. drusilla* by Wang *et al.* (2013), where the genetic distances based on COI are 0%, 0% and 0 – 1.5%, respectively. However, the intraspecific p-distance of this *R. fenestrella* is relatively low when compared to the other suborder of odonates, *Trithemis stictica* by Damm *et al.* (2010a) and *Nannophya pygmae* by Low *et al.* (2016b).

The sequences for COI and 16S rRNA genes from eight populations ranged between 1.5 – 4.6% of variability among all studied individuals reflected in 28 variable nucleotide sites in 614 sequenced base pairs from COI gene and 8 variable nucleotides in 534 base pairs from 16S rRNA gene. The base composition of the COI and 16S rRNA segment showed a significant A + T nucleotide bias which is consistent with other insect mitochondrial genes (Crozier & Crozier, 1993; Simon *et al.*, 1994; Frati *et al.*, 1997; Chippindale *et al.*, 1999; Artiss, 2004). Table 4.10 and 4.11 presented the variable sites of *Rhinocypha fenestrella* haplotypes based on COI sequence at 27 sites, and 16S rRNA sequence at 10 sites, while there was one insertion which occurred at

position 355 in the region of the 16S rRNA at the H9 haplotype from the Terengganu state. It can be concluded that polymorphism occurred in the *R. fenestrella* populations in Malaysia.

This current work provided strong evidence for intraspecific patterns of molecular variation among the geographically separated populations of *R. fenestrella* in Malaysia. Overall, many phylogeographical mechanisms had been proposed using damselflies as model organisms, but in contrast they lack detailed sampling. This finding would be useful in detecting phylogenetic relationships and phylogeography patterns to reveal common ancestor for these populations within species across the different continents.

University of Malaysia

5.4 TAXONOMIC STUDIES WITHIN THE FEMALES OF *Rhinocypha* BY UTILIZING 4 CONTRASTING TOOLS

Adults of odonates are conspicuous, easy to record and taxonomically well studied (Brown, 1991). Although there is a wealth of data available to be utilized for solving taxonomic problems, there remain existing confusions for correct identifications in closely related and sympatric species, especially in female odonates. However, the similarities in appearance of odonates, their behaviour and body size support the view that at least two species could not live in the same habitat (Khelifa *et al.*, 2013). The female of the three studied species belonged to the *Rhinocypha* genus had broadly the same appearance and slightly the same body size which made them difficult to distinguish between the species.

The detailed morphology studies from this work (Section 4.4.1) revealed that the *Rhinocypha fenestrella* had slightly longer and broader wing compared to *Rhinocypha perforata* then followed by *Rhinocypha biforata*. Similarly, the abdominal length in the order of longest to shortest; where *R. fenestrella* > *R. perforata* > *R. biforata*. Although all three species had enfumed wings, *Rhinocypha biforata* had brown marking at the tip of the wing while *Rhinocypha perforata* had more extensive yellow color at the thorax. It has been shown that coloration (Andrew, 1966), apart of flight pattern (Pajunen, 1966), affects visual recognition of adult Odonata.

The ovipositor structures are known to have an important role in determining species differences. According to Matushkina (2011), a well-developed ovipositor in Odonata are represented by three main elements: (1) the shaft of the ovipositor, including paired cutting 1st and 2nd valves; (2) paired large plates, the 3rd valves; the distal edges of the 3rd valves that bear moveable stick-like appendages, the styli

(gonostyli of 9th segment); and (3) several sclerites associated with the ovipositor valves (paired gonocoxites of 8th segment and gonanguli, unpaired internal sclerite).

The ovipositor of *Rhinocypha* spp. belonged to the endophytic type that occurred in all Zygoptera, the anisozygopteran, *Epiophlebia superstes*, and most aeshnids (Asahina, 1954; St. Quentin, 1962; Pfau, 1985; Matushkina & Gorb, 1997; Matushkina, 2004, 2008). This study, had examined the ovipositor in *Rhinocypha* spp. using the FESEM (in Section 4.4.2), focusing on three structural parts; the sensilla and setae of the anal appendages, the V3 and distal tooth, and the stylus.

The ovipositor of *R. biforata* was shown as compact basiconic sensilla and few long setae at the anal appendages, while *R. perforata* had short articulated setae and compact of short basiconic sensilla. This could be differentiated from the species *R. fenestrella* where there were more spaces between basiconic sensilla and lot of coeloconica-like sensilla in contrast to other species. Previous studies had identified that the phylogenetically informative characters might be found in microstructural features such as in the position and shape of sensilla, and serrations of valves, but this possibility would require a systematic examination of representatives of other ovipositor-bearing Odonata groups (Matushkina, 2007).

Another distinguishing feature was the shape on the carina of V3. *R. biforata* had sharp pointed carina compared to *R. perforata* with a more diagonal in projection while vertical projection in *R. fenestrella*. The teeth of V3 were fused to form a bearing edge, or carina, by which females posturally leaned against oviposition substrates during egg laying behaviour. A study on *Lestes macrostigma*, revealed that the row of teeth on the carina of V3 was functioning to hold the female abdomen on the plant surface during plant penetration (Matushkina & Lambret, 2011). The field of campaniform sensilla on the basis of the stylus on V3 responded to the stylus

inclination, when the ovipositor contacted a substrate. These two components, located symmetrically on the right and left styli, would serve as controllers of spatial characteristics of an egg clutch, such as was previously presumed for *Lestes sponsa* (Matushkina & Gorb, 2002). For *Rhinocypha* spp., the three species had a different shape and distribution for the sensilla and knobbls at the stylus. This might relate to the stylus inclination and could imply a chemosensory function.

Furthermore, the robust setae at the apex of the stylus and on the carina of V3 were in contact with the plant surface during egg laying and would probably function as mechanoreceptors, since they lack any pore on the surface area. Several knobbls, serrations and ridges, which were found on the external surface of the cutting valves, probably function in sawing of plant tissues (Matushkina & Lambret, 2011).

Geometric morphometric (in Section 4.4.3) analysis was able to differentiate the females of *Rhinocypha* spp. and also to confirm the population differences based on wing shapes. This analysis of wing shape is a useful tool and can be applied to ecological and evolutionary research in odonates (Córdoba-Aguilar 2008). According to Zelditch *et al.* (2004), an advantage in using wing shape as a discriminating character, was that wing with two-dimensional structures, made alignment of specimens for digitizing landmarks easier and more accurate compared to three-dimensional structural characters, creating possible measuring errors caused by different alignments of individual specimens.

In this study, 15 homologous landmarks were used to quantify wing shape variation. The results of the analyses indicated that morphological variation affected different parts of the wing differently, where it was found that landmarks 6, 7 and 15 of the forewings of *Rhinocypha* spp. were more variable compared to the other landmarks. Additionally, the landmarks of the *R. biforata* demonstrated more shape variation than

the other species, suggested by the percentage of variance of the principal component analysis.

The decomposition of variance components according to landmarks showed that the landmarks differed in the amount of variation for each species of *Rhinocypha*. The factor that especially stood out in this respect was directional asymmetry. Previous study suggested that this was not simply a random outcome linked to the subtlety for this effect, and this directional asymmetry were also discovered in two different species of flies (Klingenberg *et al.*, 1998).

Wing shape analysis was successfully demonstrated for population differentiation in the European *Calopteryx splendens* (Sadeghi *et al.*, 2009), variation in flight morphology in *Enallagma cyathigerum* (Bots *et al.*, 2009), wing shape evolution (Johansson *et al.*, 2009), and the effects of latitude and selection on wing shape in *Calopteryx virgo meridionalis* (Outomuro & Johansson, 2011). This landmark-based wing shape analysis were shown to be useful in discriminating damselflies in the *Euphaea* species group, such as among the *E. guerini* species complex and geographical populations of *E. masoni* on the mainland of Southeast Asia (van Tol & Rozendaal, 1995; Hämäläinen & Karube, 2001b; Toan *et al.*, 2011), and between *E. subcostalis* and *E. subnodalis* in Borneo (Orr & Hamalainen, 2003).

This study concluded that the *R. biforata* had narrower wings compared to *R. fenestrella* which had broader wing, while *R. perforata* had a broader elongated apex. As a result of this wing shape variation for this damselfly group, the three species were separated in the Canonical Variate Analysis (CVA). Each species constructed an independent cluster, making a considerable clear separation. Studies have suggested that various selective pressures, including landscape structure (Taylor & Merriam, 1995), food and predation stress (Stoks, 2001; Svensson & Friberg, 2007), latitude and sexual

selection (Outomuro & Johansson, 2011) can affect the evolution of wing shapes in damselflies. Consequently, the strength in using geometric morphometric for wing analysis was displayed in their ability to pinpoint the location and direction of specific features for presence of variation. A proper comprehensive analysis of wing shape would thus provide an insight in phenotypic variation related to flight performance, a character that should be under selection.

Finally the molecular analysis (Section 4.4.4) revealed distinct interspecific contrasts within the genus. The three *Rhinocypha* taxa formed three different clades group separated from each species based on two DNA regions; COI and 16S rRNA. The species of *R. perforata* formed a separate monophyletic clade with very high bootstrap support for both regions, whereas *R. biforata* and *R. fenestrella* together formed a monophyletic group clearly separated from the investigated *R. perforata* species. In addition, *R. biforata* species was revealed to be a sister taxon to the species of *R. fenestrella*. To date, the phylogenetic relationships of the genera and species of Chlorocyphidae are poorly understood (van Tol, 1998). However, in 2014, a group of researchers had suggested that families within Zygoptera were monophyletic such as Calopterygidae, Euphaeidae, Isostictidae, Lestidae, Lestoideidae, Platystictidae and Polythoridae, including the family of Chlorocyphidae (Dijkstra *et al.*, 2014). This finding further confirmed previous work (Rehn, 2003; Bybee *et al.*, 2008; Dumont *et al.*, 2010) that the family of Chlorocyphidae was monophyly and showed reasonable congruence with classification by Bechly (1996).

5.5 MORPHOLOGY AND CHARACTERISTICS PROPERTIES OF THE *Rhinocypha* WINGS

The initial scanning by scanning electron microscopy (SEM) for the wings of *Rhinocypha* spp. (*R. biforata*, *R. fenestrella* and *R. perforata*) had confirmed presence of two main types of joints; mobile and immobile joints as suggested by previous study (Gorb, 1999). According to Newman (1982), there were more than six different types of cross veins. The presence of the blue-fluorescing material in the vein-joints, which was revealed by the laser scanning confocal microscopy (LSCM), was known as resilin. Here, in the three species of *Rhinocypha*, distinct patches of resilin were found within the nodus dorsally and ventrally that were consistent with the results reported previously by Donoughe *et al.* (2011). These unique structures are renowned to have roles in the wing torsion (Wootton *et al.*, 1998). Moreover, resilin was found mostly in the mobile joints where cross veins met longitudinal veins that provided elasticity and not flexibility for the twisting movements of the wings (Gorb, 1999).

Mapping of resilin by LSCM has been summarized in Figure 5A, according to nodus and major longitudinal veins by Riek and Kukalova-Peck (1984) (Figure 4.27). Essentially the structures of odonate wings differed in numerous ways; however, there were the same sets of longitudinal veins (major longitudinal veins) which mainly conserved among them. This made meaningful morphological comparisons between related groups. The presence of resilin at the dorsal surface with the trailing edge vein MP, was consistent with the previous study (Donoughe *et al.*, 2011) when compared to dragonflies (Suborder: Anisoptera), however, among the damselflies (Suborder: Zygoptera), the longitudinal veins were categorized further by having the resilin on both dorsal and ventral surfaces. Variations in resilin patch distribution on either or both surfaces could well influenced flight pattern differences in these suborders for the order.

Additionally, the distributions of spikes on the wings of *Rhinocypha* spp. were also examined in this work. Spikes were suggested to play a role in limiting the free movement of the cross veins (Newman, 1982; Gorb, 1999). In addition, the spikes had a significant role in preventing material damage by inhibiting joint combinations to be too much deflected during flight (Donoughe *et al.*, 2011; Appel & Gorb, 2014), and establishing a physical contact with the adjacent vein (Rajabi *et al.*, 2016). Previous studies by Gorb (1999) and Newman (1982) initially described spike distribution concentrated at the mobile joints affecting the chord wise flexibility of the wings. As well as in this study, it was shown that these spikes were widespread throughout the wing surfaces and were generally located on both cross and longitudinal veins (Donoughe *et al.*, 2011; Appel & Gorb, 2014).

The capacities of insect flights were closely related to the physical properties of the wings (Kreuz *et al.*, 2001; Song *et al.*, 2007; Chang *et al.*, 2009; Sun *et al.*, 2009). Different sections of the wings tolerated different loads, and the mechanical properties might be dissimilar because of this adaptation (Sun *et al.*, 2007). Currently, the development of nanoindentation techniques (VanLandingham, 2003; Shuman *et al.*, 2007; Costa & Machado, 2009; Cabibboa *et al.*, 2012) made it conceivable to measure the modulus and hardness of insect parts.

However, studies on the mechanical properties, nanostructure and the basic material of the membranous wings, are still lacking (Combes & Daniel, 2003; Xiao *et al.*, 2007). To fill in this gap, this specific study investigated three sections of the wings of *Rhinocypha* spp.; the membrane of the wing, mobile and immobile joints using the SEM together with the atomic force microscopy (AFM) to obtain topography images and to determine their elasticity. Presently, AFM is the only technique able to produce high resolution and in real time images of live microbial cell surfaces, thus it can

provide information that is complementary to the information acquired from SEM (Alsteens *et al.*, 2008).

The morphologies on the wing surfaces of damselflies as revealed in this study from SEM were patterns of a series of small waves with elongated ridges was parallel to “ripple wave morphologies” reported by previous studies that may contribute to the asymmetric resistance under mechanical loading during the flight (Xiao *et al.*, 2010; Selvakumar *et al.*, 2012). Additionally, the globular nanostructures observed on the surface of the wings were similarly observed in the other species of dragonfly, *Sympetrum vulgatum* as shown by Selvakumar *et al.* (2012), thus it can be concluded that there is an absence of differentiation in wing surface morphologies between dragonflies and damselflies expanding from previous work (Selvakumar *et al.*, 2012). However, an advantage of this study was covered the whole surface of the membranous wings of the *Rhinocypha* wings, where the veins that were divided into mobile and immobile joints. Moreover, this study further examined the amino acid sequences responsible for the mechanical properties of the wings (as discussed below).

AFM analysis of the wing with a scanned area of (1 μ m x 1 μ m) clearly showed that the surface of the *Rhinocypha* wings was filled with the nanosized particles that stacked one above another. According to Selvakumar *et al.* (2012), this arrangement composed of chitin-protein and resilin, updating the results reported by Marrocco *et al.* (2010) who described that most part of the wing comprised of chitin and the joints were less stiff resilin protein. Apart from imaging capability, AFM can as well measure the elastic properties of the samples via nanoindentation measurements (Weisenhorn *et al.*, 1993; Matzke *et al.*, 2001; Touhami *et al.*, 2003; Francius *et al.*, 2008).

The mechanical characteristics of all three *Rhinocypha* spp. wings expectedly revealed the immobile joints contained harder substances in contrast to mobile joints and the membranes of the wings. This seemed to show that the vein is the main load-bearing part of the wings (Sun & Bhushan, 2012). Additionally, they have more flexible wing membranes, yet overall stiffer veins, when compared to the wing membranes and veins of the Anisoptera for the species; *Libellula depressa* (with modulus values: 1.5 ± 0.5 GPa, 2.9 ± 0.8 GPa) (Kempf, 2000) and *Aeshna cyanea* (1.5 ± 0.5 GPa, 2.8 ± 0.3 GPa) (Kreuz *et al.*, 2001). This is most likely influenced by the differences in their flight performances and wing corrugations between the dragonflies and damselflies.

Nevertheless, the structure of the wing that comprised of the nano globules may effect and caused the variation in measuring the stiffness of the wing. According to Kim *et al.* (2006) and Wang *et al.* (2013), in nanoindentation tests, both the reduced modulus and hardness depended on the indented area that may be affected by the surface roughness of the samples. Further studies by Jongerius and Lentink (2010) suggested that the corrugations could enhance the stiffness and strength of the wings and resulting in a lightweight structure enhancing aerodynamic.

The insect wing primarily composed of the cuticle arranged in tubular, supporting veins and thin connecting membranes, with a multi-layered material of chitin microfibrils fixed in a protein matrix (Dirks & Taylor, 2012). Resilin being one of the best-known cuticle protein, is a glycine- and proline- riched protein that provided the high elasticity to the cuticle on the hinge regions (Andersen & Weis-Fogh, 1964; Rauscher *et al.*, 2006; Tamburro *et al.*, 2010) and the highly dense compositions of these proteins possibly indicated the structural importance of the resilin (Van Eldijk *et al.*, 2012).

For the production of protein, amino acids were required for structural purposes such as enzymes used for transport and storage, and also as receptor molecules (Chapman, 2013). Several amino acids such as; aspartic acid, glutamic acid, serine, glycine, threonine, alanine, tyrosine, valine, phenylalanine, leucine, isoleucine, proline, hydroxyproline, lysine and tryptophan are reported in the cuticle proteins and the composition of the amino acid of resilin known to be different from other structural proteins but has not been observed under the electron microscope (Rockstein, 1978). This was due to their hydrophilicity and shared compositional characteristics with other proteins (Van Eldijk *et al.*, 2012).

The wings of *Rhinocypha* spp. here shown to contain high amounts of glycine (results obtained from amino acid analysis) that provided elasticity to the cuticle at the hinge regions (Van Eldijk *et al.*, 2012; Xiao *et al.*, 2007). Resilin is also known to be a proline-rich protein that was not detected in this study. The existence of resilin had been confirmed by the specific fluorescence derived from resilin using a laser scanning confocal microscope.

According to Rauscher *et al.* (2006), not only proline but also glycine, which was detected as the major component of resilin by the amino acid analysis in the study, can disrupt the regular protein structures. Due to the rigid conformation, proline can disrupt the secondary structure, whereas glycine, due to high flexibility, can hamper the formation of secondary structures (Rauscher *et al.*, 2006; Tamburro *et al.*, 2010). Hence, for damselflies, the function of glycine is more prominent in wing formation for flexibility.

Arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine and valine were found to be low and accounted for less than 7% of the components of all the samples. An earlier study by Bailey and Weis-Fogh (1961) reported a lack of hydrophobic amino acids such as valine, leucine and isoleucine in resilin. To date, there is no research on the properties of resilin composition in dragonfly wings, although its functional importance in flight performance is known and has opened up many areas of research aerodynamics, kinematics and morphological studies (Newman, 1982; Jongerius & Lentink, 2010; Wootton, 1991; Wootton & Newman, 2008; Kim *et al.*, 2009).

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CHAPTER 6: CONCLUSIONS

1. Seventy species were discovered from ten families from 22 localities representing the diversity of the odonates in forest reserves for Peninsular Malaysia.
2. The most dominant family was Chlorocyphidae (40.3%), Libellulidae (23.3%) and Platycnemididae (12.7%), while Megapodagrionidae was the least (0.2%).
3. The most frequently collected species have been *Rhinocypha fenestrella*, *Rhinocypha biforata*, and *Euphaea ochracea*. In terms of sampling sites occurrence, *Vestalis gracilis* and *Rhinocypha biforata* were cosmopolitan in distribution, followed by *Zygonyx iris*, *Euphaea ochracea*, *Rhinocypha fenestrella* and *Neurobasis chinensis*.
4. Forty-one species were found aggregated with the highest dispersion statistic (20.5) and 430.50 chi value of the dispersion statistic for the species of *Prodasineura humeralis*. Instead, 29 species were found randomly dispersed with the dispersion statistic >1.00 .
5. Pearson and Spearman rank order correlations for the environmental parameters showed that there were very strong positive correlations between TDS and conductivity, conductivity and water temperature, salinity and TDS, and water temperature and TDS.
6. A very strong negative correlation was observed between the humidity and ambient temperature, ammonia and water width, conductivity and elevation, and nitrate and salinity.

7. Species richness of odonate was mostly influenced by the chemical components of the water, a positive increment with levels of sulphate, ammonia, iron, nitrite and DO, while a negative relationship with the increase of water width level.
8. A disappearance or decreased in numbers for *Dysphaea dimidiata*, *Eupahea ochracea*, *Rhinocypha biforata*, *Rhinocypha fenestrella* and *Zygonyx iris* when the water chemical level increased, and contrastingly, *Orthetrum chrysis* and *Neurothemis fluctuans* able to adapt with higher levels of chemical properties and shallow water.
9. *Libellago lineata* and *Prodasineura humeralis* was associated with the highest level of conductivity, TDS and salinity.
10. The occurrence of *E. ochracea* and *R. biforata* will decrease or disappeared with the increased of ambient and water temperature and water depth, and decreased level of humidity, and oppositely goes to the species *O. chrysis* and *N. fluctuans*.
11. Presence of *P. humeralis* was positively associated with the pH, flow rate and nitrate.
12. *R. biforata* abundance increased with lower levels of phosphate and at lower elevation.
13. Genetic diversity of Malaysian *Rhinocypha fenestrella* was considered high with 26 haplotypes revealed from the COI gene and 10 haplotypes from the 16S rRNA gene. For concatenated sequences, 33 haplotypes were revealed from a both COI and 16S rRNA genes.

14. It was proposed that haplotype AB1 was the common ancestor and the most widespread haplotype of *Rhinocypha fenestrella* based on its prevalence in Peninsular Malaysia, postulated to originate from the state of Negeri Sembilan as revealed from the COI, 16S rRNA and COI + 16S rRNA genes haplotype network analysis.
15. The nucleotide composition of the COI and 16S rRNA segment showed a significant A + T nucleotide bias (A = 30.6%; 29.1%, C = 17.7%; 11.5%, G = 19.4%; 18.6%, T = 32.3%; 40.8%), respectively.
16. There was a strong evidence for intraspecific patterns of molecular variation among populations of geographically *R. fenestrella* in Malaysia, however, additional sequence data from another continent (Thailand, Burma, Laos, Vietnam and southern China), maybe useful in detecting phylogenetic relationships, phylogeography patterns, and reveal common ancestor for these populations within species between the continents.
17. Pterostigma was a distinguishing feature for the *Rhinocypha* group. In terms of wing size, the morphometric data showed that *Rhinocypha fenestrella* had slightly longer and broader wings compared to *Rhinocypha perforata* then followed by *Rhinocypha biforata*. The same was true for the abdominal length where $R. fenestrella > R. perforata > R. biforata$.
18. Ovipositor has an important role and provided informative character in determining species differences where from the FESEM, it's divided into three components, the sensilla and setae of the anal appendages, the V3 and distal tooth, and the stylus.

19. Geometric morphometric was found useful to quantify wing shape variation where it found that landmarks 6, 7 and 15 out of 15 landmarks of the forewings of *Rhinocypha* spp. were more variable than the other landmarks.
20. As a result of this unique shape variation of this damselfly group, the three species were separated from each species in the Canonical Variate Analysis (CVA) with each species constructed an independent cluster, making a considerable clear separation.
21. The genetic data also supported the generic status of *Rhinocypha*. The three *Rhinocypha* taxa formed three different clade groups separated from each other based on COI and 16S rRNA genes. The species of *R. perforata* formed a separate monophyletic clade whereas *R. bifurcata* species was a sister taxon to the species of *R. fenestrella*.
22. The initial scanning by SEM on the wings of *Rhinocypha* spp. had confirmed presence of two main types of joints; mobile and immobile joints.
23. Resilin was found mostly in the mobile joints where cross veins met longitudinal veins which provided elasticity and not flexibility for the twisting movements of the wings.
24. The distributions of spikes on the wings of *Rhinocypha* spp. were widespread throughout the wing surfaces and were generally located on both cross and longitudinal veins.

25. The morphologies on the wing surfaces of damselflies as revealed from SEM were patterns of a series of small waves with elongated ridges was parallel to “ripple wave morphologies” and AFM analysis of the wing clearly showed that the surface of the *Rhinocypha* wings were filled with the nanosized particles that stacked one above another.
26. The mechanical characteristics of all three *Rhinocypha* spp. wings expectedly revealed the immobile joints contained harder substances in contrast to mobile joints and the membranes of the wings.
27. The wings of *Rhinocypha* spp. proven to contain high quantities of glycine. Several amino acids such as arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine and valine were found to be low components.
28. Approaches that combine structural and mechanical studies on resilin would offer more convincing evidences for the relationship of proline-glycine structural function.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Research articles

1. **Mamat-Noorhidayah**, Yazawa. K., Numata, K., & Norma-Rashid, Y. (2018). Morphological and mechanical properties of flexible resilin joints on damselfly wings (*Rhinocypha* spp.). *PLoS ONE*, 13(3), e0193147.
2. Low, V. L., Norma-Rashid, Y., Yusoff, A., Vinnie-Siow, W. Y., Prakash, B. K., Tan, T. K., **Noorhidayah, M.**, Chen, C. D., & Sofian-Azirun, M. (2017). Pleistocene demographic expansion and high gene flow in the Globe Skimmer dragonfly *Pantala flavescens* Fabricius (Odonata: Libellulidae) in Peninsular Malaysia. *Zoologischer Anzeiger*, 266, 23-27.

Papers Presented

1. **Noorhidayah, M.** & Norma-Rashid, Y. (2015). Influence of environmental parameters on the distribution and diversity of the genus *Rhinocypha* (Order: Odonata) in Peninsular Malaysia. *20th Biological Sciences Graduate Congress (BSGC)*, Chulalongkorn University, Thailand.
2. **Noorhidayah, M.** & Norma-Rashid, Y. (2016). Habitat preferences and the influence of water parameters on adult dragonflies in Selangor, Peninsular Malaysia. *International Conference of the 50th Anniversary of Ulu Gombak Field Studies Centre*, University of Malaya, Malaysia.