

**HOST PLANT PREFERENCES, ECOLOGICAL
FACTOR AND MOLECULAR PHYLOGENY OF
Tetraponera rufonigra JERDON (HYMENOPTERA:
FORMICIDAE) IN EASTERN PART OF PENANG
ISLAND, MALAYSIA**

FAEZAH SYUKRIAH BINTI SABTU

**UNIVERSITI SAINS MALAYSIA
2018**

**HOST PLANT PREFERENCES, ECOLOGICAL
FACTOR AND MOLECULAR PHYLOGENY OF
Tetraponera rufonigra JERDON (HYMENOPTERA:
FORMICIDAE) IN EASTERN PART OF PENANG
ISLAND, MALAYSIA**

by

FAEZAH SYUKRIAH BINTI SABTU

**Thesis submitted in fulfilment of the requirement
for the degree of
Master of Science**

February 2018

ACKNOWLEDGEMENT

In the name of Allah, Most Gracious, Most Merciful. First and foremost, I am grateful to the Almighty God for establishing me to complete this worthy thesis. This work would not have been completed without the generosity, support and encouragement of many people. My deepest gratitude to the main supervisor, Associate Professor Dr. Abdul Hafiz Ab Majid for his continuous encouragement and guidance during my study. I am also grateful to my co-supervisor, Dr. Hamdan Ahmad for his guidance and invaluable advice throughout my research journey.

A million thanks to my beloved husband which is also one of my labmates, Mohd Fawwaz for keeping me accompanied during my sampling days across Penang Island just to obtain my samples collection. Thanks for your endless love, understanding and encouragements. My deepest appreciation also goes to my other labmates, Aina Atira, Noor Hazwani, Zulaikha Zahran, Nurul Akmar, Siti Nor Ain and Fadhlina Hazwani who never fail to offer their ideas and assistance in solving my problems through the completion of this study. Thanks for their care and joyousness they bring into my days. I also warmly thank Wan Ahmad Syahir, Abd Hafis, Muhammad Idrus and Muhd Farhan for their friendships throughout the years I spent in USM.

I owe my thanks to my loving mother and sister, Dr. Zuraidah Mokhtar and Dr. Syairah Sabtu for their countless support and inspirations. A token of appreciation also goes to my beloved family in-laws, Mr. Mohd Rahsid, Mrs. Noorhaizad and my bubbly brother- and sister-in law. Praise to God, along with their consecutive prayers and endless support, I managed to finish my Master studies with ease.

I also would like to convey my sincere gratitude to Ministry of Higher Education (MOHE) and Universiti Sains Malaysia for financial support under My Brain15 (MyMaster) Sponsorship Programme and Skim Pembantu Siswazah, respectively. To all staff School of Biological Sciences, thank you for your assistance in every part of my research. Last but not least, big thanks to everyone who has contributed, either directly or indirectly towards the success of this study.

TABLE OF CONTENTS

ACKNOWLEDGEMENT	ii
TABLE OF CONTENTS	iv
LIST OF TABLES	viii
LIST OF FIGURES	x
LIST OF PLATES	xii
LIST OF SYMBOLS AND ABBREVIATIONS	xiii
ABSTRAK	xiv
ABSTRACT	xvi
CHAPTER 1 - INTRODUCTION	1
1.1 Background of Study	1
1.2 Problem Statement	2
1.3 Significance of Study	3
1.4 Limitations of Study	4
1.5 Objectives	4
CHAPTER 2 - LITERATURE REVIEW	6
2.1 Ants: Their History, Life and Purpose	6
2.2 Ants and Plants Diversity	7
2.3 Relationships between Ants with Their Host Plant	7
2.4 Ant Ecology and Foraging Behaviour	9

2.4.1	Ants as urban pest	10
2.5	Distribution of the Genus <i>Tetraoponera</i>	11
2.6	Biology of <i>Tetraoponera rufonigra</i>	12
2.6.1	Taxonomic Classification of <i>Tetraoponera rufonigra</i>	12
2.6.2	Life Cycle and Morphological Characters of <i>Tetraoponera rufonigra</i>	13
2.6.3	Habitat and Distribution of <i>Tetraoponera rufonigra</i>	15
2.7	Application of Geographic Information System (GIS) Application for Studying Insects	16
2.8	Importance of <i>Tetraoponera rufonigra</i>	17
2.8.1	<i>Tetraoponera rufonigra</i> as Medically-Important Pest	17
2.9	Molecular Genetic Marker	18
2.9.1	Mitochondrial Cytochrome Oxidase I (COI) and its Applications as a Genetic Marker	19
2.9.2	Application of Mitochondrial Cytochrome Oxidase I (COI) for Studying Insect Population Genetics	20
CHAPTER 3 - HOST PLANT PREFERENCES OF <i>Tetraoponera rufonigra</i> AND ECOLOGICAL FACTORS AFFECTING THE DISTRIBUTION AND SHELTER PREFERENCES ACROSS URBAN AREA IN EASTERN PART OF PENANG ISLAND		22
3.1	Introduction	22
3.2	Materials and Methods	24
3.2.1	Data Collection on <i>Tetraoponera rufonigra</i> and Field Sampling Area	24
3.2.2	Environmental Data Collection on <i>Tetraoponera rufonigra</i>	27
3.3	Results	31
3.3.1	Plant Host Preferences of <i>Tetraoponera rufonigra</i>	31

3.3.2 Correlation and Regression between the Environmental Factors and <i>Tetraponera rufonigra</i> Density	42
3.4 Discussion	46
3.4.1 Plant Host Preferences of <i>Tetraponera rufonigra</i>	46
3.4.2 Correlation and Regression between the Environmental Factors and <i>Tetraponera rufonigra</i> Density	47
3.5 Conclusion	49
CHAPTER 4 - PHYLOGENETIC RELATIONSHIPS OF <i>Tetraponera</i> <i>rufonigra</i> (HYMENOPTERA: FORMICIDAE) INFERRED FROM mtDNA COI REGION	50
4.0 Introduction	50
4.1 Materials and Methods	52
4.1.1 <i>Tetraponera rufonigra</i> collections	52
4.1.2 DNA Extraction	52
4.1.3 Polymerase Chain Reaction (PCR) Amplification and DNA Sequencing	56
4.1.4 Phylogenetic Analysis	57
4.2. Results	58
4.2.1 Phylogenetic Analysis	58
4.3 Discussion	63
4.4 Conclusion	65

CHAPTER 5 - GENETIC VARIATION AND POPULATION STRUCTURE OF <i>Tetraoponera rufonigra</i> FROM SELECTED URBAN LOCATIONS IN EASTERN PENANG ISLAND, MALAYSIA	66
5.1 Introduction	66
5.2 Materials and Methods	67
5.2.1 Sampling Sites and Species Collection	67
5.2.2 Morphological Characterization	74
5.2.3 DNA Extraction and Isolation	76
5.2.4 PCR Amplification and DNA sequencing	76
5.2.5 Molecular Data Analysis	77
5.3 Results	79
5.3.1 Morphological Identification	79
5.3.2 Genetic Variation of <i>Tetraoponera rufonigra</i>	82
5.3.2 Genetic diversity of <i>Tetraoponera rufonigra</i>	84
5.3.3 Genetic Differentiation of <i>Tetraoponera rufonigra</i>	84
5.3.4 Population Genetic Structure of <i>Tetraoponera rufonigra</i>	89
5.4 Discussion	90
5.5 Conclusion	92
CHAPTER 6 - GENERAL SUMMARY AND RECOMMENDATIONS	93
REFERENCES	95
APPENDICES	
LIST OF PUBLICATIONS	

LIST OF TABLES

		Page
Table 3.1	A total number of <i>Tetraponera rufonigra</i> workers collected in each site in eastern part of Penang Island	32
Table 3.2	A total number of <i>Tetraponera rufonigra</i> workers collected on trees based on types of trashes	37
Table 3.3	Total number of individual species of trees differentiated with trash types basis corresponding with number of workers <i>Tetraponera rufonigra</i> collected	40
Table 3.4	Correlation between density of <i>Tetraponera rufonigra</i> workers and UV light, temperature and humidity	42
Table 4.1	Collection sites at Penang Island, Malaysia specimens voucher, haplotype and accession number of each <i>Tetraponera rufonigra</i> specimens in this study	54
Table 4.2	COI mtDNA primers for <i>T. rufonigra</i>	57
Table 4.3	PCR reaction mixtures used for one reaction	57
Table 4.4	PCR amplification conditions used in this study	57
Table 4.5	Pairwise genetic distance of <i>Tetraponera rufonigra</i> using Kimura two-parameter model. The analysis involved 28 nucleotides sequences	60
Table 5.1	Samples of <i>Tetraponera rufonigra</i> analyzed for DNA (cytochrome oxidase subunit 1: COI) sequence variation with GenBank accession number, locality, GPS reading, field voucher, and identified haplotypes	70
Table 5.2	Important part for morphometric measurement of <i>Tetraponera rufonigra</i> collected in this study	80
Table 5.3	Summarize morphometric measurement of <i>Tetraponera rufonigra</i> collected in this study	81

Table 5.4	Nucleotide subdivision (N_{ST}), population subdivision (F_{ST}), and gene flow (number of migrants, N_m) among six populations of <i>Tetraponera rufonigra</i>	85
Table 5.5	Genetic differentiation matrix of populations calculated by Φ_{ST} (P -values are shown in parenthesis) among populations of <i>Tetraponera rufonigra</i>	87
Table 5.6	Nucleotide diversity (π) and net nucleotide divergence (D_a) among populations of <i>Tetraponera rufonigra</i> analyzed by locations	88
Table 5.7	AMOVA analysis on geographical population differentiation in <i>Tetraponera rufonigra</i> using COI gene	89

LIST OF FIGURES

		Page
Figure 2.1	A queen and cluster of newly laid eggs of <i>T. rufonigra</i> .	15
Figure 2.2	The slightly larger queen of <i>T. rufonigra</i> is notable by her sturdy thorax and larger abdomen.	15
Figure 3.1	A preliminary target sites based on equal distance point for a survey of <i>Tetraponera rufonigra</i>	25
Figure 3.2	Collection sites of <i>Tetraponera rufonigra</i> in Eastern part of Penang Island	26
Figure 3.3	Map of distribution based on the density of <i>Tetraponera rufonigra</i> workers	34
Figure 3.4	Map of distribution based on level infestation on trees for <i>Tetraponera rufonigra</i>	35
Figure 3.5	Preferable species of trees differentiated with trash types basis corresponding with number of workers (density) <i>Tetraponera rufonigra</i> collected	39
Figure 3.7	A linear regression between density of <i>Tetraponera rufonigra</i> workers and UV light	43
Figure 3.8	A linear regression between density of <i>Tetraponera rufonigra</i> workers and temperature	44
Figure 3.9	A linear regression between density of <i>Tetraponera rufonigra</i> workers and humidity	44
Figure 4.1	Map of Penang Island showing study locations of <i>Tetraponera rufonigra</i> collected for this study. Abbreviations are listed in Table 4.1	53
Figure 4.2	Maximum Likelihood and Neighbour Joining tree representing COI gene sequences for all <i>Tetraponera rufongira</i> populations in the 27 different infested locations against an outgroup (<i>T. hespera</i> , DQ176236.1) downloaded from NCBI genebank. The tree has been build based on pairwise distances (p-distance) and 1000 bootstrap replications	62
Figure 5.1	Map of Penang Island showing study locations of <i>Tetraponera rufonigra</i> collected in this study. Abbreviations are listed in Table 5.1	68
Figure 5.2	Morphological characters and various measurements of <i>Tetraponera rufonigra</i> in frontal view	75

Figure 5.3	Morphological characters and various measurements of <i>Tetraoponera rufonigra</i> petiole dorsal view	75
Figure 5.4	Maximum likelihood [ML], Neighbour- joining [NJ] and Bayesian inference tree inferred from sequences of the mitochondrial COI gene for 59 <i>T. rufonigra</i> individuals collected in Eastern Part of Penang Island and two outgroups (<i>T. grandidieri</i> and <i>P. gracilis</i>). Abbreviations are listed in Table 1. Numbers at nodes indicate bootstrap values (%) of ML. NJ and values of Bayesian posterior probabilities replicates, obtained by 1000 replications	83
Figure 5.5	The relationship of geographical distance and percentage of net nucleotide divergence, D_a between populations of <i>Tetraoponera rufonigra</i> . Regression coefficient: $y = 0.024158$, correlation coefficient = 0.394266 and regression statistics $Y = 0.040000$	89

LIST OF PLATES

	Page
Plate 3.1 A glass jar containing a bait (tuna) located near the infested tree	27
Plate 3.2 Illustration of sampling area based on nested circles method	28
Plate 3.3 Illustration of Height of Person Method (Ontario Woodlot Association)	29
Plate 3.4 Types of trashes on the study sites	30

LIST OF SYMBOLS AND ABBREVIATIONS

°C	Celcius
%	Percentage
Fst	variance within population
N	sample size
Nm	gene flow estimates
Km	Kilometre
>	more than
<	less than
RH	relative humidity
AMOVA	analysis of molecular variance
COI	Cytochrome oxidase I
bp	base pair
dH ₂ O	distilled water
DNA	Deoxyribonucleic acid
mtDNA	mitochondrial deoxyribonucleic acid
min	Minute (s)
GIS	Geographic Information System
NJ	neighbour- joining
PCR	polymerase chain reaction
ML	maximum likelihood

**KEUTAMAAN TUMBUHAN PERUMAH, FAKTOR EKOLOGI DAN
FILOGENI MOLEKUL *Tetraponera rufonigra* JERDON (HYMENOPTERA:
FORMICIDAE) DI BAHAGIAN TIMUR PULAU PINANG, MALAYSIA**

ABSTRAK

Tetraponera rufonigra dikenali sebagai semut arboreal dwiwarna. Ia telah mendapat perhatian sebagai salah satu daripada serangga perosak perubatan terpenting di Malaysia. *Tetraponera rufonigra* dikenali kerana mempunyai sengatan yang menyakitkan dan menyebabkan anafilaksis yang teruk terutama kepada mereka yang mempunyai sejarah alergi. Satu kajian telah dijalankan untuk mengkaji spesies pokok yang diminati oleh *T. rufonigra* di seluruh kawasan bandar di bahagian Timur Pulau Pinang dengan mengumpul sampel mengikut pelbagai jenis sampah di sekeliling perimeter pokok. Kajian dijalankan di 11 daerah iaitu Bayan Lepas, Pulau Tikus, Air Itam, Jelutong, Georgetown, Gelugor, Sungai Nibong, Bayan Baru, Batu Maung, Teluk Kumbar dan Sungai Ara. Penularan aktif *T. rufonigra* dikesan di 34 kawasan dari keseluruhan 58 kawasan yang ditinjau. Tiada perbezaan yang signifikan di antara status serangan di setiap tapak dan pelbagai jenis sampah ($p > 0.05$). Kebanyakan populasi *T. rufonigra* ditemui di atas pokok dengan perimeter di sekelilingnya yang mempunyai sampah domestik dan dedaunan. Hubungan filogenetik, kepelbagaian genetik dan struktur populasi kumpulan *T. rufonigra* ditentukan berdasarkan 503 bp gen DNA mitokondria Cytochrome Oxidase I (COI). Keputusan menunjukkan satu klad utama terdiri daripada dua kumpulan iaitu Haplotaip 1 dan Haplotaip 2. Enam populasi memperlihatkan perbezaan genetik berpasangan tinggi (F_{ST}), antara 0.42 hingga 1.00 dan aliran genetik rendah (N_m), antara 0.00 hingga 0.69, kecuali bagi populasi Changkat Sungai Ara (CSA) dan

Cheeseman (CH) ($F_{ST} = 0.0$, $N_m = - 1813.68$). Analisis AMOVA menunjukkan variasi genetik dalam kalangan populasi berjumlah 99.58%, sementara hanya 0.42% disebabkan perbezaan individu di dalam populasi. Keputusan ini memberikan maklumat asas bagi ahli entomologi untuk lebih memahami perbezaan struktur genetik *T. rufonigra* pada tahap populasi dan dapat digunakan lebih lanjut untuk menganggarkan faktor-faktor penyumbang dalam perbezaan genetik spesies ini di kawasan bandar. Keputusan kajian mencadangkan bahawa struktur populasi dan aliran genetik *T. rufonigra* di beberapa kawasan yang dikaji mungkin terjejas oleh keupayaan penyebaran serangga, keadaan persekitaran, dan/atau penyebaran manusia-pengantara, manakala populasi kawasan lain mengekalkan mereka aliran genetic. Penemuan ini mungkin memberikan maklumat asas yang berguna untuk pelaksanaan pengurusan serangga perosak di seluruh kawasan serta mengetahui variasi genetik dan struktur populasi serangga yang dianggap penting dari segi perubatan ini.

**HOST PLANT PREFERENCES, ECOLOGICAL FACTOR AND
MOLECULAR PHYLOGENY OF *Tetraponera rufonigra* JERDON
(HYMENOPTERA: FORMICIDAE) IN EASTERN PART OF PENANG
ISLAND, MALAYSIA**

ABSTRACT

Tetraponera rufonigra, commonly known as the arboreal bicolored ant has gained its notoriety as one of the medically-important pest insects in Malaysia. *Tetraponera rufonigra* is recognizable by its excruciating sting, causing a severe anaphylaxis especially to those with the history of allergy. A study was conducted to survey preferable trees infested by *T. rufonigra* across urban areas in Eastern part of Penang Island by collecting samples at sites with different types of trashes on surrounding the perimeter of trees. The survey was carried out in 11 sub-districts i.e. Bayan Lepas, Pulau Tikus, Air Itam, Jelutong, Georgetown, Gelugor, Sungai Nibong, Bayang Baru, Batu Maung, Teluk Kumbar and Sungai Ara. Active *T. rufonigra* infestation was detected in 34 sites from overall 58 surveyed sites. However, no significant difference was found between infestation status in each site and types of trashes ($p>0.05$). Populations of *T. rufonigra* were mostly found on trees with the surrounding perimeter of domestic and leaf litters. Phylogenetic relationships, genetic diversity and population structure of *T. rufonigra* populations were determined based on a 503 bp of the mitochondrial DNA Cytochrome Oxidase I (COI) gene. A major clade was formed consists of haplotype 1 and haplotype 2. Six populations exhibited high pairwise genetic differentiation (F_{ST}), ranging from 0.42 to 1.00 and low gene flow (N_m), ranging from 0.00 to 0.69, except for populations of Changkat Sungai Ara CSA and Cheeseman CH ($F_{ST}= 0.0$, $N_m= -1813.68$). AMOVA

analysis revealed that genetic variation among populations accounted for 99.58% ($F_{ST} = 0.996$ $p = 0.000 \pm 0.000$) of the variation, while, only 0.42% was attributable to differences among individuals within the population. These results provided the basic information for entomologists to better understand the difference of genetic structure of *T. rufonigra* at a population level and can be further utilized to estimate the contributing factors in the genetic differentiation of this species in urban areas. Results from this study suggested that the population structure and the gene flow of *T. rufonigra* in some studied areas might have been affected by the dispersal ability of the insect, environmental conditions, and/ or human-mediated dispersal, while, populations of other areas retained their gene flow. These findings might provide useful baseline information for the implementation of area-wide pest management as well as for further characterization of genetic variation and population structure of this medically-important pest.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Tetraponera rufonigra is an arboreal ant species that inhabits dead twigs and branches. This species is commonly known as a bicoloured ant for its light-orange brown coloured body and a broad dark head and gaster. These physical characteristics are distinctive, making this species easily recognized from other ant species. Members of *T. rufonigra* are diurnal, mostly active during the daytime to forage for food sources such as small insects (Norasmah et al. 2012). They collect as much food sources as possible and later store the foods in the nest. The nesting habit is unique, of which the nest is built from assemblages of branches and dead stems of various plant species (Ward 2001a, Ward and Downie 2005). They usually colonise tropical rain forests as their natural habitats by building nests in old dead tree stumps. Hollow stems of woody plants are among other choices when suitable wood for habitation is unavailable (Torchote et al. 2010). In rare cases, they dwell in adjacent human habitations, particularly in old posts and beams.

Due to a long association between members of *T. rufonigra* and trees, they develop an ant-plant mutualism with shared benefits such as shelter, food supply and protection. Benefiting from this mutualistic interaction, plants are protected by the ants from other organisms including herbivores, pathogens and invading vegetation, and in exchange, plants provide foods (in forms of extrafloral nectar (EFN) and food bodies) and housing to ants. For example, the range expansion of *Hyposidra talaca* (caterpillar) in tea plantations in the North-eastern India has been severely affected by *T. rufonigra* due to the tactile effects from this predatory ant (Sinu et al. 2011). Other than that, *T. rufonigra* also acts as a predator to leafhopper on rice paddy field

(Labora 1977). Besides, *T. rufonigra* was found to be associated with *Aphis craccivora* on a single host plant, *L. purpureus* from the family Fabaceae, of which the ants obtained a food supply in the form of honeydew (Rakhshan 2015).

1.2 Problem Statement

Serious medical conditions caused by *T. rufonigra*-induced hypersensitivity have been reported in humans following its painful bites and stings. These bites and stings could lead to severe anaphylaxis if left untreated (Potiwat and Sitcharungsi 2015). Several cases of anaphylaxis induced by *T. rufonigra* have been reported in Penang, Thailand, Sri Lanka and India (The Star 2007, New Straits Times 2009, Norasmah et al. 2012, Potiwat and Sitcharungsi 2015). Studies into the venom of *T. rufonigra* revealed at least eight neurotoxic alkaloids and protein allergens in its body (Potiwat and Sitcharungsi 2015, Infiniti 2016). Interestingly, all reported cases were in areas surrounded with trees. There was a case in Sri Lanka where *T. rufonigra* suddenly emerged from several fallen trees, forcing the old-nested colonies of *T. rufonigra* to disperse (The Sunday Times, 2013).

Eradication of *T. rufonigra* is often difficult as the solitary workers and the nest are inside the trees. However, the eradication is possible if the infestation is minimum. Thus, it is important to identify the preferable host plants of *T. rufonigra* and the level of infestation in order to plan the control strategy. In most cases, insecticides are used as a treatment method to eradicate the infestation. For example, a combination of two insecticides managed to minimize the population of *T. rufonigra* at Hong Hock See temple in Penang. Cutting or removing any dead branches is another means to prevent a colonization of this species.

Despite its importance as a medically-important pest insect, the molecular ecology study of *T. rufonigra* is limited. Information describing the genetic structure of this species is ignored despite *T. rufonigra* is regularly used as the outgroup in phylogenetic relationships of ants from the genus *Pseudomyrmex* (Ward 2001, Ward and Brady 2003, Ward and Downie 2005, Sanchez 2011). A phylogenetic analysis on ant species in the genus *Tetraponera* in Oriental and Australian regions have revealed four species groups within the genus (Ward 2001).

Previous work have demonstrated that mitochondrial gene, cytochrome oxidase subunit I (COI) is a powerful tool in animal taxonomic and evolutionary studies (Baur et al. 1995, Ward and Downie 2005, Seri Masran and Ab Majid 2017). Mitochondrial COI gene had been widely used in phylogenetic studies because it has relatively high substitution rate and tends to evolve more rapidly among closely related species (Narain et al. 2012). The species relatedness can be established further by comparing the variation of DNA sequences. Meanwhile, population genetic analysis elucidates relevant information such as the pattern of dispersal and gene flow, genetic distance and genetic differentiation (Yeh et al. 1998, Pramual and Pangjanda 2015). By using COI region marker, the study of genetic variation between and within populations can be conducted.

1.3 Significance of Study

This study was conducted to study *T. rufonigra* infestation in selected urban areas following several cases reported in Penang Island. This study aimed to investigate the preferable host plants to *T. rufonigra* and to discover possible contributing factors (ecological or environmental) leading to *T. rufonigra* infestation. Dispersal ability of *T. rufonigra* and physical landscape are among important factors in determining the gene flow between populations. Thus, understanding the

relationships between *T. rufonigra* population and geographical distance will allow a better understanding of the species dispersal.

1.4 Limitations of Study

The survey was only on selected urban areas in Penang Island such as residential houses, recreational parks and industrial areas since no signs of infestation were observed in rural areas. Collection of *T. rufonigra* samples were limited to visual inspection and baiting method in infested trees, thus, the total number of tree samples was differed for each location. Due to difference in the total number of tree samples, only several populations were selected for the molecular work.

1.5 Objectives

The objectives in this study were divided into three parts;

Objective 1 (Chapter 3): Evaluate on the infestation level of *T. rufonigra* in selected urban locations in Eastern Penang Island, Malaysia.

1. To evaluate the trees preferable to *T. rufonigra* infestation in urban areas in Penang Island,
2. To determine the influence (if any) of biotic factors (height and diameter of trees), ecological factors (light level, air velocity, humidity and temperature) and surrounding environment on the distribution of *T. rufonigra* in Penang Island.

Objective 2 (Chapter 4): Phylogenetic relationships of *T. rufonigra* Jerdon inferred from MtDNA COI region.

1. To examine the phylogenetic relationships among *T. rufonigra* based on host plant preferences and sampling areas.

Objective 3 (Chapter 5): Genetic variation and population structure of *T. rufonigra* Jerdon from selected urban areas in Eastern Penang Island, Malaysia.

1. To identify *T. rufonigra* based on morphological characteristics,
2. To determine genetic variation and genetic differentiation within and among several populations of *T. rufonigra*.
3. To investigate the population genetic structure and the dispersal patterns of several *T. rufonigra* populations.

CHAPTER 2

LITERATURE REVIEW

2.1 Ants: Their History, Life and Purpose

Ants are eusocial insects of the family Formicidae, belonging to the order of Hymenoptera and closely related to wasps and bees. They are among the most widespread insects and are believed to be one of the major pests in the world. Numerous studies have reported that some species, such as the red imported fire ant (*Solenopsis invicta*) are regarded as invasive and a constant pest in developing areas in the southern United States. Surveys conducted by Vittum et al (1999) and (Reinert and McCoy 2010) have shown that ants may be present in any areas such as residential, sports fields, and parks. By far, ants can be differentiated with other insects based on their morphological characteristics e.g. distinctive node-like structure that forms the slender waist and elbowed antennae (Klotz et al. 2010). Many research found that their eyes are poorly-developed, causing the panoramic view of their foraging range is limited. Hence, ants rely on chemosensory to recognize their foods (Pedigo and Rice 2009).

Ants form millions of individuals that may occupy large territories. A colony of ants generally consists of individuals with different castes bearing different roles. For example, sterile and wingless females form the caste of workers and soldiers (Klotz et al. 2007). The highest caste in the ant hierarchy is the productive caste, consisting of the drones (productive males) and the queens (productive females) (Klotz et al. 2010). The queens are larger compared to other castes including drones. The role of queens is to lay eggs, while, the drones are only for a mating purpose. The drones usually die shortly after mating. The workers are the lowest caste in the hierarchy and their main role is to build up the colony (Pedigo 2004).

Some species have stingers and biting mandibles as their defense mechanism to protect the ants as well as the host plants. *Tapinoma indicum*, for instance, emits a rotten coconut odor when crushed (Na and Lee 2001, Lee 2002).

2.2 Ants and Plants Diversity

Arthropods live in or on trees are higher during rainforest replacement process compared to the secondary vegetation. The percentage of ant populations in tropical forests is approximately 90% individuals, representing 20-60% of entire arthropods (Davidson 2003, Klimes et al. 2012). Numerous factors such as the nesting sites and foods contribute to the dispensation of plant-ant colonies. Besides, disturbance factors also play role in determining the diversity of the species and distribution in a disturbed forest (Klimes et al. 2012).

Several challenges are encountered in finding the relationship between the diversity of arboreal ants with the diversity of plants. One of the challenges is to find how plant-ant variety reacts to changes in plant species variety, tree size and source of habitat. This challenge remains less know at the level of entire forest societies, although such factors have been recommended to play main role (Klimes et al. 2012).

Besides that, diversity of ant communities also influenced by the diversity of plant (Dejean et al. 2007, Klimes et al. 2012). Different trees provide different food resources which influence the ant communities that present around the trees (Dejean et al. 2007, Klimes et al. 2012).

2.3 Relationships between Ants with Their Host Plant

Ants have a good relationship with one another and with their surroundings. This is because of their large quantities and multiple different roles such as predators,

decomposers (organisms that break down dead organisms) and seed-distribution agents (Folgarait 1998, Klimes et al. 2012). Besides, they play key roles in the development of plant and animal interactions. Interactions between them could change, either due to phenotype trait inherited from older generations and/or ecological process based on the time scale (Moreau 2006).

Among the tropical plants, myrmecophytes species undergoes a series of evolution in plant-animal interactions by giving benefit to each other ('ant-plants' and 'plant-ants') (Ellison and Farnsworth 2014, Resasco et al. 2014). For example, the habitat of the arboreal ant, *Allomerus decemarticulatus* is provided by the Amazonian plant, *Hirtella physophora* (Revel et al. 2010). Another example is *T. rufonigra*, which also develop a mutualistic interaction with the host plant.

A shelter is also one of the benefits the ants usually get from this relationship. They usually shield themselves in small chambers (holes branches or thorns and leaf sac) and in the bark area which eventually cracks over the time. Moreover, as the wood soften, the ants can easily make a hollow trail on the bark of the tree. This will attract another ant species like the carpenter ants to move in and colonize the wood. They will form a satellite colony in other trees if the former colony becomes too massive. The satellite colony, however, remains connected with the original where the queens live (Ellison and Farnsworth 2014).

The plants also provide foods to ants in the forms of extra floral nectar (EFN) and food bodies (FBs). These foods make up a significant portion of the ant's diet (Ellison and Farnsworth 2014). In addition, plants have no significant effect on the diversity of ants (Machac et al. 2011, Klimes et al. 2012, 2015).

2.4 Ant Ecology and Foraging Behaviour

Ants are poikilothermic species that constantly responsive to changes in climates (Petersen 2008). Therefore, the foraging activities are largely influenced by environmental factors such as soil or surrounding temperatures, water stress, moisture radiation and wind (Chong & Lee 2009). The foraging activities of ants are also influenced by relative humidity and light intensity. Temperature is important in energy balance and chemical processes (metabolism) of ant communities (Roces & Nunez 1995). It also directly affects the oxygen usage, water reduction and transfer costs of the foraging ants (Lopez et al. 1992). According to Peakin & Josens (1987), the breathing rates of ants increase twice at every 10°C increase in temperature. Foraging activities of *Anoplolepis gracilipes* were significantly affected by temperature and relative humidity (Chong & Lee 2009). Foraging activities of *Linepithema humile* were optimum at temperatures between 15°C and 30°C (Markin 1970).

The host plant influences the foraging behavior of ants. Plants have evolved in their behavior towards ants by producing chemical and physical barriers in response to damages caused by the ants (Raine et. al. 2002, Gaume et al. 2005). Plant parts such as flowers and inflorescences could act as ant barriers or repellents. For example, floral nectar or flower parts are repellents or barriers to several ant species (Stephenson 1981). In some circumstances, extrafloral nectar could also act as baits to ants (Bentley 1977a, Gaume et al. 2005). These barriers and repellents discourage the ants from foraging on the vegetative part of plants, primarily the flower part (Wagner & Kay 2002, Gaume et al. 2005). For this reason, several ant species do not prefer certain trees.

Ants have three circadian rhythms of foraging activity which are nocturnal, diurnal and crepuscular. For diurnal species, they intensively forage foods at daytime (Chong & Lee 2009), for example, *T. rufonigra*. For nocturnal species, they actively forage foods at night, for example, *A. gracilipes*.

Foraging activity continuously evolves in order to gain more resources or when dealing with challenging prey. In general, ant workers forage for foods in groups. When a worker could not handle a larger prey, it will send a signal to its nest mates to help and work together. Furthermore, the route to and from the nest is fixed and cleared from any objects obstructing the passage of the workers (Oster & Wilson 1978). If the colony has many individual foragers, they have an advantage in terms of harvesting diffuse spatial and temporal patterns of small food sources. This shows how cooperation between the ants has benefitted them in foraging efficiently (Reinert and McCoy 2010).

According to Buczkowski and Bennett (2009), some species forage for food individually. Workers in the genus *Mymecia*, for example, forage for food individually. They forage for other food sources similar to their size and often consume some plant secretions while foraging (Oster & Wilson 1978). This type of foraging is called a 'diffuse' foraging where no or less cooperation occurs among the workers.

2.4.1 Ants as urban pest

Ants can be considered as one of the major pests worldwide. Their adaptive nature makes it nearly impossible to eliminate the entire colonies (Lee 2000, Julie & Lee 2001). Their presence can be both advantageous and disadvantageous for humans. They can be a pest to humans, causing damages structures and buildings.

For instance, the carpenter ant, *Componotus* sp. dig a tunnel through a wood, simultaneously creating holes in the wood and a rough-surfaced bark. Also, they can cause a huge loss to agricultural sectors. They destroy crops and promote the presence of other agricultural pests such as lice and mealybugs (Hedges 1998).

In Malaysia, household ants are a group of common nuisance insect pests that infest houses and buildings (Chong & Lee 1999, Lee 2000). Yap and Lee (1994) reported that household ant species in Malaysia are dominated by Pharoah ants (*Monorium pharoanis*), followed by odorous house ants (*Tapinoma sessile*) and black crazy ants (*Paratrechina longicornis*). In 1995, a questionnaire survey conducted on 814 houses in Penang revealed that ants were the main pests in houses or buildings compared to mosquitoes and cockroaches (Lee *et al.* 1999).

Ants are also medically important and have raised a public concern in certain countries as they can pose a threat to human health. They possess a stinger that can cause allergic and infectious complications. In some cases, the sting can be life-threatening as the venomous protein responsible for the allergic reaction can be transferred through the sting. The ant species of *P. chinensis* caused dermatosis epidemic in a factory in Pohang, South Korea where 12 of 73 workers experienced pruritic papules. Other than that, Klotz *et al.* (2005) reported that in Georgia and Florida, victims suffered from anaphylactic shock including swollen tongues and breathing difficulty, subsequently causing death to the victims (Nelder *et al.* 2006).

2.5 Distribution of the Genus *Tetraoponera*

Tetraoponera is one of the three genera belonging to the subfamily Pseudomyrmecinae. Members of this genus are slender-bodied, large-eyed and mostly found throughout the Old and New World Tropics. Since in the mid-90s, much of the

literature by biologists has centered on this species for studying ant-plant interactions (Ward 1991, Kistner and Klein 1996, Dejean et al. 2008, Palmer et al. 2008). Prior to the work of Ward (2001), the genus *Tetraponera* in the Oriental and Australian regions consisted of 33 species divided into four groups i.e. *allaborans*, *nigra*, *pilosa* and *rufonigra*.

The number of species in the genus *Tetraponera* is the highest in Borneo and the Peninsular Malaysia where approximately 17 to 18 species have been identified. It has been suggested that the presence of four *Tetraponera* groups in Asia and Australia regions probably due to the dispersal of earlier ancestors *via* the breakup of Gondwanaland in Mesozoic and early Cenozoic. Besides, the queens of Asian and Australian *Tetraponera* species are fully-winged and capable of dispersing over moderate distances. In 2012, Abe and colleagues published a paper describing *Tetraponera* species in various islands in the Malay Archipelago and in Krakatau after 25 years of volcanoes explosion. They managed to list out three species in this genus i.e. *T. attenuate*, *T. nitida* and *T. rufonigra*.

2.6 Biology of *Tetraponera rufonigra*

2.6.1 Taxonomic classification of *Tetraponera rufonigra*

The taxonomic classification of *T. rufonigra* according to the Integrated Taxonomy Information System (ITIS) is as follows:

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Hymenoptera

Family: Formicidae

Genus: *Tetraponera*

Species: *Tetraponera rufonigra* (Jerdon, 1851)

2.6.2 Life Cycle and Morphological Characters of *Tetraoponera rufonigra*

Like any other ants, members of *T. rufonigra* are social insects and live in colonies. They undergo complete metamorphosis based on four different stages in the developmental process, namely, eggs, larvae, pupae and adults. The eggs are small in size with white to whitish yellow in color (Figure 2.1). The larvae are legless, soft and immobile and usually requires a nursing care from adults. Later, the larvae will moult several times to form pupae. The life-cycle of ants takes about 2-3 months to complete, however, recent studies showed longer time is required, depending on food availability and environmental temperature (Chong and Lee 2010).

Adults (workers) of *T. rufonigra* are about 10- 12 mm long, fast-moving with large eyes covering almost half of the head (Norasmah et al. 2012). All workers are sterile females that forage for food and protect the colony from invaders. Each colony has approximately 300 to 500 workers (Bolton 1994). Males are occasionally produced to mate with the queens from other colonies. The queens are larger than the workers at about 13-15 mm long (Ward 2006) (Figure 2.2). Established colonies can be found nesting in old dead hardwood and extremely difficult to get into due to the small entrance. Thus, the colony is well-protected from predators (Ward 2001, Dejean et al. 2008, Dejean 2011).

Morphologically, members of *T. rufonigra* are bicolored of which the head and gaster are dark with orange-brown mesosoma and the first segment of petiole (Ward 2001, Ward and Downie 2005, Chong and Lee 2010). They also have a well-developed sting (Ward and Brady 2003). Their short legs are ideal for their arboreal life, as they forage with the entire body close to the foraging surface. The division of caste is not distinctive, and all workers attack upon disturbance.



Figure 2.1 A queen and a cluster of newly-laid eggs of *T. rufonigra*. Retrieved from <http://www.myantshop.com/Tetraponera>



Figure 2.2 The slightly larger queen of *T. rufonigra* is notably identified by her sturdy thorax and larger abdomen. Retrieved from <http://www.myantshop.com/Tetraponera>

2.6.3 Habitat and Distribution of *Tetraponera rufonigra*

Tetraponera rufonigra was once thought to have originated from Africa. However, studies have shown that the species is widely distributed in the Indian subcontinent and Southeast Asia as far as Sumatra and Java, as well as in Seychelles (Ward 2001a, Kumar and Mishra 2008, Piromrat et al. 2008, Man and Lee 2012). Retrospectively, this ant species was most likely to spread through multiple invasions of Asia from Africa with at least four west- to east transgressions of Wallace's line into the Australian region (Ward 2001). This invasion was related to plate tectonic events that facilitating such dispersal. This species has recently recognized as a medically-important urban pest and is the most successful tree-infested species, especially in urban parklands, gardens and degraded coastal hill forests (Bingham, 1903). Numerous studies have also attempted to explain the mutualism relationship of *T. rufonigra* with the host plant ('ant-plants' and 'plant-ants') (Resende et al. 2013).

They often inhabit the arboreal stratum in tropical and subtropical regions. Most pseudomyrmecinae species have generalized twig-nesting habits, occupying dead stems and branches of many kinds of plants, usually in cavities previously excavated by coleopteran or lepidopteran larvae. (Kautz et al. 2009). More than 40 species however are obligate inhabitants of specialized ant-plants (myrmecophytes). These ants colonize intrinsically hollow cavities (myrmecodomatia) in live plant tissue and in most instances, appear to provide some degree of protection to the plant (Kautz et al. 2009).

The workers of *T. rufonigra* can be rather aggressive, protecting their nesting habitat from invaders (Wu and Wang 1990). They are predators, feeding on other ants, termites, small invertebrates and small animals such as lizards. They often hunt

in solitary, but when close to the nest, they hunt in small groups. A colony of *T. rufonigra* was discovered at Mandai, Singapore in branches and trunk of *Sonneratia ovate* previously occupied by beetle-bored (Ward 2001). In India, the *T. rufonigra* nest was found in the primary stem of *Santalum album* saplings (Bodlah et al. 2016). The nest inside the stem was presumed to be the burrows of a beetle larva (most likely a cerambycid) with incomplete septa and made of a dirt apparently added by the workers. The nest was occupied by workers, eggs, larvae and a large, mature coccid.

2.7 Application of Geographic Information System (GIS) Application for Studying Insects

Geographic Information System (GIS) is a computer-generated system to analyze and display digital geo-referenced data sets as theme layers. The data can only be displayed one at a time or on top of each other (Madelaine 2001). One of its advantages is the capacity for adding a new data to the system. In insect studies, the maps of species distribution generated by the GIS can be instantly updated as soon as the new data is available. The use of GIS and the Internet allow the scientific community to have an easy access to the most recent information on studied materials. The compilation of data in the different maps allows certain important aspects of animal ecology such as species distribution, to be examined (Xavier et al 1999).

GIS is a very useful tool in entomology. In recent years, the utilization of GIS as a tool to manage diseases related to insects by public health organizations has become a necessity in line with the advancement of technology (Gong and Liang 2006, Cringoli 2008). For example, GIS was employed to analyze the temporal and spatial models such as frequency, duration and intensity of the dengue fever cases in

Kaohsiung, a metropolitan city in south Taiwan and Subang Jaya in Malaysia (Wen et al 2006, Nazri et al 2012).

GIS application has also been applied to study the preferences of microhabitat selected by ants (Davoodi and Mesgari 2015). Some ants are highly specific to the habitat selection, while, some depend on the maximum benefits they attain for nesting, mating and food availability (Menke et al. 2007). A study by Ramachandara and Ajay (2012) at Sharavathi river basin, India has revealed that ants could be used as a habitat status indicator. Ant species composition is also different in different habitats based on varying factors such as the plantations and the degree of variability in food preferences (Martin 2012).

2.8 Importance of *Tetraponera rufonigra*

2.8.1 *Tetraponera rufonigra* as Medically-Important Pest

Tetraponera rufonigra is gaining its notoriety as an important pest ant in the urban area such as schools, nursing homes, temples, parks and garden (Lee and Tan, 2004). It is known for its extremely sharp and painful sting and bites. Moreover, its venom-containing sting can cause allergic and infectious complications, causing anaphylaxis that may pose a threat to human health (Piromrat et al. 2008, Potiwat and Sitcharungsi 2015). The first case of *T. rufonigra*-induced was reported in Thailand where a 17-month-old girl suffered two episodes of urticaria, angioedema, dyspnoea, and loss of consciousness (Potiwat and Sitcharungsi 2015). Extensive studies in later cases have found that, *T. rufonigra* is the common causative species responsible for anaphylaxis to the victims, along with *Solenopsis geminate* and *Odontoponera denticulata* (Potiwat and Sitcharungsi 2015).

Several cases have also been reported in Sri Lanka due to *T. rufonigra* bite. During the physical examination, the victims had developed an itchy rash, generalized body swelling and shortness of breath (Ratnatilaka et al. 2011). A detailed study of *T. rufonigra* on its protein allergens showed that this species had a dominant active protein of approximately 120 kDa (Potiwat and Sitcharungsi 2015). Fernando et al. (2015) found that only workers and winged females had well-sclerotized and distally pointed lancet specifically modified for the stinging. This character, however, was absent in the queens and males.

2.9 Molecular Genetic Marker

Molecular markers or genetic markers are defined by Abercrombie et al. (1991) as genes or DNA sequences or any other distinguishing features of genotypes, chromosomes or karyotypes which may be used to keep track on specific chromosomes, cells or individuals. The molecular markers should at least exist in two alternative and readily identifiable forms.

Over the past 15 years, DNA markers have made a significant contribution to molecular studies of genetic relatedness, phylogeny, population dynamics or gene and genome mapping in insects (Heckel 2003). Improvements have been made to enhance reproducibility, the power of resolution and more importantly, to reduce the cost and time consumption in developing and scoring the marker loci (Behura 2006). Since then, application of DNA markers in entomology continuously experience a noticeable change to accommodate a new technology for robust and less expensive genotyping methods (Behura 2006).

Conventionally, mitochondrial DNA (mtDNA) has been a marker of choice for studying genetic variations in insect species. It has been used in phylogenetic and

population genetic studies to construct an evolutionary history of related insect species. mtDNA is relatively easy-to-work-with as it is small in size and the gene arrangements in animals is evolutionarily conserved, thus, enabling the development of universal primers to a wide variety of vertebrates and invertebrates. With the advent of polymerase chain reaction (PCR; Saiki et al. 1985) universal primers for mtDNA regions have become available (Vigilant L et al. 1989, Blatrix et al. 2013), providing a comprehensive compilation of primer sequences in insect mtDNA. Kambhampati and Smith (1995) have provided additional sequences of four new primers suitable for mtDNA amplification supplementary to the findings of Simon et al. (1994). These sequences remain among the most widely used mtDNA universal primers to date.

Compared to nuclear DNA, mtDNA is rapidly evolving that leads to the accumulation of differences between closely related species (Behura 2006). mtDNA contains 37 genes which are essential for mitochondria to function. The evolutionary rate of mtDNA is thought to be generally high. Since the mutation rates are easily measured, it has been used to track back many species with thousands of generations. Kambhampati and Smith (1995) stated that mtDNA is extremely useful for high-resolution analysis of the evolutionary process. In genetics, mtDNA is clonally and maternally inherited where it will be passed to their progeny through the egg cytoplasm (Baur et al. 1995).

2.9.1 Mitochondrial Cytochrome Oxidase I (COI) and its Applications as a Genetic Marker

The COI is a subunit of the cytochrome oxidase complex involves in electron transport chain. Hillis et al. (1996) mentioned that sequences of COI are highly conserved across the phyla, thus, it is possible to design a useful universal primer.

This molecular marker has been used to examine the phylogenetic relationship in relatively lower levels of animal classification such as in families, genera, species or populations (Hwang and Kim 1999).

Recent studies on ants have successfully employed COI on the thief ants for DNA barcoding and species identification. COI possess few advantages such as high copy number, rapid mutation rate, ease of amplification or sequencing and alignment for intra and interspecific comparisons (Baur et al. 1995). Another finding indicates that COI gene can uncover a basal polytomy between subfamilial clades (Johnson et al. 2013). Besides, COI sequence variability is low, making it possible to confidently identify the species and able to resolve most species-level differences (Ward and Downie 2005, Beltrà et al. 2012, Narain et al. 2012, Changbunjong et al. 2016).

2.9.2 Application of Mitochondrial Cytochrome Oxidase I (COI) for Studying Insect Population Genetics

COI was the first PCR-based marker and became the most widely used molecular marker for population genetic analysis. Sequences of COI are proven to be useful in identifying various animals at a species-level. However, empirical theoretical evidence argued that COI sequences may not track the species evolution and diversification (Narain et al. 2012, Beltrà et al. 2012). Nevertheless, COI sequences are continuously used to represent a standardized and universal DNA barcoding identification system for animals, of which the sequences provide a framework for species identification as well as for large-scale biodiversity assessments (Hebert et al. 2003, Ghazali et al. 2014).

Numerous researchers have used COI sequences to identify the unknown, cryptic or invasive species in entomology, botany, ornithology and ichthyology

(Chown et al. 2008, Hebert et al. 2003, Lahaye et al. 2008, Narain et al. 2012, Takeuchi et al. 2017). Prior to work of Hebert et al. (2003), public databases of DNA barcoding have been generated for species identification such as the 'Barcode of Life' and 'Genbank. DNA barcoding is useful in separating any cryptic species. In a study by Hajibabaei et al. (2006), 97.9% of 521 species in the lepidopteran families from Costa Rica had distinctive COI barcodes. Besides, DNA barcoding can be used in early detection of invasive species and their dispersal such as the big headed-ant (*Pheidole megacephala*) and red fire ants (*Solenopsis invicta*) (Fournier et al. 2008).

The COI gene sequences have also been used for studying genetic diversity and phylogenetic relationship of pest insects of economic importance including agricultural pests e.g. yellow-legged hornet, fruit fly and scale insects (Kerdelhué et al. 2014, Takeuchi et al. 2017) and structural and household pests e.g. cockroaches, termites, ants and bed bugs (Ingram and Gordon 2003, Tee et al. 2011, Seri Masran and Ab Majid 2017). The sequences are effective to determine colony association, population structure and relationships among introduced and native populations. This information is essential to provide effective management of pest species. The COI gene sequences are also reliable to understand evolutionary forces such as selection, gene flow, and genetic drift in natural populations.

CHAPTER 3

HOST PLANT PREFERENCES OF *Tetraoponera rufonigra* AND ECOLOGICAL FACTORS AFFECTING THE DISTRIBUTION AND SHELTER PREFERENCES ACROSS URBAN AREA IN EASTERN PART OF PENANG ISLAND

3.1 Introduction

Tetraoponera rufonigra has become medically important pest especially in urban locations in India and Southeast Asia countries which leads to a number of reports and publications due to its painful sting that could cause severe anaphylaxis (Ratnatilaka et al. 2011, Fernando et al. 2015). The painful sting normally is a venom that contains substances acids and alkaloids that cause toxic reactions which give local reactions and systemic reactions (anaphylaxis) (Potiwat and Sitcharungsi 2015). In Malaysia, several cases have been reported on *T. rufonigra*. There was a case in Bukit Mertajam where a student was sent to the general hospital and was admitted to Intensive Care Unit (ICU) after being severely stung by *T. rufonigra* (Kasturi 2009). There was also a case reported on the infestation of *T. rufonigra* at Hong Hock See temple at Perak Road, Penang (Priscilla 2007). The monks and devotees were bitten by *T. rufonigra*, causing a swelling that lasted for two days. In Matara, India, the infestation of *T. rufonigra* occurred at places when several large trees fell to the ground, resulting in the dispersal of *T. rufonigra* colonies (Malaka 2013).

The pioneering study of Janzen (1966) with *Acacia* trees and *Pseudomyrmex* sp. showed that this ant species could act as a biotic defense, protecting plants against herbivores and parasites. In return, plants provide benefits such as shelter and food rewards. This relationship is known as mutualistic interactions (Bronstein et al. 2006). These ant-plant interactions are based on the resources provided by plants

as rewards ranging from food bodies, extra-floral nectaries (EFN) or nesting sites (domatia). The ant-plants or myrmecophytes will be continuously inhabited by ants during major parts of their life that often associated with single and specialized species (Djipto-Lordon et al. 2007, Webber et al. 2007).

Tetraponera rufonigra behavior has its own unique nesting habit, consisting of branches and dead stems of various kinds of plants. They usually live in empty spaces inside the plants (Bharti and Akbar 2014). The host plant usually has holes inside the thorns or branches, as this host plants also providing foods and housing to the ants (Norasmah et al. 2012). In return, the ants provide protection to the host plants through their aggressive behavior towards herbivores insects and intruders. Workers of *T. rufonigra* are good climbers due to their arboreal nature and usually prefer to forage over trees and large shrubs. However, they can also be found foraging over the ground (Savitha et al. 2008, Mahalakshmi et al. 2016). These ants usually have a large colony with a large number of workers, approximately 300-500 individuals per colony (Rakhshan 2015). Having a large number of worker is advantageous to the colony as it helps them to establish their nest (Mahalakshmi et al. 2016).

In Penang, Malaysia, the early study on foraging behavior and nutrient preferences of *T. rufonigra* were conducted by Norasmah et al. (2012). They found that this ant species was diurnal and preferred protein-based foods as their food sources. Nevertheless, the distribution and host plants preferences of *T. rufonigra* in Peninsular Malaysia has not been studied before. Therefore, the objectives of this study were to investigate the host plants preferences of *T. rufonigra* across the eastern part of Penang Island and to determine the ecological factors affecting the distribution and its shelter preferences.

3.2 Materials and Methods

3.2.1 Data Collection on *Tetraodon rufonigra* and Field Sampling Area

A total of 58 sites in eastern part of Penang Island comprises of total 243 trees were visually surveyed. The sites were divided into 11 sub-districts as follows: Pulau Tikus, Georgetown, Jelutong, Air Itam, Gelugor, Paya Terubong, Sungai Nibong, Bayan Baru, Batu Maung, Bayan Lepas and Teluk Kumbar. Surveys were mainly targeted in the urban and suburban areas since based on the earlier survey, no *T. rufonigra* was discovered in rural areas. At first, the areas were divided into smaller divisions. Next, the selected areas were randomly pointed on the map to mark the study sites by using Google Earth (2015). To minimize the bias, the distance of each point (target sites for sampling) was about the same with each other (Figure 3.1) (Baker 2010). Since the sampling was randomly conducted, only one visit was required in this survey (Zulaikha et al. 2016).