DIVERSITY AND MOLECULAR CHARACTERIZATION OF TERMITE GUT SYMBIONTS IN *Globitermes sulphureus* Haviland (BLATTODEA: TERMITIDAE)

NURUL AKMAR BINTI HUSSIN

UNIVERSITI SAINS MALAYSIA

2020

DIVERSITY AND MOLECULAR CHARACTERIZATION OF TERMITE GUT SYMBIONTS IN *Globitermes sulphureus* Haviland (BLATTODEA: TERMITIDAE)

by

NURUL AKMAR BINTI HUSSIN

Thesis submitted in fulfillment of the requirements

for the degree of

Doctor of Philosophy

March 2020

ACKNOWLEDGEMENT

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

$	Dollar sign
%	Percentage
$\times$ g	Times gravity
°C	Degree Celcius
†	Indicate name proposed for fossil taxa
a	Percentage of relative abundance
bp	Base pair
cm	Centimeter
g	gram
g/L	Gram per liter
Gb	Gigabase pair
GB	Gigabyte
h	Hour
kb	Kilobase pair
L	Liter
m	Meter
m^2	Square meter
Mb	Megabase pair
MB	Megabyte
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
N	Normality

nm	Nanometer
pg	Picogram
sec	Second
U/mL	Unit per millilitre
β	Beta
μg/mL	Microgram per millilitre
μL	Microliter
μM	Micromolar
μmol	Micromol

LIST OF ABBREVIATIONS

BI	Bayesian inference
BLAST	Basic local alignment search tool
BP	Bakti Permai
cDNA	Complementary DNA
COG	Cluster of orthologous groups
COI	Cytochrome c oxidase I
COII	Cytochrome c oxidase II
DGGE	Denaturing gradient gel electrophoresis
dH$_2$O	Distilled water
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic acid
DV	Durian Valley
EggNOG	Evolutionary genealogy of genes: non-supervised orthologous groups
EPNs	Entomopathogenic nematodes
FISH	Fluorescence in situ hybridisation
GFP	Green fluorescent protein
GH	Glycosyl hydrolase
GO	Gene ontology
HCl	Hydrochloric acid
HCN	Hydrogen cyanide
IK	Indah Kembara
KEGG	Kyoto encyclopedia of genes and genomes
ML	Maximum likelihood
NA	Nutrient agar
NaOH	Sodium hydroxide
NB	Nutrient broth
NCBI	National centre for biotechnology information

NGS	Next-generation sequencing
NJ	Neighbor-joining
NPV	Nuclear polyhedrosis virus
OD	Optical density
ORF	Open reading frame
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
pH	Potential hydrogen
PHA	Polyhydroxyalkanoate
PM	Padang Minden
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
rRNA	Ribosomal RNA
RT-qPCR	Reverse transcription qPCR
SRA	Sequence read archive
TH	Tasik Harapan
T-RFLP	Terminal restriction fragment length polymorphism
US	United States
USD	United States dollar
USM	Universiti Sains Malaysia
WEGO	Web gene ontology annotation plot

KEPELBAGAIAN DAN PENCIRIAN MOLEKULAR SIMBION GASTROUSUS ANAI-ANAI *Globitermes sulphureus* Haviland (BLATTODEA: TERMITIDAE)

ABSTRAK

Globitermes sulphureus (Haviland) adalah anai-anai kumpulan tinggi dan dikenali sebagai serangga perosak utama di kawasan pertanian terutamanya di ladang kelapa dan kelapa sawit. *Globitermes sulphureus* juga telah dijumpai menyerang bangunan berstruktur kayu di kawasan luar bandar dan bandar. Walaupun statusnya sebagai serangga perosak, maklumat mengenai serangga tersembunyi ini dari segi genetik dan gastrousus simbionnya masih terhad. Dalam kajian ini, jujukan COII kepunyaan *G. sulphureus* telah diamplifikasi untuk mengkaji hubungan filogenetik di antara lima koloni anai-anai di dalam kawasan kampus USM, Penang. Analisis *Maximum Likelihood* (*ML*) dan *Neighbour-Joining* (*NJ*) menunjukkan persamaan topologi dan parafili di antara populasi *G. sulphureus*. Lima koloni tersebut termasuk dalam kumpulan adik beradik. Kemudian, gastrousus daripada lima koloni tersebut telah dipencilkan menggunakan teknik kultur-bergantungan dan dikenalpasti melalui teknik jujukan gen 16S rRNA. Keputusan menunjukkan, kepelbagaian bakteria di antara kasta pekerja dan askar adalah hampir sama untuk sesebuah koloni tetapi sedikit berbeza jika dibandingkan di antara koloni-koloni tersebut. Filum bakteria yang paling banyak dipencilkan daripada lima koloni anai-anai adalah Firmicutes, diikuti dengan Proteobakteria dan Actinobakteria. Simbion daripada gastrousus *G. sulphureus* telah dicirikan lagi menggunakan *high-throughput sequencing* iaitu 16S metagenomik. Kawasan V3-V4 dalam gen 16S rRNA telah diamplifikasi dan dijujukan menggunakan pentas Illumina HiSeq 2500. Hasil jujukan tersebut dianalisis

menggunakan *pipeline* Qiime v1.9.1. Keputusan menunjukkan filum bakteria yang mendominasi adalah Spirochaetes, Fibrobakteres, Firmicutes, Proteobakteria, dan Bakteroidetes. Tambahan lagi, kasta pekerja dan askar berkongsi filum bakteria yang sama tetapi berbeza dari segi genera bakteria. Selain daripada gastrousus, genetik anai-anai itu sendiri juga telah dikaji. Kepala daripada kasta pekerja telah digunakan untuk *de novo* transkriptom dengan menggunakan mesin jujukan Illumina HiSeq X10. Bacaan-bacaan telah dicantumkan secara *de novo* dengan menggunakan perisian Trinity dan telah menghasilkan sebanyak 243,057 transkrip dan 193,344 gen. Transkrip-transkrip tersebut dianotasi menggunakan *pipeline* Trinotate. Secara keseluruhan, 27,061 transkrip telah berjaya dianotasi dengan menggunakan BLASTX terhadap pengkalan data SwissProt dan 17,816 gen telah dipadankan kepada 47,598 terma GO. Sebanyak 14,233 transkrip telah dikelaskan kepada COG dan menghasilkan 25 kumpulan fungsi anotasi. Kemudian, sebanyak 12,194 gen telah dipadankan kepada laluan KEGG dan 392 laluan metabolic telah diramalkan. Dua enzim selulase; β-glukosidase dan endo-β-1,4-glukanase telah disahkan menggunakan qPCR dan tahap ekspresi mereka telah dibandingkan di antara kasta pekerja dan askar. Akhir sekali, salah satu daripada bakteria yang telah berjaya dipencilkan daripada gastrousus anai-anai iaitu *Bacillus licheniformis* telah digunakan untuk bioasai anai-anai. *Bacillus licheniformis* telah diinduksi untuk penghasilan kitinase dan ekstrak mentah enzim ini seterusnya digunakan sebagai biotermitisida melawan *G. sulphureus*. Keputusan menunjukkan, kitinase telah menyebabkan kematian ketara anai-anai selepas 48 jam apabila ia bersentuhan dengan kutikel anai-anai. Secara keseluruhan, tesis ini telah menyediakan pengetahuan asas mengenai sosiogenomik dan digestomik untuk *G. sulphureus* dan juga kaedah alternative untuk mengawal anai-anai.

DIVERSITY AND MOLECULAR CHARACTERIZATION OF TERMITE GUT SYMBIONTS IN *Globitermes sulphureus* Haviland (BLATTODEA: TERMITIDAE)

ABSTRACT

Globitermes sulphureus (Haviland) is a higher group termite and is known as a major pest in agricultural areas especially in coconut and palm oil plantations. *Globitermes sulphureus* was also found in infesting the wood building structure in rural and urban areas. Despite its pest status, the information about this cryptic insect in term of genetic and gut symbionts are still limited. In this study, the COII sequences of *G. sulphureus* was amplified to study the phylogenetic relationship between five termite colonies in the USM Penang campus. Maximum Likelihood (ML) and Neighbour-Joining (NJ) analysis showed identical topology and paraphyly of *G. sulphureus* species. The five colonies were sister groups with each other. Then, the gut symbionts from these five colonies were isolated using the culture-dependent method and identified using the 16S rRNA gene sequencing method. From the results, worker and soldier castes had almost similar bacterial diversity within the same colony but a little bit different if compared among five colonies. The most isolated bacteria phyla from five termite colonies were Firmicutes, followed by Proteobacteria and Actinobacteria. The gut symbionts of *G. sulphureus* were further characterized using a high-throughput sequencing; 16S metagenomic method. The V3-V4 region of the 16S rRNA gene was amplified and sequenced using the Illumina HiSeq 2500 platform. The sequencing output was analyzed using Qiime pipeline v1.9.1. The results showed the most dominant bacterial phyla were Spirochaetes, Fibrobacteres, Firmicutes, Proteobacteria, and Bacteroidetes. Furthermore, worker and soldier castes shared

similar bacterial phyla but different in bacterial genera. Other than gut symbionts, the genetic of the termite itself also were studied. The head of the worker caste was used for the *de novo* transcriptome using the Illumina HiSeq X10 sequencing machine. Reads were *de novo* assembled using the Trinity software and generated 243,057 transcripts and 193,344 putative genes. The transcripts were annotated using the Trinotate pipeline. In total, 27,061 transcripts were successfully annotated using BLASTX against the SwissProt database and 17,816 genes were assigned to 47,598 GO terms. 14,223 of transcripts were classified into COG classification, resulting in 25 groups of functional annotation. Next, a total of 12,194 genes were matched in the KEGG pathway and 392 metabolic pathways were predicted based on the annotation. The two cellulase genes; β-glucosidase and endo-β-1,4-glucanase were validated using qPCR and their expression level was compared between worker and soldier castes. Lastly, one of the isolated bacteria from termite gut which was *Bacillus licheniformis* was used for termite bioassay. *Bacillus licheniformis* was induced for chitinase production and this crude extract enzyme was later used as biotermiticide against *G. sulphureus*. The results showed the chitinase had caused significant termite mortality after 48 hours. Overall, this thesis has provided the basic knowledge of sociogenomics and digestomics of *G. sulphureus* and an alternative method for controlling this termite.

CHAPTER 1

INTRODUCTION

1.1   Background Study

Termite (Blattodea) is known as soil engineer and nutrient cycler as they consume organic matters, and then release back the mineral nutrients to the ecosystem through their feces, salivary secretions, corpses, and predators (Jouquet et al., 2011). However, from 3,106 species of termite in the world, about 371 of them reported as destructive and 104 are considered a serious threat (Krishna et al., 2013). In Malaysia, there are 7 genera (*Coptotermes*, *Macrotermes*, *Microtermes*, *Globitermes*, *Odontotermes*, *Schedorhinotermes*, and *Microcerotermes*) of the termites that have been reported as destructive pests in the literature (Ab Majid & Ahmad, 2011; Lee et al., 2007). Termite can consume a variety of food that contains cellulose such as woods, leaf litter, grass, soil, humus, dung, cloth, and paper (Krishna et al., 2013; Mikaelyan et al., 2015). The problems arise when the termites attacked the wood structure of the man-made buildings and the crops in the agriculture plantations. This destruction has caused economic losses in Malaysia and the cost of termite preventive control measures has reached almost 12 million (Kuswanto et al., 2015).

1.2   Problem of Statements

Globitermes sulphureus is a major pest in the agriculture plantations especially in the coconut and oil-palm plantations (Bakaruddin et al., 2018; Lee et al., 2003; Neoh et al., 2011). This species is also commonly found infesting the wood-building structures such as door and window frames (Ngee & Lee, 2002). Since the introduction of baiting systems, the occurrence of secondary pests in the infestations has increased. *Globitermes sulphureus* was found infested the same premises that previously have

been treated with termite bait (Lee et al., 2007; Neoh et al., 2011). Higher group termites such as *Macrotermes*, *Globitermes*, and *Odontotermes* are not easily eliminated with baiting systems (Iqbal et al., 2017; Lee et al., 2007). Due to this issue, the liquid chemical pesticide is preferable when controlling higher group termites. However, the chemical pesticide can give a negative impact on the environment such as water contamination, soil contamination, and it can hit non-target vegetation (Aktar et al., 2009). Thus, biological control could be an alternative to liquid pesticides. There are a variety of biological control agents including botanical extracts (Bakaruddin et al., 2018), fungi, bacteria, and viruses (Chouvenc et al., 2011a; Qasim et al., 2015; Verma et al., 2009).

Globitermes sulphureus (Haviland) is a higher group termite which only has bacteria and archaea in the gut. Unlike lower group termite where they have protists, bacteria, and archaea in their guts (Waidele et al., 2017). The gut microbes play an important role in cellulose and lignocellulose digestion. However, there is evidence that termite also produces their own cellulase in salivary glands and midgut epithelium (Franco Cairo et al., 2016; Lima et al., 2014; Ni & Tokuda, 2013). These abilities have attracted many scientists to conduct several types of research in order to understand the collaboration between the termites and their gut symbionts. Other than living in a caste system, the survivorship, and fitness of termite colonies are obligately dependent on association with the microbes. These microbes must be able to tolerate a variety of biological and chemical stressors as the termite host go foraging, feeding, and nesting. There are studies show that disruption of gut symbionts gave negative impacts on termite fertility, longevity, and fitness (Rosengaus et al., 2011; Sen et al., 2015). Therefore, it is crucial to include the gut symbionts in the termite study. Despite the status of *G. sulphureus* as a pest, the fundamental knowledge of this species is very

limited especially in terms of the composition of the bacteria in their gut and their genetic sequences. With this basic information, strategies for controlling this termite can be improved in the future.

1.3   Significant of the Study

The research was conducted to investigate the genetic diversity, the phylogenetic relationship of *G. sulphureus* and the bacterial species inhabit the termite gut within and between the *G. sulphureus* colonies in Universiti Sains Malaysia, Penang campus. The culture-dependent method and culture-independent method (16S metagenomic) were combined in order to get the overall bacterial community in this termite species. The gut bacteria (*Bacillus licheniformis* USMW10IK) isolated from *G. sulphureus* was chosen as biological agents against termite. In addition, the genomic sequence resources of *G. sulphureus* in the database are very scarce. Only a few sequences have been published of which correspond to mitochondrial (Bourguignon et al., 2017) or ribosome fragments (Yeap et al., 2007). The development of high-throughput sequencing such as Illumina sequencing has improved the sensitivity of gene profiling and speed up the process (Loman et al., 2012). Thus in this study, we used Illumina sequencing to characterize the head transcriptome of *G. sulphureus*.

1.4   Limitations of the Study

This study involved the use of high-throughput sequencing which was Illumina sequencing for microbial and genomic discoveries. This technology comes with particular limitations. High-throughput technologies generate a huge amount of data, which required specific analysis and correct interpretation that is relevant for the studies. In particular, the short-read sequences are sometimes hard to assemble or map.

Furthermore, the data obtained from next-generation sequencing technologies have certain computational requirements for their analysis. High computational resources and more complex bioinformatics analyses are necessary for bigger datasets generated. In addition, some of the analyses required a very long time to run and a large data storage for archive and processing the data.

1.5   Hypothesis

Different termite colonies and different termite castes have different microbial resided in termite guts. Different termite castes also have different expression levels of cellulase enzymes. Termite gut symbionts can be manipulated to kill its own host.

1.6   Objectives

This thesis aims to provide fundamental information of *G. sulphureus* regarding gut symbionts and it's genomic data. In addition, to provide an alternative method to control this termite using a biological agent. In order the achieve these, the following objectives were conducted:

Objective 1 (Chapter 3): To determine the phylogenetic relationships and genetic diversity of *G. sulphureus* populations in the USM main campus by using the COII gene as a genetic marker.

Objective 2 (Chapter 4): To isolate and identify the bacteria from the worker and soldier gut of *G. sulphureus* from five colonies using culture-dependent techniques.

Objective 3 (Chapter 5): To characterize the bacterial composition in the worker and soldier gut of *G. sulphureus* by using the culture-independent technique (16S metagenomic).

Objective 4 (Chapter 6): To characterize the worker's head of *G. sulphureus* using *de novo* transcriptome and to analyze the relative expression level of two cellulases genes between worker and soldier castes.

Objective 5 (Chapter 7): To produce crude chitinase enzyme from *Bacillus licheniformis* USMW10IK and to evaluate the virulence of this enzyme in killing termite.

CHAPTER 2

LITERATURE REVIEW

2.1   Termite

Termites are eusocial insects that fall under order Blattodea (previously order Isoptera) (Inward et al., 2007). The phylogenetic study has confirmed that termites were closely related to wood-eating cockroaches (genus *Cryptocercus*) (Inward et a., 2007). The termites are synapomorphies with *Cryptocercus*, for example, proctodeal trophallaxis, specific hindgut protists, xylophagy, and biparental care (Wang et al., 2017).

There are three types of termites in this world: dampwood, drywood, and subterranean. The dampwood termites (wood-dwellers) under family Termopsidae are usually eating rotten and wet wood and can only be found at a cold, high altitude (700-2500m) environment (Lacey et al., 2010). The drywood termites (wood-dwellers) under family Kalotermitidae are eating and nesting in the dry wood (arid environment) without necessary contact with soil (Thompson et al., 2000). This termite can be found in the southwestern and southern of the United States (Austin et al., 2012) and also Australia (Thompson et al., 2000). Whereas, the subterranean termites (ground-dwellers) must be connected with the ground as the soil provides moisture reservoirs (Kamble, 1991; Ptáček et al., 2013). This termites live and breed in the ground and some colonies nesting in trees or mounds (Krishna et al., 2013). The subterranean termites are widespread throughout tropical, subtropical, and temperate regions of the world (Kuswanto et al., 2015; Vargo & Husseneder, 2009).

6

From the three types of termites, the drywood and the subterranean termites were reported as a significant pest as they frequently attack human-made structures (Austin et al., 2012; Heather, 1971; Lee et al., 2007; Thompson et al., 2000).

2.1.1 Termite diversity

As of 2011 until 2013 there are about 3,106 species of termites are identified in the world, and these termites are classified into 12 recognized families. The families are (1) †Cratomastotermitidae, (2) Mastotermitidae, (3) †Termopsidae, (4) Hodotermitidae, (5) Archotermopsidae, (6) Stolotermitidae, (7) Kalotermitidae, (8) †Archeorhinotermitidae, (9) Stylotermitidae, (10) Rhinotermitidae, (11) Serritermitidae, and (12) Termitidae (Engel, 2011; Krishna et al., 2013). Daggers (†) indicate names proposed for fossil taxa. All the names presented here generally follow the format of Engel and Krishna (2004). The first 11 families are referred to as the lower termites, while the last one is known as the higher termites.

The †Cratomastotermitidae is a fossil family with one species was found in the Neotropical region in Brazil (Krishna et al., 2013). The Mastotermitidae is a primitive family with one sole wood-feeding species (*Mastotermes darwiniensis*) found only in Australia while other genera are extinct (Krishna et al., 2013; Ni & Tokuda, 2013). The †Termopsidae is a primitive family normally known as dampwood termites where they eat on wet dead logs (Lacey et al., 2010). The Hodotermitidae is a harvester termite that primarily feeds on dry grass but sometimes it also consumes trees and shrub materials (Symes & Woodborne, 2011). The Archotermopsidae and Stolotermitidae were long considered a single family, the Termopsidae. However, the phylogenetic analysis has led the Archotermopsidae as a family name for northern termopsids and the Stolotermitidae for southern termopsids (Cameron et al., 2012;

Engel et al., 2009). Unlike Termopsidae, Kalotermitidae prefers dry habitats and dry woods for a living (Li et al., 2015; Thompson et al., 2000). Archeorhinotermitinae was previously a primitive subfamily in Rhinotermitidae, and later was removed and put under the Archeorhinotermitidae family (Engel et al., 2009; Krishna & Grimaldi, 2003). The Stylotermitidae consists of one extant genus *Stylotermes* and two fossil genera *Parastylotermes* and *Prostylotermes* (Wu et al., 2018). *Stylotermes* and *Parastylotermes* have been classified under Rhinotermitidae but later changed to Stylotermitidae because of their distinctive trimerous tarsi (Liang et al., 2017). The phylogenetic analysis also has shown that Stylotermitidae as a sister group of all remaining Neoisoptera (Rhinotermitidae, Serritermitidae, and Termitidae) (Wu et al., 2018). Rhinotermitidae consumes woods and three genera from this family were reported as the most invasive pest which is *Reticulitermes*, *Heterotermes*, and *Coptotermes* (Evans et al., 2013). Serritermitidae contains two genera: *Serritermes* and *Glossotermes* which are all single-piece nesters (eating and nesting in single wood) and found only in Brazil (Bourguignon et al., 2014; Emerson & Krishna, 1975; Hanus et al., 2012). Termitidae representing over 85% of all termite genera, and the most ecological and behavioral diversity of all termite families (Krishna et al. 2013).

Termitidae consists of eight subfamilies, namely Macrotermitinae, Sphaerotermitinae, Foraminitermitinae, Apicotermitinae, Cubitermitinae, Termitinae, Nasutitermitinae, and Syntermitinae (Bourguignon et al., 2014; Ni & Tokuda, 2013).

2.1.2 Caste system in termites

Termites are eusocial insects with a well-developed caste system. Eusocial insects are true insects with three characteristics: cooperative brood care, the overlap of two or more generations with offspring assisting with brood care, and reproductive

division of labor (Zablotny, 2009). Castes of termites are divided into two types of individuals: fertile (reproductives) and sterile individuals (neuters). The sterile castes consist of workers, pre-soldiers, and soldiers, while the fertile ones are primary reproductive (also alates) and secondary reproductive (Watanabe et al., 2014). Every caste has different morphologies specialized in tasks that are allocated in the colonies.

The primary reproductive is consists of a king, queen and fully developed winged adults (alates). The reproductive caste possesses compound eyes, fully developed wings and body pigmentation (Vargo, 2019). The queen is responsible for laying eggs for the colony while the king is mating with the queen for life (Keller, 1998). The queen has a swollen abdomen for the mass production of eggs (Horwood, 2005). The alates (females and males) are responsible for establishing new colonies soon after the nuptial flight (Horwood, 2005; Vargo, 2019).

The secondary reproductive or neotenic (usually found in subterranean termite: Rhinotermitidae) is produced upon the death of the primary king and queen (Costa-Leonardo et al., 2004; Maekawa et al., 2008; Matsuura et al., 2010). There are two types of neotenic: brachypterous neotenic and apterous neotenic. Brachypterous neotenic are nymph-derived neotenic, have wing pads whereas apterous neotenic are worker-derived neotenic without wing pads (Matsuura et al., 2010; Vargo, 2019).

The soldier caste has a big-headed structure and large mandible and varies according to species. The scientific identification (taxonomy) of termite species usually based on the characteristics of the soldier caste (Horwood, 2005). Soldiers are wingless and blind and the main function is to defend the colony from the enemies especially ants (Horwood, 2005; Zablotny, 2009). Some species of termites

(*Macrotermes* spp.) possess minor and major soldiers and both of them are female sterile castes (Neoh & Lee, 2009).

The worker caste is the most abundant caste in the termite colony. Workers are responsible for brood, nest maintenance, foraging for foods, feeding other castes, and waste removal (Horwood, 2005; Zablotny, 2009). Like soldiers, workers also blind and wingless (Ptáček et al., 2013). Workers can be differentiated into three, two or one types or worker caste depending on the species. *Hospitalitermes medioflavus* (Holmgren) and *H. umbrinus* (Haviland), have three worker castes (minor, medium and major) that perform different tasks during food harvesting (Cornelius, 2012). In the fungus-growing termite, *Macrotermes gilvus* and *M. carbonarius* have two worker caste: major workers (male and larger) and minor workers (female and smaller) (Neoh & Lee, 2009). Besides that, drywood and dampwood termites have no true workers (also known as pseudergates) in their colonies (Zablotny, 2009). The pseudergates are developed from late-instar larvae, and may later develop into secondary reproductive or soldiers (Watanabe et al., 2014). The damage to the structural wood is performed by the worker caste.

2.1.3 Life cycle

Termites are diploid hemimetabolous (incomplete metamorphosis) unlike order Hymenoptera such as ants, bees, and wasps that undergo holometabolous (complete metamorphosis) (Korb et al., 2015). Hemimetabolous is having several larval stages that molting becomes more adult-like with each transition, while holometabolous is having the final larval stage develops via a pupa into adulthood (Terrapon et al., 2014). Worker termites are diploid individuals of males and females sexes and develop from fertilized eggs. In contrast, the workers in Hymenoptera are

females (diploid) and develop from fertilized eggs while drones are males (haploid) and develop from unfertilized eggs (Korb, 2008). Depending on species, male and female workers may have different roles in a termite colony (Cornelius, 2012).

The developmental pathways of termites are divided into two patterns: a linear pathway or a bifurcated pathway (Fig. 2.1) (Watanabe et al., 2014). The family Kalotermitidae employs the linear pathway and other families such as Mastotermitidae, Termitidae, and Rhinotermitidae utilize the bifurcated pathway (Maekawa et al., 2008). The bifurcated pathway is divided into lines: the 'imaginal' and 'apterous' lines. The life cycle of a termite begins with eggs, then the larvae are hatched from the eggs. These larvae develop into workers or nymphs. In the 'imaginal' line, nymphs develop into alates equipped with wings and eyes that disperse and become as primarily reproductive or nymphs develop into secondary reproductives. The 'apterous' line, workers can transform into secondary reproductive, remain workers, or become presoldiers that later molt into soldiers (Vargo & Husseneder, 2009).
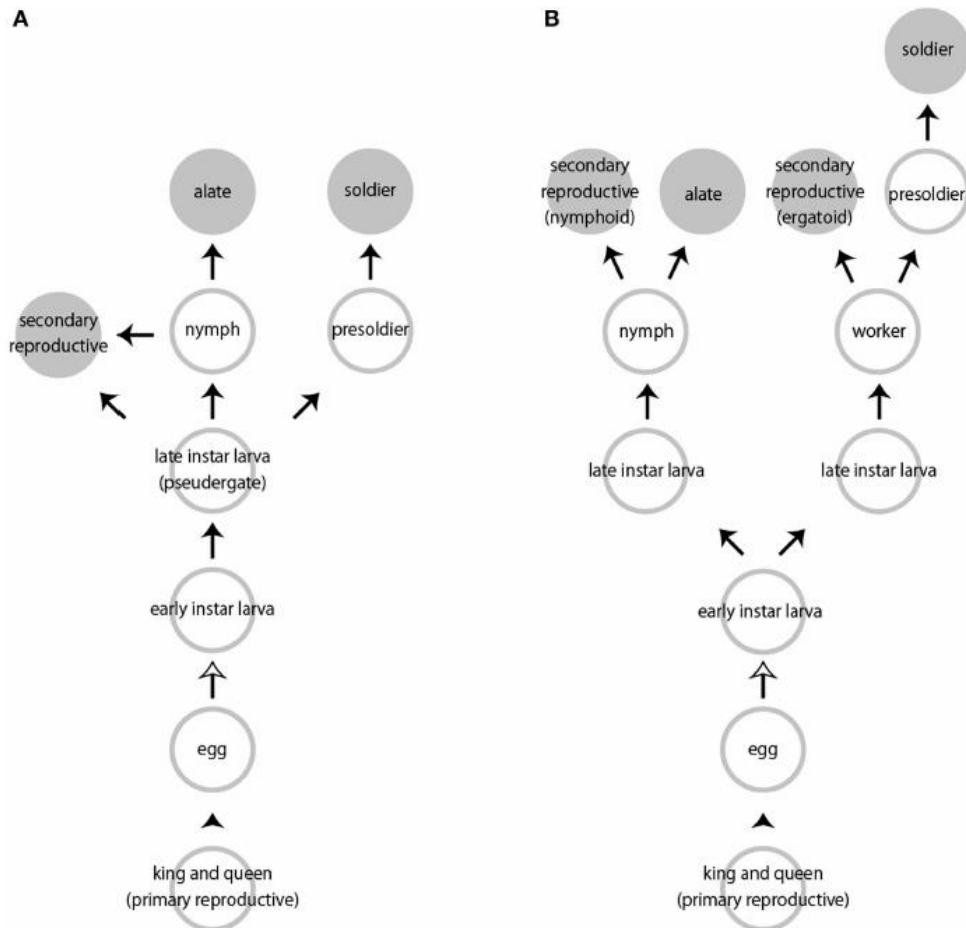
Figure 2.1: Diagram of life-cycle in termites. (A) Linear pathway. (B) Bifurcated pathway. Arrowheads indicate mating and oviposition. White and black arrows indicate hatching and molts, respectively. Not all pseudergates or workers molt into subsequent developmental stages. Reproduced from Watanabe et al., (2014).

2.1.4   Termite Nest

A termite colony can be differentiated either as wood-dwellers (damp- and drywood termites) or ground-dwellers (subterranean termites). The wood-dwellers termites nesting in the same wood where they are eating without necessary contact with soil. On the contrary, the ground-dwellers need soil for living and breeding (Ptáček et al., 2013). Subterranean termites construct runways of soil (also known as mud tube) and some are builders of spectacular mounds (Kuswanto et al., 2015).

Termite nests have a variety of sizes and forms according to species and colony age. They can be located completely underground, above the ground (arboreal) such as trees and buildings where they maintain the connection with the soil through mud tubes, and on the surface of the ground (epigeal) (Aiman Hanis & Abu Hassan, 2011; Claggett et al., 2018; Horwood, 2005; Kuswanto et al., 2015).

Termite nest is important for humidity, protection against enemies (such as ants) and rain, for keeping food and provide proper temperature (Jouquet et al., 2011; Korb, 2011; Noirot & Darlington, 2014; Ptáček et al., 2013). Building materials of the nest are made from partly digested plant materials, wood, and soil that mixed with saliva and feces, depending upon the termite species (Fall et al., 2007; Noirot & Darlington, 2000).

2.1.5 Feeding habits

Termites are well-known as efficient lignocellulose decomposers in the ecosystems (Ni & Tokuda, 2013; Ohkuma, 2003; Scharf et al., 2011; Wang et al., 2016). Their ability to degrade lignocellulose is based on the collaboration of the termites as host with the microbial symbionts harbored in their intestinal tracts (Franco Cairo et al., 2016; Scharf et al., 2011).

Lower termites possess cellulolytic protists, archaea, and bacteria while higher termites lack protists and contain exclusively archaea, bacteria and fungi in the hindgut (Mikaelyan et al., 2017; Rossmassler et al., 2015). Lower termites have a relatively short and simple compartment of guts and look similar to those of cockroaches and fungus-cultivating higher termites (such as Macrotermitinae) in both morphology and physicochemical conditions. In other higher termites subfamilies, the hindgut is longer and more compartmentalized (Brune, 2014). With the simple gut of lower termites,

their normal food is wood. Whereas higher termites have shown variation in their feeding behavior.

Majority of higher termites feeding on the soil (Apicotermitinae and Termitinae) (Mikaelyan et al., 2015). Some feed on lignocellulose including sound or decayed wood or dry grass (Termitinae, Nasutitermitinae), others on litter or humus or herbivore dung (Termitinae, Nasutitermitinae, and Syntermitinae) (Bujang et al., 2014; Mikaelyan et al., 2015). Whereas Macrotermitinae is a fungus-growing termite, where they cultivate and consume basidiomycete fungi (Termitomyces sp.) in their nests (Bourguignon et al., 2014; Mikaelyan et al., 2015; Ohkuma et al., 1999).

2.1.6 Termite and their gut symbionts

Termites have a close association with microbes in their intestinal system. Lower termites harbored flagellated protists (unicellular eukaryotes), bacteria, and archaea in their hindgut while higher termites typically lack flagellated protists (Peterson & Scharf, 2016a). The mutual relationship between termites and their gut symbionts produce essential nutrients and substrates for energy metabolism and that sustain both the symbionts and their host (Nalepa, 2015). The lignocellulose requires a set of enzymes for catalyzing its breakdown. There are studies reported that termites have synergistically collaborated with their symbionts for lignocellulose digestion (König et al., 2013; Scharf et al., 2011; Scharf & Tartar, 2008; Tartar et al., 2009).

Termites produce several enzymes glycosyl hydrolases (GH1 and GH9) such as β-glucosidase, endo-β-1,4-endoglucanase, and protist in lower termites hindgut produces enzymes exo-glucanase (GH7), endoglucanase (GH5 and GH45), and xylanase (GH10, and GH11) (Franco Cairo et al., 2016). These enzymes are important in cellulolytic activity. In higher termites, the cellulolytic activity is more variable

depending on the ecological and feeding-habit of the termite species (Ni & Tokuda, 2013). The digestive enzymes are encoded by the termite genome or produced by gut symbiotic bacteria, such as Spirochetes and Fibrobacters (Warnecke et al., 2007). Metagenomic analysis has reported the presence of diverse cellulase genes in the hindgut microflora of higher termites *Nasutitermes* spp. such as GH5, GH8, GH9, GH44, GH45, GH51, and GH74 that acting as endoglucanase, whereas GH1 and GH3 participated in beta-glucosidase (Warnecke et al., 2007).

Other than lignocellulose digestion, gut symbionts are needed for diverse metabolic processes including nitrogen fixation, acetogenesis, methanogenesis, and degradation of lignin phenolics (Peterson & Scharf, 2016a). Besides that, despite grooming and hygienic behavior of termite, symbiotic bacteria provide additional protection against pathogens and parasites for termite immunity (Peterson & Scharf, 2016a). Furthermore, disruption of gut symbionts with an antibiotic has reduced the longevity and fecundity of termites (Rosengaus et al., 2011).

The intestinal tract of termites is basically divided into three parts; foregut, midgut, and hindgut. The hindgut has several dilated compartments that harbored a diverse gut microbiota (Berlanga, 2015). The diversity of hindgut microbiota depends on several factors, including microecology of the termite gut (oxygen, hydrogen and pH level, the structure of the gut, and redox gradient), diet, habitat, and phylogeny of the termite (Abdul Rahman et al., 2015; Berlanga, 2015; Brune & Friedrich, 2000; Köhler et al., 2012; Mikaelyan et al., 2015; Su et al., 2016).

Every caste of termites possesses microbial symbionts in their guts but the composition of the bacterial community is different. The alate caste of *Reticulitermes flavipes* has a lower abundance of *Treponema* and *Endomicrobia* compared to worker

caste (Benjamino & Graf, 2016). The soldier caste of *Coptotermes formosanus* has a lower abundance and diversity of Bacteroidetes and slightly higher in Spirochaetes when compared to worker caste (Xiang et al., 2012). Furthermore, comparison of bacterial symbiont among castes and ages of fungus-growing termite *Macrotermes gilvu*s have shown that clustering pattern was clearly more related to a difference in age than to caste (Hongoh et al., 2006). The newly molted worker, young and old worker of this termites showed significant differences in the composition of the bacterial community in their gut (Hongoh et al., 2006). These data suggest that the gut microbiota has co-evolved with the termite host and was shaped according to termite taxonomy, diet, caste, and age within a termite species (Benjamino & Graf, 2016; Hongoh et al., 2006).

Additionally, the acquisition of gut symbionts in termites can be acquired through (i) vertical transmission from parent to offspring, (ii) horizontal transmission from colony-to-colony transfer, and (iii) "mixed-mode" transmission which combined previous two transmissions (Bourguignon et al., 2018). Maintaining of microbial populations in the guts of individual termites can be achieved via proctodeal and stomodeal trophallaxis and by the obligatory recolonization after molting. Worker caste transfer food via stomodeal (by regurgitation) and/or proctodeal (by excretion of the hindgut contents) containing microbes to other colony mates and castes (Abdul Rahman et al., 2015; Berlanga, 2015).

2.1.7 Pest Species and Their Economic Importance

Termites are important decomposers in the ecosystems as they digest wood and other plant materials. They contribute significantly to the nutrient turnover and aeration of soil as they tunneling (Rust & Su, 2012; Verma et al., 2009). However,

some species of termites have become important pests when they feed on wooden structures of humans' buildings or furniture, agricultural crops and plantations, forests, and other commercial products (Evans et al., 2015; Kuswanto et al., 2015).

As in 2013, there are about 371 of termites species have been reported as destructive and only 104 of them are considered as serious pests (Krishna et al., 2013). Four of termite families are considered to be economically important: Kalotermitidae, Hodotermitidae, Rhinotermitidae, and Termitidae (Qasim et al., 2015). Subterranean termite pests accounted for 38 of the serious pests, with the genus *Coptotermes* containing the largest number of species (18 spp.) followed by *Macrotermes*, *Reticulitermes* and *Odontotermes* (Kuswanto et al., 2015; Rust & Su, 2012). For drywood termite (Kalotermitidae), the majority of pests belong to the genus *Cryptotermes* (eight species are considered serious pests), followed by *Incisitermes* spp. (Rust & Su, 2012).

The most notorious pests are generally introduced into new geographical areas, usually caused by human activity, and become invasive (Krishna et al., 2013). South and Southeast Asia (Indo-Malaya) are the source of the highest number of invasive species (seven), followed by South America (six), Australia (five), Africa (two), North America (two), the Caribbean Islands (one), East Asia (one), and Europe (one) (Evans et al., 2013). All invasive termites typically eat woods, nesting in wood, and easily produce the secondary reproductives which combined to create propagules for transport (Evans et al., 2013). Most of the woods are traded and transported from the tropical regions of South and Southeast Asia (Indo-Malaya) and South America (Evans et al., 2013).

The global damage caused by termites was estimated at the US $ 22 billion to US $ 40 billion worldwide (Rust & Su, 2012). In Malaysia, the cost of termite control in 2003 was USD 10-12 million (Kuswanto et al., 2015). Subterranean termites attack accounted for 90% of the total economic loss and about 70% of the damage to the construction (Kuswanto et al., 2015).

2.2 *Globitermes sulphureus*

Globitermes sulphureus (Haviland) under subfamily Termitinae is a subterranean termite species and commonly found in the Southeast Asia region. *G. sulphureus* was first identified by George D. Haviland. Haviland was the curator of the Kuching Museum and contributor to the Kew Herbarium (Krishna et al., 2013).

2.2.1 Characteristics of *Globitermes sulphureus*

Globitermes sulphureus is a higher group termite that can be easily recognized by the bright yellow colored abdomen of its soldier termite (Fig. 2.2) due to the defensive secretion in a frontal gland. This defensive gland is separated from the salivary gland and located from thorax until the abdominal cavity (Bordereau et al., 1997). The soldiers of *G. sulphureus* use autothysis mechanism where this defensive secretion exudes after the rupture of the glandular wall and the integument (Bordereau et al., 1997). In addition, the soldiers also use their large mandible as defensive behavior against their enemies.

Globitermes sulphureus is a mound-building termite that forms a dome-shaped structure (Fig. 2.2) with a smooth outer space of mound (Ahmad, 1965). The mound can be up to 80 cm in height and 60 cm in diameter (Neoh et al., 2011). *Globitermes sulphureus* can go foraging distance from 3.5 m up to 16 m and the foraging area can

reach 530.40 m2 depending on the foraging populations (Ab Majid & Ahmad, 2011; Lee et al., & Jaal, 2003; Ngee & Lee, 2002).

Figure 2.2: *Globitermes sulphureus* colony: a) mound, b) arrows 1 and 2: workers, c) arrow 3: presoldier and arrow 4: soldier, d) arrow 5: nymphs, and e) queen.

2.2.2 *Globitermus sulphureus* as pests

Globitermes sulphureus was reported as an economic pest in Malaysia, Singapore, Thailand, and Vietnam (Ahmad, 1965; Bordereau et al., 1997; Kuswanto et al., 2015; Lee et al., 2007; Ngee & Lee, 2002). It is a significant pest in agricultural crops especially coconut and palm oil plantations (Ab Majid & Ahmad, 2011; Lee et al., 2003; Neoh et al., 2011). Although *G. sulphureus* is an important pest in agriculture, this species has been found infested the residential premises and man-made structures in the rural, disturbed forest (developed for ecotourism), and urban areas (Aiman Hanis et al., 2014; Lee et al., 2007; Lee et al., 2003; Ab Majid & Ahmad,

2009). Most of the infested urban areas come from plantations areas that have been cleared and developed to become residential premises and complexes (Ngee & Lee, 2002). *G. sulphureus* was found infesting door, window frames, wooden walls, and wooden pillars, and carpet grippers (Ab Majid & Ahmad, 2009; Ngee & Lee, 2002). Besides that, this species was also found in premises that previously been treated with termite bait to eliminate *Coptotermes* spp. (Neoh et al., 2011).

2.2.3   Controlling the colonies of *Globitermus sulphureus*

Previously, several data have been published on controlling *G. sulphureus* using chemical termiticides, baiting, biological controls, and plant extracts. The research using imidacloprid (nonrepellent termiticide) have shown that *G. sulphureus* colony was successfully eliminated after six weeks of the treatment (Ab Majid et al., 2007) while Fipronil (slow-acting nonrepellent termiticide) managed to ceased the colony after three weeks of the treatment (Ab Majid & Ahmad, 2011). Baiting systems using chlorfluazuron (chitin synthesis inhibitor) conducted by Peters et al., (2005) and (2008) have taken around four months to eliminate the *G. sulphureus* colonies. This results in similar to data reported by Neoh et al., (2011) when they used Bistrifluron as bait.

Even though chemical termiticides have been proven in termite colonies elimination, their use has declined due to awareness of environmental and health issues (Evans & Iqbal, 2015). In addition, difficulties in creating and maintaining a soil barrier system can cause failure in termite control (Ab Majid & Ahmad, 2011; Peters et al., 2008). Therefore, multiple commercial baiting systems are now available in the markets as an alternative to the chemical termiticides (Evans & Iqbal, 2015). The baiting system inhibits the synthesis of chitin during termite molting (Dhang, 2011).

However, few studies have reported that baiting has been less effective against higher termites. Higher termites required more total bait consumption and longer time for elimination than that required of rhinotermitids (Dhang, 2011; Lee et al., 2014; Neoh et al., 2011; Peters et al., 2008).

Above all methods, the biological agents and plant extracts have also been considered for termite control. Biological agents consist of bacteria, fungi, and nematodes (Verma et al., 2009). A few studies have reported termite mortality when using fungi and plant extracts for controlling *G. sulphureus* (Azmi et al., 2016; Bakaruddin et al., 2018). However, how far these biological agents and plants extracts can be used in the field remain unclear.

2.3   Phylogenetic tree

A phylogenetic tree, also known as a phylogeny, is a diagram that represents evolutionary relationships of different species, organisms, or genes that derive from a common ancestor (Baum, 2008). Phylogenetic trees are reconstructed by a method called "phylogenetic systematics" where it clusters groups of organisms together based upon shared, derived characters from their ancestors called synapomorphies (McLennan, 2010). Phylogenetic trees are used for 1: understanding the relationships among the species represented by sequences (DNA or protein sequences) 2: understand the pattern and processes of evolution, and 3: estimate time of divergence between a group of organisms that share a common ancestor (Baum, 2008; Hall, 2013; Soltis & Soltis, 2003).

There are four distinct steps for building a phylogenetic tree: (i) identify and acquire a set of homologous DNA or protein sequences, (ii) align those sequences, (iii) estimate a tree from the aligned sequences, and (iv) present that tree (Hall, 2013).

21

Several approaches can be used in phylogeny reconstruction of molecular sequences: maximum parsimony (MP), maximum likelihood (ML), distance-based methods such as neighbor-joining (NJ), and Bayesian inference (BI) (Soltis & Soltis, 2003). All of these methods have strengths and weaknesses.

Phylogenetics has been widely used in termite studies. For example, understanding the relationship of termite symbionts with the roles and diet of termite hosts based on 16S rRNA genes identification (Abdul Rahman et al., 2015; Rossmassler et al., 2015; Tai et al., 2015). Then, phylogenetic relationships among termite species by using cytochrome oxidase I and II genes, or mitochondrial genes: 12S rRNA, 16S rRNA, 28S rRNA (Li et al., 2009; Liang et al., 2017; Murthy et al., 2015; Singla et al., 2016; Thompson et al., 2000; Yeap et al., 2007). Also, comparing the phylogenetics of termite symbionts with termite nests (Fall et al., 2007) and some researchers focused on phylogenetic of specific genes such as nitrogen fixation (Ohkuma et al., 1999), and endo-β-1,4-glucanase (Bujang et al., 2014).

2.4   Cytochrome oxidase II (COII) gene

Cytochrome c oxidase is a protein in the mitochondria function as the terminal complex in the respiratory chain (Lunt et al., 1996; Soto et al., 2012; Zhang et al., 2013). Cytochrome c oxidase is composed of three mitochondria-encoded subunits (I–III) and four subunits encoded by the nuclear genome (Frati et al., 1997). Mitochondrial cytochrome oxidase I, II (COI, COII) genes are suitable as molecular markers because of the rapid evolution compared to nuclear genes especially the COII gene (Yeap et al., 2007). Thus these genes have a relatively high degree of genetic variation and therefore good markers for phylogenetic studies such as species relationships within a genus (Aly et al., 2012; Singla et al., 2016).

The COII gene has been used for many phylogenetic studies of termites from different geographical areas (Singla et al., 2016). For example, *Coptotermes gestroi* and *Coptotermes vastator* from Malaysia, Singapore, Thailand, Indonesia, Japan, and the Philippines (Yeap et al., 2007). *Coptotermes formosanus* and *C. gestro*i in Taiwan (Li et al., 2009), *Reticulitermes urbis* in Europe (Leniaud et al., 2010) and drywood termites in Australia (Thompson et al., 2000).

2.5 Culture-dependent method

The culture-dependent method is typically used for screening the cultivable microorganisms. This method involved several techniques for the isolation of individual microorganisms species such as bacteria and fungi in pure culture. The techniques are serial dilutions, spread plate method, pour plate method, and streak plate method (Sanders, 2012). The pure culture of individual colonies is later used for species identification by using a conventional method which is a series of biochemical test or proceeds with molecular techniques: DNA extraction, polymerase chain reaction (PCR), and sequencing (Ramin et al., 2009; Ramin et al., 2008). Culture-based methods require sterile workplaces, solutions, agar media, and equipment to avoid possible contamination from the laboratory (Sanders, 2012).

2.5.1 Importance of cultivable microorganisms

Cultivation of microorganisms is essential because, it enabling us to characterize the morphological of individual cells as well as to determine their metabolism and biochemical properties (Collin & Lyne, 2004). Pure culture will be an invaluable asset in public health, agricultural, and industrial sectors. For example, live bacteria from the clinical samples are useful for searching the virulence or antibiotic resistance traits in order to cure the patient from infectious diseases (ten Hove et al.,

2017). Whereas, bacteria isolated from the termite gut of *Coptotermus formosanus* were used for paratransgenesis research in order to control termite populations (Tikhe et al., 2017). In addition, polyhydroxyalkanoate (PHA) accumulating bacteria isolated from the gut of *Macrotermes carbonarius* were evaluated for their potential use in the bioplastics production (Tay et al., 2010).

2.5.2   Limitations of culture-dependent methods

Growing of microorganisms in the laboratory is highly dependent on the medium used for isolation and the incubation conditions. Nutrient agar/broth generally can grow typical genera clinically important microorganisms (Collin & Lyne, 2004). However certain bacteria such as slow-growing and fast-growing need specific nutrients, pH conditions, incubation temperatures or levels of oxygen in the atmosphere (Vartoukian et al., 2010). Moreover, some bacteria may also be outcompeted when a mixed culture present in the same media (Ndoye et al., 2011). Hence, multiple methods of cultivation should be used to present the actual microbial diversity from the environment. Furthermore, the identification until species level requires multiple series of biochemical tests, thus culture-based techniques are very time consuming and tedious processes (Ndoye et al., 2011; Vartoukian et al., 2010).

2.6   Culture-independent methods

From the total populations of bacteria on Earth, only 1% of them can be cultivated in vitro and most of the microorganisms are hardly distinguishable based on the morphology (Rahman, 2015; Vartoukian et al., 2010). The emergence of ribosomal RNA genes as molecular markers (Woese and Fox, 1977) and in conjunction with the Sanger sequencing in 1977 (Sanger et al., 1977) had revolutionized the study of microbial ecology (Escobar-Zepeda et al., 2015). The cultivation of the microbial is