THE EFFECT OF CHITIN SYNTHESIS INHIBITORS (CSI) ON THREE SUBTERRANEAN TERMITE SPECIES (BLATTODEA: RHINOTERMITIDAE, TERMITIDAE) TOWARDS THE TERMITE GUT SYMBIONTS

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by

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%	Percentage
°C	Degree Celsius
△	Increase
▽	Decrease
1X	One-fold concentration
bp	Base pair
cm	Centimeter
E	To the east (coordinate)
g	Gram
L	Liter
ml	Milliliter
µl	Microliter
N	To the north (coordinate)
w/w	Weight concentration of a solution

LIST OF ABBREVIATIONS

AIs	Active ingredients
ANOVA	Analysis of variance test
BLAST	Basic local alignment search tool
BLASTN	Basic local alignment search tool
CHOU1	Species richness measurement calculation
CSI	Chitin Synthesis Inhibitor
DNA	Deoxyribonucleic acid
ddNTPs	Dideoxyribonucleotides
FASTA	Sequence similarity searching tool
FLASH	Fast length adjustment of short reads tool
HTS	High-throughput sequencing technology
IBM SPSS	Statistical analysis tool
NGS	Next-generation sequencing
OTUs	Operational taxonomic units
PCR	Polymerase chain reaction
QIIME	Microbiome bioinformatic platform
rDNA	Ribosomal deoxyribonucleic acid
USM	Universiti Sains Malaysia
WGS	Whole-genome sequencing

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KESAN PERENCAT SINTESIS KITIN (CSI) TERHADAP TIGA SPESIES ANAI-ANAI BAWAH TANAH (BLATTODEA: RHINOTERMITIDAE, TERMITIDAE) TERHADAP SIMBION USUS ANAI-ANAI

ABSTRAK

Serangan anai-anai bawah tanah pada awalnya dijumpai selepas penubuhan ladang getah pada abad ke-20 di Semenanjung Malaysia. Setelah itu, penyelidikan tentang anai-anai melonjak naik dan Perencat Sintesis Kitin (CSIs) telah diperkenalkan sebagai salah satu agen untuk membasmi anai-anai daripada kelas atas dan kelas bawah. Kebanyakan terbitan mengenai penyelidikan tersebut tertumpu kepada tahap keberkesanan dalam pembasmian koloni dengan tiadanya anai-anai di dalam umpan dan stesen pemantauan. Namun, kajian seperti penilaian aktiviti pemakanan dan kematian dengan kaedah biocerakin makmal dan pengaruh agen tersebut terhadap komposisi simbion isi perut anai-anai adalah terhad. Lantaran itu, penyelidikan ini dilaksanakan pada anai-anai kelas bawah (*Coptotermes gestroi*) dan anai-anai kelas atas (*Globitermes sulphureus* dan *Macrotermes gilvus*) menggunakan tiga umpan CSIs dalam penilaian biocerakin 'Tiada pilihan'. Setiap umpan ini juga mengandungi bahan aktif yang tersendiri dan digunakan secara meluas oleh syarikat pembasmian haiwan perosak seperti chlorfluazuron, bistrifluron dan hexaflumuron. Dapatan daripada pemerhatian aktiviti pemakanan dahulunya dikesan pada umpan yang dimakan oleh *C. gestroi*, kemudian diikuti pada umpan oleh *G. sulphureus* dan akhir sekali oleh *M. gilvus*. Hasil analisis tersebut menyatakan bahawa perbandingan aktiviti pemakanan antara spesies anai-anai yang telah dirawat berbeza secara signifikan ($p < 0.05$). Namun hal ini tidak bagi umpan chlorfluazuron yang mana hanya *C. gestroi*

menunjukan perbezaan secara signifikan daripada spesies yang lain ($p < 0.05$). Seterusnya, hasil tindak balas daripada semua umpan mendedahkan purata kematian tertinggi dimiliki oleh *M. gilvus*, kemudian diikuti oleh *C. gestroi*, dan akhir sekali oleh *G. sulphureus*. Dapatan hasil kajian juga mendapati purata kematian antara semua spesies anai-anai yang dirawat adalah berbeza secara signifikan ($p < 0.05$). Namun begitu, hal ini berkecuali bagi anai-anai yang dirawat menggunakan hexaflumuron. Dapatan analisis menggunakan HSD Tukey kemudian mendedahkan hanya purata kematian anai-anai *G. sulphureus* dan *M. gilvus* adalah berbeza secara signifikan ($p < 0.05$) daripada hasil tindak balas umpan chlorfluazuron dan bistrifluron. Seterusnya, pengaruh umpan CSI pada komposisi simbion isi perut anai-anai telah disiasat menerusi kaedah kebergantungan kultur dan empat filum didapati memiki komposisi yang tertinggi. Antara filum tersebut ialah Proteobakteria dan diikuti oleh Firmikutes. Empat puluh tujuh spesies bakteria juga telah dikenal pasti dan perbandingan bilangan bakteria pada plat agar antara semua anai-anai yang dirawat dan tidak dirawat didapati tidak konsisten. Antara spesies anai-anai yang telah dirawat dan memiliki bilangan bakteria pada plat agar yang berkurangan secara signifikan ($p < 0.05$) ialah *C. gestroi* daripada hasil tindak balas chlorfluazuron dan bistrifluron, serta *G. sulphureus* daripada hasil tindak balas oleh kesemua umpan. Selanjutnya, simbion isi perut daripada semua anai-anai dirawat dan tidak dirawat telah dianalisis menggunakan penjujukan daya pemprosesan tinggi (16S Illumina metagenomik bakteria) yang menyasarkan kawasan hiper boleh berubah iaitu V3 hingga V4. Data yang telah dikuantifikasikan menggunakan analisis bioinformatik menampilkan perbezaan yang signifikan dalam kepelbagaian Unit Operasi Taksonomi (OTU) antara semua spesies anai-anai dirawat dan tidak dirawat. Terdapat juga ketidakselarasan dalam kelimpahan relatif filum yang dikongsi bersama. Antara filum yang berbeza secara signifikan itu

ialah Aktinobakteria, Desalfobakterota, Proteobakteria dalam *C. gestroi*, Proteobakteria dan Rs-k70 kumpulan anai-anai dalam *G. sulphureus*, serta Desalfobakterota dan Proteobakteria dalam *M. gilvus*. Tambahan pula, antara bakteria yang memiliki komposisi tertinggi ialah *Azobacteroides* sp. dalam *C. gestroi*, *Anaerovorax* sp. dalam *G. sulphureus* dan *Alistipes* sp. dalam *M. gilvus*. Hasil penyelidikan ini mewartakan maklumat asas yang sebelumnya tidak diketahui akan kesan terhadap simbion isi perut apabila anai-anai dipengaruhi oleh rawatan pengumpanan PSK dan penambahbaikan dalam formula PSK bagi membasmi anai-anai daripada pelbagai kelas.

THE EFFECT OF CHITIN SYNTHESIS INHIBITORS (CSI) ON THREE SUBTERRANEAN TERMITE SPECIES (BLATTODEA: RHINOTERMITIDAE, TERMITIDAE) TOWARDS THE TERMITE GUT SYMBIONTS

ABSTRACT

The infestation subterranean termites were first encountered after establishment of rubber plantation during 20st century in Peninsular of Malaysia. Subsequently, interest has been devoted in termite research and Chitin Synthesis Inhibitors (CSI) has been introduced as one of numerous agents to against lower and higher termite infestation. Majority of published studies focused on efficacy and considering colony elimination in the absence of termites in the bait and monitoring station. However, there was dearth of studies assessing the impact on the feeding activity and mortality in laboratory bioassay, and the bait influence towards gut symbiont composition. Therefore, this study was performed on the lower (*Coptotermes gestroi*) and higher termites (*Globitermes sulphureus* and *Macrotermes gilvus*), using three CSI baits through 'No-choice' bioassay. Each of these baits also contained respective active ingredients which is widely used by pest companies in Malaysia, and they are chlorfluazuron, bistrifluron, and hexaflumuron. The feeding activity was initially detected in *C. gestroi*, followed by *G. sulphureus*, and lastly *M. gilvus*. The results determined that there were substantial differences ($p < 0.05$) in the feeding activity among the treated termites. However, unlike chlorfluazuron, only *C. gestroi* was differed significantly from the other treated species. In response to all baits, *M. gilvus* exhibited the highest mean mortality, followed by *C. gestroi*, and *G. sulphureus*. Except for hexaflumuron, the mean mortality of all treated termite species showed significant differences across all baits ($p < 0.05$). Tukey's HSD further revealed there were significant differences in the responses of only *G. sulphureus* and *M. gilvus* to chlorfluazuron and bistrifluron ($p < 0.05$). Determination on the effect of CSI baits upon the gut symbionts between untreated control and bait treated termites was performed via culture-dependent method. Four phyla were identified, with Proteobacteria being the most abundant and Firmicutes being the second most abundant. Forty-seven bacteria species were identified entirely and there was inconsistency in the bacterial count on the agar plate of all treated termites when compared to the untreated controls. The bacterial counts which significantly reduced after treatment were *C. gestroi* in response to chlorfluazuron and bistrifluron, and *G. sulphureus* in response to all baits. High-throughput sequencing (Illumina 16S bacterial metagenomic) was performed to evaluate the gut symbiont of all treated and untreated termite species which focus on the hypervariable area spanning from V3 to V4. The quantification of data using bioinformatics analysis revealed a stark contrast in the alpha diversity of Operational Taxonomic Units (OTUs) across all termite species between treated and untreated samples. There were also inconsistencies in the relative abundance of the shared phyla whereby those had significant difference were Actinobacteria, Desulfobacterota, Proteobacteria in *C. gestroi*, Proteobacteria and Rs-k70 termite group in *G. sulphureus*, and Desulfobacterota and Proteobacteria in *M. gilvus*. In addition, those identified bacteria that has the highest composition are *Azobacteroides* sp. in *C. gestroi*, *Anaerovorax* sp. in *G. sulphureus* and *Alistipes* sp. in *M. gilvus*. This study provides a previously unidentified foundation for the effect of termite susceptibility to CSI bait treatment on gut symbiont and an insight to improve CSI bait formula in combating multi group of subterranean termite infestation.

CHAPTER 1

INTRODUCTION

1.1 Background Study

Termites are eusocial insects that formerly classified separately in the well-established Dictyoptera lineage, along with cockroaches and mantids (Inward et al., 2007). Lo et al. (2000) then revealed the termites embedded in larger clade of cockroaches, indicating that the wood-feeding cockroach, *Cryptocercus* represents the evolutionary transition between primitive unsocial taxa and eusocial termites. Further research through morphology and molecular characteristic, and gut microbiome characterization also reflected this finding, which in turn shifted the termite taxonomic nomenclature under the order of Blattodea (Klass et al., 2008; Nalepa, 2015; Peterson et al., 2015; Schauer et al., 2012). Approximately 3106 termite species have been currently documented worldwide, with 180 of those species being found in Malaysia (Gitanjali, 2019; Kakkar & Gupta, 2017). Early research interest in termite taxonomy was driven by the infestation of rubber trees that had been introduced in Peninsular Malaysia during the beginning of the 20th century (Wong et al., 2000). To date, several termite genera have been recognised as important pests not only in agriculture but also in urban environments and forestry (Freymann, 2008; Jones & Nutting, 1989; Kirton, 2005; Wan Umar & Ab Majid, 2020).

The early control strategies in Malaysia were heavily reliant on soil termiticide and fumigants due to the cost-effectiveness and volatility (Hu, 2011; Lee et al., 2007; Wong et al., 2000). However, numerous studies revealed that these methods were deemed hazardous for both the environment and human (Oi, 2022; Su, 2005). Several substitutes, consequently, were introduced as termite management strategies and bait treatment effectively suppresses the subterranean termite infestation in several tests by monitoring the foraging activity on the lower termites such as *Coptotermes* and *Schedorhinotermes*

(Su, 2019; Vahabzadeh et al., 2007). The bait that is commonly utilised by pest company is Chitin Synthesis Inhibitors (CSIs) where this agent contains active ingredients that works as toxicant (Su & Scheffrahn, 2000). And this toxicant will respond by inhibiting the chitin biosynthesis during ecdysis phase, which eventually cause the death (Tunaz & Uygun, 2004). Although, various species responded differently in terms of time taken to reduce the foraging behaviour. Differences in foraging territory measurement, feeding rate, active ingredient response, and the toxic-targeted-species type of baits all are contributing to this outcome (Chung & Lee, 1999; Lee et al., 2007; Sajap et al., 2000).

The susceptibility of bait treatment towards higher subterranean termites resulted to be ineffective as it took extended period to be controlled than the lower subterranean termites (Lee, 2002; Su & Scheffrahn, 1988). On the other hand, the higher subterranean termites were also stated to re-infest same premises after the lower subterranean termites were successfully eliminated (Sajap et al., 2000). A study reports the multi genera of subterranean termite management became a significant problem in urban areas (Lee et al., 2007). It showed that the bait treatment was ineffective in controlling all subterranean termites especially for higher group termite, which cost a lot to consumers to prevent the infestation. Studies by Brune (2014), and Peterson and Scharf (2016) determined the diversity of symbiont community in subterranean termite gut between lower and higher termite causes different responses to a specific diet and environment. Similar studies also proved the symbionts composition in the gut of subterranean termite corresponded to the host historical effects and dispersal. In conjunction with these findings, different species of subterranean termite consist of varying composition and density of gut symbiont community which might contribute to various effects on the host's body systems.

1.2 Problem statement

A considerable amount of research has been conducted on baiting treatments, in which several Chitin Synthesis Inhibitors baits were employed in the field to eradicate subterranean termites in order to assess their effectiveness. However, there is lack of research performed in laboratory bioassay to determine the effects such as feeding activity and mortality, and gut microbiome composition. Furthermore, research characterizing the composition of the gut symbionts of termites susceptible to CSI baits is limited as well.

1.3 Significant of the study

Therefore, in this research, the subterranean termites, were bait-fed (treated) with three CSIs baits with respective active ingredients (AIs) in CSI namely, bistrifluron, chlorfluazuron, and hexaflumuron according to ‘No-choice’ bioassay whereby, only one food option was given at a time. This assessment of the mean feeding activity and the mean mortality was performed within four weeks and the results were compared between untreated controls and treated termites. Comparison between treated termites species also was executed to identify the differences. Then, the gut symbionts of untreated controls and treated termites were extracted for culture dependent and metagenomic study. This was due to determine the effect of CSI baits towards the gut symbiont composition of the termites species. Differences of gut symbionts between lower and higher group, and untreated controls and treated termites were analysed and discussed.

1.4 Research Objective

Objective 1 (Chapter 3): To determine the effect of three CSI baits with respective active ingredients towards higher and lower subterranean termites in feeding activity and mean mortality by using ‘No-choice” bioassay.

Objective 2 (Chapter 4): To determine the bacterial count of untreated controls and treated subterranean termites by using culture dependent technique.

Objective 3 (Chapter 5): To characterize the bacterial composition of untreated controls and treated subterranean termites by using 16SrRNA metagenomic approach.

CHAPTER 2

LITERATURE REVIEW

2.1 Termite

Termite was once classified under the taxonomic rank of Isoptera however, subsequent research then confirmed this insect classified within similar cockroach order, Blattodea (Inward et al., 2007; Klass et al., 2008; Nalepa, 2015; Schauer et al., 2012). They are commonly recognized as ‘white ants;’ however, true ants belong to the order of Hymenoptera (Grimaldi & Engel, 2005). About 3106 species of termites were recorded worldwide, and 435 species were recognised in Asia (Kakkar & Gupta, 2017; Su, 2003). Termites can be broadly categorised into three groups according to their ecology: subterranean, damp wood, and dry wood. Damp wood and dry wood termites inhabit above ground or in rotting wood. They acquire water content from wet rotting wood, and the water content from the dry wood digested. Unlike subterranean termites, they live underground and venture out for their food and water by tunnelling into the soil for a moist environment and potential food source (Baker & Marchosky, 2005; Woodrow et al., 1999). Based on the microbial association, subterranean termites are classified into two groups which are lower (Rhinotermitidae) and higher (Termitidae). Termitidae composed of 75% of all the termite species and recognised to have no protozoa with a few bacteria species in the intestines, whilst the lower termites contain protozoa and most bacteria in the intestines (Breznak & Brune, 1994; Kambhampati & Eggleton, 2000).

2.2 Economic importance of subterranean termite

Termites serve a crucial role in preserving the ecosystem by decomposing organic waste materials such dead wood, decaying plants, leaf litter, and herbivore excrement (Freymann, 2008; Jones & Nutting, 1989; Kirton, 2005). Although, extensive growth of the colony and human population caused termite to become a significant pest in forestry, urban and agriculture ecosystems (Wan Umar & Ab Majid, 2020). Approximately of 180 species of termites composing 42 genera recorded in Malaysia and 10% of species are determined as significant pest in the human ecosystem (Tho, 1992). According to Lee et al. (2007), 12 genera of subterranean termite were found namely, *Coptotermes* spp., *Macrotermes* spp., *Microtermes* spp., *Globitermes* spp., *Odontotermes* spp., *Schedorhinotermes* spp. and *Microcerotermes* spp. infesting buildings and structures in Malaysia and Singapore. The damage ensues in various manner, both on small and large scales, to the human habitation. Timber-framed buildings, for example, will be severely impacted. This situation is very dissimilar from buildings made of bricks, concrete, and cement.

Subterranean termites are likely to be transported to new areas due to their reliance on continual moisture for tunnelling and foraging (Cornelius & Osbrink, 2010; Su & Puche, 2003). Unbeknownst to many, these circumstances result in further severe repercussions and substantial monetary losses. In United States, subterranean termites' management was spent approximately US$1.5 billion per year in 1993 and predicted to climb until 50% in six years during 1999. However, the expense of termite control is outweighed by the expected cost of repairing the damaged structures, as stated by the New Orlean Mosquito and Termite Control Board, 4:1. (repair against control). Su (2002) posited that the expense of repair and management is subjected to the subterranean termite species present as well as the regional living standards.

Jones and Nutting (1989) determined that subterranean termites feed on leaf litter, decaying plants, herbivore faeces, and deadwood under natural conditions in the desert. As opposed to urban areas, where the resources are scarce, subterranean termite were compelled to consumed wood, sheetrock, wallpaper, wood panel, furniture, textile materials, polymers (polytene, polyvinyl chloride), and metal foils (Howse, 1984; Su & Scheffrahn, 2000). Many areas in Malaysia that were once oil palm or rubber plantations were cleared because of difficulties with the cost of lodging and converted to residential areas. Although the trunks of every tree were removed, the roots remained underground. This suggests that subterranean termites successfully sustained their food sources until the construction of new residences, resulting in the termites subsequently disrupting human settlements (Lee, 2002). Subterranean termites, according to Lee (2002), be inclined to harm structures even though they do not feed on inorganic materials like cement, fibreglass, and stucco. Nevertheless, they utilise these materials to support their line and shelter tubes.

2.2.1 Subterranean termite as pest in Malaysia

Urban locations of Southeast Asia are more susceptible to the devastating effects of subterranean termites than dry wood termites. There have been reports of building infestations by subterranean termites in the following countries namely, Myanmar, Indonesia, Thailand, Peninsular Malaysia, Singapore, the Philippines, Brunei, and Indochina (Burma) (Kirton, 2005). According to research conducted in Malaysia by Yap and Lee (1996), the cost of termite management was estimated to be around US$5 million in 1995 and was projected to levitate to between US$8 and US$10 million by 2000. Termite control and related services accounted for 40% of the total revenue of the pest management company in 1995; that proportion climbed by 50% by the year 2000. Lee (2002) asserted that there was a dearth of study pertaining to termites in urban areas, which necessitates further investigation. In the meantime, Kirton and Wong (2000) asserted that termite infestation in rubber trees transplanted to Peninsular Malaysia at the turn of the twentieth century has prompted extensive termite research since 1986. Tho (1992) conducted an exhaustive analysis of the history of termites and concluded that subterranean termites constitute a significant pest concern in residential areas. In a subsequent publication in 2002, Lee provided a proportional breakdown of the intensity of full pest control services. Residential areas accounted for 65% of the services, while industrial sectors contributed 20%, commercial structures 10%, and other sites 5%.

2.3 *Coptotermes gestroi* termite

The genus *Coptotermes* from Rhinotermitidae (lower group of termites) is widely distributed in tropical and subtropical regions and classified under lower group termite (Rhinotermitidae). *Coptotermes gestroi* (Wassman), Asian subterranean termite (AST) originated from Indo-Malaya region, notably a native distribution covering Assam to Myanmar (previously known as Burma), Thailand to Malaysia, and Indonesia Archipelago (Lee, 2002; Lertlumnaphakul et al., 2022; Venkatesan et al., 2021). Because of their size and white morphological characteristics, which derive from the coloration of the defensive liquid secretion in the frontal gland, *C. gestroi* are frequently referred to as "white ants" (J. Chen et al., 1999; Colon et al., 2016). The soldier caste of *C. gestroi* possessed frontal glands for defensive activities which often a gluelike white liquid secreted between the open mandibles (Figure 2.1) (Chen et al., 1999; Da Silva et al., 2019; Ohta et al., 2007).

2.3.1 *Coptotermes gestroi* as pest

Among 21 *Coptotermes* species, sixteen are considered structurally important pests in which addressing this genus has the highest number of termite pests (Jenkins et al., 2007; Su & Scheffrahn, 2000). The wood-feeding termite, *C. gestroi* is a native termite species from the Indo-Malaya region, Southeast Asia (Lee et al., 2007). However, there are numerous research discovered the infestation was encountered in Philippine, Taiwan, Hawaii, South Florida, West-indies, and Brazil (Acda, 2004a; Costa-Leonardo & Barsotti, 1998; Li et al., 2009; Manjula et al., 2016; Su & Scheffrahn, 2000; Woodrow et al., 1999). The pest status, invasiveness, and potential for damage have similarities to its relative, *Coptotermes formosanus*, which has been linked to substantial global economic losses of up to US$32 billion (Rust & Su, 2012). Given that the habits of *C. gestroi* construct cellulose carton material for their nests and fill aboveground voids and quarries, human movement seemed a more likely means of dispersing the species (Jenkins et al., 2002). *Coptotermes* also can produce neotenic reproductive, which can either supplement or replace primary reproductive. Hence, they provide alternatives for dispersal through alates flight and population control over time (Jenkins et al., 2007).

Figure 2.1 Soldier of *Coptotermes gestroi* secreted white gluelike liquid between the mandibles as a defence mechanism.

2.4 *Globitermes sulphureus* termite

Globitermes, which consists of two species, *Globitermes globosus* and *Globitermes sulphureus*, and this genus is classified within the Termitidae family, a higher group of termites. *G. sulphureus* is restricted to Indo-Malayan regions and this species is easily identified by the bright yellow-colour abdomen of the soldier caste (Figure 2.2) (Lee et al., 2003). The coloration of this species is due to liquid stored in the abdomen and thorax, and secreted through frontal glands, once threatened (Hussin & Ab Majid, 2017; Neoh et al., 2011). This autothysis act determined as suicidal defensive behaviour as they ruptured by violent contraction of the abdominal wall of the sternal thoracic integument (Bordereau et al., 1997; Shorter & Rueppell, 2012). Unlike other termite species, *G. sulphureus* creates a dome-shaped mound with a smooth outer wall surface and a distinct darker shade (Ahmad, 1965). It may also reach a maximum height of 80 cm (about 2.62 ft) and a maximum diameter of 60 cm (Neoh et al., 2011). The foraging distance of *G. sulphureus* can be in the range 3.5m to 15.0 m depending on the colony age and size, and environmental conditions (Ab Majid & Ahmad, 2011; Lee et al., 2003; Ngee & Lee, 2002).

2.4.1 *Globitermes sulphureus* as pest

The mound-building termite, *G. sulphureus* commonly found, infesting crop plantation especially coconut and palm oil plantation (Harris, 1961; Lee et al., 2003; Neoh et al., 2023). Nevertheless, this species also invades urban areas, manufactured structures in rural area, and disturbed forest (ecotourism) due to development of complexes and residential without soil treatment beforehand (Ab Majid & Ahmad, 2011; Aiman Hanis et al., 2014; Hussin et al., 2018; Lee et al., 2007). Other cases, *G. sulphureus* was reported to be re-infesting same premises after the elimination of primary termite pest such as *C.*

gestroi (Lee et al., 2007). Hence, this species is classified as a significant secondary termite pest in urban environments.

Figure 2.2 Soldier of *Globitermes sulphureus* possessed bright yellow abdomen and thorax.

2.5 *Macrotermes gilvus* termite

Macrotermes is the biggest genus among all termite genera that exhibits dimorphism in both soldier and worker caste (major and minor) with respective roles in the colony (Acda, 2004b). This genus is classified under Termitidae family (higher group of termites) and known as fungus-growing termite due to their foraging behaviour in forming fungus balls for their food (Chung et al., 2021). *Macrotermes* are widely dispersed throughout Africa and Southeast Asia, though only *Macrotermes gilvus* and *Macrotermes carbonarius* are often found in Southeast Asia (Neoh & Lee, 2009). *M. gilvus* able to visibly identify through the morphology of the major soldier caste, which has a reddish-brown with sub-rectangle head and stout mandibles (Figure 2.3) (Ahmad, 1965). Depending on the size of the population, the mound of this species can grow up to

3m in height and 10m in basal circumference (Inoue et al., 1997; Lee et al., 2012). The foraging territory ranges from 16m to 48m distance (Acda, 2004b).

2.5.1 *Macrotermes gilvus* as pest

Macrotermes gilvus is commonly found in forests and rural areas. It has been reported that this species seldomly cause infestation in buildings and structures, despite being in the housing parameter (Dhang, 2011; Lee et al., 2012). However, there was research determined this species reinfest same premises after elimination of the lower termite using bait treatment (Lee, 2002). Several studies also revealed the infestation occurred by attacking trees and building structure in Philippine, Thailand, Malaysia, and Singapore (Acda, 2007; Iqbal & Evans, 2017; Lertlumnaphakul et al., 2022). Therefore, *M. gilvus* has been classified as a secondary pest in urban and suburban areas and control measures are needed to control this species (Dhang, 2011).

Figure 2.3 Soldier of *Macrotermes gilvus* has distinct characteristic compared to other species.

2.6 Diversity of gut microbiomes in subterranean termites

Termites rely heavily on the gut microbiota for cellulose digestion, fundamental nutrients acquisition, nitrogen fixation, and vitamins and amino acids synthesis (Brune, 2014; Taylor et al., 2016). These biological processes represent a complex synergistic interaction as any changes on the microbials may lead to various effects on the termites(Brune, 2014; Peterson et al., 2015; Rosengaus et al., 2011). Furthermore, Tai et al. (2015) reported that termite phylogeny can be determine through gut symbiont community in the hindgut due to vertical inheritance as the historical effects. Additionally, the presence of symbiotic bacteria provides termite immune protection against pathogens and parasites (Peterson & Scharf, 2016).

Former research in termite and wood-feeding cockroach phylogeny also discovered the gut flagellates may have inherited from their ancestors early in the history of groups (Frank, 2007; Inward et al., 2007; Lin & Michener, 1972). The microecology of the termite gut (oxygen, hydrogen, pH level, gut structure, and redox gradient), diet, habitat, and termite phylogeny are some of the factors that influence the variety of hindgut microbiota (Abdul Rahman et al., 2015; Berlanga, 2015). Many researchers concluded termites are a good model to study evolutionary transition as they are also divided into two distinct categories, lower and higher according to the gut microbiome composition (Figure 2.4).

2.6.1 Lower subterranean termite

Termites are feed on cellulose materials obtained from wood, grass, leaf litter, herbivorous faeces, and any substantial made of cellulose or fibre (Bignell et al., 2011; Evans et al., 2013). Therefore, lower termites (Rhinotermitidae family) depend on complex symbiotic interactions with eukaryotic flagellate protozoa and bacteria to produce enzymes to digest the biomass into biofuel (Auer et al., 2017; Ni & Tokuda, 2013). Enzymes such as exo-glucanase (EC 3.2.1.91), endoglucanase (EC 3.2.1.4), and xylanase (12.4.5) are produced by protozoa of lower termites for cellulolytic activity (Franco Cairo et al., 2016). Nevertheless, not all castes in the colony feed directly to the wood whereby, the newly hatched nymphs and soldiers rely on workers to be fed via trophallaxis or proctodeal feeding (Nalepa, 2015). Older workers in the colony predominate all the given tasks such as foraging, nest repair, and feeding other castes (Crosland et al., 1997). This type of feeding also helps in transferring protozoans down the lineage (Nalepa, 2015; Nalepa & Mullins, 2009).

2.6.2 Higher subterranean termite

Despite the termite great reliance on protozoans in digesting recalcitrant cellulose materials, there are also termites that host a constrained number of protozoan species such as the higher termites in Termitidae family (Chouvenc et al., 2021). The reduction or loss of protozoan in Termitidae, repurposing the bacterial and fungal nutritional mutualist in nutrition acquisition (Bignell, 2016). Higher termites mutualistically shift the lignocellulose and cellulose digestion exterior of their termite hindgut to a nutritional comb (Chouvenc et al., 2021). Two sister lineages, Sphaerotermitinae and Macrotermitinae build bacterial comb, and fungal comb (*Termitomyces* sp.) for lignocellulose digestion, and amino acid and metabolic water production (Garnier-Sillam et al., 1989; Pierre-Paul et al., 1984). Another successive event in the formation of the Termitidae family is their transition to a soil-like diet, as this is a trait of precursor termitids, allowing the soil-feeders to digest their food internally (Bourguinon et al., 2018; Chouvenc et al., 2021). Most termitids, other than Sphaerotermitinae and Macrotermitinae, that feed on highly decaying wood which are either soil-like or fully in humus form are identified as soil-feeders (Donovan et al., 2001).

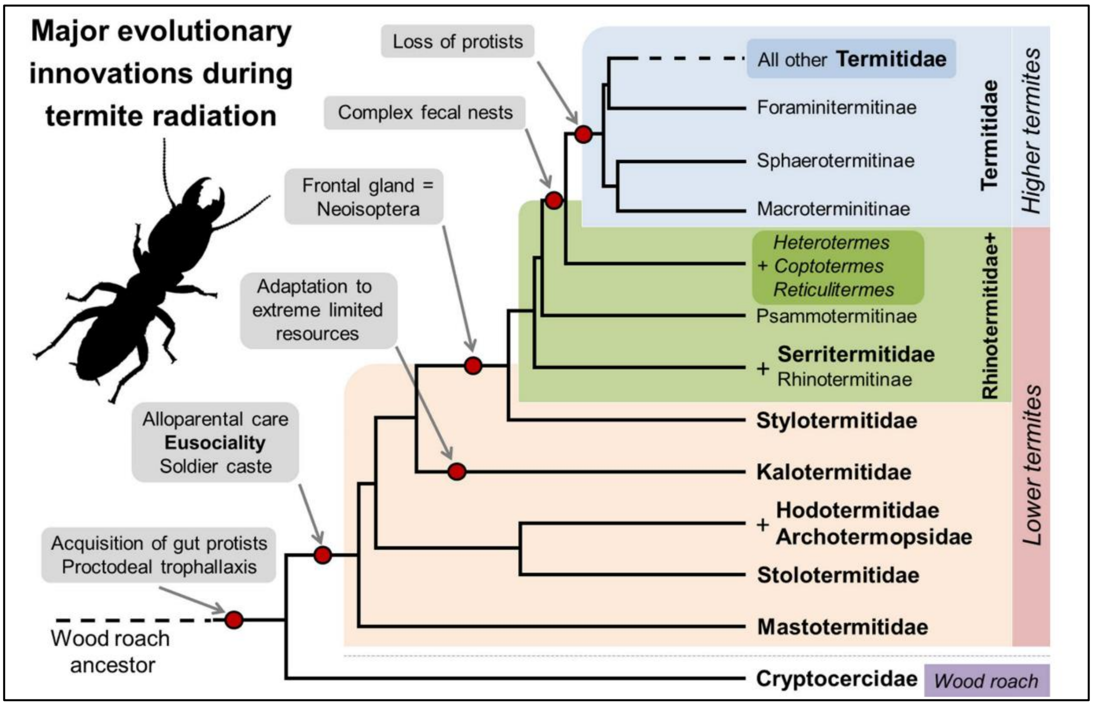

Figure 2.4 A simplified evolutionary tree that illustrates several novel characteristics that the termites developed over time. Retrieved from Chouvenc et al., (2021).

2.7 Subterranean termite control strategies

Soil treatment has been a major component of subterranean termite control strategy since it is accessible to pest control companies and requires less cost efficacy (Chung & Lee, 1999; Su, 2005). Using soil termiticide around and beneath the building helps form a barrier that prevents termites from penetrating the structure to forage (Su & Scheffrahn, 1988). There are variety of control techniques including trenching, dusting, deployin mechanical barriers, and applying soil termiticide can be used either before or after construction (Ibrahim & Adebote, 2012). Arsenic trioxide is widely used in dusting even though this chemical item is stated to be unregistered in Malaysia. Chlorpyrifos-based termiticides are highly markets covering 80% of the range, followed by chlordane and other pyrethroids (15%) and cypermethrin (5%). Liquid termiticide is another alternative to remedy the soil-borne termite infestation. However, a tedious and proper procedure is needed to apply this item to the soil, such as drilling the floor and pumping copious quantities of liquid termiticide through the drilled bore (Su, 2002). The extensive range of foraging subterranean termite activity yet causes soil termiticide to be less affecting the entire population of subterranean termites. The existing subterranean termites remain, reproduce, and infesting neighbouring areas.

2.7.1 Insect growth regulators

The population control was against bait treatment to suppress or eliminate the subterranean termite population near the structures. Su (1982) emphasised using slow-acting and non-repellent active ingredients (AIs) in baiting treatment. The item used as bait known as the Insect Growth Regulators (IGRs). There are two classes of IGRs:

2.7.1(a) Chitin Synthesis Inhibitors (CSIs)

The chitin layer of insect and crustacean is essential as it serves as a support structure in the gut lining, respiratory systems, some gland duct, and form as the exoskeleton. CSIs, formerly known as larvicides used to treat insects in the molting process and turned out, failed due to inhibition of chitin (cuticle layer) biosynthesis during the ecdysis phase (Tunaz & Uygun, 2004). This item, therefore, become an important component in insect pest control. Benzoylphenylurea diflubenzuron, for instance, was the first CSIs introduced in the market (Racke et al., 1997). Other CSIs such as hexaflumuron and sulfluramid are also used and claimed to have different efficacy in pest control (Su, 2002). Hexaflumuron first applied in October 2002 in Malaysia and stated in many studies to be an effective bait to eliminate *Coptotermes* spp. within 70 days (about 2 and a half months) of deployment (Lee, 2002). Sajap et al. (2002) mentioned *Coptotermes curvignathus* impacted when the colonies consumed 137.5-395 mg of the hexaflumuron bait within two to eight weeks, depending on the colony's size. *Coptotermes formosanus* shows a slower response to reduce the foraging activity (Sajap et al., 2002). Different response for each species is due to the differences in population sizes and foraging activity between tropical and subtropical termites-*Coptotermes curvignathus* has smaller foraging territory and population size than *Coptotermes formosanus* (Su & Scheffrahn, 1988). Other factors that might contribute are the species response to the active compound in the bait content and feeding rate of the termites (Lee, 1999; Sajap et al., 2000). Lee et al. (2007) found that CSIs are also toxicant to targeted species, which drives the suppression of certain termite colonies that are most susceptible to the baits, such as *Coptotermes* and *Schedorhinotermes*.

2.7.1(b) Juvenile Hormone Mimics (JHM) and Juvenile Hormone Analogues (JHA)

Juvenile hormone was first topical applied to control succeeding multiplication of insects and metamorphosis inhibition in 1956. The hormone was acquired from the removal of abdominal crude extracted from male moths, *Hyalophora cecropia* (Racke et al., 1997). They also stated Juvenile hormone mimics (JHM) were later discovered by Harvard researchers from a paper towel that contained an active ingredient known as juvabione. The paper towel was made from a plant, namely Balsam fir, *Abis balsamea*, and became a key component in controlling the hemipteran family. Racke et al. (1997) also stated numerous studies were made ever since this plant-derived used to produce insect growth regulators according to biochemistry and physiology of insect growth understanding. Juvenile hormones function to control growth processes in many insects, such as embryogenesis, molting and metamorphosis, reproduction, diapause, pigmentation, silk production, phase transformation, and, for subterranean termite, caste differentiation. Research by Hrdy (1972) found that three types of juvenile hormone analogue namely, juvenoids hydroprene, 11-chloro farnesoic acid and tetrahydrofuryl of Methoprene, caused high number of pseudergates-soldier (false soldier) excessively than worker caste termite during differentiation of larvae and pseudergates. Subterranean termites merely depend on worker caste for food; therefore, this circumstance caused food scarcity in the colony, leading to high mortality.

2.8 Culture-dependent method

The culture-dependent method is widely used to cultivate natural isolates for numerous screening test according to research interests, namely enzyme activities, antibiotic resistance, microbial or fungal identification, and antibacterial or entomopathogenic fungal activities (Goettel, 1984; Vester et al., 2015). The techniques involve taking samples from the field, incubation, serial dilution, and streaking or spreading plate method (Salih, 2015). Pure colonies are an important study component in terms of morphology, physiology, and biochemical characteristics in bacterial isolation (Franco-Duarte et al., 2019). To obtain pure cultures, application of streaking and spreading on agar plates is the optimal method. And to preserve them for future purposes, sub-cultivating in broth and freeze-drying them with glycerol is one of appropriate approaches (Ruangpan & Tendecia, 2004).

Bacteria derived from culture-dependent technique identified via phenotypic methods which involve morphology and physiology characterisation, protein profiling, and carbohydrate fermentation patterns (Temmerman et al., 2004). They also mentioned 90% of the isolates could only be recognised to genus level which explain the combination of those methods is necessary due to low taxonomic resolution of this technique (Gevers et al., 2003; Temmerman et al., 2004). Nevertheless, the major drawback is extensive cost obliged to high labour and workload which consequently, stimulated the popularity miniatured identification system such as API (BioMerieux) or even molecular technique such as Sanger sequencing (Bhumbla et al., 2020; Sorescu & Stoica, 2021).

2.8.1 Importance of culture-dependent methods

Cultivation dependent method is important in characterizing microorganisms and fungi that are inevitably associated with biotechnology, food sciences, medicines, agriculture, and industrial sector (Clarke, 2004; Franco-Duarte et al., 2019). This method is used in multi-production such as antibiotics, hormones, amino acids, and enzymes used for lignocellulose decomposition (Bisen et al., 2012). As example, a study has determined 18 isolated bacteria from yogurt, raw milk, boza, cheese, and whey, capable of suppressing pathogenic and degrading microorganisms by producing natural antimicrobials and antifungal properties (Kanak & Yilmaz, 2021). Culture-dependent method also was used in pathogenicity screening whereby, a fungus, *Fusarium equiseti* caused wilt symptoms on the leaves of *Pythium aphanidermatum* (Al-Jaradi et al., 2018). Subsequently, bacterial, and fungal characterisation derived from isolation, identification, following taxonomic classification of biological material and interactions remain significant in industrial application and infection treatment (Bisen et al., 2012).

2.9 Sanger sequencing

Sanger sequencing method was developed by Frederick Sanger and team in 1977, involving selective integration of chain-terminating dideoxynucleotides by DNA polymerase through in vitro DNA replication (Sanger et al., 1977). For many years, Sanger sequencing has been the gold standard for sequencing. It not only serves as the grounds for automated and modern techniques, but it is also the most widely used method for sequence verification, assay monitoring, and phylogenetic analysis (Crossley et al., 2020). In classical Sanger sequencing, several components namely, DNA template, a sequencing primer, DNA polymerase, deoxynucleotide triphosphates (dNTPs), dideoxynucleotides triphosphates (ddNTPs) are needed for annealing, labeling, and termination which are performed in four separated reactions. Addition of dNTPs to 3'end chain, will continue the chain extension whereas, addition of ddNTPs, will terminate the chain extension (Men et al., 2008).

In modern Sanger sequencing, all ddNTPs are combined in single reaction and each of four dNTPs are labelled with distinct fluorescence that are detected by laser following the capillary electrophoresis. By taking the weight of DNA, capillary electrophoresis is used to visualize DNA separation in laser-based fragment detection, which eventually builds a high-throughput Sanger sequencing as of today (Dovichi, 1997). The process of sending a DNA template for sequencing analysis entails identifying, isolating, purifying, and quantifying the desired DNA region in the amplicon through DNA extraction and PCR reactions. Quantification involves the process of confirming DNA concentration and purity ratio by using spectrophotometric analysis or ultraviolet fluorescence tagging, to obtain a success sequencing reaction (Sambrook & Russel, 2001).

2.10 Next generation sequencing (NGS)

Unlike Sanger sequencing where it only sequences one amplicon at a time, Next generation sequencing (NGS) can massively parallel or deep sequencing millions of DNA amplicon fragments simultaneously and captures broader spectrum of mutations (Behjati & Tarpey, 2013; Lakdawalla & VanSteenhouse, 2008). Besides, NGS also allows culture independent method whereby, it can access and identify diverse microorganisms and fungi on earth, including species that were previously inaccessible through culture-dependent methods (Lind, 2018). NGS is a high-throughput sequencing technology that offers both DNA and RNA sequencing using different modern sequencing platforms such as Illumina sequencing, Ion Torrent and 454 sequencing (Meyer et al., 2008; Quail et al., 2012). There are two types of sequencing which are metagenomic for DNA sequencing and transcriptomic for RNA sequencing.

2.10.1 Metagenomic sequencing

Metagenomic sequencing allows determination of microbial diversity through genomic DNA of a sample, derived from bacteria, fungi, archaea, viruses, or combination of these present in a sample (Roux et al., 2021; Yang et al., 2018). Obtaining highly pure DNA is important to acquire free from any inhibitors and contaminants, and genomic DNA must be accurately quantified using fluorometric methods (Grieb et al., 2020). There are two library preparations in metagenomic sequencing which are Targeted library preparation and Shotgun library preparation.

Targeted library or Targeted amplicon-based preparation, selects and amplifies single targeted sequences (region) in the amplicon which should be informative and be escorted by other sequence variants that aid in the identification of the species present (Harismendy et al., 2009). Metabarcoding sequencing is an alternative term for metagenomic sequencing. Illumina 16S metagenomics demonstrated protocols for bacterial rRNA and Illumina fungal metagenomics demonstrated protocols (ITS) for fungus samples are examples of Illumina metagenomic platforms (Kennedy et al., 2014; Shaffer et al., 2022).

Shotgun library preparation, as opposed to targeted amplicon-based, encompasses the entire region of the amplicon that is present in the genomic DNA sample in sequencing (Hamady & Knight, 2009). Preparation of shotgun libraries is also called Whole-genome sequencing (WGS). Shotgun sequencing is a technique that assembles smaller DNA fragments into larger contigs, which develop into chromosomes, by overlapping them simultaneously (Quince et al., 2017).

CHAPTER 3

LABORATORY BIOASSAY ON IMPACT OF CSIs BAITING TREATMENT AGAINST *Coptotermes gestroi, Globitermes sulphureus,* AND *Macrotermes gilvus*[1]

3.1 Introduction

The previous control strategies to eradicate subterranean termite infestations in Malaysia, comprised the use of soil termiticides and fumigants (Castillo et al., 2013). Thereafter, several control techniques, including Chitin Synthesis Inhibitors (CSI), have been introduced for baiting treatment. CSIs are known for their active ingredients that work as toxins to inhibit or reduce the synthesis of chitin during the moulting phase of immature subterranean termites (i.e larvae, nymph, and worker) (Merzendorfer, 2013). Worker caste is the key component of transmitting the active ingredients since they are tasked with the obligation of foraging and feeding other castes via trophallaxis. It was found that a negligible quantity of active ingredients in the bait is sufficient to eradicate an entire colony of termites via transmission of trophallaxis and grooming (Chouvenc, 2018).

Previous research majorly focused on the effectiveness of CSI baits against several species of termites in the field. Nevertheless, there is lack of study that observed the effect of CSI baits towards subterranean termite conducted in laboratory bioassay, even though bioassay is an important method to determine the physiology, toxicological, pathology, and behaviour of termites with limited biological relevance (Chouvenc, Bardunias, et al.,

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[1] Shamsuri, Q. A. S., & Ab Majid, A. H. (2024). Laboratory efficacy and performance of several type of chitin synthesis inhibitors (CSIs) towards three species of subterranean termite (Blattodea:Rhinotermitidae, Termitdae). *International Journal of Tropical Insect Science.*

2011; Leong et al., 2020). In this research, lower termite (*Coptotermes gestroi*) and higher termites (*Globitermes sulphureus* and *Macrotermes gilvus*) were tested by using three baits that contain respective active ingredients (AIs) namely, bistrifluron, chlorfluazuron and hexaflumuron. The evaluation was performed by using 'No-choice' bioassay whereby, the termites were force-fed with one type of food in petri dish within four weeks. The observation on feeding activity and mean mortality were analysed to determine the effect of three baits with respective AIs towards these three species of termites.

3.2 Materials and methods

3.2.1 Sampling site

Universiti Sains Malaysia (USM), Penang has spread over of 416.62 hectares which lies in northern region of Peninsular Malaysia. This campus, which was situated in urban area, contained mixture of habitats including, specialist garden, secondary forest, and amenities specify for research. Additionally, Penang has an equatorial climate with year-round warmth and humidity, which may account for the abundance of subterranean termite species discovered in this study area. Three locations within this university compound were used to collect the three species of subterranean termites, either by employing bait stations or excavating the termite mound (Table 3.1). The location also illustrated in Figure 3.1.

Table 3.1 Sampling site information for Figure 3.1

Species	Locations	Coordinate	Colour code
Coptotermes gestroi	Pusat Islam USM	5°21'14.5" N, 100°18'01.1" E	
Globitermes sulphureus	School of Pharmacy	5°21'23.1" N, 100°17'52.4" E	
Macrotermes gilvus	Padang Kawad	5°21'19.3" N, 100°17'37.0" E	

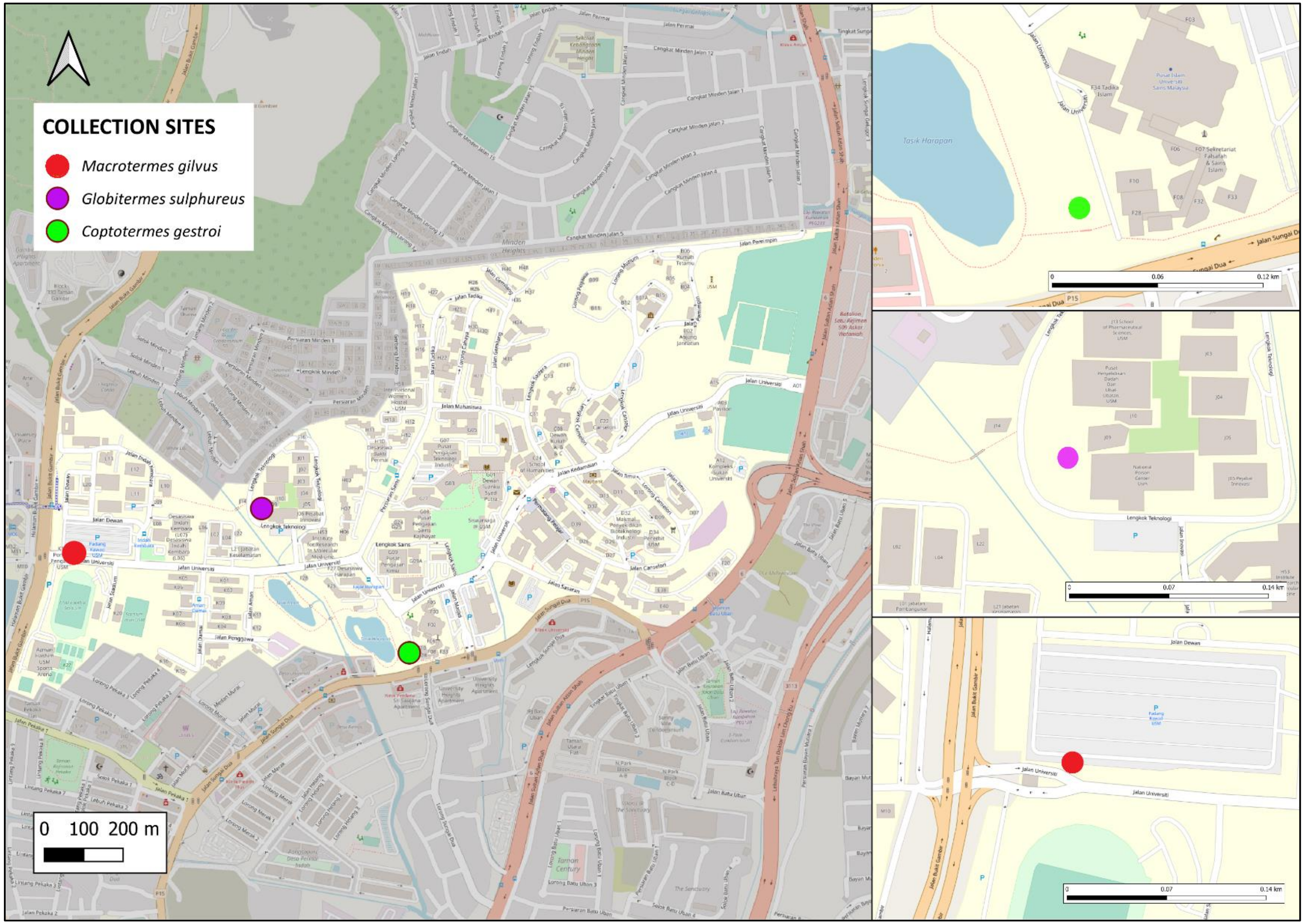

Figure 3.1 Three collection sites according to subterranean termite species in Universiti Sains Malaysia. Map generated using QGIS. Basemap: OpenStreetMap.