POPULATION STRUCTURE AND GUT FLORA DIVERSITY IN *COPTOTERMES GESTROI* IN SOUTHEAST ASIA

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Declaration

I hereby declare that thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

Zhang Manping

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Summary

Coptotermes gestroi (Wasmann) (Insecta: Isoptera: Rhinotermitidae), is one of the most widespread and invasive pest termite species in Southeast Asia. The species is believed to have originated in Southeast Asia and spread to many other geographic regions. Despite the importance of *C. gestroi*, our knowledge about its dispersal pattern and essential gut symbionts is limited. In this thesis, I discover significant factors that determine the population genetic structure of *C. gestroi* in its native region, as well as important factors that shape the gut bacterial community of this species. Both of them contribute to our understanding of spread and adaptation of this invasive species and provide valuable information for pest management.

First, in chapter 2, I investigated the genetic structure of *C. gestroi* populations across Southeast Asia. I found that native colonies of the species were highly structured and with high genetic differentiation. Three genetic clusters were revealed: 1) Northern population, including colonies from Cambodia and Vietnam; 2) Southern population, including colonies from Thailand, Malaysia, Singapore and Indonesia; and 3) Philippines population, including all Philippines samples. These three populations had further genetic substructure. The genetic structure of all of these native colonies in the three populations showed evident spatial pattern and isolation by distance, suggesting significant association between geography and genetic structure across Southeast Asia. The majority of the native colonies consisted of extended families

with highly genetically related nestmates. The inbred colony structure may partly explain the great genetic differentiation of populations, as amplifies the effect of distance. Although invasive termites rely on human transport for dispersal, natural dispersal seems to be the primary factor in determining the distribution of *C. gestroi* across Southeast Asia.

In chapter 3, I explored the correlation between the gut bacterial community of *C. gestroi* and the host phylogeny at the colony level. I detected no correlation between the two and, thus, gut bacterial community may not be a good indicator of the genetic relationships of the host colonies. However, I found that gut community was associated with habitat of host termites.

In the last chapter, I surveyed the diversity and structure of gut bacterial community of *C. gestroi* and the impact of habitat and host tree species on this diversity. In general, both habitat and host tree species had a significant influence on gut bacterial diversity and community structure. Diversity of gut bacteria correlated with diversity of food resources provided by habitat. However, I discovered 19 bacterial genera common to all 30 samples, regardless of habitat and host tree species. These 19 genera may be obligate symbionts, and so be essential for *C. gestroi* digestion and survival.

In sum, geographic features are of great importance in determining the population genetic structure of *C. gestroi* across Southeast Asia. Natural dispersal rather than

human transport is the major drive in distribution of native colonies. Meanwhile, habitat and host tree, rather than colony genotype, are more important in shaping the gut bacterial community of *C. gestroi*. Differentiation of gut bacterial community of *C. gestroi* may be a good indicator of host habitat but not of host's genetic relationships. My results provide a contrast with two traditional concepts regarding invasive termites: 1) That their dispersal relies primarily on human transport. 2) That the impact of the habitat and diet on gut flora community is not significant. My results contradict these two notions and provide a new perspective to understand spread and adaptation of invasive termites.

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Chapter 1: Thesis Introduction

1.1 Invasive species

Invasive species are nonindigenous species capable of establishing and spreading in new environments (Les and Mehrhoff 1999; Simberloff 2013). While invasive species represent a small proportion of living organisms, they have attracted great attention because they can threaten the balance of native ecosystems, and they can affect human health and the economy (Kolar and Lodge 2001; Stachowicz and Tilman 2005). They often compete with native organisms for resources because they occupy the same niches, or act as new predators or spread diseases and parasites (Davis 2009). Some species threatened by invasive ones are of great economic value, potentially affecting local economies. Bacteria, viruses and parasites carried by invasive species may threaten humans and thus public health (Stachowicz and Tilman 2005).

Invasion of new habitats by a species is not a novel issue, nor one that is driven by humans exclusively. But human transport and commercial trade in the past 200 years have spurred the invasion of more species at a global scale (Mack et al. 2000). Physical or topographic barriers, such as mountains, rivers and oceans, used to be significant factors that limit the distribution of species. However, human-mediated dispersal has overcome these barriers, making long distance migration more common (Gaston 2003; Crispo et al. 2011).

1.2 Invasive termites

1.2.1 Current status of invasive termites worldwide

Although a relatively small order (Isoptera), termites are one of the most diverse and abundant animals in tropical and warm temperate regions. There are more than 2700 described termite species and a recent review recognized 27 of them as invasive species (Evans et al. 2013). A previous review, made 47 years ago, reported 17 invasive termite species worldwide, indicating that 10 new species have since expanded their distribution (Gay 1969; Evans et al. 2013).

The largest geographic source of invasive termites is South and Southeast Asia, with a total of seven species. Other geographic origins of invasive termite species include South America, Australia, Africa, North America, Caribbean, East Asia and Europe (Evans et al. 2013). Most of the invaded habitats are islands. The islands in the Pacific Ocean were invaded by 13 species and those in the Caribbean Sea by 9 species. Some invasive termites, however, have intruded deeper into the mainland of some continents. The most well-known case is *Coptotermes formosanus* in the USA. *C. formosanus* was first found in the coastal region and then dispersed across ten states in US by infesting railway ties (Su 2003).

Humans facilitate the spread of invasive termites. Wood trade is believed to be the major driver of termite invasions globally (Evans 2011). It was speculated that *Cryptotermes brevis* was introduced from Peru to the Caribbean and Central America through the Spanish trade, as early as the European colonial era (Scheffrahn et al. 2009). Several invasive species might have landed on New Zealand, Fiji, and New Guinea inside of Australian log cargoes (Bain and Jenkin 1983; Evenhuis 2007; Thistleton et al. 2007).

1.2.2 Common traits of invasive termites

Traits that are shared by all invasive termites include their feeding on wood, nesting in wood, and they all producing secondary reproductives (Evans et al. 2013). Termites can be categorized into various feeding types; they can eat non-decomposed plant material, decomposed plant material, and mineral soil (Donovan et al 2001). Although around half of the described termite species eat decomposed plant and soil, all invasive species eat non-decomposed wood exclusively.

Similarly, termite species have diverse nesting types (Abe 1987). Single-piece nesters eat and dwell in one single piece of wood, dispersing only by flight. Intermediate nesters are similar, however, as well as flight, they can disperse into a new home and food resource, on foot, once they finish eating the previous one (Grace et al. 2009). Separate nesters live in one or more pieces of wood and eat different pieces of wood, keeping their nests and food separate. There are some species that move continuously, and don't have a fixed nest. Again, only a few particular types of nesters are invasive species; all of them are single-piece nesters or intermediate nesters. The feeding and nesting habits of invasive termites enable them to survive for several weeks or months as they travel within wood cargo across hundreds or thousands miles (Gulmahamad 1997).

Finally, invasive termites have a great capacity to generate secondary reproductives. All termite species produce primary reproductives (Evans et al. 2013). They form from alates (winged adults), which disperse by flight from their natal nests, and mate and establish a new colony independently. Some termite species, however, have the potential to produce

secondary reproductives. These form from nymphs, workers, and pseudergates (or even soldiers in the Termopsidae). These wingless castes turn into replacement reproductives, typically when the primary ones are distant or absent (Myles 1999). Secondary reproductives have been observed for some invasive species when they colonized a new location (Evans 2011; Myles 1999). For these invasive termites, the presence of several individuals, regardless of caste, is sufficient for the establishment of a mature colony.

1.3 *Coptotermes* as an invasive genus

The genus *Coptotermes* (Insecta: Isoptera: Rhinotemitidae), which means 'cut termite' in Greek (referring to the appearance of the fontanelle on the soldier's head, which resembled the base of a horn cut off the head of the termite), was first recorded by Wasmann (1896) as a sub genus of *Termes* (Wasmann 1896). In 1901, Silvestri raised it to genus level (Silvestri 1909). Distribution of *Coptotermes* mainly covers tropical and sub-tropical areas. *Coptotermes* may have the largest number of species; Snyder recorded 44 species and Roonwal recorded 48 (Snyder 1949; Roonwal and Chhotani 1962). However, both estimates could be inaccurate because termite taxonomy is still contentious, as I will elucidate below in 1.4.2 (Chouvenc et al. 2015).

Su and Scheffrahn (2000) identified the genus *Coptotermes* as containing the most pest species impacting human constructions. This genus is also the most invasive genus, containing 26% of the recorded invasive species (Evans et al. 2013). These invasive species are *Coptotermes acinociformis*, *C. curvignathus*, *C. frenchi* Hill, *C. sjostedti* Holmgren, *C. formosanus* shiraki

and *C. gestroi* (Wasmann) (Evans et al. 2013). Their distribution covers almost all the continent except Arctic and Antarctic. Among them, Grace (2014) claimed that *C. formosanus* and *C. gestroi* were the widest-spread species.

1.4 *Coptotermes gestroi*

1.4.1 Basic information on *Coptotermes gestroi*

Coptotermes gestroi, given the name 'Asian subterranean termite' by the Entomological Society of America, was first described in Myanmar in 1902 by Wasmann (Wasmann 1902) (Figure 1.1). It is a tropical species, with distribution limited to 27 degrees latitude north to 9 degrees south (Hapukotuwa and Grace 2012). Workers comprise the majority of the colonies and are responsible for housework. Soldiers mainly carry out colony defense and secret white, milk-like glue when they are attacked. Both workers and soldiers are sterile; secondary reproductives are derived from nymphs (Costa-Leonardo et al. 2004). The nest system of *C. gestroi* is hierarchical, and comprises a main nest containing a king and queen and satellite nests connected to it (Janei and Costa-Leonardo 2015a).

Figure 1.1. *Coptotermes gestroi*. Individuals with a brown head are soldiers. The others are workers. Adopted from http://www.termiteweb.com/

1.4.2 Taxonomy of termites

Coptotermes species have been poorly described, documented and thus the taxonomy and species distribution are unclear (Yeap et al. 2007; Yeap et al. 2010; Grace 2014; Chouvenc et al. 2015). Termite species identification is mostly based on soldier morphology, and alates are also occasionally used as references. Occurrence of alates is seasonal, so in most cases only soldiers were used (Li 2000). But soldiers are not good reference for species identification because their morphological characters are unclear: they either vary too much within a species or too little within a genus (Kirton 2005; Chouvenc et al. 2015). Morphological characters of termites are influenced by colony stage, age and environment which lead to distinguishable diversity between soldiers of the same species from different colonies. Meanwhile, morphological difference between different *Coptotermes* species can be subtle sometimes. All

in all, morphological based identification is problematic (Chouvenc et al. 2015).

Recently, with the facility of molecular evidences, some *Coptotermes* species have been confirmed as the synonyms of *C. gestroi*. Yeap et al (2007, 2010) synonymized *C.vastator* Light and *C. heimi* with *C. gestroi* based on COII sequences. Other synonyms include *C. parvulus* Holmgren, *C. havilandi* Holmgren, *C. pacificus* Light, *C. javanicus* Kernner, *C. obliquus* Xia and He and *C. yaxianensis* Li (Grace 2014). Further work will be needed to resolve the vexed issue of *Coptotermes* taxonomy

1.4.3 Origin, distribution, and destruction caused by *C. gestroi*

The Indo-Malay region is regarded as the origin of *C. gestroi*, though its distribution is not limited to that region (Yeap et al. 2011). The distribution of *C. gestroi* expands when the distribution of all the synonymized species are included. They have invaded or been introduced into Asia, North America, South America, Europe, islands on Pacific Ocean, Caribbean Ocean and Indian Ocean (Evans et al. 2013) (Table 1.1). Grace (2014) stated that both *C. gestroi* and *C. formosanus* are subterranean termites with widest distribution among all invasive termites.

C. gestroi is a destructive pest. In Singapore and Malaysia, it is estimated to cause 85% of all termite destruction to human construction in urban areas, with costs approaching $400 million annually (Lee 2002; Lee et al. 2007). It can also pose a great threat to the public. *C. gestroi* frequently infests street trees, and because it lives deep inside the trunk, it is hard to detect the

termites until the trees are badly damaged. This can lead to trees falling suddenly and potentially harming pedestrians and cars. However, relatively little research has paid attention to *C. gestroi*, compared to *C. formosanus*. Considering its prevalent distribution and capacity for destruction, *C. gestroi* deserves more research concern and effort.

Table 1.1. Global distribution of *C. gestroi.*

Geographic area	Region
Asia	Myanmar, Malaysia, Singapore, Thailand, Philippines, Indonesia, India, China, Pakistan
Pacific Ocean	French Polynesia Guam, Midway Is, Marquesas Is, Hawaii
North America	Mexico, USA-Florida
Europe	Italy, Germany
Caribbean Sea	Virgin Gorda, St. Kitts, Cuba, Grand & Little Cayman, Jamaica, Puerto Rico, Antigua, Barbados, Barbuda, Nevis, Monserrat
South America	Brazil
Indian Ocean	Mauritius & French Reunion

1.4.4 Traits contributing to the invasiveness of *C. gestroi*

Like other invasive termites, *C. gestroi* is wood-feeding and an intermediate piece nester (Evans et al. 2013). Besides its feeding and nesting habits, *C. gestroi* possesses some unique characteristics that may contribute to its invasiveness.

C. gestroi is a notable city invader. Not only is the species dispersed through shipments and cargo, but it also takes full advantage of urbanization. Human heating may allow *C. gestroi* to invade in high latitudes, beyond the limitation of 27 degree north; for example, it has been found in Italy, nesting near a heating system on a yacht (Ghesini et al. 2011). Global warming may additionally spur further expansion of the species (Robinet and Roques 2010).

C. gestroi can produce secondary reproductives, and non-functional neotenics have been discovered in a colony of an invasive population, even with the presence of a primary reproductive (Costa-Leonardo et al. 2004). Costa-Leonardo et al (2004) speculated that the presence of non-functional neotenic reproductives might be related to the species' colony-breeding strategy in non-native regions, which allows rapid establishment.

Preliminary fusion among different colonies has also been reported in the lab (Guaraldo and Costa-Leonardo 2009). If colony fusion can happen in the field as well, then super-colonies may exist and become dominant in a foreign ecosystem.

Water maintenance is critical for termite survival because they can die quickly due to water loss. A lab experiment conducted by Janei and Costa-Leonardo (2015b) demonstrated that *C. gestroi* is more tolerant of desiccation than *R. flavipes* and can rehydrate after water stress. This implies that more *C. gestroi* individuals would survive transport, and sufficient propagules would be introduced.

1.5 Population and colony genetic studies of invasive termites

Population genetic tools are a powerful approach to provide information about invasive insects. They help reveal spatial and temporal dispersal patterns, species histories, and colony and population dynamics of invasive species (Sakai et al. 2001; Ascunce et al. 2011). Moreover, a population genetic strategy is useful for source and origin identification, which offers valuable

suggestions for the management of invasive insects (Sakai et al. 2001).

Of all the genetic markers available in population genetic studies, a microsatellite marker is a good choice to investigate the genetic and colony structures of social insects due to their high variability (Vargo and Husseneder 2011). A microsatellite, also known as a 'simple sequence repeat' or a 'short tandem repeat', is a short, non-coding DNA fragment containing repeated sequence motifs (Oliveira et al. 2006). The mutation rate of a microsatellite is high, which renders it a good resolution to reveal the intraspecific differentiation within several thousand generations.

There are myriad studies using microsatellite markers to investigate the colony and population genetic structures of invasive termites (Vargo et al. 2003; Dronnet et al. 2005; Husseneder et al. 2005; Husseneder et al 2008; Yeap et al. 2011; Vargo and Husseneder 2011). *Reticulitermes* and *Coptotermes* are two of the most-studied genera (Vargo and Husseneder 2009). Microsatellite markers are also applied to identify the sources of introduced termites (Vargo and Husseneder 2011). They have been used to evaluate pest-control treatments for infestations in buildings, by helping to discover the colony turnover after eradication (Vargo 2003; Husseneder et al. 2007). Population genetic studies have additionally shed light on the attributes correlated with a successful termite invasion. Reduced genetic variation has been found in introduced colonies of both *Reticulitermes* and *Coptotermes* (Vargo 2003; Dronnet et al. 2005; Husseneder et al. 2005; Vargo et al. 2006a).

Only a few genetic studies of invasive termites focus on the colony and population genetic structures of the species in native regions, and of these, even fewer of *C. gestroi* (Vargo and Husseneder 2011). Even though it is regarded as the most economically important invasive pest, our knowledge about it is quite limited (Rust and Su 2012). To date, there is only one paper adopting the microsatellite method to reveal the population genetic structure of *C. gestroi* in its native region (Yeap et al. 2011). Yeap et al (2011) found that *C. gestroi* in South and Southeast Asia was genetically homogeneous but provided no information about colony structure. Considering the limited flying capacity of *Coptotermes* (Messenger and Mullins 2005), the genetic structure identified in their study resembled the structure pattern of introduced species, although *C. gestroi* Asia is native to this region (Yeap et al. 2011). Perhaps most importantly, 84% of the samples in Yeap et al (2011) came from Peninsula Malaysia and Singapore, making it unlikely that their results represent the actual population genetic structure of *C. gestroi* across all of Southeast Asia.

Termites are special insects because they do not inherit only genes from their parents, but also gut flora, directly or indirectly (further explained in 1.7). Gut flora are transferred between nestmates in termite colonies, which may induce coevolution between termites and symbiotic gut flora (Brune and Dietrich 2015). A few papers reported that diversity and structure of gut microbes reflected phylogenetic relationship of hosts, at least to some extent (Dietrich et al. 2014; Tai et al. 2015; Ranhman et al. 2015; Brune and Dietrich 2015). One study compared the gut bacterial community of three lab colonies of *Reticulitermes flavipes*, suggesting that variation in bacterial composition may be related to colony genetics (Boucias et al 2013).

However, no research to date has studied whether termite gut flora have a specific correlation with colony genotypes and can reflect the genetic relationships of intraspecific colonies the way microsatellites or other genetic markers do. Given the high diversity of gut microbes, confirming this will provide an effective facilitated tool for genetic study.

1.6 General information about flora communities in the termite gut

There are seven families of termites: Mastotermitidae, Termopsidae, Hodotermitidae, Kalotermitidae, Serritermitidae, Rhinotermitidae and Termitidae (Krishna et al. 2013). These seven families can be divided into two informal groups: higher and lower termites. All termite species harbor bacteria and archaea in their guts; but lower termites also have flagellated protists in their guts, while higher termites do not. Termitidae is the only member of higher termites, which is phylogenetically apical and account for three quarters of termite species. The rest six families consist of lower termites and are phylogenetically basal.

Termites are one of the few animals that can digest cellulose, hemicellulose, and lignin, which are the main constituents of plant cell walls, and particularly abundant in wood and other structural plant tissues (Matsui et al. 2009). Higher and lower termites have different ways to digest this recalcitrant food. Higher termites are classified into two types: fungus-cultivating and non-fungus-cultivating. Fungus-cultivating termites grow a symbiotic fungus in their nest, which helps decompose wood, while non-fungus-cultivating termites rely on symbiotic bacteria in the gut for wood digestion (Radek 1999; Aanen et al. 2002). For lower termites, protists play the crucial roles in food digestion. Termites ingest and grind wood material into

small pieces using their mandibles and gizzards (Noirot 1995). Then, endogenous enzymes, such as endoglucanase and B-glucosidase, produced by the salivary gland and/or midgut, partly break down the particles into glucose (Watanabe and Tokuda 2010). The major work of food degradation is accomplished by protists in the hindgut, with hindgut bacteria contributing mainly to the cellodextrin degradation that follows cellulose breakdown (Tokuda et al. 2014; Brune and Dietrich 2015). Because *Coptotermes gestroi* is a typical lower termite, this thesis focuses on the microbial community of lower termites.

A single gut of a lower termite possesses 10^3 to 10^5 protist cells, occupying more than 90% of the volume of the hindgut and generally presenting 1 to 20 morphologically distinct species (Hongoh 2010). These species are categorized into either the Parabasalia or Preaxostyla phylum, of the order Oxymonadida. Some gut protists of lower termites are also found in the wood-feeding cockroach *Cryptocercus*, implying that both insect groups acquire the gut protists from a common ancestor (Ohkuma 2008).

A single gut also has 10^6 to 10^8 prokaryote cells, fewer than 10% of which are archaea (Hongoh 2010). The most common archaea are methanogens, and the *Methanobrevibacter* species is dominant (Hongoh and Ohkuma 2010). The largest proportion of prokaryotic organisms is bacteria, which have gone unrecognized for a long time because most of them are not cultivable in the lab (Hongoh 2011). However, due to the development of culture-free technology such as pyrosequencing, researchers now have a better understanding of the diversity of gut bacteria (Brune and Dietrich 2015; Berlanga and Guerrero 2016).

More recent studies show that the gut of lower termites harbors hundreds of bacteria phylotypes, many of which are specialized to the termite gut (Hongoh 2010). The Spirochaetes, Bacteroidetes, Firmicutes, and Proteobacteria phyla compose most of the bacterial community, and Spirochaetes is specific to termites (Berlanga and Guerrero 2016). Furthermore, the genus *Treponema* in the Spirochaetes phylum, the order Bacteroidales in the Bacteroidetes phylum, and the class Clostridia in the Firmicutes phylum are all dominant in the gut bacteria community. Endomicrobia in Elusimicrobia, the TG3 phylum, and the Fibrobacteres phylum are also prevalent in some species (Hongoh et al. 2003; Hongoh et al. 2005). Free-swimming bacteria are uncommon, and most are stationary and clustered in certain locations, such as the gut wall, luminal fluid, and the surface and interior of protists (Ohkuma 2008; Hongoh 2011).

In fact, many bacterial species are endo- or ectosymbionts of protists (Brune and Stingl 2005; Hongoh and Ohkuma 2010). One-to-one specific symbiosis has been observed between *Azobacteroides* and *Pseudotrichonympha* protists, *Candidatus endomicrobium* and *Trichonympha* protists, and *Candidatus Armantifilum* and devescovinid protists (Noda et al. 2007; Ikeda-ohtubo and Brune 2009; Desai et al. 2010). Other studies have shown non-specific symbiosis between bacteria and protists (Okuma 2008; Hongoh and Ohkuma 2010).

In lower termites, protists make major contributions to cellulose digestion and generate acetate, which is the main energy and carbon source of the species (Yamin 1979; Bandi et al 2000). It

is now recognized that bacteria also play a role in cellulose digestion (Tokuda et al. 2014). Besides that, bacteria have other key functions to support the survival of host termites.

One major function of bacteria is nitrogen capture. Wood as a food resource is essentially carbohydrate, so low in protein and therefore nitrogen, thus it is crucial for termites to efficiently obtain and preserve sufficient amounts of nitrogen. The complete genomic sequencing of phylotype Rs-D17, belonging to *Candidatus endomicrobium*, indicates that it has the ability to synthesize amino acids (Ohkuma 2008; Ayayee et al. 2015). Fifteen amino acids and various cofactors can be synthesized by upgrading nitrogenous compounds like NH_3 for use by termites and protists (Hongoh 2011). Similar genomic sequencing of endosymbiotic phylotype CfPt1-2, belonging to the order Bacteroidetes, predicts that it can produce amino acids and recycle elements from the nitrogen waste of host protists (Hongoh et al. 2008a). Additionally, genes related to nitrogen fixing such as *nifH* genes have been reported in the genus *Treponema* and other members of the phylum Fibrobacteres (Warnecke et al. 2007).

Bacteria participate in the metabolism of hydrogen and acetate in the termite gut. Protists are the dominant producers of H_2 in lower termites (Hongoh 2011). The accumulation of H_2 restrains fermentation, which produces acetate and changes the H_2 gradient in the gut paunch (Ohkuma 2008). Both ectosymbiotic spirochaetes and endosymbiotic *Bacteroidales* consume H_2, and the former produce acetate as well (Inoue et al. 2008). Bacteria help maintain the H_2 balance in the termite gut. Moreover, the endosymbiotic *Candidatus endomicrobium* can turn sugar to acetate through substrate-level phosphorylation (Hongoh et al. 2008b). It is estimated

that bacterial acetogenesis contributes to one-quarter of all acetate.

Apart from material metabolism, gut bacteria may serve other functions such as preventing the introduction of alien bacteria (Engel and Moran 2013). Termites and their gut microbe communities have evolved a mutually beneficial network on multiple levels (Figure 1.2). Termites provide shelter and a favorable micro-niche for gut microbes, while microbes enable the insects to survive on nutrient-poor but abundant foods, which allows for the exploitation and high abundance of the hosts. This reciprocal relationship lays the foundation of coevolution for gut microbes and termite hosts.

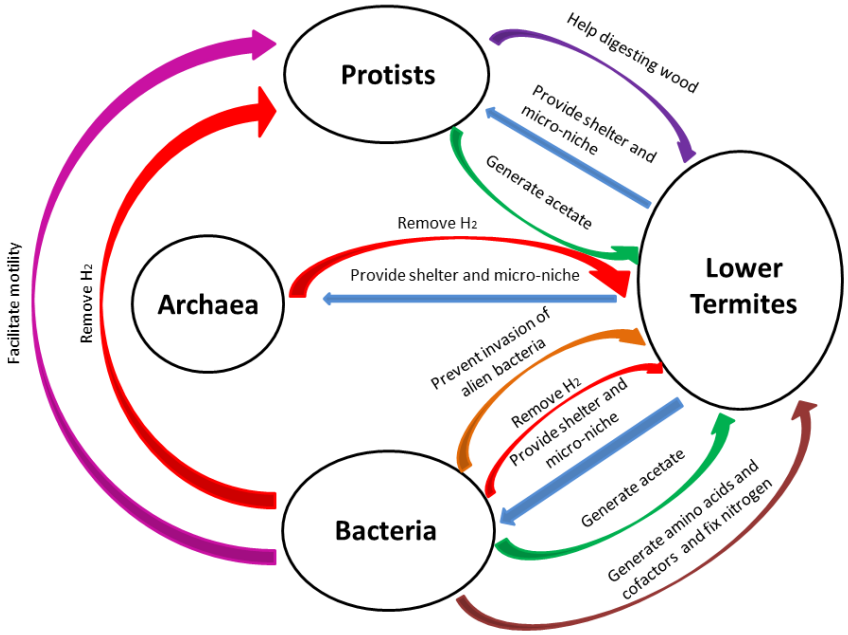


Figure 1.2. Interaction among termites, protists, archaea and bacteria.

1.7 Coevolution of termites and the gut flora community

Termite gut flora are strongly associated with their hosts through functional reciprocity. Vertical transmission of gut flora within the colony enhances the correlation and may lead to the

coevolution of termites and symbiotic microbes (Eggleton 2006; Engel and Moran 2013; Nalepa 2015; Brune and Dietrich 2015). Vertical transmission begins when founding termites start a new colony. In the initial stage of the colony, the founding adults feed the first few instars on hindgut fluids via proctodeal trophallaxis (Nalepa 2015; Brune and Dietrich 2015). The young instars are nutrient dependent before the third instar and rely on repeated trophallactic input to obtain nutrition and gut symbionts. When the colony is established, trophallactic behavior shifts from being parental to alloparental, which means newly born termites receive their hindgut content from their sibling nestmates (Nalepa 2015). Moreover, lower termites lose gut flora during their molting cycle and re-acquire the microbes though proctodeal trophallaxis (Nutting 1956; Nalepa 2015). In sum, the gut flora of lower termites are transferred either from parents to offspring or between nestmates, and this stable vertical transmission may contribute to the impact of the hosts' evolutionary factors observed in the termite gut community.

Studies have reported significant phylogenetic signals in the structure of termite gut flora (Hongoh et al. 2005; Reid et al. 2014; Dietrich et al. 2014; Tai et al. 2015; Rahman et al. 2015; Brune and Dietrich 2015). Dietrich et al (2014) demonstrated that the structure pattern of the gut bacterial community reflected a major evolutionary division of host termites, and the phylogenetic trees of several higher termite species almost matched the cladogram of the bacterial community. Tai et al (2015) found that the composition of protists, parabasalids, and several phyla of gut bacteria in lower termites were structured by host phylogeny. Moreover, another investigation that includes seven higher and nine lower termite genera showed that the

trees of the host phylogeny and the bacterial community were congruent (Rahman et al. 2015). These studies reflect the importance of host phylogeny to the gut community and support coevolution between host and microbial symbionts. Some of these results illustrate that the clustering of the gut flora community corresponds to the family or genera clustering of the host termite, but it has not been investigated whether it also corresponds to colony clustering.

1.8 Impact of habitat and diet on shaping termite gut flora

Some research that demonstrates the importance of host phylogeny also suggests that it might not be the exclusive factor in shaping the gut community; habitat and diet may also be involved (Rahman et al. 2015; Brune and Dietrich 2015). A gut community profile suggested that the cockroach, which is evolutionarily close to termites, might acquire microbes from their habitat (Dietrich et al. 2014). Although no such evidence exists for termites, some lab experiments indicate that diets can change their gut flora community. Huang et al (2013b) has reported that corn stover (i.e. stalks and leaves) decreased, while wood increased, the microbial diversity in *Reticulitermes flavipes* when provided as food in the lab for several days. In a 30-day test, Tanaka et al (2006) observed a 40% reduction of gut microbial diversity in wood-fed *C. formosanus* compared to cellulose-fed ones. Changes occurred probably because termites need to adapt to the new diets. However, no research to date has focused on environmental and dietary factors using a termite colony in the field; a lab experiment may not accurately represent the natural situation of a termite gut community.

1.9 Thesis aim and objectives

In this thesis, I investigated the population genetic structure and colony structure of *Coptotermes gestroi* across Southeast Asia. I also investigated the diversity and structure of gut bacterial community of *C. gestroi* and their correlation with genotype of host colony and habitat. My research objectives includes: 1) determining the most significant factor affecting the population genetic structure of *C. gestroi* in its native region; 2) studying the relative importance of natural dispersal compared to human transport in distribution of *C. gestroi* across its native region; 3) investigating the correlation between host phylogeny at colony level and gut flora community and; 4) investigating roles of habitat and diet in shaping gut flora community of *C. gestroi* in nature. My aim was to reveal the important aspects in spread and adaptation of this invasive species and provide valuable information for pest management.

1.10 Overview of chapters

In chapter 2, I studied the population and colony genetic structure of *Coptotermes gestroi* across Southeast Asia. I revealed the correlation between geographic features, such as distance, mountains and oceans, and population genetic structure of the species in its native region. I tried to figure out roles of natural dispersal and human transport in distribution of *C. gestroi* in Southeast Asia.

In chapter 3, I investigated the possible correlation between gut bacterial community of *C. gestroi* and host phylogeny at colony level. I examined whether gut community could reflect the genetic relationship of *C. gestroi* colonies and the potential of gut bacterial community as facilitated tool in genetic study.

In chapter 4, I surveyed the diversity and structure of gut bacterial community of *C. gestroi* and determined impact of habitats and host trees on shaping the gut community. In chapter 3, I found structure of gut bacterial community seemed to be correlated with habitat rather than host phylogeny. I confirmed the impact of habitats in this chapter and revealed how gut bacterial community responded to host habitat. I also listed bacterial genera shared by all *C. gestroi* colonies, regardless habitats and host trees. These genera may be the essential bacteria for survival of *C. gestroi* and I discussed the potential function of these genera.

Chapter 5 is the final conclusion of thesis by synthesizing all previous chapters.

Chapter 2: Population genetic diversity and structure of *Coptotermes gestroi* across Southeast Asia

2.1 Introduction

Population genetic studies try to quantify the genetic variation of natural populations and provide clues for the potential evolutionary and ecological factors that lead to variation. Social insects are interesting systems for population genetic studies because they have an organized and independent social structure. The population of social insects is colony-based rather than individual, so population genetic structure is correlated with colony genetic structure (Vargo and Husseneder 2011).

Much research has been done on the population genetic structure of termite species because termites are social insects and some are invasive (Vargo et al. 2003; Dronnet et al. 2005; Husseneder et al. 2005; Husseneder et al 2008; Yeap et al. 2011; Vargo and Husseneder 2011). Within the most recent centuries, modern transport has promoted the dispersal and genetic flow of most invasive termites. As a result, anthropogenic activity could be another important factor influencing termite population genetic structure (Evans et al. 2013). Although many studies have been conducted, most population genetic studies of invasive termites involve populations in their introduced ranges rather than their native areas and focus on *C. formosanus* and *R. flavipes* (Vargo et al. 2003; Dronnet et al. 2005; Husseneder et al. 2005; Vargo and Husseneder 2009; Vargo and Husseneder 2011).

Coptotermes gestroi is a destructive and invasive termite, whose native populations have been studied little (Yeap et al. 2011). There is one paper about the population genetic structure of *C. gestroi* that uses microsatellite markers (Yeap et al. 2011). Yeap et al (2011) sampled 85 colonies of *C. gestroi* across the species' native area, Southeast Asia, and included several introduced colonies from Taiwan and Hawaii. She reported moderate genetic differentiation and admixture of populations in Peninsular Malaysia and Singapore. Four clusters were identified for all 13 putative populations, but the entire population did not seem to be structured. The revealed population genetic pattern resembles that of an invasive species, although *C. gestroi* is native to Southeast Asia. Moreover, most of the samples were collected from Singapore and Malaysia, while a large part of Southeast Asia was not covered in that study. Another study by Li et al. (2012) used COII and 16S gene sequences and reported three phylogenetic clades: Clade I (Thailand, Malaysia, Singapore and Indonesian populations), Clade II (Hainan, Taiwan and one Philippines samples) and Clade III (two Philippines samples) (Li et al. 2012). However, they had only 19 samples in their paper. Consequently, the colony structure of *C. gestroi* in Southeast Asia has never been studied and remains unknown.

In this study, I collected 103 *C. gestroi* samples from 7 Southeast Asia countries and 2 introduced districts. My sampling covers a larger native range than that covered by both studies above and includes colonies from Cambodia and Vietnam for the first time. This study investigates the colony and population genetic structure of *C. gestroi* in Southeast Asia; the correlation between geographic features and genetic structure; and the role of human transport

in the dispersal of *C. gestroi* across Southeast Asia. I hypothesized that geographic factors would be most important in determining the population structure of native *C. gestroi* colonies and that natural dispersal, rather than human transport, mainly contributed to the distribution of the species in its native region.

2.2 Material and methods

2.2.1 Sample collection

Sampling sites were displayed in Figure 2.1. *C. gestroi* samples were obtained from 9 countries and collected from human constructions, street trees, regrowth or secondary forests on 'wastelands', urban parks and natural forests (Appendix 1). Both worker and soldier caste were included in the samples. All termites were preserved in 100% ethanol immediately after collection on location, and stored at -20℃ until extraction.

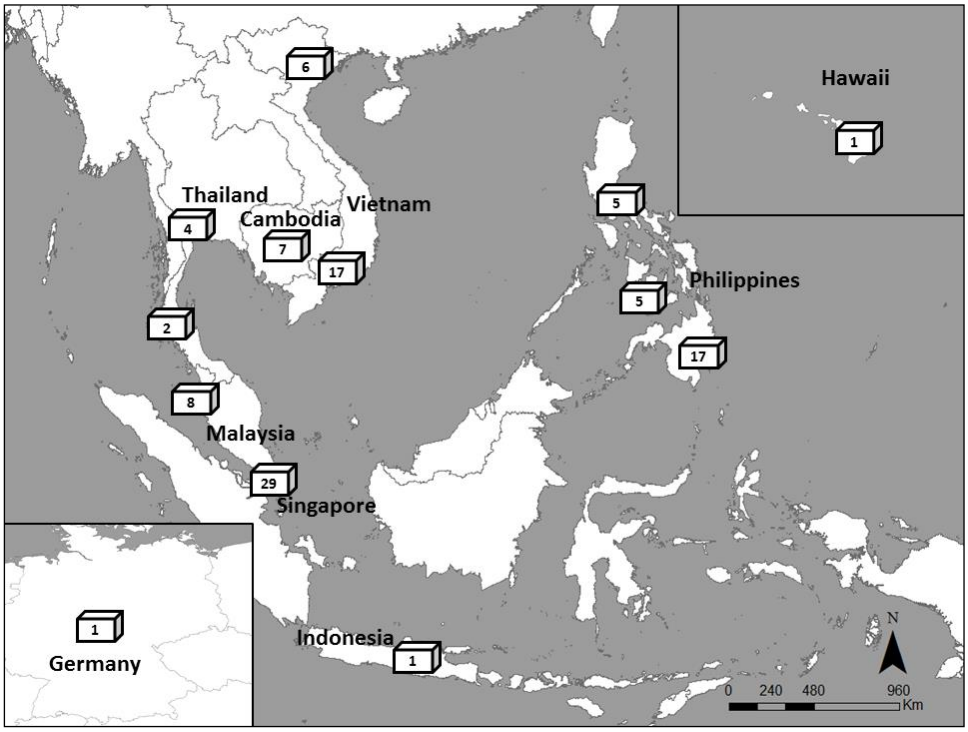


Figure 2.1. Map of sampling location of *Coptotermes gestroi* across Southeast Asia.

2.2.2 DNA extraction, amplification, species confirmation and genotype analysis

I extracted genomic DNA from individual workers and soldiers using the CTAB extraction method (Winnepenninckx et al. 1993). I crushed the whole body of workers, or the head of soldiers (we did not use the bodies of soldiers to avoid defensive compounds, which may interfere with the DNA extraction), with beads and OMNI bead Ruptor. I identified species using morphological characters (Bouillon and Mathot 1965), and the 1kb portion of cytochrome oxidase subunit II (CO2) gene. I amplified the genomic DNA with the primer set C2F2:5/B-tLys, sequenced the PCR products, and used a BLAST on the NCBI database to confirm species identify (Li et al. 2009). I used only confirmed *C. gestroi* samples in all subsequent analyses.

I genotyped 17 to 24 individuals from each colony at nine microsatellite loci; four loci were identified from *C. gestroi* and five were from *C. formosanus* (Table 2.1). I used a fluorescent labeled forward primer in each primer set, and I amplified seven of the loci in three groups by multiplexing: CG6 and CG21; CG26 and CG33; CF4:1A2-5, CF4-10 and CF10-5. I amplified CF4-9A and CF8-4 independently as both were incompatible with the others. Polymerase chain reaction of CG primer sets was conducted with a modified version of Yeap et al (2009): 2 min of initial denaturation at 95℃，followed by 35 cycles of three steps PCR at 95 ℃ for 30 sec, 58 ℃ for 30 sec and 72 ℃ for 1 min and a final extension at 72℃ for 10 min. loci cf8-4 and cf4-9 were amplified separately according to the touch-down program of Vargo (2000) with slightly changes: initial denaturation at 95℃，followed by six cycles of PCR at 95℃ for 30

sec, 60 ℃for 30 sec and 72℃ for 30 sec, and then ramping down annealing temperature 1 per cycle, and 30 cycles at 54 ℃ for annealing temperature. Final step was extension at 72 for 5 min. For group 3, I used Qiagen multiplex kit and followed the standard protocol. PCR products were sequenced using the ABI3730 sequencer, with a GeneScan-600 LIZ size standard. I scored allele sizes with Genemapper.

Table 2.1. The nine microsatellite markers used for *Coptotermes gestroi*. All CG loci from Yeap et al. (2009); all CF loci from Vargo (2000).

Locus	Repeat	Size	Dye	Primers(5'-3')	Accession no
CG6	$(GT)_{12}$	160-188	NED	F:CACCCGTTGAAATTGACCTT	GQ412734
				R:AGACCGTTCCCAGCAACTTA	
CG21	$(CCAA)_9(CCAT)_6$	159-197	VIC	F:TACCTACCGACCGAACGAAC	GQ412737
				R:TCCTGTTACAGCCCCAAAAG	
CG26	$(CT)_6(GTCT)_7$	188-228	NED	F:AAGCTCATTACGCGCAACTT	GQ412739
				R:GTGAAGCCTCGACAATGAGG	
CG33	$(CAA)_{16}$	185-224	PET	F:TTTCATCGAAAGTGCAGGTG	GQ412742
				R:TGTCGCATGAGGAAGATGTC	
CF4:1A2-5	$(CAA)_{10}$	141	FAM	F:TCGGACTCCAGGTACTACCAA	AF247459
				R:GATTGCCGTTCCTTCCTTCT	
CF4-10	$(CAT)_{11}$	229	NED	F:GCAAGTTTTGCCCTGTCAGT	AF247465
				R:GAAAAACAGCGACTGCTTCC	
CF10-5	$(GAT)_8$	295	VIC	F:CAGCTATATTGGGCACAGCA	AF247470
				R:CACGACGGACTGAAGTGGTT	
CF4-9A	$(TCA)_{11}$	283	FAM	F:GTGTGGGATTTGAGGTGGAC	AF247464
				R:GAAAAACAGCGACTGCTTCC	
CF8-4	$(CTA)_9(CTC)_{15}$	221	VIC	F:TCTGTGGAACGTGGTGTGAT	AF247468
				R:CCTCTCTGTGCCTGCTTAGG	

2.2.3 Genetic patterns

Since individual termites within the colonies are close kin and not genotype independent, one individual per colony was used to test deviation from Hardy-Weinberg equilibrium (HWE) and pairwise linkage disequilibrium (Yeap et al. 2011; Huang et al. 2013a). I conducted the check with exact test using GENETIC DATA ANALYSIS version 1.1 of 3200 shufflings (GDA;

Lewis and Zaykin 2001). I performed 3 replications with one randomly chosen individual per colony for above tests. Observed heterozygosity and expected heterozygosity were also calculated with single individual per colony using GDA and average values of 3 replications were obtained. I collated other basic statistic information across all loci and colonies using Genalex6.5 (Peakall and Smouse 2012). I checked for colony affiliation by means of G-test implemented in GENPOP4.3 (Raymond and Rousset 1995).

I searched for any recent genetic bottlenecks in three populations using BOTTLENECK v.1.2.02 (Piry et al. 1999) with one individual per colony, based on the clusters assigned by STRUCTURE. A population usually displays significant heterozygosity excess after passing through a genetic bottleneck, such as a small initial population. I used a Wilcoxon sign-rank test under Stepwise Mutation Model (SMM) to detect the excess heterozygosity.

2.2.4 Genetic spatial structure

I investigated genetic relationships among colonies in two ways. First, I used Bayesian model based clustering software STRUCTURE (Pritchard et al. 2000). Three runs were performed in STRUCTURE analysis, each using different randomly resampled data set with one individual per colony (Yeap et al. 2011; Huang et al. 2013a). For all colonies, I assessed the potential number of populations, genetic clusters (K), from 1 to 20, with ten replicates each, and all simulations were run under an admixture model with 50,000 replicates burn-in length and 100,000 for Markov chain Monte Carlo. Three populations were discovered. Then for each putative population, STRUCTURE analysis was performed with 200,000 replicates of burn-in

length and 200,000 for Markov chain Monte Carlo and assumed genetic clusters was from 1 to 10. The most likely K of all analysis was then determined by Structure Harvester (Earl and vonHoldt 2012), and I visualized results using DISTRUCT (Rosenberg 2004). Second, I used Principal Coordinate Analysis (PCoA) to show the genetic differentiation of all colonies and within each putative population. I created a scatterplot based on the matrix of mean genetic differentiation values from all pairwise comparisons. Each colony was represented by mean values of all individuals in that colony. ANOSIM was adopted to evaluate the significance of clustering in PCoA using vegan package in R version 3.2.0 (R Development Core Team 2008).

I assessed the potential relationship between geographic distance and genetic differentiation of three genetic populations suggested by STRUCTURE, using isolation by distance analysis. I calculate genetic differentiation as $F_{ct} / (1 - F_{ct})$, where we obtained pairwise F_{ct} between all colonies using GenAlEx (Peakall and Smouse 2012). I calculated Pearson's correlation coefficient for genetic differentiation and the logarithm (ln) of geographical distance, and assessed significance with a Mantel test with 9,999 replicates, also implemented in GenAlEx.

2.2.5 Colony and population genetic structure

I explored colony and population genetic structure using fixation indices (a.k.a. F-statistics; Wright 1949) following Weir & Cockerham (1984), and we calculated relatedness of colony members after Queller & Goodnight (1989), both in Fstat (Goudet 2001). I assessed significance by calculating the 95% confidence intervals (CIs) by bootstrapping over loci with 10,000 replicates to both F-statistic and relatedness. I followed the notation of Thorne et al (1999) and Bulmer et al (2001), so that F_{it} replaced the standard inbreeding coefficient, F_{is},

indicated inbreeding level of the individual relative to the total; F_{ct} represented F_{st}, the genetic differentiation among colonies; and F_{ic} was the coefficient of inbreeding between individuals within colony, which is strongly associated with number of reproductives.

I investigated the family type of each colony by calculating the deviation from expected Mendelian ratios by G test of goodness-of-fit for all loci and all colonies. I considered a colony to be a simple family, i.e. possessing one pair of reproductives, when I observed no significant deviation in all loci. I considered a colony to be an extended family, i.e. to have more than two related reproductives, when any of the following scenarios occurred: (1) any locus had more than four genotypes, (2) any locus had more than three homozygote genotypes, and (3) at least one locus showed significant deviation ($P < 0.05$). I considered a colony to be a mix family, i.e. having more than two unrelated reproductive, when one or more locus have more than four alleles (Vargo and Husseneder 2011).

Analysis which is not specifically indicated used 17 to 24 individuals in each colony.

2.3 Results

2.3.1 Basic genetic information

A total of 2,273 individuals of *C. gestroi* from 103 colonies were genotyped and analyzed over nine loci (Appendix 1). Locus pairs CF4-10 and CF4-9 showed a linkage disequilibrium, so CF4-9 was excluded from subsequent analyses (Table 2.2). No linkage disequilibrium and deviation from Hardy Weinberg equilibrium (HWE) were detected for the remaining loci.

Genotype differentiation based on a G-test (all pairwise $P < 0.05$) confirmed that 103 samples were distinct colonies. The average numbers of alleles and gene diversity over eight loci were 19.75 (range of 13 to 30 alleles) and 0.53, respectively, demonstrating that alleles are quite diverse across Southeast Asia (Table 2.3).

Based on STRUCTURE assignment, Southeast Asian colonies were divided into three populations: Northern, Southern, and Philippines. Subpopulations were also identified for each population based on STRUCTURE assignment. The expected heterozygosity of all populations and subpopulations was larger than observed as indicated by a two-tailed t-test (Table 2.3, Table 2.4) ($P=0.00004$). No bottleneck was detected in any of the three populations (Northern: $P=0.677$; Southern: $P=0.843$; Philippines: $P=0.723$). The average allele per locus and the gene diversity of the Philippines population were lower than those of the Northern and Southern populations, but the differentiation was not significant, as indicated by a two-tailed t-test (average allele: $P=0.676$, $P=0.745$; gene diversity: $P=0.841$, $P=0.655$). For six out of eight loci, the Southern population covered the majority of the alleles in the allele pool (Figure 2.2). On average, the Northern and Philippines populations contained similar proportions of alleles for each locus. Table 2.4 shows the shared and unique alleles of three subgroups for each locus. The Northern and Philippines populations had fewer shared alleles on average, compared to the other two pairs, which indicates that these two groups might be less similar. The Southern population had the most unique alleles.

Table 2.2. Sampling number for each location across Southeast Asia.

Country	Region	N[1]
Thailand	Chombueng Distict, Ratchaburi Province	2
	Ban Takhun Distict,Surat Thani Province	1
	Mueng Distict,Surat Thani Province	1
	Bangkok	1
	Samut Sakorn	1
Vietnam	Hanoi	6
	Saigon	17
Cambodia	Phnom Penh	7
Singapore	Singapore	29
Malaysia	Penang	8
Philippines	Manila	5
	Bacolod	5
	Davao	17
Indonesia	Bogor	1
Germany	Germany	1
America	Hawaii	1
Total		103

1. Numbers of colonies.

Table 2.3. Allelic statistical data for *Coptotermes gestroi* in Southeast Asia for the microsatellite loci.

Loci	No. alleles	Frequency of most common allele	Allelic richness	Gene diversity	He[1] ±SE	Ho[2] ±SE
CG6	30	0.138	0.14	0.59	0.93±0.01	0.78±0.03
CG21	15	0.429	0.43	0.48	0.77±0.02	0.64±0.04
CG26	21	0.244	0.24	0.48	0.89±0.01	0.6±0.04
CG33	14	0.248	0.25	0.54	0.85±0.01	0.71±0.04
CF4:1A2-5	25	0.215	0.22	0.56	0.9±0.01	0.72±0.04
CF4-10	13	0.251	0.25	0.51	0.85±0.01	0.68±0.04
CF10-5	16	0.243	0.24	0.57	0.85±0.01	0.75±0.03
CF8-4	24	0.275	0.28	0.53	0.88±0.01	0.72±0.04
MEAN	19.75	0.255	0.26	0.53	0.87±0.01	0.7±0.04

1. He = expected heterozygosity

2. Ho = observed heterozygosity

Table 2.4. Allelic data for *Coptotermes gestroi* for the colonies in Southeast Asia. * indicates a significant difference between He and Ho (p = 0.00004)).

	Allele/Loci	Gene diversity	He[1] (SE)	Ho[2] (SE)
Native colonies				
All colonies	2.75	0.54	0.86 ±0.01	0.71 ±0.01*
northern population	2.74	0.55	0.79 ±0.01	0.72 ±0.02*
Hanoi	2.86	0.55	0.69 ±0.01	0.65 ±0.02
Phnom Penh +Saigon	2.71	0.55	0.77 ±0.01	0.74 ±0.04
Southern population	2.82	0.55	0.81 ±0.01	0.73 ±0.03*
Thailand	2.92	0.58	0.84 ±0.02	0.74 ±0.04*
Malaysia+Indonesia	2.46	0.45	0.72 ±0.01	0.60 ±0.05*
Singapore	2.9	0.58	0.80 ±0.01	0.77 ±0.02
Philippines population	2.66	0.5	0.83 ±0.01	0.66 ±0.02*
Davao1[3]	2.83	0.54	0.76 ±0.04	0.70 ±0.02
Bacolod	2.13	0.34	0.63 ±0.01	0.51 ±0.03*
Manila+Davao2[4]	2.71	0.53	0.85 ±0.02	0.69 ±0.03*
Introduced colonies				
German	1.63	0.15		
Hawaii	3.38	0.61		

1 He = expected heterozygosity

2 Ho = observed heterozygosity

3 Davao1: all Davao colonies except PHI33, PHI35 and PHI39.

4 Davao2: PHI33, PHI35 and PHI39

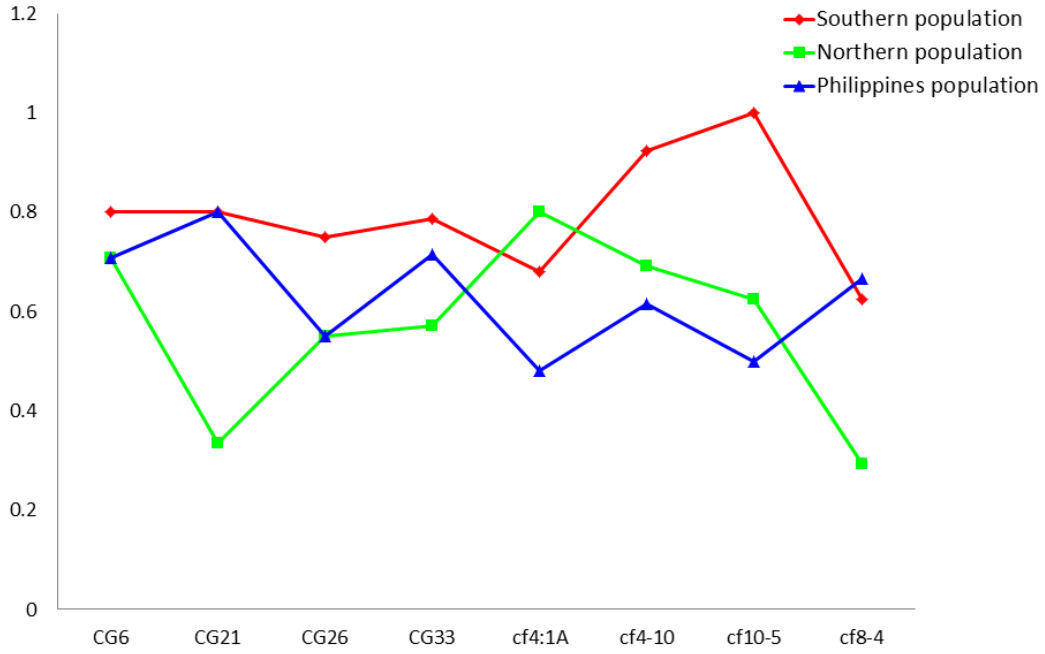


Figure 2.2. Coverage rate of alleles for each loci of 3 populations. Ratio= allele numbers of that particular population/total allele numbers of that loci, so the maximum value is one (all alleles across all locations are found in that population).

Table 2.5. List of shared and unique allele in Southeast Asia.

	CG6	CG21	CG26	CG33	CF4:1A2-5	CF4-10	CF10-5	CF8-4	AVERAGE
Shared alleles									
all three populations	7	4	4	6	7	5	7	5	5.63
Northern and Southern populations	4	1	3	1	5	3	3	2	2.75
Southern and Philippines populations	8	5	2	1	2	1	1	2	2.75
Northern and Philippines populations	2	0	3	1	4	0	0	0	1.25
unique alleles for each population									
Southern population	5	2	6	3	3	3	5	6	4.13
Northern population	4	0	1	0	4	1	0	0	1.25
Philippines population	0	3	1	2	0	0	0	9	1.88

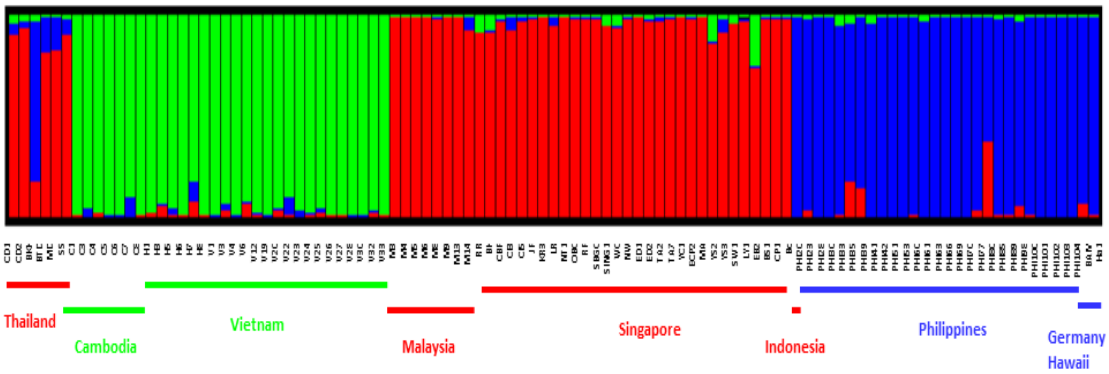
2.3.2 Genetic spatial structure

All three runs found three genetic clusters, based on deltaK in Structure Harvester (highest ΔK = 103; Appendix 2a) (Figure 2.3a). For 101 native colonies, colonies from Cambodia and Vietnam were generally in one cluster, which was the Northern population. The Southern population included colonies from Thailand, Malaysia, Singapore, and Indonesia, and the Philippines population included all of the Philippines samples. The two colonies introduced from Germany and Hawaii were clustered with the Philippines population, indicating that they may have originated from the Philippines. The clustering of the colonies showed spatial patterns; geographically close colonies were classified as one genetic group.

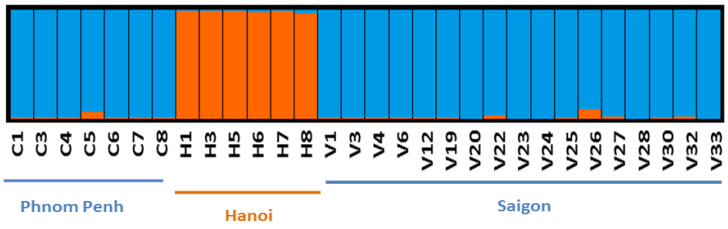
A STRUCTURE analysis of the Northern and Philippines populations also displayed sub-structure and geographic correlations. All three runs suggested two genetic clusters in the Northern population and three clusters in the Philippines population (Appendix 2b,d) (Figure 2.3b,d). Phnom Penh colonies were grouped with Saigon colonies, while colonies of Hanoi were in a distinct group. This is probably because Saigon is closer to Phnom Penh than to Hanoi. In the Philippines population, colonies from each island were in individual genetic cluster, except for PHI33, PHI35, and PHI39, which were clustered with the Manila colonies. Structure Harvester suggested three genetic clusters in the Southern population for all three runs (Appendix 2c). Colonies in Thailand and Malaysia seemed to be two genetic clusters, but genetic flow was observed among colonies in the Southern population (figure 2.3c).

The PCoA result generally corroborated the STRUCTURE assignment (Figue 2.4 a-d).

Sub-populations in the Southern population overlapped with each other (Figure 2.4c). Two notable points are that the Indonesia colony was closer to the Malaysia colonies, and that the Manila colonies seemed to be closer to the Davao colonies than to the Bacolod ones, although they are closer to Bacolod in terms of geographic distance. ANOSIM confirmed that the grouping in all PCoA plots was statistically significant ($p = 0.01$).



a



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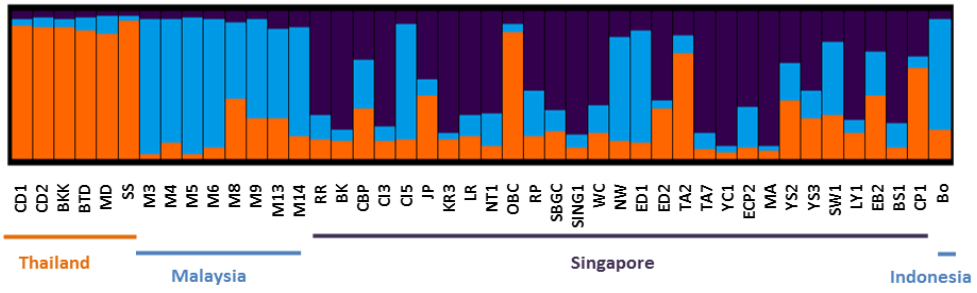

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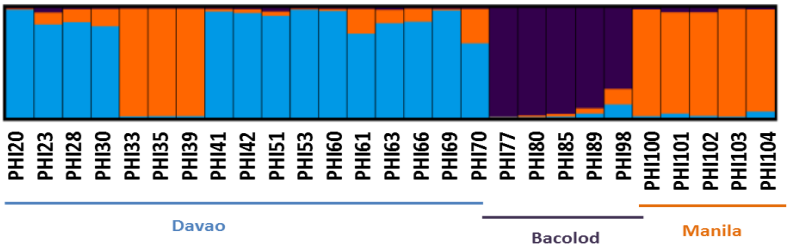
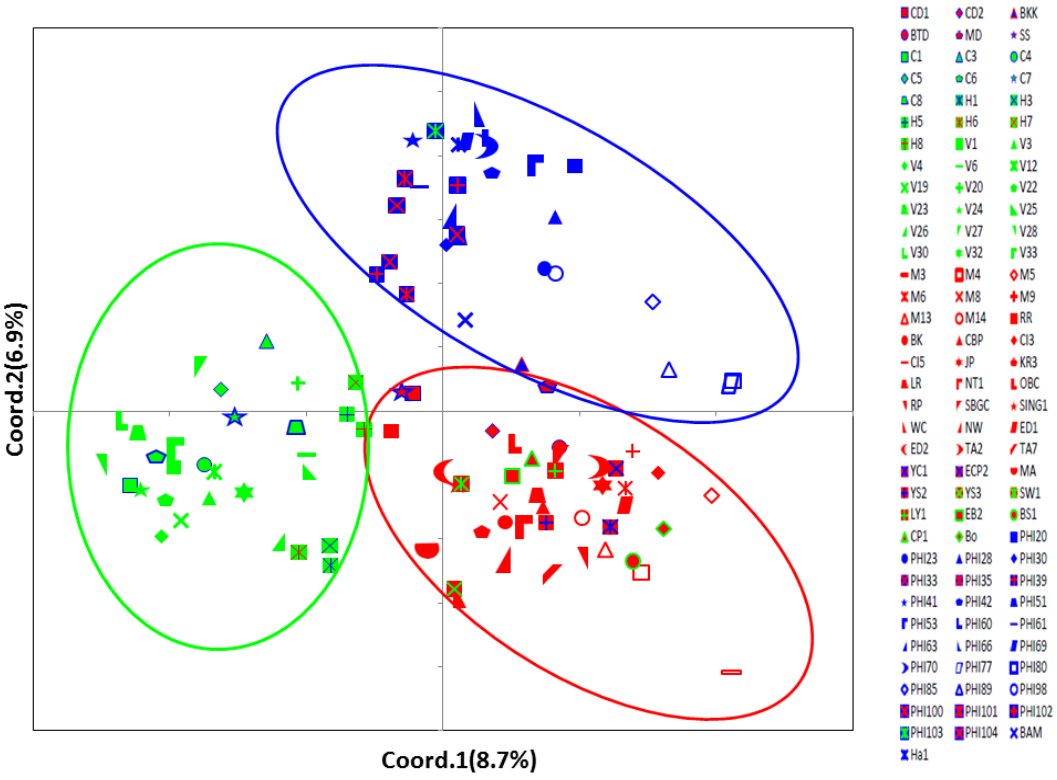
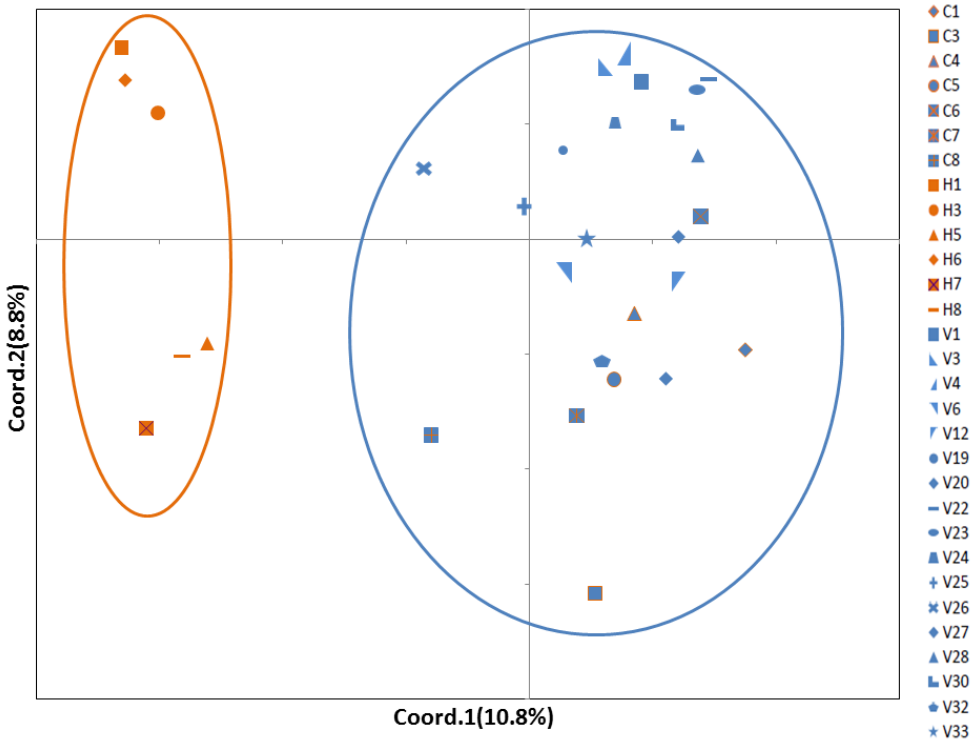


d

Figure 2.3. STRUCTURE plot showing the assignment of the 2273 individuals from the 103 *Coptotermes gestroi* colonies across Southeast Asia to genetic clusters. a) all colonies, K=3; b) Northern population, K=2, 30 colonies, 661 individuals; c) Southern population, K=3, 44 colonies, 979 individuals ; d) Philippines population, K=3, 27 colonies, 586 individuals


Coord.2(6.9%)
Coord.1(8.7%)
CD1 CD2 BKK
BTD MD SS
C1 C3 C4
C5 C6 C7
C8 H1 H3
H5 H6 H7
H8 V1 V3
V4 V6 V12
V19 V20 V22
V23 V24 V25
V26 V27 V28
V30 V32 V33
M3 M4 M5
M6 M8 M9
M13 M14 RR
BK CBP CI3
CI5 JP KR3
LR NT1 OBC
RP SBGC SING1
WC NW ED1
ED2 TA2 TA7
YC1 ECP2 MA
YS2 YS3 SW1
LY1 EB2 BS1
CP1 Bo PHI20
PHI23 PHI28 PHI30
PHI33 PHI35 PHI39
PHI41 PHI42 PHI51
PHI53 PHI60 PHI61
PHI63 PHI66 PHI69
PHI70 PHI77 PHI80
PHI85 PHI89 PHI98
PHI100 PHI101 PHI102
PHI103 PHI104 BAM
Ha1

a


Coord.2(8.8%)
Coord.1(10.8%)
C1
C3
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V4
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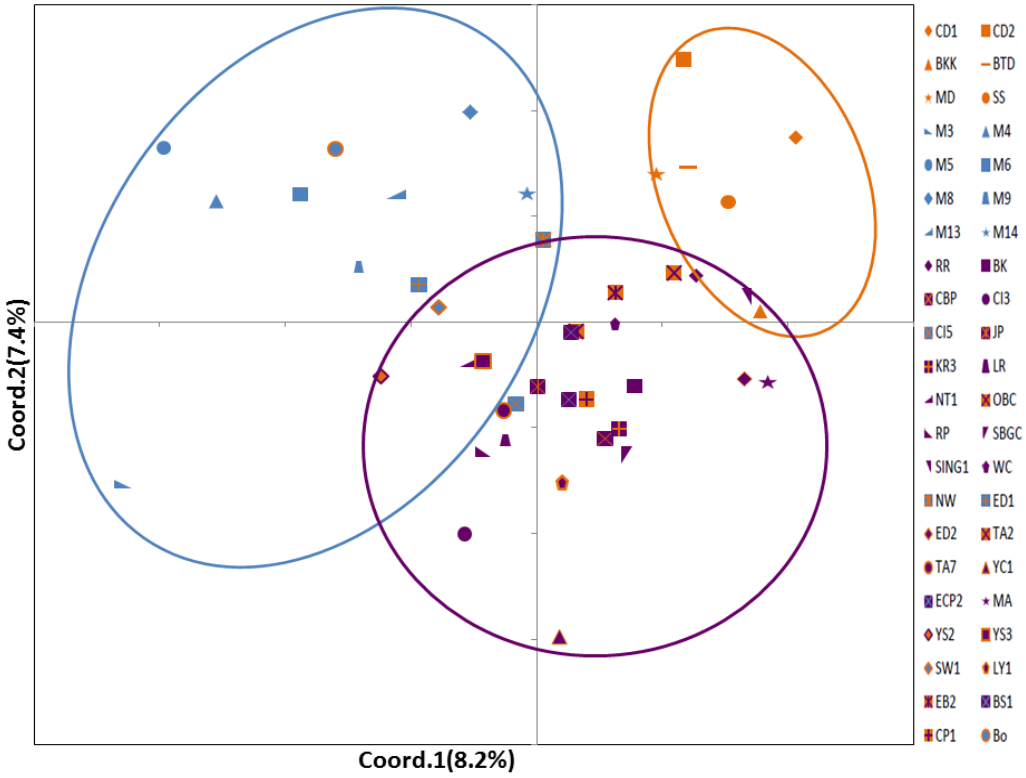
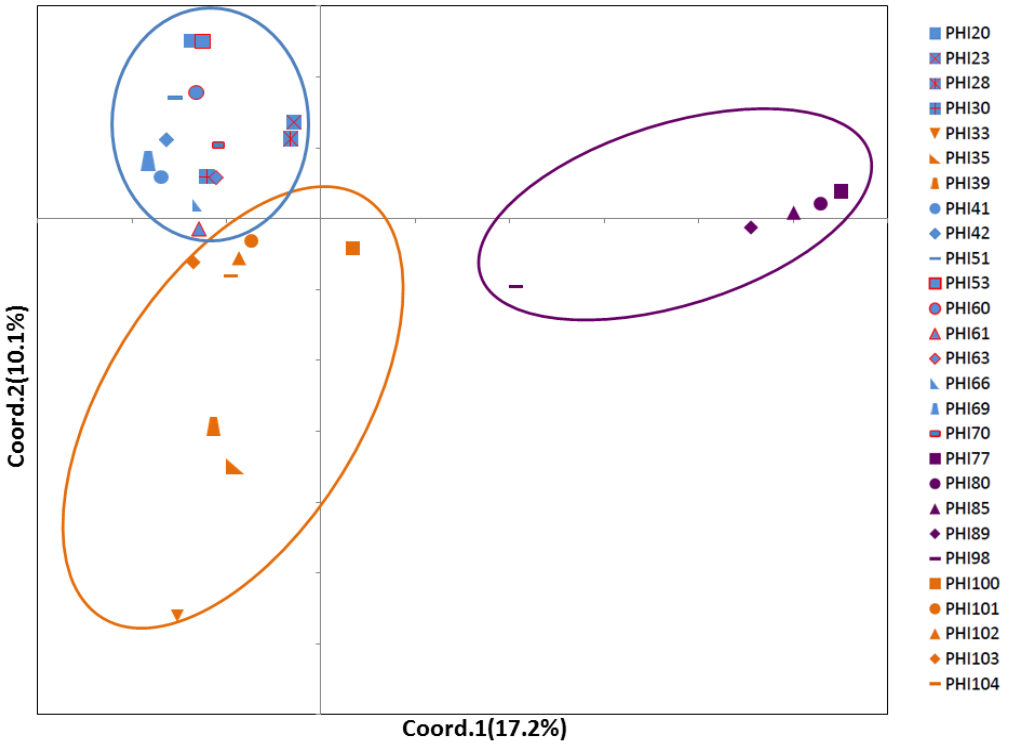
V32
V33

b



c



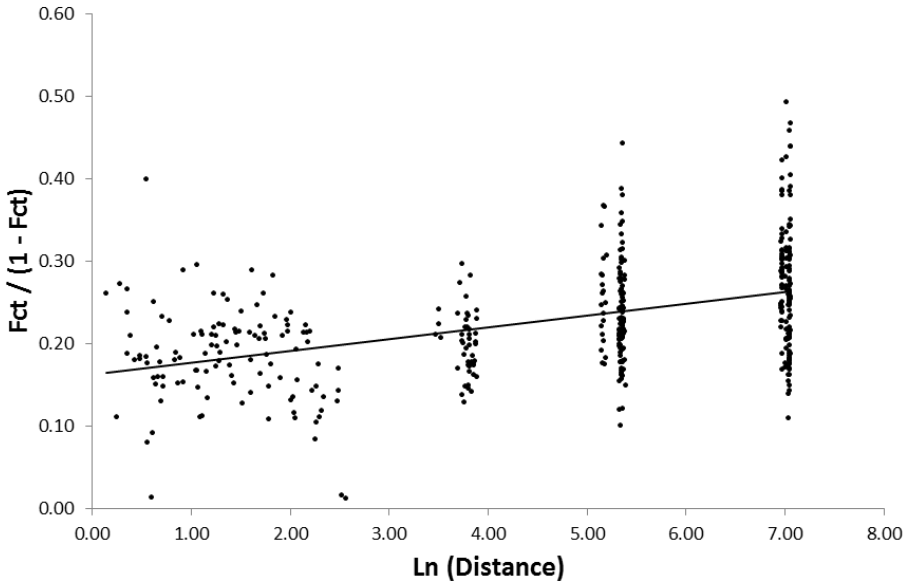
d

Figure 2.4. The principal coordinates analysis of the 103 *Coptotermes gestroi* colonies across Southeast Asia. The percentage of variation explained by the first two co-ordinates (the axes) is shown in brackets. Colonies are labelled by colors corresponding genetic clusters assigned by STRUCTURE. a) All colonies (ANOSIM, P = 0.001); b) Northern population (P = 0.001);

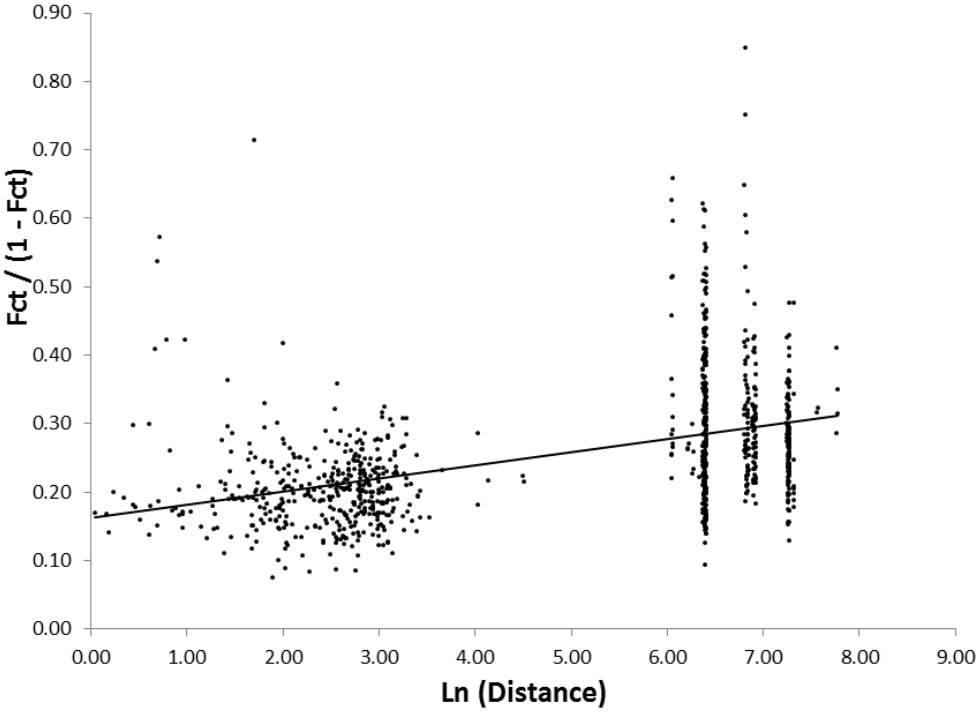
c) Southern population (P = 0.001); d) Philippines population (P = 0.001)

2.3.3 Isolation by distance

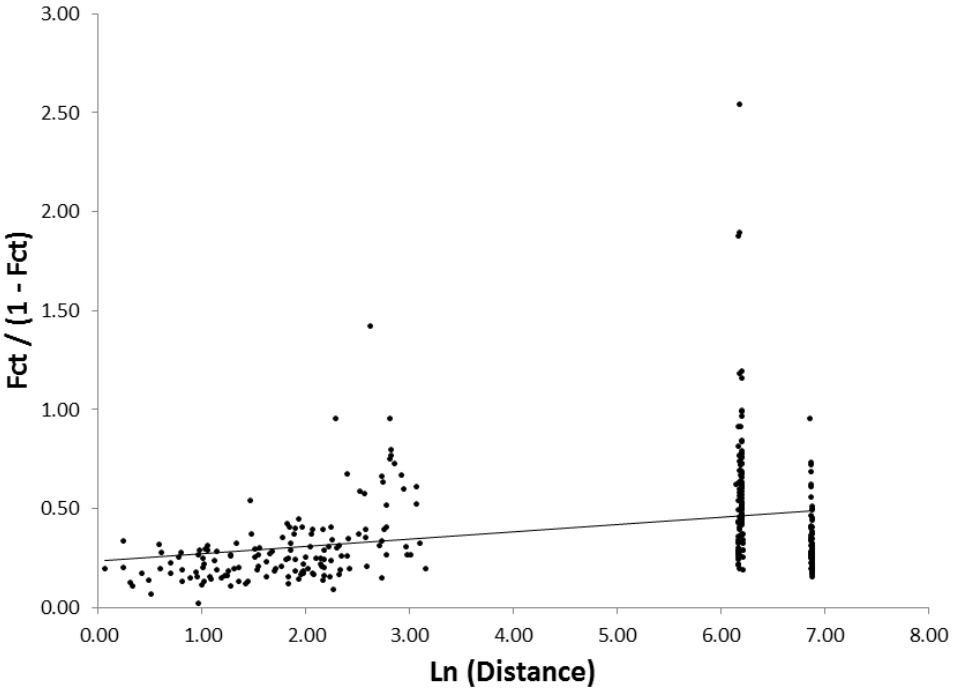
There was a strong and significant correlation between geographic distance and genetic differentiation (r = 0.46, P = 0.0001) for the Northern population (Figure 2.5a). Colonies in the Southern population also exhibited strong and significant isolation by distance (r = 0.44, P = 0.0001) (Figure 2.5b). Colonies in the Philippines population showed moderate and significant isolation by distance (r = 0.34, P = 0.0003) (Figure 2.5c).



a



b



c

Figure 2.5. Plot showing geographical distance and genetic distance [= F_{ct} / (1-$F_{ct)}$] between colonies of Northern population (a), Southern population (b) and Philippines population(c).

2.3.4 Colony features and population genetic structure

F_{ct} for all colonies was high (0.38), which implies a significant genetic differentiation across Southeast Asia (Table 2.6). The total F_{it} was moderate (0.19), suggesting some inbreeding for these samples. The negative value of F_{ic} demonstrates low numbers of reproductives in each colony. The relatedness coefficient, however, was considerably high (0.64). As a result, I speculated that colony members were genetically close. Comparing the F-statistic among three population groups revealed some variation. The Philippines population exhibited high level of inbreeding (F_{it} = 0.23), strong colony member relatedness (r = 0.66), and the most significant genetic differentiation (F_{ct} = 0.41). Considering that these colonies were from isolated islands in the Philippines, the F-statistic result may reflect the geographic features of their locations. F-statistical indexes of Northern and Southern populations were similar.

Within each population, colonies from Saigon and Phnom Penh showed stronger genetic differentiation (F_{ct} = 0.29) and member relatedness (r = 0.5) than colonies from Hanoi (Table 2.6). Colonies from Singapore had lower inbreeding levels (F_{it} = 0.04) and genetic differentiation (F_{ct} = 0.27) than colonies from Penang and Indonesia. Colonies from Davao1 (all Davao colonies except PHI33, PHI35 and PHI39) showed lower inbreeding (F_{it} = 0.06), lower genetic differentiation (F_{ct} = 0.28), fewer numbers of reproductives (F_{ic} = -0.31), and lower nestmate relatedness (r=0.53) than colonies from Manila and Davao2 (PHI33, PHI35 and PHI39).

In general, 75.2% of colonies were extended families, 20.8% were simple ones, and only 4% were mixed families (Table 2.7). The composition of family types was different among the

three populations. The Philippines population possessed the highest percentage of extended (85.2%) and mixed families (7.4%). It had the lowest proportion of simple families, which was 7.4%, while simple families in the Northern population were up to 30%. Additionally, the Northern population had the lowest percentage of extended families (66.7%).

Within each population, Hanoi colonies had one mixed family but no simple family (Table 2.7). Singapore colonies possessed the lowest percentage of extended families (69%) and the highest percentage of simple families (27.6%). The only mixed family in the Southern population also came from Singapore. The two mixed families in the Philippines population came from Davao, and all Bacolod colonies were extended families.

Table 2.6. F-statistics and relatedness coefficients (r) for colonies in Southeast Asia. Confidence intervals of 95% are shown in bracket. Non-overlapping confidence intervals indicate significant difference between colonies or populations.

	N	Fit (95%CI)	Fct (95%CI)	Fic (95%CI)	R (95%CI)
All colonies	103	0.19 (0.16 - 0.24)	0.38 (0.36 - 0.41)	-0.31 (-0.33 - -0.29)	0.64 (0.62 - 0.66)
Native colonies	101	0.19 (0.15 - 0.23)	0.38 (0.36 - 0.40)	-0.31 (-0.33 - -0.29)	0.64 (0.62 - 0.66)
Northern population	30	0.07 (0.04 - 0.12)	0.3 (0.29 - 0.32)	-0.33 (-0.36 - -0.29)	0.57 (0.55 - 0.58)
Hanoi	6	-0.02 (-0.07 - 0.05)	0.2 (0.16 - 0.24)	-0.26 (-0.34 - -0.17)	0.4 (0.32 - 0.48)
Phnom Penh +Saigon	24	0.04 (0.01 - 0.08)	0.29 (0.27 - 0.31)	-0.35 (-0.38 - -0.31)	0.55 (0.53 - 0.58)
Southern population	44	0.11 (0.06 - 0.15)	0.32 (0.30 - 0.33)	-0.31 (-0.34 - -0.27)	0.57 (0.56 - 0.59)
Thailand	6	0.1 (0.01 - 0.17)	0.32 (0.28 - 0.35)	-0.33 (-0.39 - -0.27)	0.58 (0.56 - 0.62)
Malaysia+Indonesia	9	0.18 (0.13 - 0.22)	0.34 (0.29 - 0.38)	-0.24 (-0.28 - -0.21)	0.58 (0.52 - 0.63)
Singapore	29	0.03 (-0.02 - 0.10)	0.27 (0.25 - 0.29)	-0.32 (-0.36 - -0.28)	0.52 (0.51 - 0.54)
Philippines population	27	0.23 (0.17 - 0.29)	0.41 (0.36 - 0.46)	-0.3 (-0.33 - -0.26)	0.66 (0.61 - 0.71)
Davao1[1]	14	0.06 (0.02 - 0.10)	0.28 (0.26 - 0.30)	-0.31 (-0.36 - -0.27)	0.53 (0.50 to 0.57)
Bacolod	5	0.13 (-0.05 -0.32)	0.33 （0.20 - 0.47)	-0.29 (-0.35 - -0.20)	0.59 (0.41 - 0.71)
Manila+Davao2[2]	5	0.23 (0.16 - 0.30)	0.39 (0.33 - 0.45)	-0.26 (-0.28 - -0.25)	0.64 (0.57 - 0.69)

1.Davao1: all Davao colonies except PHI33, PHI35 and PHI39.

2. Davao2: PHI33, PHI35 and PHI39

Table 2.7. Statistic of family types of colonies across Southeast Asia. Note that the number is followed by the percentage in brackets.

	simple family	extended family	mix family
Native colonies			
All colonies	21 (20.8%)	76 (75.2%)	4 (4%)
Northern population	9 (30%)	20 (66.7%)	1 (3.3%)
Hanoi	0	5 (83.3%)	1 (16.7%)
Phnom Penh +Saigon	9 (37.5%)	15 (62.5%)	0
Southern population	10 (22.7%)	33 (75%)	1 (2.3%)
Thailand	1 (16.7%)	5 (83.3%)	0
Malaysia+Indonesia	1 (11.1%)	8 (88.9%)	0
Singapore	8 (27.6%)	20 (69%)	1 (3.4%)
Philippines population	2 (7.4%)	23 (85.2%)	2 (7.4%)
Davao1	0	12 (85.7%)	2 (14.3%)
Bacolod	0	5 (100%)	0
Manila+Davao2	2 (25%)	6 (75%)	0
Introduced colonies			
German	0	1(100%)	0
Hawaii	0	1(100%)	0

2.4 Discussion

2.4.1 Genetic diversity

All remaining eight microsatellite loci were polymorphic with an average of 19.75 alleles per locus, which was higher than that of Yeap et al (2011) (15.4 alleles per locus). The mean gene diversity over eight loci was 0.53, ranging from 0.51 to 0.59. Thus, it is fair to conclude that these eight markers had substantial power in uncovering the colony and population structure of *C. gestroi* and support the claim that Southeast Asia is the origin of the species.

The genetic diversity of three native populations was similar based on the statistical results of gene diversity and allele numbers per locus. The Northern and Philippines populations shared the fewest common alleles, which might indicate that they have least genetic exchange. The Southern population contained the most unique alleles and had the highest coverage of alleles in six loci, probably because the Southern population includes the most colonies (n=44) or because the regions in Thailand and Malaysia are the center of the origin of *C. gestroi*.

For all three populations, there was no significant difference in genetic diversity among subpopulations based on a two-tailed test.

The genetic diversity of colonies in Southeast Asia was higher than that of the German colony, which was expected because the German colony was introduced while Southeast Asian colonies were native. Introduced termite colonies usually experience bottleneck and lose genetic diversity (Vargo 2003; Vargo et al. 2003, 2006a; Dronnet et al. 2005; Vargo et al. 2006b; Husseneder et al. 2005, 2008). However, the Hawaii colony was an exception and had higher genetic diversity than native colonies. The Hawaii colony might have been introduced for a long time, and genetic mutation might have resulted in high genetic diversity, but since there was only one Hawaii colony, this exception was suspicious. More samples from Hawaii may resolve this confusion.

2.4.2 Genetic spatial structure

C. gestroi colonies in Southeast Asia are highly structured. I found three evident populations and a clear spatial pattern across Southeast Asia. The genetic clustering of the colonies was strongly associated with their geographic locations across Southeast Asia. This spatial structure was also found in the native populations of the European subterranean termite *Reticulitermes grassei*, fire ants, and Argentine ants (Tsutsui et al. 2001; Ahrens et al. 2005; Ross et al. 2007; Dronnet et al. 2015).

Further STRUCTURE and PCoA analyses of the three genetic populations revealed sub-populations. In the Northern population, Hanoi formed one distinct genetic cluster, while Saigon and Phnom Penh formed another. Hanoi is separated from Saigon and Phnom Penh by 1,765 km of mountain ranges. The genetic structure of the Northern population may be attributed to physical barriers such as mountains and distance, in this case. Colonies on each island of the Philippines population were basically one distinct genetic cluster. For the Philippines population, the ocean may serve as an important physical barrier which impedes genetic flow. For the Southern population, colonies from Thailand seemed to form one genetic cluster, and those from Penang and Indonesia seemed to form another. There was no sharp discontinuity in the genetic structure of the Southern population, and genetic flow was observed within the population. Yeap et al (2011) reported admixture clusters in the Peninsular Malaysia and Singapore populations. Here, I found a similar pattern but with lower levels of admixture.

Significant isolation by distance was discovered in all three populations, which suggests

population viscosity within each population. A correlation between genetic differentiation and geographic distance was also reported in the native populations of *Reticulitermes grassei* and *Mastotermes darwiniensis* (Goodisman and Crozier 2002; Dronnet et al. 2015). In contrast, introduced populations of *Coptotermes formosanus* in Japan, New Orleans, and Oahu lacked population viscosity (Vargo et al .2003). Tsutsui et al 2001 also concluded that geographically close colonies of Argentine ants tend to be genetically similar in their native region, while such a pattern was not observed in the introduced population of Argentine ant. This is probably because the dispersal of introduced colonies is often facilitated by human transport, and human-mediated transport would attenuate the signal of isolation by distance (Evans et al. 2013; Huang et al. 2013a).

I conclude that geographic features, such as distance and physical barriers, are the most important factors in determining the population genetic structure of *C. gestroi* in Southeast Asia, suggesting that the dispersal of these colonies may depend mainly on natural migration. There were some exceptions. Three Davao colonies, PHI33, PHI35, and PHI36, were clustered with Manila colonies, and the Indonesia colony seemed to be from Penang, which disrupts the spatial structure and suggests an anthropogenic transport of these colonies. However, the genetic structure of the majority of the colonies shows spatial patterns and corresponds to natural dispersal.

2.4.3 Colony features and their influence on population structure

In terms of family type, 20.5% of all colonies were simple families, 4% were mix families and 75.2% were extended families. Simultaneous occurrence of all three types of families

has also been reported in native populations in other termite species (Goodisman and Crozier 2002; Aldrich and Kambhampati 2007; Atkinson and Adams 1997). Most native *C. gestroi* colonies are extended families. Colonies of extended families have genetically related reproductives descended from the founding primary reproductive, which corresponds to a moderate F_{it} and high nestmate relatedness. These colonies possess a low number of reproductives (<6), since F_{ic} is smaller than -0.2. No budding was discovered because F_{ic} were negative values (Thorne et al. 1999; Bulmer et al. 2001). The discovery of mixed families implies the possibility of colony fusion of *C. gestroi* in its native area, although mixed families were not common.

In contrast to results of Yeap et al (2011), significant genetic differentiation was discovered in colonies across Southeast Asia (F_{ct}= 0.38) and in the three populations (F_{ct}=0.3, 0.32, 0.41). Husseneder et al (2012) also found great genetic differentiation among *C. formasonus* colonies from China. Significant genetic differentiation was additionally reported in endemic populations of other social insects such as fire, Argentine, and wood ants (Ahrens et al. 2005; Pedersen et al. 2006; Vanhala et al. 2014; Vogel et al. 2009). The great genetic differentiation indicates restricted genetic flow, supporting the theory of natural dispersal because human transport usually promotes genetic flow on a large geographic scale.

Termite colony features such as colony breeding structure influence the species' dispersal and population structure as well (Vargo and Husseneder 2009, 2011). Considering the flight capacity of *Coptotermes alates* and the colony breeding structure discovered in this study, I

speculate that the natural genetic exchange of *C. gestroi* colonies in Southeast Asia seem to be restricted to a small scale, which would partly explain the great genetic differentiation at population level of all Southeast Asian colonies and the three populations (F_{ct}=0.3, 0.32, 0.41) (Messenger and Mullins 2005). Habitat fragmentation caused by the ocean may enhance the genetic differentiation of the Philippines population (F_{ct}=0.41).

2.5 Conclusion

Colonies of *Coptotermes gestroi* across Southeast Asia are highly structured and possess great genetic differentiation. Three genetic populations have been revealed: the Northern population, which includes colonies from Cambodia and Vietnam; the Southern population, which includes colonies from Thailand, Malaysia, Singapore, and Indonesia; and the Philippines population, which includes all colony samples from the Philippines. These three populations have sub-structures. The genetic structures of all Southeast Asian colonies and the three populations show evident spatial patterns and significant isolation by distance, suggesting a significant correlation between geographic factors and genetic structure across Southeast Asia. Inbreeding colony structure may partly explain the great genetic differentiation of populations, and some colonies may also be transported by anthropogenic activity. However, geographic features, such as physical barriers and geographic distances, have been found to be the most important factor in determining the population genetic structure of native *C. gestroi* colonies, and the distribution of these colonies may be mainly attributed to natural dispersal

Chapter 3: Investigation of possible correlation between gut flora community of *C. gestroi* and host phylogeny at colony level

3.1 Introduction

It has been suggested that host phylogeny is the primary factor in structuring gut flora community of termites due to the vertical transmission of gut flora (Dietrich et al. 2014; Brune and Dietrich 2015; Rahman et al. 2015; Tai et al. 2015). Several studies have indicated that the gut flora community similarity of composition corresponded to the phylogenetic relationships of the termites at family or genus level (Rahman et al. 2015; Brune and Dietrich 2015). In other words, the closer two termite species were phylogenetically, the more similar their gut flora communities.

Other researchers have suggested that termite gut microbe communities could be colony specific (Matsuura 2001; Minkley et al. 2006; Boucias et al. 2013). For example, Minkley et al. (2006) reported subtle but distinct differences in the gut microbe community among different intraspecific colonies of the lower termite, *Hodotermes mossambicus.* Boucias et al (2013) found colony specific gut bacterial communities in *Reticulitermes flavipes*. They ran an experiment in which the termites were fed different artificial diets, yet they found no significant impact of the different diets, but a moderate but significant differentiation of the gut community among different colonies and speculated that it may be correlated with colony genetics. Despite these intriguing preliminary results, No study to date has tried to investigate correlation between the gut floracommunity and colony genotype.

Termites have hundreds of phylotypes of microbes in their gut; Do et al (2014) documented 1,460 bacterial species in the gut of *C. gestroi*. Thus, if the gut flora community is found to have a specific correlation with the genotype of its host colony, the gut flora(specifically the bacterial) community may be considered as an alternative and effective markers to microsatellites (or other molecular marker) based on the termite DNA, and also reveal genetic relationships of termite colonies, or perhaps termite populations. Such a finding would provide further support for the important role that host phylogeny plays in shaping the gut flora community.

In this chapter, I compared the phylogenetic structure of 23 *C. gestroi* colonies with that of their gut bacterial communities. I investigated the phylogenetic structure of the colonies using microsatellite markers as described in Chapter 2 and of the gut bacterial communities using 16S rDNA barcoding. My specific aim was to test whether gut bacterial community co-varied with colonies of different genetic makeup in *C. gestroi*.

3.2 Materials and Methods

3.2.1 Samples

Twenty-three colonies of *C. gestroi* were included; two were from Vietnam and twenty-one were from Singapore. Collecting information of these colonies is shown in Table 3.1.

Table 3.1. Collecting information of 23 *C.gestroi* colonies

	Country	Habitat
V23	Vietnam	Street tree
V32	Vietnam	Urban park
YS3	Singapore	Street tree
YS2	Singapore	Street tree
YC1	Singapore	Street tree
TA7	Singapore	Street tree
TA2	Singapore	Street tree
SW1	Singapore	Street tree
SING1	Singapore	Secondary forest in nature reserve
SBGC	Singapore	Secondary forest
RR	Singapore	Secondary forest
NW	Singapore	Street tree
LY1	Singapore	Street tree
KR3	Singapore	Street tree
ED2	Singapore	Street tree
ED1	Singapore	Street tree
ECP2	Singapore	Urban park
EB2	Singapore	Street tree
CP1	Singapore	Street tree
CI5	Singapore	Secondary forest on island
CI3	Singapore	Secondary forest on island
CBP	Singapore	Urban park
BS1	Singapore	Street tree

3.2.2 Investigation of genetic relationship among colonies

The genetic information of 23 *C. gestroi* colonies was obtained as outlined in Chapter 2 and adopted for analysis in this study. I generated a principal coordinate analysis to illustrate the genetic relationship and similarity among colonies. The principal coordinate analysis (PCoA) was carried out as described in 2.2.4.

3.2.3 16s rDNA pyrotag sequencing of gut bacteria

I pulled the entire gut from 30 live workers per colony with sterilized forceps, and preserved the

guts in a vial of 100% ethanol, usually within six hours, and always within 24 hours, of collection from the field. I homogenated all 30 preserved guts by bead- beating (45 seconds, 2 cycles), and then extracted whole DNA using CTAB (Winnepenninckx et al. 1993).

To amplify the gut bacterial DNA, I adopted two prokaryote universal primers, 343Fmod (TACGGGWGGCWGCA) and 784Rmod (GGGTMTCTAATCCBKTT); this pair of primers targeted the V3-V4 region of bacterial 16S rDNA (Kohler et al. 2012). This modified primer set has been used to improve the coverage of the taxonomy of gut microbes in termites and cockroaches (Kohler et al. 2012). I sent the PCR products to AITbiotech for library preparation and MiSeq sequencing on Illumina.

3.2.4 Quality control of sequences and investigation of gut bacterial community relationships

Raw reads data went through quality control using both trimmomatic v0.36 and Mothur software suit (version 1.36.1) (Schloss and Handelsman 2005; Bolger et al. 2014). First, adapter sequences and poor quality bases (<Q30) were trimmed using trimmomatic. Second, I removed primer sequences and selected reads according to the following criteria: read length between 395bp and 435bp; reads with ambiguous bases were discarded, and reads with more than 8 homopolymers were removed. Third, I classified remaining sequences using Naïve Bayesian Classifier in Mothur with termite and cockroach specified bacteria reference database, DictDb v. 2.3 with 60% cutoff (Dietrich et al. 2014). Finally, I removed reads with chimeras. Each unique sequence then became an operational taxonomic unit (OTU), which is a utilitarian

proxy for microbial species (Blaxter et al. 2005)

Prior to statistical analysis, I normalized all 23 samples to 16,541 reads each, which was the smallest number of sequence reads for any sample.

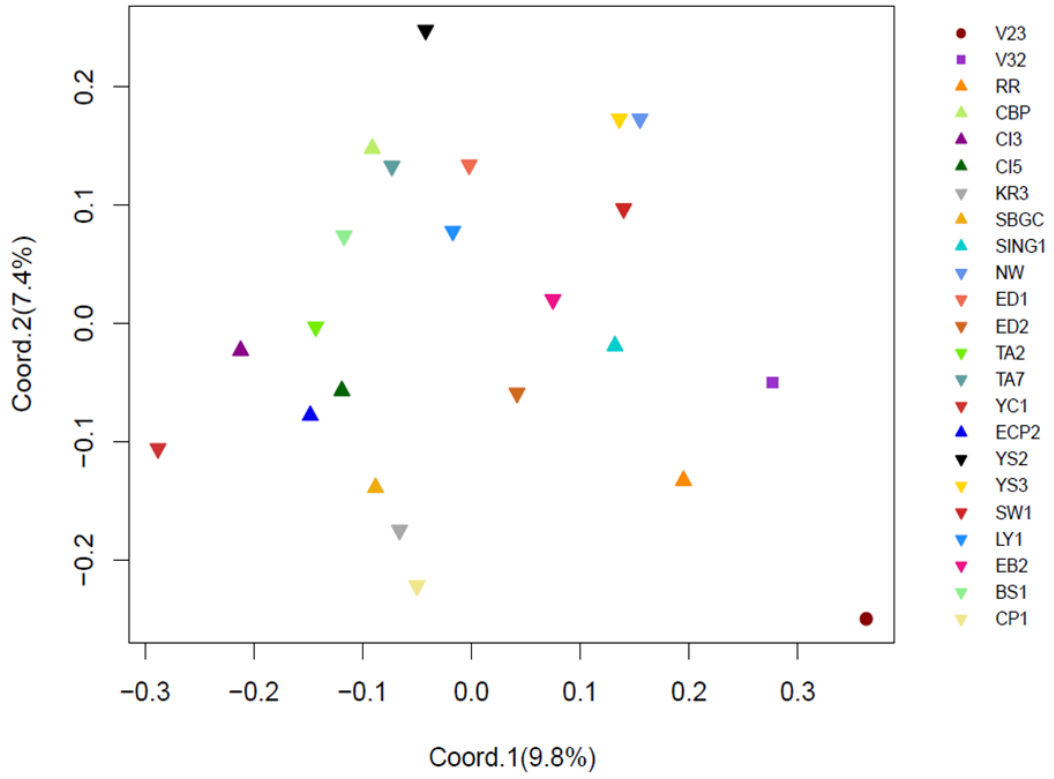
For the statistical analysis, I analyzed pairwise dissimilarity between the structures of gut flora communities of all 30 samples using Thetayc calculator. Thetayc calculator gives the dissimilarity between the structure of two communities based on relative abundance of OTUs (Yue and Clayton 2005). This dissimilarity index is related to the Jaccard index, but unlike the Jaccard Index it includes not only the proportion of shared species, but also the proportion of non-shared species in each population, with a non-parametric maximum likelihood estimate. It is now widely used for microbial community similarity comparisons (WAlker et al 2011; Griffith et al. 2012; Kong et al 2012; Morris et al 2013; Kistler et al 2013) Principal coordinate analysis (a type of multidimensional scaling computation, MDS) was conducted using dissimilarity matrix, to illustrate the relationship among gut bacterial communities (Zuur et al. 2007). PCoA plot of gut bacterial communities was compared with PCoA plot of host colony using Mantel test from the vegan package, as implemented in R version 3.2.0 (R Development Core Team 2008).

3.3 Results

3.3.1 Comparison of PCoA plots

Comparing the PCoA plot of genetic similarity of host colonies with the plot of similarity of

their gut bacterial communities revealed that the genetic relationship of host colonies was not reflected in their gut bacterial communities. The observed patterns of the PCoA plot of genetic distance of colonies and that of gut bacterial communities were clearly different (Figure 3.1a, b). The Mantel test indicated an absence of a correlation between PcoA plot of *C. gestroi* colonies and that of their gut bacterial community (P= 0.774) (Figure 3.1b). As shown in figure 3.1a, two Vietnamese colonies (V23,V32) were close to each other in genetic distance, as were Singapore colonies, reflecting the general pattern in the PcoA plot that that distribution of points on the plot was related with their collection locations. However, Figure 3.1b showed a different pattern. Distribution of points on plot was not correlated with their geographic locations (P= 0.774); instead they seemed to be related to other factors. The most likely factor appears to be the habitats from which their termite hosts were collected. This is because most of the bacterial communities of colonies from street trees were clustered together, as were those from the other habitats (forest, parks etc). .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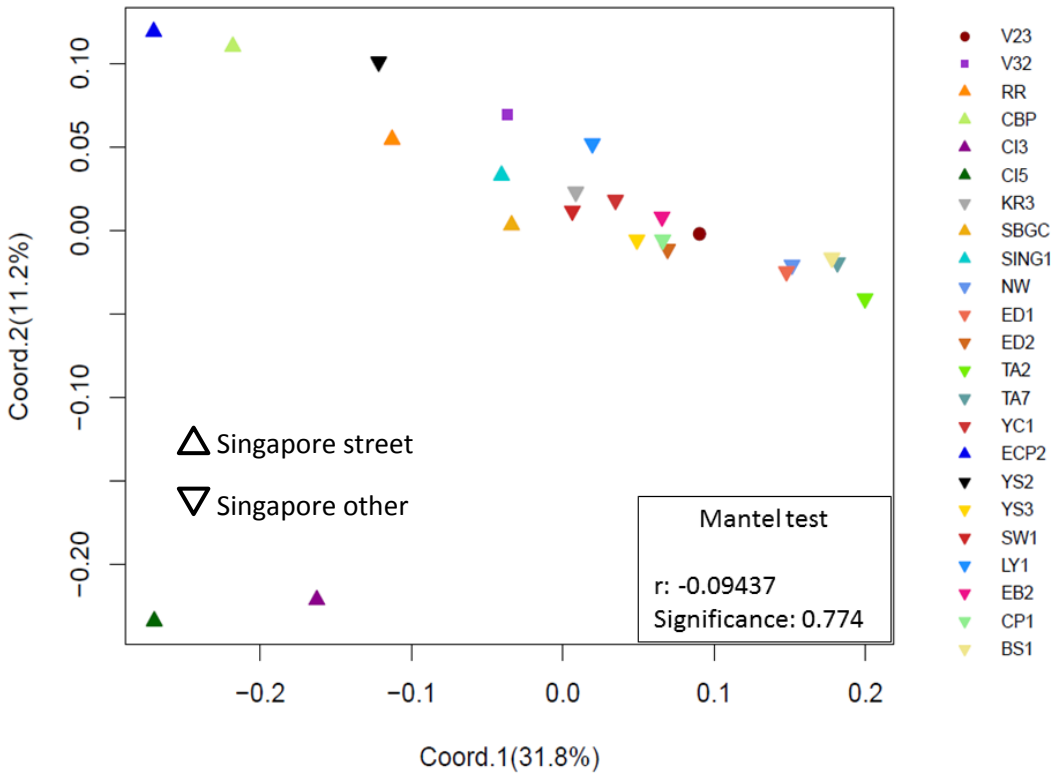
0.2
0.1
0.0
−0.1
−0.2
−0.3
−0.2
−0.1
0.0
0.1
0.2
0.3
Coord.1(9.8%)
V23
V32
RR
CBP
CI3
CI5
KR3
SBGC
SING1
NW
ED1
ED2
TA2
TA7
YC1
ECP2
YS2
YS3
SW1
LY1
EB2
BS1
CP1

a


Coord.2(11.2%)
0.10
0.05
0.00
−0.10
−0.20
−0.2
−0.1
0.0
0.1
0.2
Coord.1(31.8%)
Singapore street
Singapore other
Mantel test
r: -0.09437
Significance: 0.774
V23
V32
RR
CBP
CI3
CI5
KR3
SBGC
SING1
NW
ED1
ED2
TA2
TA7
YC1
ECP2
YS2
YS3
SW1
LY1
EB2
CP1
BS1

b

Figure 3.1. a) PCoA of the 23 *Coptotermes gestroi* colonies from Vietnam and Singapore using genetic distance. b) PCoA of 23 gut bacterial samples using similarity values calculated by Thetayc calculator. The percentage of variation explained by the first two co-ordinates (the axes) is shown in brackets. Samples represented by point down triangles were collected from street trees in Singapore and samples represented by point up triangles were collected from other habitats in Singapore. Box in Figure 3.1b shows result of Mantel test.

3.4 Discussion

Host termite phylogeny has been considered as the most important determining factor in shaping termite gut flora communities, due to the potential coevolution of termites and their (vertically transmitted) symbiotic gut flora community. Several studies have observed some measure of correlation between the host phylogeny and their gut microbe communities. Dietrich et al (2014) found phylogenetic tree of several termite species was mostly consistent with the cladogram of gut bacterial community similarity. Tai et al (2015) concluded that gut bacterial communities clustered based on the phylogeny of their hosts after comparing the PCoA plots of bacterial communities and host termite species. Bacterial communities were significantly more similar to each other if their host termites were phylogentically closer (Tai et al. 2015). These studies compared gut bacterial communities of several termite species and/or families (Hongoh et al. 2005; Reid et al. 2014; Dietrich et al. 2014; Tai et al. 2015; Rahman et al. 2015; Brune and Dietrich 2015). However, when I compared gut bacterial communities within one single termite species, *C. gestroi*, I found no correlation between host phylogeny (at colony level) and gut community similarity. The PcoA plot of host colonies obtained from genetic distance did not match that of gut bacterial communities obtained from dissimilarity values. A Mantel test also indicated no correlation between colony genotype and gut bacterial community.

Thus, within one termite species, it would appear that colony relatedness (or genotype) does not explain the variation of its gut bacterial community. This may be due to the amount of variation in both the host genetics and their gut microbial communities. Clearly there are lower levels of variation within a species than between species, even less when compared with variation within a family. For example, I found 303 OTUs in *C. gestroi* in this current study, where as previous studies found 1291 OTUs on average in 8 lower termite species(Diethrich et al. 2014). It may be possible that this relative lack of variation within species is merely an artifact of the reduced variation; only further work on other termite species will reveal whether this is so.

There is another explanation, based on another factor. Dissimilarity of gut bacterial communities did not reflect the host colony genetic relationships in *C. gestroi*, but seemed to be correlated with the habitat of the termites. As seen in figure 3.1, most of the bacterial communities of termite colonies from street trees were clustered together, as were those from other habitats (forests and parks). Further investigation of this observed correlation between gut bacterial community and habitat will be elucidated in Chapter 4.

For some insect species, other factors rather than host phylogeny are found to be related with gut bacterial community and may contribute to structure of the community. These factors included diet and host plants. For example, the gut bacteria of gypsy moth caterpillars were discovered to be highly dependent on the diet (Broderick et al, 2004); gut microbial communities of moth larvae fed the same diet had similarity. Surveys of *Helicoverpa*

armigera in the field displayed that gut bacterial community was associated with host plants (Priya et al. 2012). There are reports that gut flora of termites changed due to diet shifts (Tanaka et al. 2006; Huang et al. 2013b). Therefore factors other than host phylogeny, should be considered when investigating gut flora community diversity in termites. Relative importance of host phylogeny and other factors to termites' gut community should be reevaluated at different phylogenetic levels of host termites.

3.5 Conclusion

This study found that the relationship of gut bacterial community of *C. gestroi* was not consistent with the genetic relationship of host colonies. No significant phylogenetic signal at colony level was detected in the structure of gut bacteria. It appears that habitat may be associated with gut bacterial community of *C. gestroi*.

Chapter 4: Diversity and structure of the gut flora community of *C. gestroi* from different habitats

4.1 Introduction

Termites are one of the very few organisms that have evolved to digest cellulose; the most abundant biomolecule on earth (Norkrans 1963; Béguin and Aubert 1994; Dixon et al. 1994). Therefore, they are very abundant and important ecologically, due to their role in decomposition and nutrient recycling (Eggleton and Tayasu 2001; Bignell 2006; Evans et al. 2011; Jouquet et al. 2011), consuming up to 90% of the wood in some habitats (Buxton 1981; Bignell 2006; Jouquet et al. 2011; Brune 2014), and economically important, as pests of timber in forestry and human construction (Su and Scheffrahn 2000).

In order to thrive on recalcitrant wood and plants, termites harbor flora in their guts to help them in two major tasks; first, digest cellulose, and second, fix nitrogen and turn the nitrogenous compounds into amino acids and proteins for biological use. Cellulose is a recalcitrant molecule as very few organisms have the enzymes to break it down into glucose, the smaller, constituent molecules from which it is made. Termites do have their own endogenous cellulases, but they rely more heavily on cellulases produced by their gut microbes (Watanabe et al 1998; Nakashima et al. 2002; Zhou et al. 2007). Glucose and cellulose contain no nitrogen, therefore termites require another source. Some of the gut bacteria in lower termites appear to play a major role in nitrogen fixation, and thereby provide essential amino acids and cofactors for the host termites (Desai and Brune 2012; Hongoh et al. 2008a; Hongoh

et al. 2008b).

Flora in termite guts are also involved in hydrogen metabolism and oxygen consumption. Hydrogen and oxygen in the termite gut are two important factors that influence survival of the hosts (termites) and their symbionts (gut flora). Hydrogen is maintained at a high partial pressure inside the termite's gut to help remove the reducing equivalents produced in cellulose fermentation (Ohkuma 2008). This hydrogen appears to originate from parabasalian protists, which are able to generate hydrogen even under high partial pressures (Inoue et al. 2005). Other bacteria, such as *Bacteroidales* endosymbionts, consume hydrogen, creating a dynamic system for hydrogen balance in the termite gut (Inoue et al. 2007).

Oxygen in the termite gut is harmful to the host. In the termite gut, some protists generate acetate, which is the main energy and carbon source for the termites (Yamin 1979; Bandi et al 2000). Oxygen is harmful because it reduces the yield of acetate. Therefore, it needs to be removed to promote production of acetate. Oxygen can be reduces with oxygense enzymes. Genome sequencing has shown oxygenases exist in some termite gut spirochaetes, which indicates that these bacteria may help termites to lower oxygen levels in the gut (Lucey and Leadbetter 2014). Thus as a community, the gut flora appear to interact in order to maintain a specific microenvironment in the termite intestine, which in turn supports the survival of their own, other gut microbes, and the termite host.

Gut flora are critical symbionts for termites. There appears to be very broad evolutionary relationships between termites and their microbe symbionts: the lower termites

(Mastotermitidae, Stolotermitidae, Hodotermitidae, Kalotermitidae, Rhinotermitidae and Serritermitidae) form symbiosis with protists and some bacteria and archaea, whereas the higher termites (Termitidae), rely entirely on bacteria and archaea (Breznak and Brune 1994; Ohkuma and Brune 2011; Brune 2014) with the exception of the fungus-growing termites (Termitidae: Macrotermitinae), which have evolved a unique symbiotic relationship with a wood rot fungus (Hyodo et al. 2000; Aanen et al. 2002).

The factors that determine the community structure of gut flora remain contentious, with host phylogeny, diet, and habitat under consideration (Brune and Dirtrich 2015). Because of the vertical transmission of gut flora within the termite colony, it is widely accepted that host phylogeny ought to be an (perhaps the most) important factor. Vertical transmission refers to termite gut flora are passed within termite colonies, from generation to generation. It has been reported that abundance change of some gut flora lineages corresponded to the major evolutionary divisions of host termite species. Thus, it is believed that composition of termite gut flora is strongly associated with host termite phylogeny. For example, some studies reported that gut flora composition of congeneric termites was similar (Kohler et al. 2012; Dietrich et al. 2014; Tai et al 2015; Rahman et al. 2015).

The influence of diet on termite gut microbe community has received more and more attention in recent years. One study used massive parallel sequencing of field collected termites and uncovered some influence of diet (Mikaelyan et al. 2015). Some lab experiments, on the other hand, have demonstrated that changes in artificial diets shift the gut microbe community in some lower termites (Tanaka et al. 2006; Huang et al. 2013b).

However, these studies are not sufficient to demonstrate the ubiquity of the importance of diet on the gut communities of lower termites. This is for several reasons. The massive sequencing study of Mikaelyan et al. (2015) focused on several higher termite species, whereas the lab experiments of Tanaka et al. (2006) and Huang et al. (2013b), focused on lower termites. Furthermore, the lab experiments used only one type of food source, the numbers of termites were low relative to natural colonies (reducing the size of the 'communal gut'), and the changes of gut microbe community were reported after a short time only, and therefore may have been temporary. In contrast, termites in the field under natural conditions have the opposite conditions. They have a greater variation in their food sources, many individuals in the colony and thus a larger communal gut, which temporal changes in gut microbe community are small. It seems likely that the results from lab-raised termites may not be applicable to termites in the field.

Finally, the third main factor proposed to influene termite gut microbe communities is the habitat (Rahman et al. 2015; Brune and Dietrich 2015). However, no research to date has studied the influence of habitat on termite gut microbes. Furthermore, few studies have investigated the gut flora of lower termite colonies from the field using a detailed method, even though it can be an effective way to reveal the relative importance of host phylogeny, diet, and environmental factors to termite gut flora.

In this study, I surveyed the gut bacterial community of *Coptotermes gestroi* using Illumina sequencing, based on 16s rRNA genes. I collected 30 samples from different habitats and

different tree species, sequenced gut extracts, and examined the influence of different diets and habitats on the gut bacteria community.

In this Chapter, with a more detailed investigation, my specific aims were to measure gut flora diversity in *C. gestroi* from different habitats; to identify species of gut microbes found across all termite samples (suggesting they are essential and obligate microbes for *C. gestroi*); to identify species of gut flora shared by all termites feeding on the same tree species (suggesting they may be essential for digesting that tree species); and to confirm the influence of diet and habitat on the gut flora community structure of *C. gestroi*.

I hypothesized that the diversity and structure of gut flora from *C. gestroi* collected in different habitats would differ and correspond to the diversity of food in the habitat; that there would be bacteria common to all *C. gestroi*, regardless of habitats and host trees, which may be essential to digestion; that there would be gut flora common to termites feeding on the same tree species, which may be essential to digestion for that tree species; and that diet and habitat were important factors in shaping the gut flora community of *C. gestroi.*

4.2 Material and methods

4.2.1 Termite sampling and pyrotag sequencing

I sampled *Coptotermes gestroi* from various habitats. I collected two colonies that were kept in the laboratory (lab colony P1 was from forestand has been kept in the laboratory for 4 months, and lab colony BO was from secondary forest and kept for two years), 16 colonies

from street trees (each street had only one species of tree), and 12 colonies from parks, secondary forests in 'waste woodlands' and primary forest in nature reserves (Table 4.1).

I aimed to investigate influence of two factors on termite gut bacteria community: habitat and diet. Habitat of the termites determines diversity of food to which they have access. I divided habitats of all collected samples to three types: lab, street trees and multiple tree species habitat. Habitat type was determined based on diversity and complexity of food sources that environment can provide. Colonies in lab fed on pine wood exclusively, which was the least diverse food. Generally, only one tree species was planted along the whole street, within the foraging range of *C. gestroi*. So colonies found inside street trees usually feed on that one particular tree species along the street, plus whatever urban wood (construction wood, waste wood, or wood products, such as cardboard) they can find. Colonies found in multiple tree species habitat have more than one tree species as food sources and have the highest diversity of food choice. Colonies from parks, secondary forests in 'waste woodlands' and primary forest in nature reserves were all in multiple tree species group.

I also investigated diet impact on termite gut flora community, by which I mean the impact of one food type on gut flora. In general, *C. gestroi* feed on and nest inside one tree and that particular tree contributes to most its diet. I compared gut bacterial community of 5 colonies (= nests) in *Casuarina equisetifolia* trees, 6 in *Samanea saman* trees and 6 in *Peltophorum pterocarpum* trees to reveal the influence of food type. I choose these trees

because they are widely planted in streets in Singapore, are attacked by termites relatively often and represented a range of phylogenetic relatedness.

DNA extraction of gut community and 16s rDNA sequencing followed method described in 3.2.3.

Table 4.1. Collecting information of 30 samples.

Sample	Country	Habitat	Host tree species
Lab			
BO	Indonesia	Lab	fed with pine wood
P1	Singapore	Lab	fed with pine wood
Street tree			
YC1	Singapore	Street tree	*Casuarina equisetifolia*
EB2	Singapore	Street tree	*Peltophorum pterocarpum*
ED1	Singapore	Street tree	*Peltophorum pterocarpum*
LY1	Singapore	Street tree	*Peltophorum pterocarpum*
SW1	Singapore	Street tree	*Peltophorum pterocarpum*
TA7	Singapore	Street tree	*Peltophorum pterocarpum*
YS2	Singapore	Street tree	*Peltophorum pterocarpum*
NW	Singapore	Street tree	*Samanea saman*
TA2	Singapore	Street tree	*Samanea saman*
BS1	Singapore	Street tree	*Samanea saman*
CP1	Singapore	Street tree	*Samanea saman*

ED2	Singapore	Street tree	*Samanea saman*
YS3	Singapore	Street tree	*Samanea saman*
KR3	Singapore	Street tree	NA
V23	Vietnam	Street tree	NA
V31	Vietnam	Street tree	NA
Multiple tree species habitat			
CBP	Singapore	Park	*Casuarina equisetifolia*
CI3	Singapore	Socondary forest island	*Casuarina equisetifolia*
CI5	Singapore	Secondary forest island	*Casuarina equisetifolia*
ECP2	Singapore	Park	*Casuarina equisetifolia*
ECP	Singapore	Park	*Cerbera odollam*
RR	Singapore	Secondary forest waste woodland	*Hevea brasiliensis*
SBGC	Singapore	Secondary forest	*Hevea brasiliensis*
PHP1	Singapore	Park	*Tamarindus indica*
DO	Singapore	Secondary forest waste woodland	NA
JR	Singapore	Secondary forest waste woodland	NA
SING1	Singapore	Secondary forest in natural reserve	NA
V32	Vietnam	Park	NA

4.2.2 Preprocessing and basic statistical information of sequences

Preprocessing of sequences followed 3.2.4. Numbers of sequences, operational taxonomic units (OTUs) and Good's coverage, which estimates percentage of total OTUs representing in a sample, were obtained with Mothur (Schloss and Handelsman 2005). Rarefaction curve of

observed OTUs was plotted.

4.2.3 Comparison of diversity and structure among different habitat and tree species groups

I subsampled gut bacterial sequences of all 30 samples to 14,355 reads, which was the sequence number of the smallest sample. I used the Shannon diversity index to elucidate community diversity and Shannon evenness to elucidate community evenness. The diversity index takes both genus richness, the number of genus presents in sample, and relative abundance of each genus into consideration. The evenness index refers to how equal the abundances of different genera are. I performed all calculations in Mothur (Schloss and Handelsman 2005). I applied ANOVA in R version 3.2.0 to test whether the gut samples of colonies from the different habitats and different diets (tree species) significantly differed in richness and diversity.

Then I tested whether the gut samples of colonies from the different habitats and different tree species significantly differed in gut bacterial community structure. First, I got pairwise dissimilarity values of the gut flora communities of all 30 samples using Thetayc calculator (as in 3.2.4 above). Pairwise dissimilarity values were calculated based on shared and unique bacterial OTUs in each pair of samples. Subsequently, I applied both UniFrac significance test (weighted) and AMOVA test on pairwise dissimilarity values to check whether habitats and tree species significantly influence gut bacterial community structure. I visualized pairwise dissimilarity matrix with 3D PCoA, in R package rgl. ANOSIM was adopted to

evaluate the significance of different groups in PCoA using vegan package in R.

4.2.4 Shared bacterial genera

I compared the gut flora communities from all colonies and identified those taxa that are common to all colonies. Similarly, I identified the common and unique genera of bacteria from termites collected from the three urban tree species.

4.2.5 Characteristic OTUs differentiating groups

I used a linear discriminant function analysis to discover OTUs that are important factors in differentiating groups using LefSe function in Mothur Platform (Segata et al. 2011). I used a Kruskal-Wallis test (alpha value of 0.05) to test the relative abundance of OTUs, and thereby to identify important OTUs based on their relative abundance. I then ranked these OTUs by the effect size using linear discriminant analysis model. The threshold for logarithmic linear discriminant analysis score for discriminative OTUs was 2.

4.2.6 Pairwise differentiation

I displayed pairwise differentiation of gut bacteria communities based on pairwise dissimilarity matrix using heatmap drawn in R package gplot. The pairwise dissimilarity matrix was obtained using Thetayc calculator.

4.3 Results

4.3.1 Basic statistical information of sequences

I obtained a total of 642,887 reads of high-quality sequence of gut bacterial DNA, with an average of 21,430 reads and 304 OTUs at the bacterial genus level. The Good's coverage ranged from 98.8% to 99.4%, indicating that the majority of bacterial diversity was uncovered (Table 4.2). The rarefaction curve also supported that majority of bacterial phylotypes were uncovered (Figure 4.1).

Table 4.2. Characteristics of 16S rDNA libraries of 30 gut samples.

Sample	Sequence number	OTUs	Coverage[1]	Shannon[2]	Shannoneven[4]
Lab					
BO	24086	333	0.991	3.25	0.56
P1	19858	267	0.992	2.72	0.49
street tree					
BS1	29305	291	0.994	3.6	0.63
CP1	24639	253	0.992	2.76	0.5
EB2	14596	277	0.991	2.85	0.51
ED1	24442	253	0.994	3.37	0.61
ED2	33566	313	0.992	3.43	0.6
KR3	18471	261	0.993	3.37	0.61

LY1	22302	330	0.993	3.76	0.65
NW	17088	320	0.99	3.01	0.52
SW1	14397	371	0.988	3.44	0.58
TA2	29188	233	0.994	2.94	0.54
TA7	23678	219	0.994	3.58	0.66
V23	16865	321	0.992	3.39	0.59
V31	22029	276	0.993	3.28	0.58
YC1	22868	323	0.993	4.02	0.7
YS2	26095	254	0.993	3.56	0.64
YS3	14355	346	0.988	3.06	0.52
Multiple tree species habitat					
CBP	20901	287	0.991	3.32	0.59
CI3	22579	394	0.991	4.02	0.67
CI5	20273	349	0.992	4.1	0.7
DO	17883	400	0.992	4.1	0.68
ECP	19081	268	0.993	2.97	0.53
ECP2	17961	313	0.99	3.39	0.59
JR	15879	328	0.992	3.74	0.65
PHP1	24812	366	0.991	3.87	0.66
RR	22444	286	0.994	3.91	0.69
SBGC	19949	267	0.994	3.59	0.64
SING1	20274	373	0.991	3.75	0.63
V32	23023	244	0.994	3.52	0.64

1. Good's coverage.
2. Shannon diversity.
3. Inverse Simpson diversity.
4. Shannon evenness.
5. Simpson evenness.

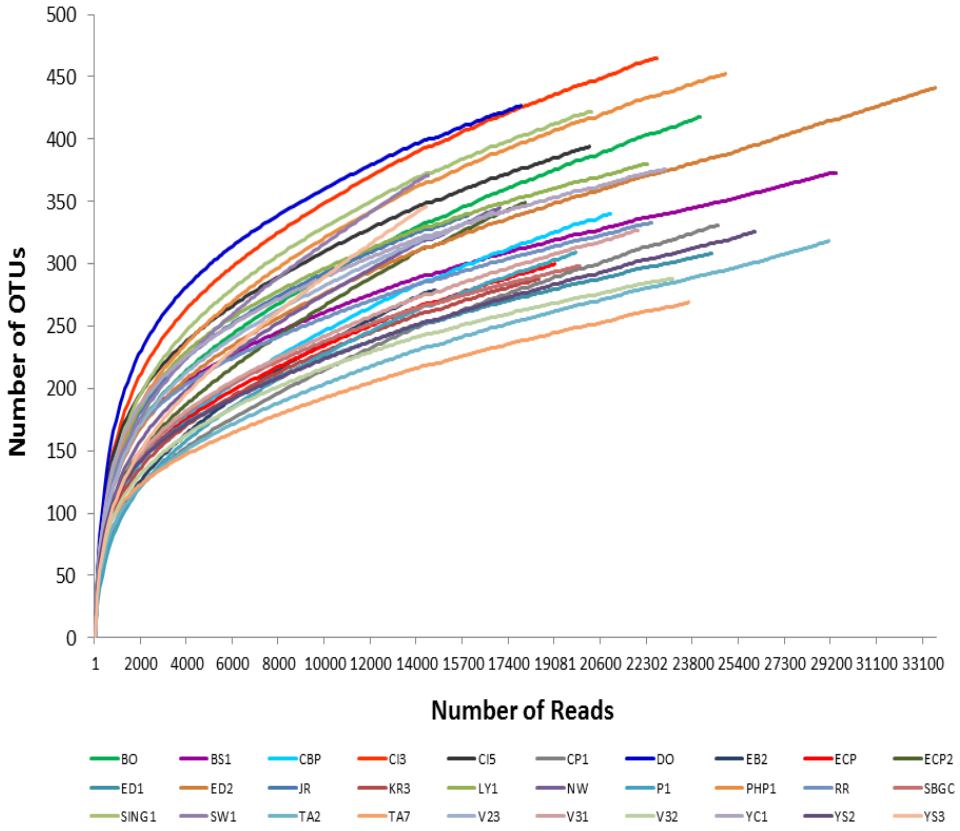
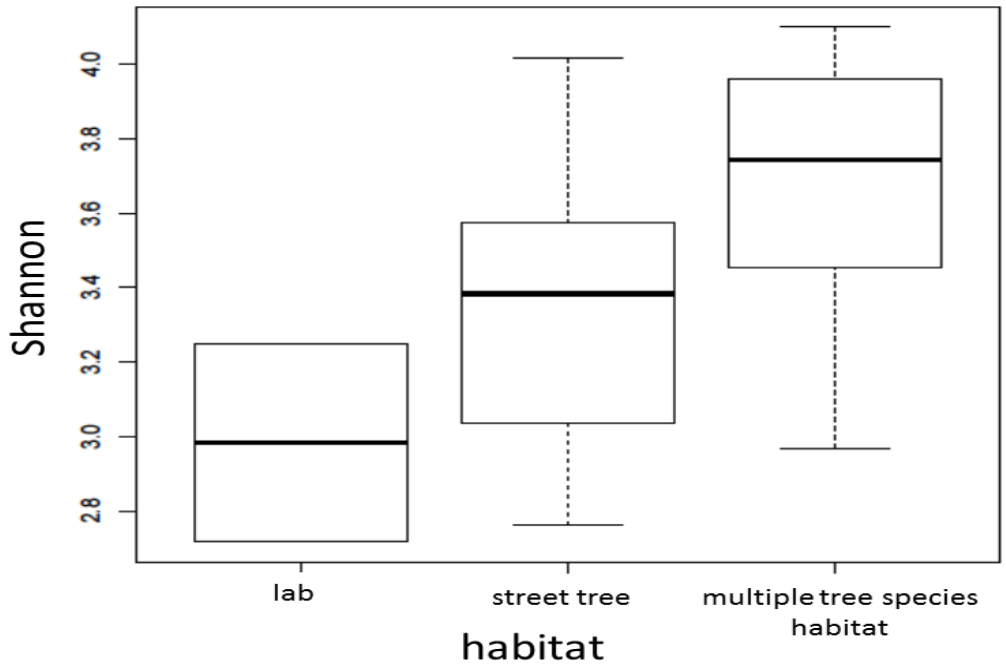


Figure 4.1. Rarefaction curves. Rarefaction curves comparing the number of reads with number of OTUs detected in 30 termite gut samples.

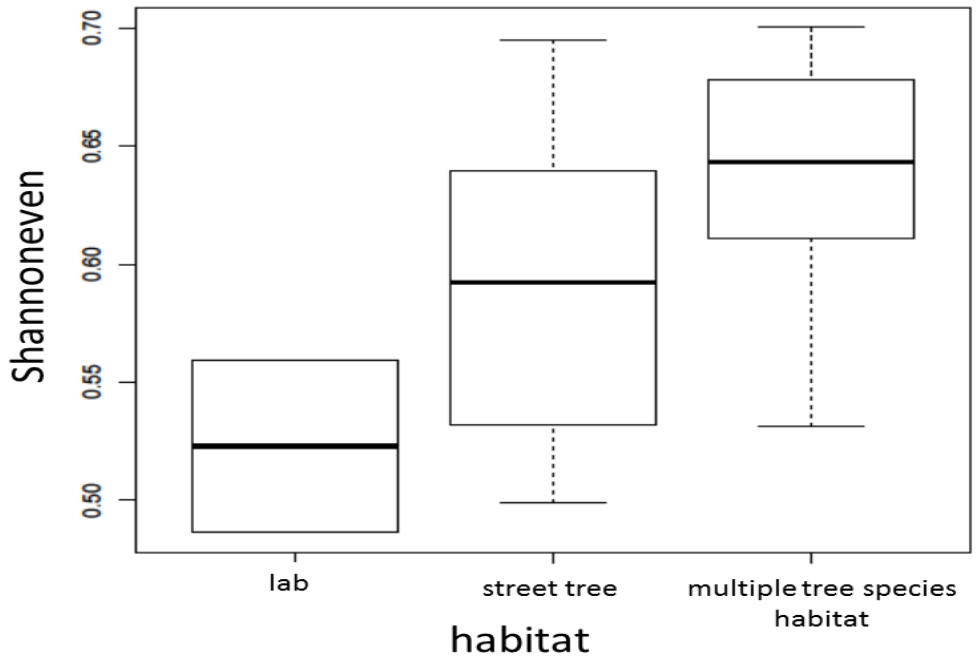
4.3.2 Comparison of diversity and structure among habitat and tree species groups

In general, OTUs in termite guts from a habitat with multiple tree species were more diverse (322 +/- 53.25)) than from one with urban street trees (290 +/- 43.44) and the laboratory colonies (300 +/- 46.67)) (Table 4.2). Both Shannon diversity ($F = 5.51$, $df = 2$, $P = 0.01$) and Shannon evenness ($F = 5.12$, $df = 2$, $P = 0.013$) of three habitat groups were significantly different. Diversity and evenness of termite gut flora from the multiple tree species habitat were significantly higher than those from street trees and those from the laboratory colony (Figure 4.2a-b) (two tailed T-test; multiple tree species vs street trees: $P = 0.012$; multiple tree species vs lab: $P = 0.021$)

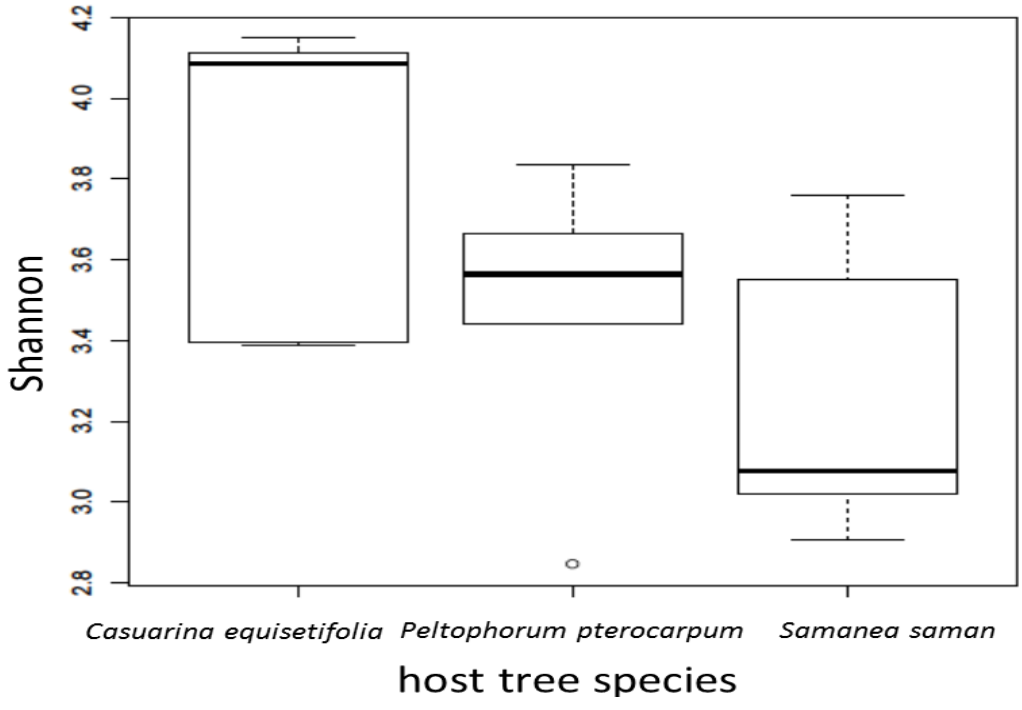
In general, OTUs in termite gut samples from *Casuarina equisetifolia* (333+/- 40.60) were more diverse than from *Peltophorum pterocarpum* (284 +/- 56.25) and*Samanea saman* (292 +/- 42.76)\ (Table 4.2). Shannon diversity (F = 3.74, df = 2, P = 0.05) of three tree species groups were significantly different. However, Shannon evenness (F = 2.80, df = 2, P = 0.09) of three tree speices groups were not significantly different. Termite gut microbial communicties from *Casuarina equisetifolia* were more diverse than those from *Peltophorum pterocarpum* and *Samanea saman* (Figure 4.2c)) (two tailed T-test; *Casuarina equisetifolia* vs *Peltophorum pterocarpum*: P = 0.03; *Casuarina equisetifolia* vs *Samanea saman*: P = 0.021) Shannon diversity and evenness of all colonies are displayed in Table 4.2.



a



b



c

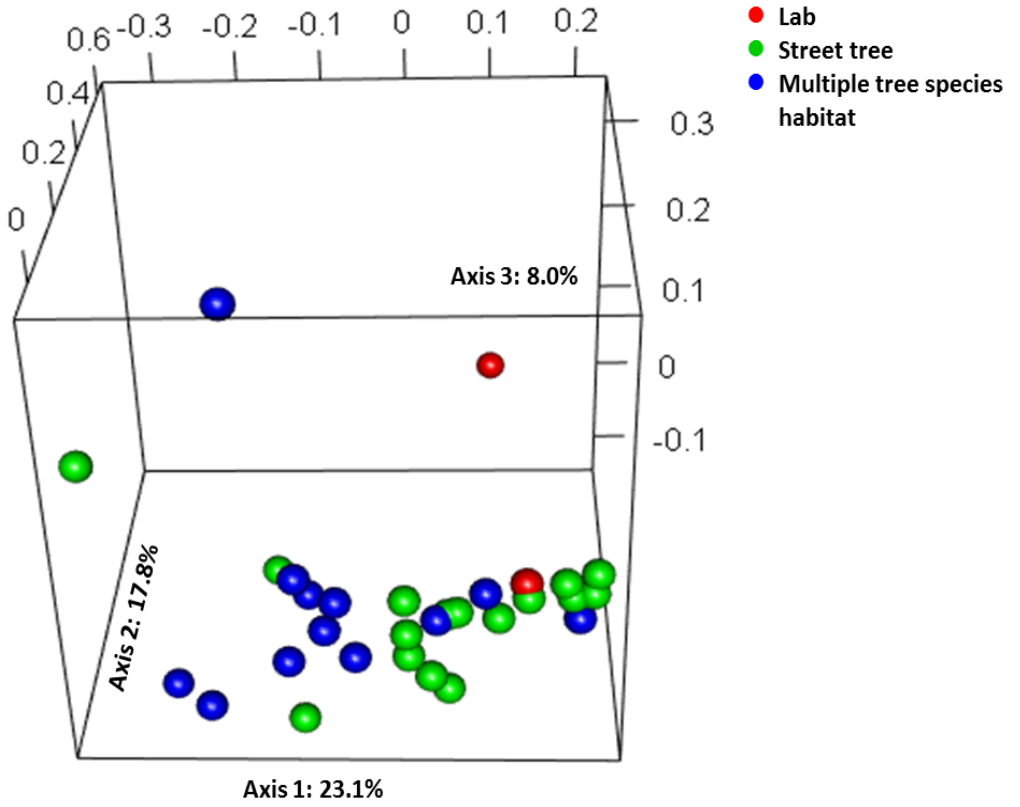
Figure 4.2. Plot of shannon diversity and shannoneven indexes of different habitats and street trees: a) Shannon diversity of different habitats b) Shannon evenness of different habitats c) Shannon diversity of three host tree species. Shannoneven of different street trees is not shown here because it is not statistically significant.

I found significant differences in the gut community structure analyses among different habitats

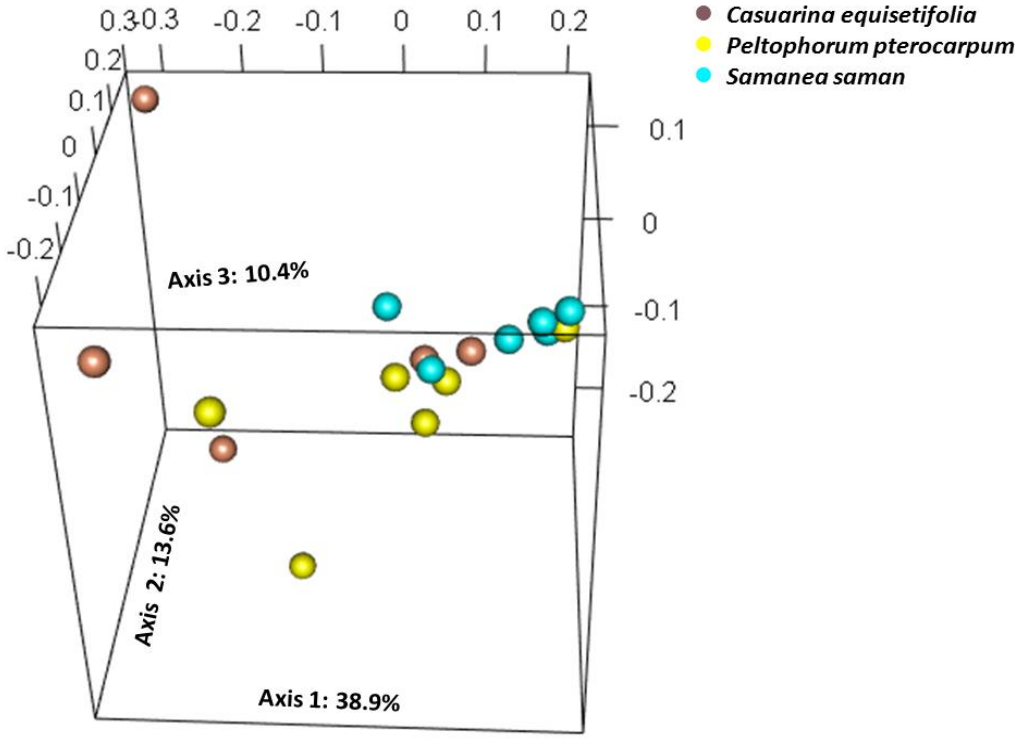
and tree species. There were significant difference among three habitat groups and three tree species groups estimated both by AMOVAs (habitat: Fs = 3.22, P = 0.008; tree species: Fs= 2.79, P= 0.024) as well as UniFrac significance tests (habitat: WScore = 0.6 , WSig < 0.001; tree species: WScore = 0.8, WSig < 0.001).

The three dimensional Principal Coordinate Analysis found similarity of termite gut bacterial community structure from three habitats based on pairwise dissimilarity matrix (Figure 4.3a). ANOSIM test of PCoA supported that there were three habitat groups: lab, street trees and multiple tree species habitat. As indicated by figure 4.3a, most gut communities from multiple tree species habitat were more similar to each other within the group than across the group. Moreover, most gut communities from street tree were more similar to each other within the group than across the groups. Community structure of lab samples were more similar to most street tree samples, compared with samples from multiple tree species group.

Also, three dimensions Principal Coordinate Analysis demonstrated similarity of bacterial community structure of colonies from three tree species. ANOSIM test of PCoA supported that there were three habitat groups: *Casuarina equisetifolia* , *Peltophorum pterocarpum* and *Samanea saman*. However, as indicated by figure 4.3b, it seemed to be hard to conclude that gut bacterial community of samples were more similar to each other within the group than across the groups.


Lab
Street tree
Multiple tree species habitat
0.6
0.4
0.2
0
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.2
0.1
0
-0.1
Axis 3: 8.0%
Axis 2: 17.8%
Axis 1: 23.1%

a


Casuarina equisetifolia
Peltophorum pterocarpum
Samanea saman
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.2
-0.1
0
0.1
0.2
0.1
0
-0.1
-0.2
Axis 3: 10.4%
Axis 2: 13.6%
Axis 1: 38.9%

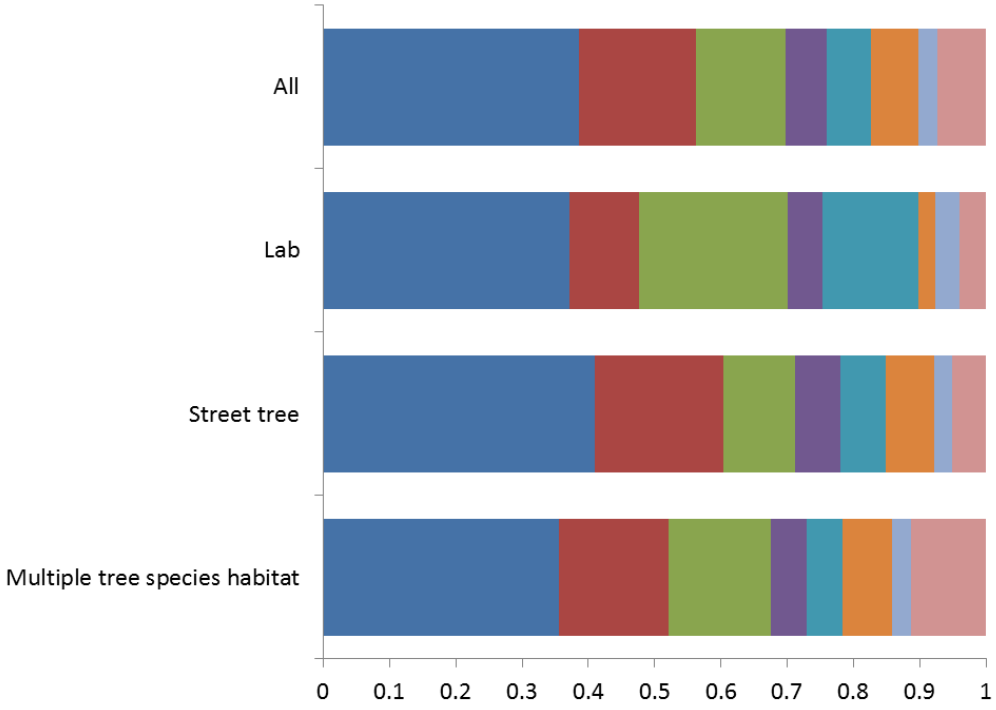
b

Figure 4.3. Three dimensional plot of the Principal Coordinate Analysis of the bacterial community structures of colonies from three habitats and three tree species. The PCA is based on pairwise dissimilarity matrices of the communities, which was calculated using thetayc calculator. Percentages of variation explained by the first three axes are shown in brackets. a) The three habitat groups (P=0.009, ANOSIM); b) the three host tree species groups (P= 0.04, ANOSIM).

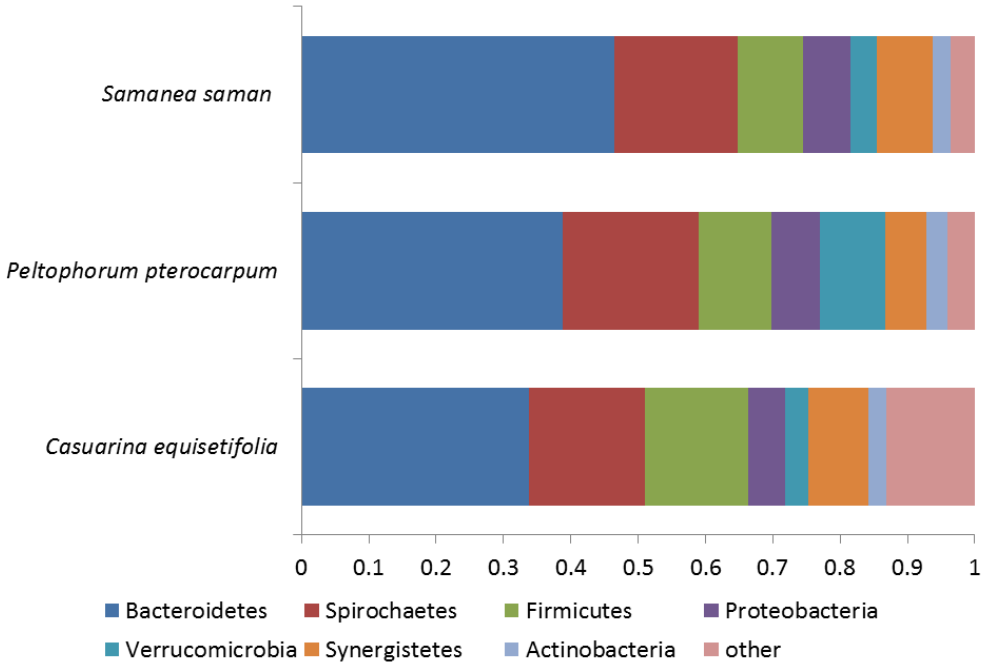
4.3.3 Comparison of phylum structure

The comparison of phylum composition of gut bacterial communities of all 30 samples found there was composition differentiation among three habitat and three tree species groups. Over all samples, Bacteroidetes (38.6%) was the most predominant phylum in the gut bacterial community of *C. gestroi*, followed by Spirochaetes (17.7%) and Firmicutes (13.4%), which together accounted for 69.7% of total diversity (Figure 4.4a). Among the three habitat groups, bacterial communities from street tree habitats contained the highest proportion of Bacteroidetes (41%) and the lowest proportion of Firmicutes (10.8%). Lab communities contained the lowest proportion of Spirochaetes (10.5%) and the highest proportion of Firmicutes (22.5%) and Verrucomicrobia (14.6%) (Figure 4.4a). Gut bacterial communities from the multiple tree species habitat had the highest proportion of other phyla (11.3%).

Among the three urban tree groups, bacterial communities from *Samanea saman* had highest proportion of Bacteroidetes (46.4%) while *Casuarina equisetifolia* had highest proportion of Firmicutes (15.3%) and other phyla (13.1%) (Figure 4.4b).



a



b

Figure 4.4. Phylum-level differences of diversity among *C.gestroi* colonies from different habitats and street tree species. Other represents sum of all other phyla. a) all communities

and three habitat groups. All presents all 30 communities. b) Three street trees groups.

4.3.4 Shared bacterial genera

Table 4.3 lists the 19 bacterial genera shared by all 30 samples, regardless of habitat and host tree; their ubiquity suggests that these genera may be essential for *Coptotermes gestroi*. Two of these genera are in the phylum Actinobacteia; four in Bacteroidetes; four in Firmicutes; six in Spirochaetes; and one each in Proteobacteria, Synergistetes, and Verrucomicrobia. Figure 4.5 shows the proportion of shared sequences in each phylum. Bacteroidetes contained the highest proportion of shared genera, which accounted for 34.7% of the Bacteroidetes phylum. Spirochaetes had the second-highest proportion (14.4%), followed by Synergistetes (7.1%), Verrucomicrobia (4%), and Firmicutes (3%). In total, the 19 shared genera accounted for 65.8% of the analyzed reads.

Shared genera among communities of the three urban tree species are shown in Figure 4.6. Twenty-four genera could be found in all three tree groups. The genera *Gut_cluster_9*, *Termite_group_aa*, *Porphyromonadaceae_Cluster_V_unclassified*, *Flavobacteriaceae_2_unclassified* and *Enterobacteriaceae_unclassified* were found in all three tree groups but not shared by all 30 colonies. *Casuarina equisetifolia* had the most unique genera, with the majority from Firmicutes. *Samanea saman* contained no unique genera.

Table 4.3. Shared bacterial genus by all 30 samples

Phylum	Class	Family	Genus
Actinobacteria	Actinobacteria	Termite_cluster_2	*Termite_cluster_2_unclassified*
		Coriobacteriaceae	*Uncultured_10*
Bacteroidetes	Bacteroidia	Porphyromonadaceae_1	*Dysgonomonas*
		Porphyromonadaceae_3	*Cluster_IV*
		Porphyromonadaceae_Cluster_V	*Candidatus_Azobacteroides*
			Termite_Cockroach_cluster
Firmicutes	Clostridia_1	Family_XIII_Incertae_Sedis	*Termite_cockroach_cluster_1*
		Lachnospiraceae	*Gut_cluster_13*
		Ruminococcaceae	*Uncultured_12*
			Gut_cluster_1
Proteobacteria	Deltaproteobacteria	Desulfovibrionaceae	*Termite_cluster_II*
Spirochaetes	Spirochaetes	Spirochaetaceae	*Spirochaeta*
	Spirochaetes	Spirochaetaceae_Treponema	*Treponema_II*
		Spirochaetaceae_Treponema_I	*Spirochaetaceae_Treponema_I_unclassified*
			Treponema_Ia
			Treponema_Ib
			Treponema_Ig
Synergistetes	Synergistia	Synergistaceae	*Termite_cockroach_cluster*
Verrucomicrobia	Opitutae	Opitutaceae	*Opitutus*

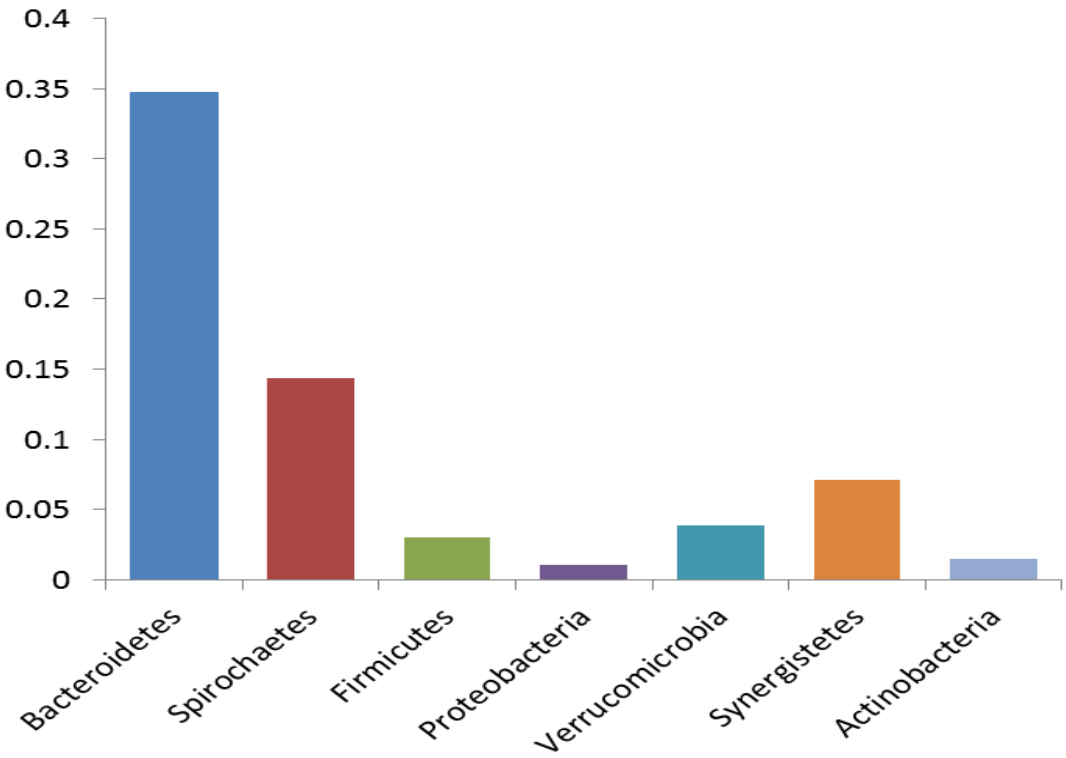


Figure 4.5. Proportion of sequences shared by all 30 colonies in each Phylum. Colors are consistent with Figure 4.4.

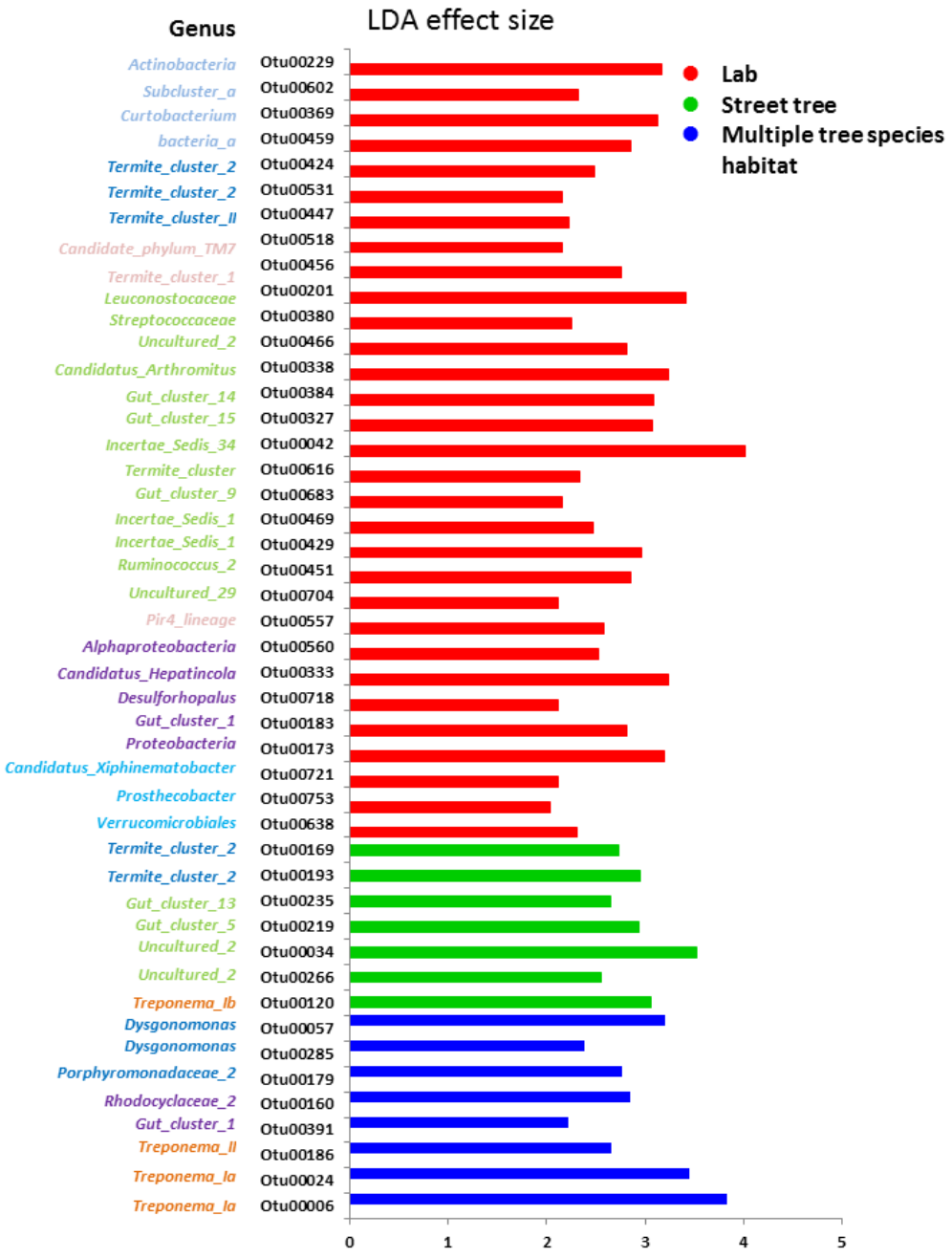
Figure 4.6. The shared bacterial genera found in guts of termites from the three street tree types in Singapore. Genera within circles or unions of circles were found in termite guts of the three street tree species. Pink circle encloses genera shared in all five samples collected from *Casuarina equisetifolia*; yellow circle encloses samples from *Peltophorum pterocarpum*; blue circle encloses samples from *Samanea saman*. Genera in the black square are shared by all 30 termite samples from all street trees. Five genera were found in all three tree groups but not shared by all 30 colonies: *Gut_cluster_9*, *Termite_group_aa*, *Porphyromonadaceae_Cluster_V_unclassified*, *Flavobacteriaceae_2_unclassified* and *Enterobacteriaceae_unclassified*. Color of the genera indicates the Phylum it belongs to and corresponds to the legend of Figure 4.4.

4.3.5 Characteristic OTUs differentiating groups

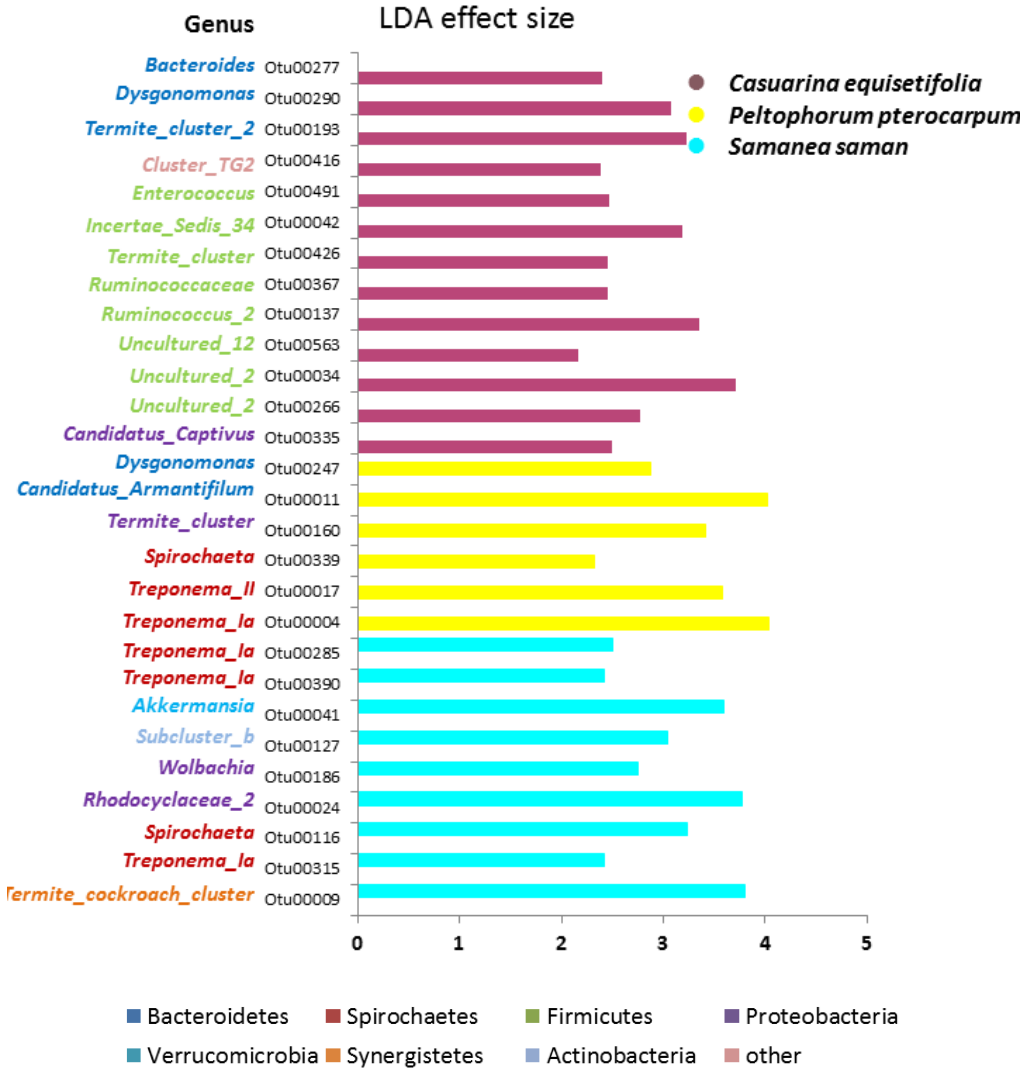
There were 46 characteristic OTUs among the three habitat groups, and 28 OTUs among three street tree species groups, as detected by LEfSe ($P < 0.05$ and LDA >2) (Figure 4.7). Further analysis revealed that, for habitat groups, the majority of the detected OTUs (n = 37) were enriched in the lab group; most of these belonged to the Firmicutes. Similarly, the

majority of the enriched OTUs found in the street tree group belonged to the Firmicutes. However, for the multiple tree species group, no enriched OTUs were in the Firmicutes; instead most of the enriched OTUs were those found in all 30 gut samples.

For the street tree species groups, most of characteristic OTUs were enriched in *Casuarina equisetifolia* and majority of these belonged to the Firmicutes (Figure 4.7). The Spirochates was the dominant phylum of OTUs detected in *Peltophorum pterocarpum* and *Samanea saman.*


Genus
LDA effect size
Lab
Street tree
Multiple tree species habitat
Actinobacteria Otu00229
Subcluster_a Otu00602
Curtobacterium Otu00369
bacteria_a Otu00459
Termite_cluster_2 Otu00424
Termite_cluster_2 Otu00531
Termite_cluster_II Otu00447
Candidate_phylum_TM7 Otu00518
Termite_cluster_1 Otu00456
Leuconostocaceae Otu00201
Streptococcaceae Otu00380
Uncultured_2 Otu00466
Candidatus_Arthromitus Otu00338
Gut_cluster_14 Otu00384
Gut_cluster_15 Otu00327
Incertae_Sedis_34 Otu00042
Termite_cluster Otu00616
Gut_cluster_9 Otu00683
Incertae_Sedis_1 Otu00469
Incertae_Sedis_1 Otu00429
Ruminococcus_2 Otu00451
Uncultured_29 Otu00704
Pir4_lineage Otu00557
Alphaproteobacteria Otu00560
Candidatus_Hepatincola Otu00333
Desulforhopalus Otu00718
Gut_cluster_1 Otu00183
Proteobacteria Otu00173
Candidatus_Xiphinematobacter Otu00721
Prosthecobacter Otu00753
Verrucomicrobiales Otu00638
Termite_cluster_2 Otu00169
Termite_cluster_2 Otu00193
Gut_cluster_13 Otu00235
Gut_cluster_5 Otu00219
Uncultured_2 Otu00034
Uncultured_2 Otu00266
Treponema_Ib Otu00120
Dysgonomonas Otu00057
Dysgonomonas Otu00285
Porphyromonadaceae_2 Otu00179
Rhodocyclaceae_2 Otu00160
Gut_cluster_1 Otu00391
Treponema_II Otu00186
Treponema_Ia Otu00024
Treponema_Ia Otu00006
0
1
2
3
4
5

a)



b)

Figure 4.7. The LefSe plot of characteristic operational taxonomic units (OTUs) in three habitat groups. All taxa with statistically significance (P<0.05) are showed. Genera of OTUs are listed on the left and LDA effect sizes are plotted on the right. Color of OUT name represents its phylum. Red bars represent OTUs enriched in lab habitat; green bars represent OTUs enriched in street trees and blue bars represent OTUs enriched in habitat of multiple tree species. Effect size indicates magnitude of variation of OTUs among three groups; larger effect size means that the OTU is more abundance in that particular group compared with the other two. b) LefSe plot of characteristic OTUs in three tree species habitat groups. Dark pink bars represent OTUs enriched in *Casuarina equisetifolia;* yellow bars represent OTUs enriched in *Peltophorum pterocarpum* and light blue bars represent OTUs enriched in *Samanea saman.*

4.3.6 Pairwise differentiation

Figure 4.8 uses a heatmap to show the pairwise differentiation of the gut bacteria communities of all 30 samples. P1, the lab community fed with pine wood for four months, was distinctly different from the others, and the gut bacterial diversity of P1 was low (Table 4.2). These results suggest that gut bacteria communities of lab-raised colonies may differ from those of field colonies and are not an ideal system for termite gut bacterial study. Other colonies with moderate differences include: JR (higher diversity of gut microbes from multi-tree habitat), LY1 and YC1.

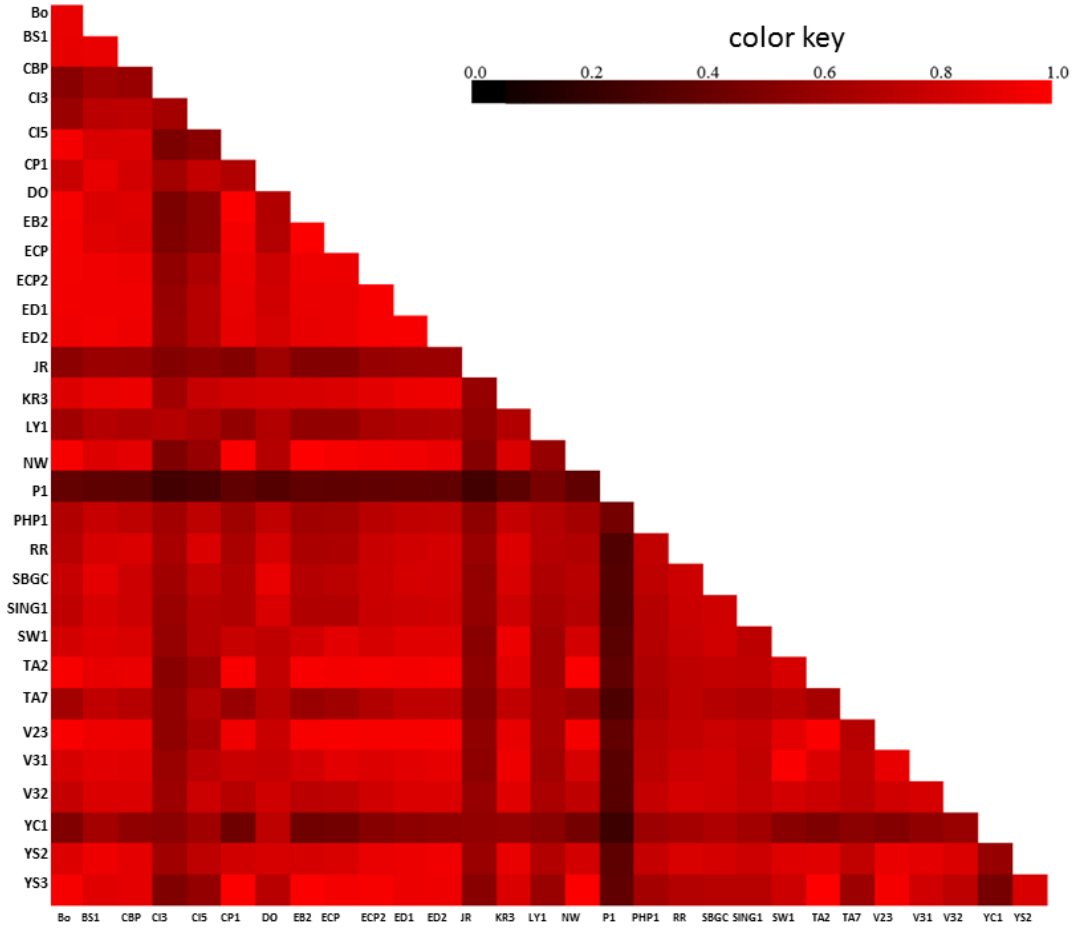


Figure 4.8. Heatmap of pairwise differentiation of gut bacteria community based on dissimilarity matrix calculated by Thetayc calculator.

4.4 Discussion

4.4.1 Impact of habitat and diet on gut bacterial community of *C. gestroi*

Most previous studies showed that phylogeny of host termite was the most important factor in structuring termite gut bacterial community, at least at taxonomic classification above species level. However, the results from this research provide a new perspective on termite gut communities, within a species. Colman et al (2012) surveyed the gut bacterial community of 58 insect species and reported that termite gut communities were highly similar, compared to those in other insect orders. In addition, they reported that the closer the host termites were phylogenetically, the more similar their gut flora communities. Reid et al (2014) studied three termite species and found that the gut bacterial communities of *Stolotermes ruficeps* and *Stolotermes inopinus* were clustered based on host phylogeny, irrespective of collecting location. Their results were consistent with a previous study, in which Hongoh et al. (2005) suggested that termite gut bacterial community was structured by host genotype, regardless of the habitats of the colonies.

However, when I focused on the gut community of one particular termite species using 30 samples from a variety of habitats, I discovered that the community structure and diversity were different. Both habitats and host trees had a significant influence, implying that the importance of habitat and diet to termite gut bacterial communities may be underestimated, at least with one species. Previous studies compared gut communities among several distinct insect or termite species, and it is likely that the great phylogenetic differentiation of host masked the signal of habitat and diet.

In this study, both weighted uniFrac and AMOVA analyses discovered significant differences in gut community structure among different habitat and host tree samples. Anosim test of PCoA plot also supported three habitat (lab, street tree and multiple tree habitats) and three street tree species (*Casuarina*, *Peltophorum* and *Samanea*) groups. The diversity of gut bacterial community from these different habitats and host tree groups differed significantly. The evenness of different habitats differed significantly, whereas that of host tree groups did not. Thus, I concluded that habitats and host trees—which determine termite colonies' food sources and diets, to some extent—are shaping the general gut bacterial community of *C. gestroi*.

My results are consistent with those from some other studies. Habitat and host plants have been reported to influence some insect species (Priya et al. 2012; Rahman et al. 2015), and diet is the predominant factor in shaping the human gut community (Priya et al. 2012; Engel and Moran 2013; Jandhyala et al. 2015) – notably these studies are all from one host species as well. My studies reveal similar results about the importance of habitat and diet, which also seem to be a more important determinant than host phylogeny at colony level for *C. gestroi*.

My investigation shows that bacterial communities from termites living in habitats with multiple tree species are the most diverse. Those from street trees have intermediate diversity, whereas those from lab colonies maintained in the laboratory are the least diverse. Habitats with multiple tree species possess many potential food sources, while street tree habitats

usually have a monoculture (i.e. all the trees planted along the street belong to the same species), with some other wood products used in the buildings (but low volumes, as buildings in Singapore are mostly steel-reinforced concrete). The two lab colonies of *C. gestroi* had the lowest diversity of food, as they were fed with pine wood exclusively. These results support the hypothesis that the diversity of gut bacteria corresponds to the diversity of food resources provided by their habitat.

How did habitat and diet alter the diversity and structure of the gut bacterial community? There are two potential hypotheses that may explain the correlation between habitat diversity and gut bacterial community diversity. 1) Having fewer food choices or merely one kind of food may selectively enhance the abundance of some microbes, because these microbes are better adapted at digesting that particular food. As a result, the other bacterial species decline and become difficult to detect. In support of this hypothesis was the pattern observed in lab colonies. Lab colonies had the least diverse bacterial communities and most characteristic enriched OTUs as indicated by LEfse, suggesting that several genera were dominant in the community, possibily the result of one food source. More studies on genera with high LDA effect size may help reveal the interaction between termite gut bacterial community and environment. 2) In a more diverse habitat, there are likely to be a greater diversity of bacteria, and therefore termites are exposed to more bacterial species and thereby acquire a greater diversity of species from the environment. That may explain why bacterial community of habitats with multiple tree species, including forests and urban parks, had the highest proportion of other bacterial phyla.

These two hypotheses are not mutually exclusive. It is likely that habitat and diet do not change the bacteria directly, but alter the diversity and abundance of their symbiotic protists. Most gut bacterial species are the endo- or ectosymbionts of gut protists, and the disappearance of any protist species leads to the reduction of its bacterial symbionts (Noda et al. 2003; Noda et al. 2005; Hongoh et al. 2007a; Hongoh et al. 2007b; Sato et al. 2009). A diet shift resulted in the disappearance of two protists in *Coptotermes formosanus* and changed the gut bacterial community (Tanaka et al. 2006).

4.4.2 Characteristic bacterial phyla and genera in the *C. gestroi* gut community and their function

Several characteristic bacterial phyla were detected in gut bacterial community of *C. gestroi*. Bacteroidetes was the most predominant phylum in gut bacterial community of *C. gestroi*, followed by Spirochaetes and Firmicutes. Bacteroidetes and Firmicutes are common in the guts of many animals and human intestines (Breznak 2000; Engel and Moran 2013; Douglas 2015; Jandhyala et al. 2015). It is interesting to note that some phyla, such as Bacteroidetes and Firmicutes, are constantly present in animal guts, regardless of the host species, indicating some commonalities among animal guts. A high proportion of Spirochaetes was discovered in lower termites and wood-feeding termites (Dietrich et al. 2014). Spirochaetes is considered as the most characteristic phylum in the termite gut community.

There were 19 genera found in the gut communities of all *C. gestroi* colonies, regardless of

habitats and host trees (Table 4.2). I speculate that these bacteria may be essential microbes for *C. gestroi* and are inherited from nestmates. They may also play a major role in wood digestion and nutrient uptake for the host termite. *Dysgonomonas* has been reported to be involved in lignocellulose decomposition (Sun et al. 2015). Cellulolytic enzyme complexes were found in Clostridia, suggesting that Clostridia might have a function in cellulose digestion (Demain et al. 2005). *Dysgonomonas* and *Candidatus azobacteroides* contain genes for nitrogen fixing (Inoue et al. 2015; Hongoh et al. 2008a). A metagenomic and function study of the gut community in the wood-feeding termite revealed that *Treponema* contains hydrogenase and glycoside hydrolases and might also be involved in CO_2-reductive acetogenesis, suggesting the potential of *Treponema* to produce H_2 and acetate and to digest glycoside (Warnecke et al. 2007; Lilburn et al. 2001).

Besides digestion and nutrient provision, termite gut bacteria may have other functions, such as detoxification, influencing host physiology, and protection. Woody material usually contains a variety of phenolic compounds, which are toxic and are generated by plants in order to deter herbivorous animals from eating them. Recent studies have shown that human gut bacterial species in the genera *Bacteroides*, *Clostridium*, and *Lactobacillus* are capable of degrading various phenolic compounds, which suggests the plausibility of gut bacterial detoxification (Jandhyala et al. 2015). Interestingly, this study found that the gut community from *Casuarina equisetifolia* had more unique Firmicutes species and characteristic OTUs from Firmicutes. Considering that the tree species has phenolic acids and some members of Firmicutes are able to detoxify these compounds, it seems plausible that these bacteria might help their termite

hosts to deal with the challenge of toxins (Narhi and Fulco 1986; El-Tantawy et al. 2013).

In addition, gut bacteria has been reported to participate indirectly in the development of its host insect. Studies of *Drosophila melanogaster* showed that bacteria modulated the activity of midgut stem cells, and the renewal extent of epithelial cells was proportional to the concentration of midgut bacteria (Buchon et al. 2009a; Buchon et al. 2009b; Jiang et al. 2009). Moreover, a few studies have proven that the colonization of gut bacteria protects the host from parasite invasion (Dillon et al. 2005; Koch and Schmid-Hempel 2011; Chambers et al. 2012). More functional studies of termite gut flora may reveal more diverse roles, and their importance to host termites may exceed our current expectations.

4.5 Conclusion

This is the first study to reveal the impact of diet and habitat on the gut bacteria community of the lower termite *Coptotermes gestroi* in the field. Habitat types and tree species were generally found to have significant influence on gut bacterial diversity and community structure. Bacterial communities from multiple tree species habitats are most diverse, followed by street tree communities and then lab communities. The diversity of gut bacteria corresponds to the diversity of food resources of the habitat. I reported that within the same species, diet and habitat are more important factors in shaping the gut bacterial community than the host phylogeny, which refers to colony genotype here. I discovered 19 bacterial genera common to all 30 samples, regardless of habitat and host tree species, which accounted for 65.8% of all reads obtained. These 19 genera may be essential for *C. gestroi* digestion and survival.

Finally, the heatmap of pairwise differentiation shows that the gut bacteria community of lab colony P1 was distinct from the others, implying lab colonies of termites may not be a good model for termite gut bacterial study. Differentiation of gut bacterial communities from different habitats and host trees may shed light on strategy that invasive termites take to adapt to new environment.

Chapter 5: Final conclusion

In this thesis I explored the colony features and population genetic structure of *Coptotermes gestroi* across Southeast Asia using microsatellite markers. *Coptotermes gestroi* was believed to be native to Southeast Asia and the high polymorphism of microsatellite locus (average=19.75) discovered in colonies from this region, compared with those of colonies from introduced regions, supported this claim (Chapter 2). I found three genetic clusters across Southeast Asia: 1) Northern population, including colonies from Cambodia and Vietnam; 2) Southern population, including Thailand, Malaysia, Singapore and Indonesia; and 3) Philippines population, including all Philippines samples. These three populations had substructure. Genetic structure of all native colonies and three populations showed evident spatial pattern and isolation by distance, suggesting significant correlation between geographic factors and genetic structure across Southeast Asia.

Invasive termites are typically moved between cities by humans. I collected the majority of my samples from cities, nevertheless my results indicated that natural dispersal rather than human- mediated dispersal was the primary factor determining the distribution of *C. gestroi* in Southeast Asia. Southeast Asian cities were built on forests that contained local colonies of *C. gestroi*; these may have expanded as the cities were built, and prevented the introduction of *C. gestroi* colonies from elsewhere. I speculate that the importance of anthropogenic activity to current invasive termite distribution patterns may be overestimated within SE Asia. In the environment where population size of a particular species has reached a balance, like in its native region, it might be hard for human transport to introduce more individuals of that species

into the environment. Similar scenarios might be found in regions where the species has been introduced a long time ago, such as in Brazil for *C. gestroi*.

Additionally, my project on *Coptotermes gestroi* may provide various insights on management of invasive termites. *C. gestroi* is invasive in many islands in the Pacific Ocean, in Mexico and islands of the Caribbean Sea, and is widespread in Brazil (Evans et al. 2013). The two invasive colonies found in Hawaii and Berlin were clustered with native populations in the Philippines, suggesting the possibility that Philippines may be an important source of introduced *C. gestroi*. Also, Yeap et al (2011) reported two Taiwan colonies probably originated from Philippines. Thus, closer scrutiny of wooden exports from the Philippines is recommended to reduce risk of further spread. The Philippines was a very important source of timber during the Spanish and American colonial period, which may when *C. gestroi* was spread to the Americas (Liu et al. 1993; Bankoff 2007)

I surveyed the diversity and structure of gut bacterial community of *C. gestroi* using 16s rDNA pyrotag sequencing. I did not find correlation between host phylogeny at colony level and gut bacterial community. On the contrary, I reported significant impact of habitat and host trees on the gut bacterial community. Diversity of gut bacteria corresponded to the diversity of food resources provided by its habitat. Moreover, I listed bacterial genera shared by all *C. gestroi* colonies, regardless of habitats and host trees, which comprised about 65.8% of all bacteria. These genera may be the essential bacteria for the survival of *C. gestroi*.

It appears that the gut flora community of invasive termites was more flexible than expected

and reflected an interaction with environment. This could be important for both the successful invasion and urban adaptation of the species. For example, termites collected from urban street trees, which have a limited diet of a single tree species (and perhaps some discarded wood from buildings or fences), had some unique gut bacteria genera. The bacterial communities found in termites eating *Casuarina equisetifolia* were different from those eating *Samanea saman* and *Peltophorum pterocarpum*, which are more related to each other (both Order Fabales, Family Fabaceae) than to *Casuarina* (Order Fagales, Family Casuarinaceae) (Figure4.3b). Also, *Casuarina* contains some secondary metabolites that function as toxins, so perhaps the higher diversity of bacteria in termites feeding on *Casuarina* are there to de-toxify the food.

A better knowledge of gut flora may provide novel pest management methods. For example, the 19 essential genera of gut bacteria may be a better target for insecticides than the insects themselves. It may be possible to incorporate an antibiotic specific to one or more of the 19 essential bacterial genera as a biological active ingredient into baits, further reducing the non-specific chemical insecticides used in termite management (Evans and Iqbal, 2015).

Future directions

Genetic structure of *C. gestroi* is still poorly understood. Very few researchers have research its genetic structure, either in native or introduced lands. It will be interesting to survey the global population genetic and colony structure of introduced colonies, especially in Brazil and the islands in the Pacific of *C. gestroi* using microsatellite markers, and incorporate this information with data of native populations. Comparing introduced with native populations may reveal some interesting patterns, which would improve our understanding of introduction pathway of this invasive species. For example, the Spanish occupied the Philippines for centuries, and shipped products from Manilla across the Pacific to Acapulco in Mexico (the 'Galeón de Manila' and 'La Nao de la China'), and may explain the early introduction of *C. gestroi* to Hawaii (Bjork1998; Fish 2011)

On the other hand, I sampled the largest number of colonies (103) across the greatest geographical range (seven countries) of any study of *C. gestroi* in this thesis, and so I have established a good database for source identification of introduced colonies. Genetic analysis including both native and introduced colonies will clarify the routes that *C. gestroi* took and explain its current global distribution. This larger analysis may uncover a role of antropopgenetic activity in global distribution of invasive termites and provide important references for pest control. For example, results from my research show *C. gestroi* from Hawaii and Germany were likely originally from the Philippines, even though the introductions of these termites were likely many years, perhaps centuries, apart. National quarantine / biosecurity officers could pay more attention to wood cargo from potential source

regions of introduced colonies.

It was shown in this thesis that habitat and host plant influence the diversity and composition of gut bacterial genera of *C. gestroi*. However, reasons for the differentiation of gut community from different habitats and host trees are not clear. I proposed two hypotheses. First, the habitat and/or host tree may selectively enhance some bacterial genera, due to performing a certain function, which then increases the fitness of the host termite. A profile of functional genes of gut community from different habitats and host tree could be used to test this hypothesis. My second hypothesis is that *C. gestroi* may acquire different amounts and types of bacteria from different environments, relative to the existing bacterial diversity in those environoments. A comparison between gut bacterial and environmental bacterial communities may address this hypothesis. Also, considering that protists are essential symbionts for lower termites, and that many bacteria are the endo- or ectosymbionts of protists, it would be important to reveal interactions between the environment and both protists and bacteria. Both pyrosequencing and Fluorescence In Situ Hybridization (FISH) would be powerful tools to investigate the specific role of gut flora for termites in difference habitats, and indeed to consider their effect in new, invaded habitats. In sum, to grasp a better understanding of invasive termites, both population genetic and gut flora studies are needed. The former helps us to know how the invasive termites spread and the latter may shed light on why they are so successful in invading new environments.

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Appendix

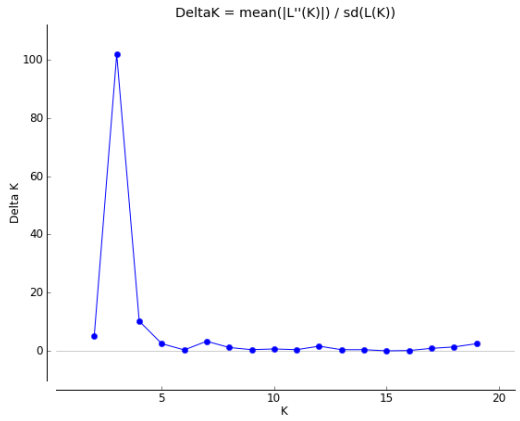
Appendix 1. Sample list

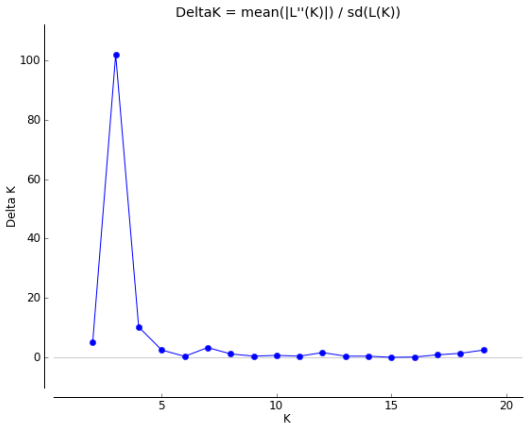
sample	country	individuals sampled	location	lantitude	longtitude
CD1	Thailand	23	Chombueng Distict, Ratchaburi Province	13º36’7.95”N	99º39’40.99”E
CD2	Thailand	23	Chombueng Distict, Ratchaburi Province	13º36’7.96”N	99º39’43.99”E
BKK	Thailand	20	Bangkok	13º46’23.55”N	100º28’53.21”E
BTD	Thailand	18	Ban Takhun Distict,Surat Thani Province	8º57’41.864”N	98º48’39.17”E
MD	Thailand	21	Mueng Distict,Surat Thani Province	9º6’4.98”N	99º21’48.91”E
SS	Thailand	21	Samut Sakorn	13º34’39.78”N	100º10’58.79”E
C1	Cambodia	23	Phnom Penh,sovana shopping center	11º32’44.68”N	104º54’4.64”E
C3	Cambodia	24	Phnom Penh,sofitel	11º32’47.97”N	104º56’0.58”E
C4	Cambodia	23	Phnom Penh,#138D Norodom Blvd	11º33’20.44”N	104º55’41.93”E
C5	Cambodia	19	Phnom Penh,diamond island	11º32’40.65”N	104º56’11.95”E
C6	Cambodia	24	Phnom Penh,diamond island	11º32’49.27”N	104º56’16.19”E
C7	Cambodia	24	Phnom Penh,camko city	11º35’39.03”N	104º53’37.83”E
C8	Cambodia	22	Phnom Penh,preah trasak paem street	11º33’9.32”N	104º55’23.03”E
H1	Vietnam	23	Hanoi,west lake	21º3’41.75”N	105º49’23.44”E
H3	Vietnam	24	Hanoi,Linh Dam park	20º58’31.74”N	105º53’55.77”E
H5	Vietnam	20	Hanoi,west lake	21º2’48.30”N	105º48’45.45”E
H6	Vietnam	18	Hanoi,west lake	21º4’1.34”N	105º49’22.90”E
H7	Vietnam	20	Hanoi,Thong Nhat park	21º4’40.80”N	105º30’18”E
H8	Vietnam	23	Hanoi,west lake	21º2’29.80”N	105º49’44.90”E
V1	Vietnam	22	Saigon,nguyen van troi	10º47’37.95”N	106º40’41.84”E
V3	Vietnam	23	Saigon,nguyen van troi	10º47’37.94”N	106º40’41.83”E
V4	Vietnam	22	Saigon,le van tam	10º47’17.37”N	106º41’35.47”E
V6	Vietnam	23	Saigon,Thao Can Vien	10º47’17.37”N	106º42’23.62”E
V12	Vietnam	22	Saigon,pai 103	10º48’26.72”N	106º41’21.50”E
V19	Vietnam	24	Saigon, van kiep	10º48’3.42”N	106º41’36.32”E
V20	Vietnam	24	Saigon, ham nghi	10º46’14.87”N	106º42’18.52”E
V22	Vietnam	20	Saigon, ham nghi	10º46’15.78”N	106º41’59.51”E
V23	Vietnam	21	Saigon,phu my hung	11º6’33.08”N	106º28’22.15”E
V24	Vietnam	23	Saigon,phu my hung	11º7’32.28”N	106º27’2.60”E
V25	Vietnam	22	Saigon,phu my hung	11º8’7.75”N	106º27’23.92”E
V26	Vietnam	21	Saigon,cong vien hoang van thu	10º48’11.27”N	106º39’51.03”E
V27	Vietnam	22	Saigon,tranhung dao	10º48’11.27”N	106º41’25.53”E
V28	Vietnam	21	Saigon,ben xe men dong	10º48’52.48”N	106º42’36.52”E
V30	Vietnam	23	Saigon, duong xuan hong	10º47’39.59”N	106º39’8.65”E

V32	Vietnam	22	Saigon,phu lam park	10º44’49.97”N	106º37’27.12”E
V33	Vietnam	19	Saigon,phu lam park	10º44’52.31”N	106º37’33.30”E
M3	Malaysia	23	Penang,siam road	5º24’51.06”N	100º19’17.63”E
M4	Malaysia	20	Penang,kek lok si	5º23’57.95”N	100º16’25.56”E
M5	Malaysia	22	Penang,kek lok si	5º24’1.62”N	100º16’25.98”E
M6	Malaysia	24	Penang,barkok street	5º25’57.83”N	100º20’13.56”E
M8	Malaysia	23	Penang,clan jetty	5º24’44.28”N	100º20’23.7”E
M9	Malaysia	23	Penang,northam beach caf é	5º25’41.41”N	100º19’21.79”E
M13	Malaysia	23	Penang,persiaran gunrney	5º25’53.65”N	100º19’8.11”E
M14	Malaysia	24	Penang,pengkakn weld	5º24’46.90”N	100º20’22.65”E
RR	Singapore	22	riffle range Road	1º20’32.19”N	103º46’46.83”E
BK	Singapore	22	brqanksome	1º18’11.72”N	103º53’9.85”E
CBP	Singapore	22	changi beach park	1º23’28.01”N	103º59’18.42”E
CI3	Singapore	24	corney island	1º24’54.33”N	103º55’2.45”E
CI5	Singapore	23	corney island	1º24’39.24”N	103º55’20.18”E
JP	Singapore	21	jalan pari dedap	1º19’57.88”N	103º56’34.47”E
KR3	Singapore	21	National university of Singapore	1º17’45.15”N	103º46’40.07”E
LR	Singapore	24	loyang rise	1º21’44.45”N	103º57’52.94”E
NT1	Singapore	22	National university of Singapore	1º18’21.93”N	103º46’26.72”E
OBC	Singapore	17	pulan ubin	1º24’31.45”N	103º57’18.54”E
RP	Singapore	23	punggol rubber plantation	1º25’1.45”N	103º54’24.72”E
SBGC	Singapore	24	Singapore botanic garden	1º18’31.58”N	103º49’4.76”E
SING1	Singapore	21	bukit timah nature reserve	1º20’48.37”N	103º46’45.05”E
WC	Singapore	22	west coast plaza	1º18’31.58”N	103º49’4.76”E
NW	Singapore	24	newtown circus	1º18’9.96”N	103º45’55.13”E
ED1	Singapore	23	Pasir ris close	1º22’36.75”N	103º57’22.61”E
ED2	Singapore	21	Pasir ris close	1º22’36.84”N	103º57’23.85”E
TA2	Singapore	22	tropica condor	1º20’58.04”N	103º55’40.23”E
TA7	Singapore	24	tampines street 33	1º21’12”N	103º57’28”E
YC1	Singapore	23	yuan ching Road	1º20’16.89”N	103º43’28.70”E
ECP2	Singapore	21	east coast park	1º17’41.56”N	103º53’6.66”E
MA	Singapore	24	marsiling lane	1º26’40”N	103º46’39”E
YS2	Singapore	22	yishun avenue 6	1º26’19”N	103º50’25”E
YS3	Singapore	23	yishun avenue 6	1º26’1”N	103º50’40”E
SW1	Singapore	22	sembawang Drive	1º27’29”N	103º48’52”E
LY1	Singapore	24	lok yang way	1º19’28”N	103º41’22”E
EB2	Singapore	22	Elithabeth moutain hospital	1º18’24.87”N	103º50’9.25”E
BS1	Singapore	23	bedok south avenue3	1º19’12”N	103º56’38”E
CP1	Singapore	24	city plaza	1º18’54.52”N	103º53’36.18”E
Bo	Indonesia	21	Bongor,Bongor Agriculture university	6º33’32.77”S	106º43’31.69”E
PHI20	Philippines	23	Davao	7º3’25.13”N	125º33’38.23”E
PHI23	Philippines	20	Davao	7º3’49.86”N	125º35’31.88”E
PHI28	Philippines	19	Davao	7º3’6.52”N	125º35’40.7”E

PHI30	Philippines	20	Davao	7º4’0.98”N	125º36’59.11”E
PHI33	Philippines	24	Davao	7º7’7.39”N	125º27’9.11”E
PHI35	Philippines	22	Davao	7º7’15.24”N	125º39’8.24”E
PHI39	Philippines	19	Davao	7º8’16.51”N	125º38’46.64”E
PHI41	Philippines	24	Davao	7º8’22.63”N	125º38’44.55”E
PHI42	Philippines	21	Davao	7º5’37.36”N	125º36’7.88”E
PHI51	Philippines	20	Davao	7º4’33.96”N	125º35’54.2”E
PHI53	Philippines	23	Davao	7º5’4.81”N	125º35’56.11”E
PHI60	Philippines	21	Davao	7º4’37.56”N	125º35’9.13”E
PHI61	Philippines	21	Davao	7º6’14.83”N	125º35’50.64”E
PHI63	Philippines	23	Davao	7º5’25.91”N	125º37’6.06”E
PHI66	Philippines	21	Davao	7º2’42”N	125º34’19.38”E
PHI69	Philippines	17	Davao	7º3’31.50”N	125º31’56.28”E
PHI70	Philippines	21	Davao	7º3’50.76”N	125º31’19.63”E
PHI77	Philippines	24	Bacolod	10º39’2.81”N	125º56’14.78”E
PHI80	Philippines	22	Bacolod	10º39’12.31”N	125º57’13.28”E
PHI85	Philippines	24	Bacolod	10º39’18.14”N	125º58’37.81”E
PHI89	Philippines	23	Bacolod	10º41’9.56”N	125º58’23.56”E
PHI98	Philippines	23	Bacolod	10º28’38.1”N	122º56’24.03”E
PHI100	Philippines	23	Manila	14º35’41.26”N	121º7’23.86”E
PHI101	Philippines	23	Manila	14º34’36.94”N	121º8’22.26”E
PHI102	Philippines	23	Manila	14º35’50.45”N	121º7’23.62”E
PHI103	Philippines	22	Manila	14º35’45.06”N	121º7’20.82”E
PHI104	Philippines	20	Manila	14º35’36.51”N	121º7’8.47”E
BAM	Germany	24	NA	NA	NA
Ha1	Hawaii	23	NA	NA	NA

Appendix 2. The delta K values given by Structure Harvester of three runs in each test. a) All colonies; b) Northern population; c) Southern population; d) Philippines population

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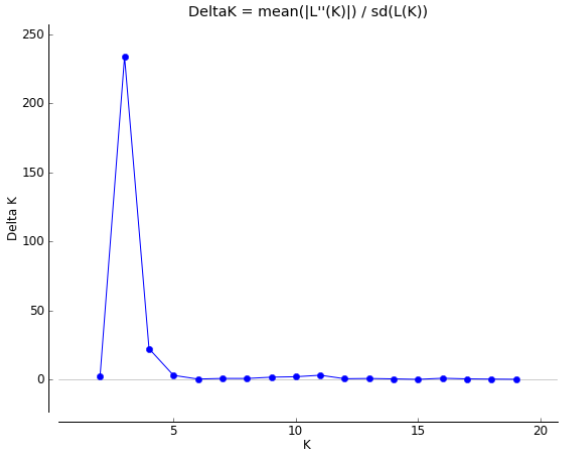
Delta K
K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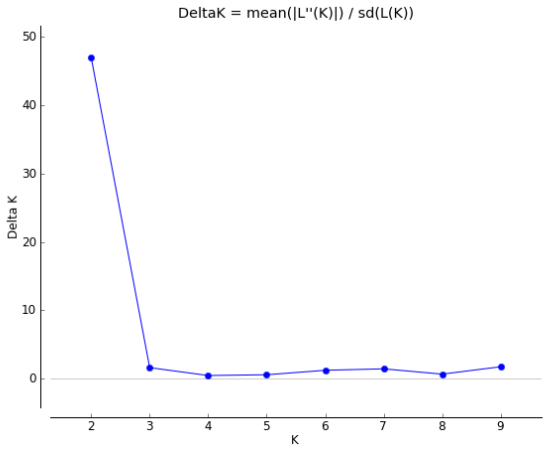
K

a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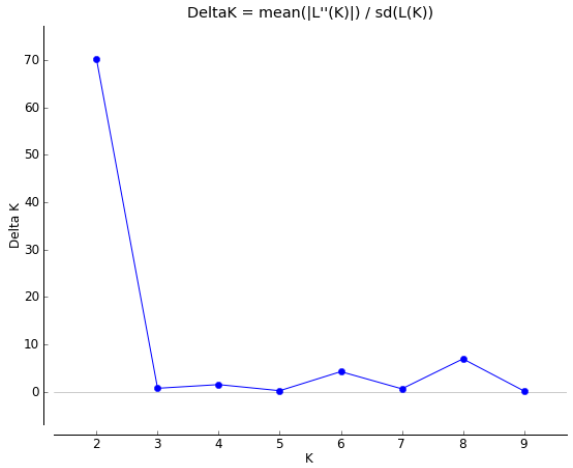
K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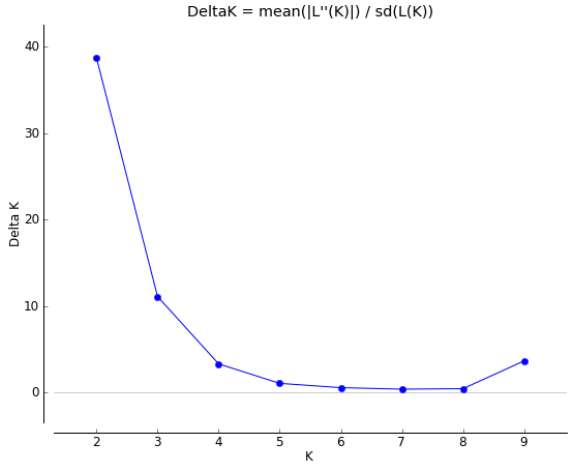
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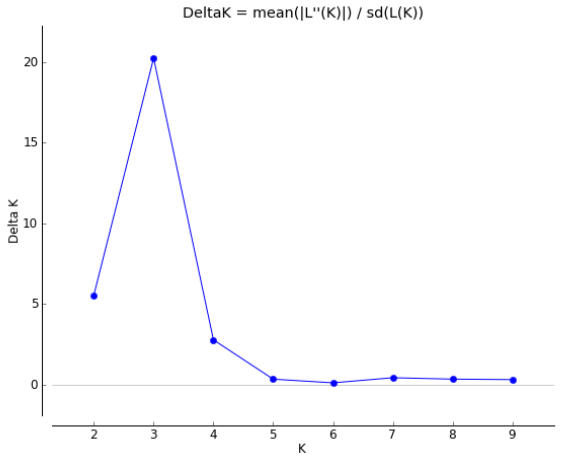
Delta K
K

b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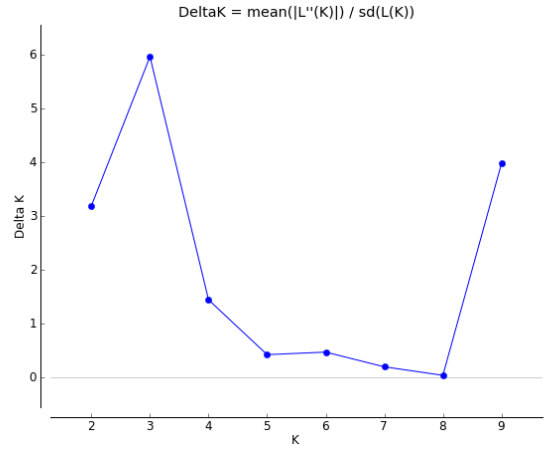
K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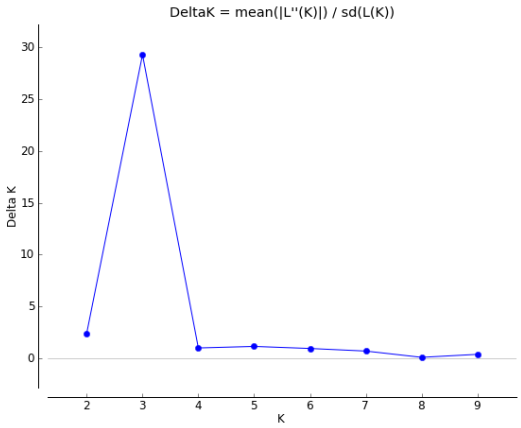
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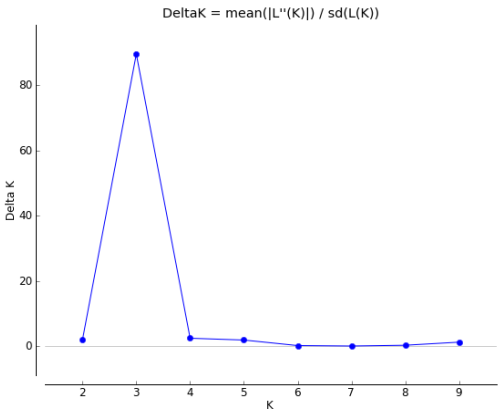
K

C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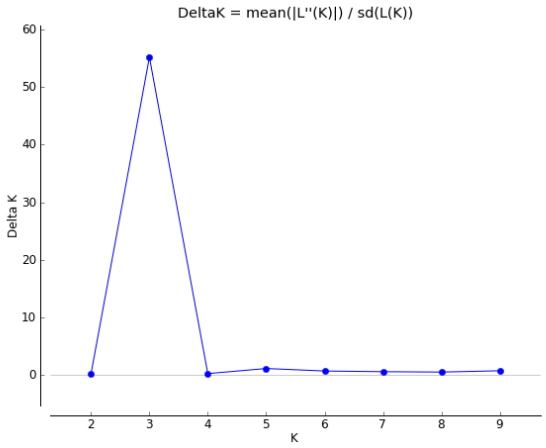
Delta K
K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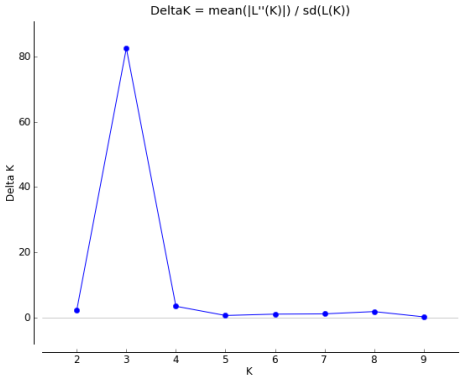
Delta K
K
60
50
40
30
20
10
0
2
3
4
5
6
7
8
9


DeltaK = mean(|L''(K)|) / sd(L(K))
Delta K
K
80
60
40
20
0
2
3
4
5
6
7
8
9

d