Inter and intra termites colonies comparisons of gut microbial diversity from worker and soldier caste of *Globitermes sulphureus* (Blattodea: Termitidae) using 16S rRNA gene

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ABSTRACT

Aims: The present study aims at isolation and identifying termite gut bacteria from different *Globitermes sulphureus* mounds using a molecular approach based on 16S rRNA genes.

Methodology and results: The bacteria from the whole gut of soldier and worker castes of the termite *G. sulphureus* was isolated and identified. The diversity of the bacteria was compared between five different colonies in Universiti Sains Malaysia, Penang, Malaysia. The isolated bacteria were identified by using 16S rRNA gene sequencing method and subsequently used for phylogenetic analysis. Sequences analyses of identified bacteria were found to be affiliated with the phyla Firmicutes, Proteobacteria, and Actinobacteria. Soldiers and workers seemed to have little differences in bacterial species from the same colonies. We noted some bacteria which were detected in soldiers were not detected in workers.

Conclusion, significance and impact of study: Differences in the culturable bacteria composition were not significant between termite colonies. However, the phylogenetic analysis revealed that most of the bacteria from one colony were slightly but distinctly different with bacteria from other colonies.

Keywords: termite gut, microbial diversity, *Globitermes sulphureus*, higher termites, 16S rRNA

INTRODUCTION

Termites are well known as social insects where they live in a caste system consist of royal pairs (king and queen), or supplementary reproductive, soldiers, workers, and nymphs (Sen *et al.*, 2013). Different castes have different morphological features suitable for their functions. The subterranean termite, *Globitermes sulphureus* (Haviland) of the subfamily Termitinae is known as secondary pest and occurs at high densities in the Indo-Malayan region, from Myanmar to peninsular Malaysia (Roonwal, 1970; TROVE, 1992; Abdul Hafiz *et al.*, 2007; Abdul Hafiz and Abu Hassan, 2011; Neoh *et al.*, 2011). It is also a common pest to agriculture crops such as oil palm and sometimes attacks building structures, particularly in rural and suburban areas (Ngee and Lee *et al.*, 2002; Abdul Hafiz *et al.*, 2011; Neoh *et al.*, 2011). This species can be controlled using imidacloprid, fipronil, and bistrifluron (Abdul Hafiz *et al.*, 2007; Abdul Hafiz and Abu Hassan, 2011; Neoh *et al.*, 2011). As a higher group termite, *G. sulphureus* harbor some morphologically and biochemically diverse bacteria in their gut, unlike lower group termite where they retain motile protists in their gut

(Ni and Tokuda, 2013). Termite and gut microbiota are mutually symbiotic for digestion of lignocellulose and production of the nutrients. However, there are evidences to show termites themselves produce cellulase, hemicellulase, and lignases that contribute in collaboration in lignocelluloses digestion (Scharf and Tartar, 2008; Scharf *et al.*, 2011).

Because of the vital role and diversity of bacteria in termite gut, the researchers use bacteria as a good target for aiming at termite control. The initial work was done by targeting the bacteria with antibiotics. The antibiotic rifampin significantly alters the termites' bacteria, reproduction, and colony lifespan (Rosengaus *et al.*, 2011). The gut bacterium *Enterobacter cloacae* was genetically engineered with a recombinant plasmid (pEGFP) containing genes encoding ampicillin resistance and green fluorescent protein (GFP). This transgenic bacterium was successfully used as a shuttle detrimental gene into *Coptotermes formosanus* colony without risking detection and elimination by the termites' natural defenses (Husseneder and Grace, 2005). In addition, researchers in

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the LSU AgCenter are in progress to prove the concept that natural phages can disrupt the termites' bacterial community and thus can kill a termite colony (Husseneder *et al.*, 2015). To date, the complete genome of *Citrobacter Phage* CVT22 isolated from gut of *Coptotermes formosanus* Shiraki was successfully sequenced (Tikhe *et al.*, 2015).

In termite colony, worker caste is responsible for feeding other castes. The feeding process is known as trophallaxis, meaning the direct transfer of alimentary liquids, including suspended particles and symbiont bacteria from one nestmate to another via proctodeal and stomodeal (Fujita *et al.*, 2001). In this research, we used a culture-dependent technique to compare the whole gut bacteria of soldier and worker castes from the wood-feeding termite *G. sulphureus*. Even though they have different roles in the termite colony, we expected they harbor similar bacterial species in the gut because of trophallaxis activities. In addition, we also investigated the bacterial diversity from different termite colonies of the same species. Different colonies might have different bacteria diversity depending on the food source available in a period of time.

MATERIALS AND METHODS

Collection of termites

Termites (*G. sulphureus*) were collected freshly from the field from five different colonies in the Universiti Sains Malaysia, Penang (colony 1: Durian Valley (DV), colony 2: Indah Kembara (IK), colony 3: Padang Minden (PM), colony 4: Tasik Harapan (TH), colony 5: Bakti Permai (BP). Soldier and worker castes were used in this study.

Isolation of bacteria

Ten individuals of the worker and soldier caste from each colony were analyzed. The termites were surface sterilized with 70% ethanol and rinsed with sterile distilled water. The whole gut of the termites was removed using sterilized forceps and then placed in 500 μ L of 1x phosphate buffered solution (pH 7.2). The guts were homogenized using micropestle. The 500 μ L of the suspension was mixed with 4.5 mL medium 1 which contained 5 g/L carboxymethylcellulose CMC and 0.2 g/L yeast extracts (Ramin *et al.*, 2008). The mixture was incubated at 30 °C for 3 days. Ten-fold serial dilutions were made from the bacteria cultures with sterile distilled water and then plated on nutrient agar medium. The pure colonies were obtained by several subsequent culturing and plating.

DNA extraction and PCR amplification

Bacterial DNA from each pure colony was extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to manufacturing protocols. The 16S rRNA gene was amplified using the bacterial specific primer pair 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-

GGGCGGWGTGTACAAGGC-3') (Marchesi *et al.*, 1998). The 50 μ L PCR reactions contained a final concentration of 1.5 mM MgCl₂, 0.2 μ M of each primer, 1.25 units TopTaq DNA polymerase (Qiagen), 1x PCR buffer, 200 μ M of each dNTP, and 10-100 ng of bulk DNA template. The PCR cycling was performed by initial denaturation at 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 90 sec, and a final elongation step at 72 °C for 10 min. The PCR products were purified with MEGAquick-spin™ Total Fragment DNA Purification Kit (iNtRON Biotechnology) according to manufacturer protocols.

16S rRNA gene sequencing and phylogenetic analysis

The purified PCR products were sent to First Base Laboratories Sdn. Bhd., Malaysia for sequencing. All sequence data were analyzed with FinchTV 1.4 (www.geospiza.com) and aligned using T-Coffee (Notredame *et al.*, 2000). Sequences were compared to GenBank using BLASTN algorithm (<https://blast.ncbi.nlm.nih.gov>) and sequence similarity $\geq$ 99% for identification to the species level were used. The sequence data were submitted to the GenBank (<http://www.ncbi.nlm.nih.gov/>). Phylogenetic and molecular evolutionary analyses were carried out with MEGA version 7 (Kumar *et al.*, 2016).

The phylogenetic tree was constructed using the maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). A discrete Gamma distribution was used and bootstrap values are based on 1000 replicates. The branch lengths of the tree were measured in the number of substitutions per site.

RESULTS

Three phyla (Firmicutes, Proteobacteria, and Actinobacteria) were successfully identified by using culture-dependent techniques. Among the phyla, Firmicutes was the dominant followed by Proteobacteria and Actinobacteria (Figure 1). The predominant Firmicutes were *Bacillus cereus*. Actinobacteria was only identified from TH colony.

Figure 1: Percentage of bacterial phylotypes from the whole gut of soldier and worker castes of *G. sulphureus*.

From five different colonies of *G. sulphureus*, we have been able to isolate about fifty three bacterial strains. All the 53 sequences had $\geq 99\%$ sequence similarity to sequences found in GenBank except for strain USMS16BP and USMW5BP only 92% similarity with *Lactococcus* spp. in the GenBank. It is possible to consider these strains as novel bacteria. There were little differences in microbial diversity between soldiers and workers castes within same colony. For example, *Enterobacter asburiae* was only found in the gut of soldiers from TH colony and *Microbacterium azadirachtae* was only found in workers gut of TH colony (Table 1). *Staphylococcus hominis* was detected in soldier gut from BP colony and detected from neither worker caste nor other colonies. *Bacillus cereus* was frequently found from worker and soldier gut of all colonies.

Phylogenetic analysis (Figure 2) involved 71 nucleotide sequences and all the bacteria were clustered together according to genera. NR_074540, NR_113991, NR_117473, NR_043242, NR_113265, NR_074923, NR_036956, NR_042072, NR_115724, NR_112882, NR_113802, NR_113767, NR_113577, NR_116502, NR_024640, NR_026078, NR_042636, and KR872395 are the accession number of the bacterial species from the GenBank.

DISCUSSION

To date, we have been able to isolate about fifty bacterial strains from five different mounds of *G. sulphureus*. The guts of wood-feeding termites typically contain hundreds of microbial species (Berlanga *et al.*, 2011). Termite gut bacteria require certain environmental conditions for survival and reproductions, the isolation of many bacterial species by culturing technique are often restricted. However, isolate and culture of live bacteria from termite gut is important to investigate their physiology, ecology and possible contribution to the survival of termite host, e.g., by genetic engineering (Husseneder and Grace, 2005). In addition, being able to isolate novel bacteria would be an advantage in this research. For example, bacteria strain USMS16BP and USMW5BP were isolated from the gut of *G. sulphureus*. The sequence was 92% similarity with *Lactococcus* spp. in the GenBank thus can be considered as novel strains. *Lactococcus* spp. was also reported from higher termite *Nasutitermitis hainanensis* with the given species name *Lactococcus nasutitermitis* (Yang *et al.*, 2016). *Lactococcus* spp. was under the order Lactobacillales (lactic acid producing bacteria). These bacteria were also found from the gut of *C. formosanus* (Husseneder *et al.*, 2005). Lactic acid metabolites serve to recycle carbon and nitrogen via metabolizing uric acid (Potrikus and Breznak, 1981). Lactic acid also maintains the microoxic zones within termite gut environment (Bauer *et al.*, 2000).

The variation of the gut flora within a species can vary at different levels of social organization because they live in colonies system and the variation between termites within the same colony is supposed to be low (Minkley *et*

al., 2006), since colony members live under the same conditions and commonly use the same nutrition source (Matsuura, 2001). There were little differences in microbial diversity between soldiers and workers' castes within same colony. For example, *Enterobacter asburiae* was only found in the gut of soldiers from TH colony and *Staphylococcus hominis* was only found in workers gut from TH colony. Berlanga *et al.* (2011) found no significance differences in phyla of microbial gut from worker and soldier caste of *Reticulitermes grassei* except for phyla Alphaproteobacteria where genus *Wolbachia* was only detected from soldier's gut. Additionally, the isolation of bacterial populations in the guts of individual termites is constantly counteracted by the exchange of gut fluids containing microbes via proctodeal and stomodeal trophallaxis between colony mates and by the obligatory refaunation after molting (McMahan, 1969; Thorne, 1997).

There were differences in bacteria diversity from five different mounds of *G. sulphureus* might due to termites of different colonies usually do not exchange microbial flora. In addition, geographically separated termite colonies might be subjected to different ecological conditions and use different nutrition sources. Different chemical components in the food might favor different microbial groups becoming predominant (Mannesmann 1972; Husseneder *et al.*, 2009). Wood-feeding termites were clearly separated from humus and soil feeder termites where diet is the primary determinant of bacterial community structure. However, gut communities of termites within each diet group were more similar from the same subfamily compared those of distantly related species (Mikaelyan *et al.*, 2015).

Overall, the dominant phyla (Figure 1) of bacteria from all mounds was represented by Firmicutes (80%), followed by Proteobacteria (10%) and Actinobacteria (5%). Most of the data from 16S rRNA gene sequencing showed different phyla composition in every species of termite. For example, six phyla within the domain Bacteria: Proteobacteria, Spirochaetes, Bacteroidetes, Firmicutes, Actinobacteria and Endomicrobia were recorded from *Reticulitermes flavipes* (Fisher *et al.*, 2007). The bacterial diversity from *Reticulitermes grassei* were from phyla Spirochaetes, Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Synergistetes, Verrucomicrobia, and candidate phyla Termite Group 1 (TG1) and Termite Group 2 (TG2) (Berlanga *et al.*, 2011). Husseneder *et al.* (2005) reported that, the bacteria identified with culture-dependent technique were different with bacteria identified with culture-independent techniques. Thus, it is recommended to use both methods in a way to describe the microbial diversity in the termite gut. From metagenomic analysis, Firmicutes and Spirochaetes were the most abundant phyla in *Amitermes wheeleri*, in contrast to *Nasutitermes corniger* and *Microcerotermites* where Spirochaetes and Fibrobacteres were dominated (Köhler *et al.*, 2012; He *et al.*, 2013).

The dominant genus in *G. sulphureus* termite gut was *Bacillus*. *Bacillus cereus* was present in gut of worker and soldier castes from all the five mounds. This data can

Table 1: Bacteria strains isolated and cultured from the gut of *Globitermes sulphureus*.

Bacterial species	Strains	Location	Mound	Source	Accession no.	BLASTN identity (%)
<i>Bacillus cereus</i>	USMS1DV	5°21'34.9"N 100°18'19.3"E	Durian Valley, USM Penang, Malaysia	Soldier	KX037102	100
<i>Bacillus cereus</i>	USMW14DV			Worker	KX037105	100
<i>Bacillus megaterium</i>	USMS10DV			Soldier	KX037114	99
<i>Bacillus pumilus</i>	USMS12DV			Soldier	KX037116	100
<i>Bacillus pumilus</i>	USMW3DV			Worker	KX037113	100
<i>Bacillus subtilis</i>	USMS5DV			Soldier	KX037119	100
<i>Lysinibacillus fusiformis</i>	USMW15DV			Worker	KX037123	100
<i>Lysinibacillus</i> sp.	USMW4DV			Worker	KX037126	99
<i>Lysinibacillus</i> sp.	USMW20DV			Worker	KX037127	99
<i>Paenibacillus</i> sp.	USMS2DV			Soldier	KX037135	99
<i>Paenibacillus</i> sp.	USMW2DV			Worker	KX037136	99
<i>Bacillus cereus</i>	USMS1PM	5°21'34.6"N 100°18'29.4"E	Padang Minden, USM Penang, Malaysia	Soldier	KX037103	100
<i>Bacillus cereus</i>	USMW14PM			Worker	KX037107	100
<i>Bacillus licheniformis</i>	USMW2PM			Worker	KX037111	100
<i>Brevibacillus reuszeri</i>	USMW4PM			Worker	KX037121	100
<i>Lysinibacillus</i> sp.	USMW13PM			Worker	KX037124	97
<i>Lysinibacillus</i> sp.	USMW3PM			Worker	KX037130	99
<i>Lysinibacillus</i> sp.	USMW1PM			Worker	KX037125	99
<i>Lysinibacillus</i> sp.	USMW5PM			Worker	KX037156	99
<i>Lysinibacillus sphaericus</i>	USMS8PM			Soldier	KX037129	100
<i>Paenibacillus</i> sp.	USMW7PM			Worker	KX037137	99
<i>Bacillus cereus</i>	USMW1TH	5°21'14.5"N 100°18'01.1"E	Tasik Harapan, USM Penang, Malaysia	Worker	KX037108	100
<i>Bacillus cereus</i>	USMW13TH			Worker	KX037118	100
<i>Bacillus cereus</i>	USMS7TH			Soldier	KX037104	100
<i>Bacillus cereus</i>	USMS10TH			Soldier	KX037115	100
<i>Enterobacter asburiae</i>	USMS9TH			Soldier	KX037122	99
<i>Lysinibacillus</i> sp.	USMW5TH			Worker	KX037128	99
<i>Microbacterium azadirachtae</i>	USMW9TH			Worker	KX037131	99
<i>Pseudomonas</i> sp.	USMW7TH			Worker	KX037139	99
<i>Pseudomonas</i> sp.	USMS2TH			Soldier	KX037138	99
<i>Bacillus cereus</i>	USMS12IK	5°21'20.0"N 100°17'35.2"E	Indah Kembara, USM Penang, Malaysia	Soldier	KX037101	100
<i>Bacillus cereus</i>	USMW14IK			Worker	KX037106	100
<i>Bacillus licheniformis</i>	USMS2IK			Soldier	KX037109	100
<i>Bacillus licheniformis</i>	USMW10IK			Worker	KX037110	100
<i>Bacillus pseudomycoides</i>	USMW3IK			Worker	KX037112	100
<i>Bacillus</i> sp.	USMS6IK			Soldier	KX037117	99
<i>Brevibacillus agri</i>	USMS9IK			Soldier	KX037120	100
<i>Paenibacillus</i> sp.	USMS10IK			Soldier	KX037134	99
<i>Paenibacillus alvei</i>	USMS15IK			Soldier	KX037132	99
<i>Paenibacillus alvei</i>	USMW7IK			Worker	KX037133	99
<i>Bacillus cereus</i>	USMS4BP	5°21'30.7"N 100°18'04.9"E	Bakti Permai, USM Penang, Malaysia	Soldier	KX037142	100
<i>Bacillus cereus</i>	USMW3BP			Worker	KX037149	100

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suggest that *Bacillus cereus* is the true symbiont and necessary for the host's survival. Moreover, Bacilli form a significant portion of the intestinal microbial community of soil invertebrates, especially among cellulose degraders (König, 2006).

CONCLUSION

The present work indicates that the bacteria in the soldier and worker gut of *G. sulphureus* are similar. Even though, worker caste does all the foraging and feeding works but trophallaxis activities between nest mates could explain such similarity. Different mounds of termites also harbor similar but distinct bacteria because usually termites do not exchange foods between different colonies. However, foraging of the termite and chance encounter with other termite colonies might result in related bacterial species in the gut. Although many researchers have reported the diversity of gut bacteria from termites but this is the first report of culturable bacterial diversity from *G. sulphureus*.

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