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Review Article

Diversity of Nitrogen Fixing bacteria Associated with Various Termite Species

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ABSTRACT

Nitrogen is one of the vital elements for the growth and survival of many organisms. Atmospheric N_2 can only be used by certain organisms like microbes that supply inorganic form of nitrogen to their host, insects, or plants via symbiotic or non-symbiotic interaction. Arthropods are diverse species on the earth, whose guts are inhabited by microbes that help in many physiological activities like N_2 fixation. As N_2 fixing bacteria ecologically play vital roles, many studies have demonstrated the presence of N_2 fixing bacteria in termite gut. Study on termite's gut omics has also supported a complex systemic understanding of gut digestome that is imperative in understanding the termite holobiome. This review gathers a variety of information from multifarious research which has been done on the isolation and diversity of N_2 fixing bacteria in various termite species.

Keywords: Acetylene reduction, *nifH*, nitrogen, nitrogen fixation, termite gut

INTRODUCTION

Nitrogen is an essential component of amino acids and proteins. N_2 is abundant in the atmosphere but atmospheric N_2 can only be used by certain organisms like microbes that supply inorganic form of N_2 to their host, insects, or plants via symbiotic or non-symbiotic interactions (Khan, Mohiuddin, & Rahman, 2008). Thus, N_2 fixation process is vital in providing fixed N_2 to organisms. Biological N_2 fixation (BNF) is the conversion of atmospheric N_2 into ammonia (Eskin, Vessey, & Tian, 2014). About 90 genera of microbes are able to fix N_2 by utilizing nitrogenase enzyme (Gaby & Buckley, 2012).

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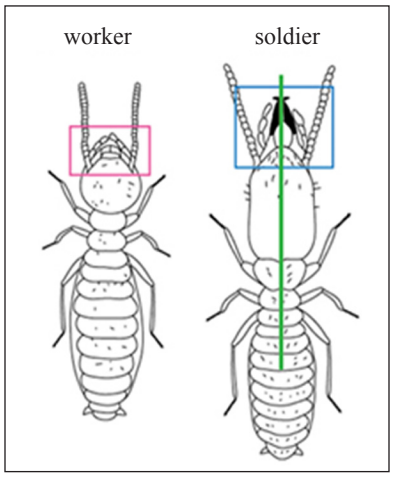
N_2 fixing bacteria are classified into two categories; symbiotic and non-symbiotic bacteria. They can be found in soils, gut of arthropods, or insects and root nodules. Arthropods are diverse species on the earth, whose guts are inhabited by microbes that help in many physiological activities like N_2 fixation and cellulose degradation (Bashir et al., 2013). Since N_2 is one of the limiting nutrients in insects' diet, most of the insects depend on mutualistic bacteria having N_2 metabolism in order to obtain sufficient amount of N_2 (Engel & Moran, 2013). N_2 fixing bacteria have symbiotic interaction in insects' guts, like in termites, cockroaches, and beetles. As N_2 fixing bacteria ecologically play vital roles, many studies have demonstrated the presence of N_2 fixing bacteria in termite gut that are capable of utilizing nitrogenous wastes excreted by termite and convert them into high-value N_2 (Ohkuma, Noda, & Kudo, 1999). This review gathers a variety of information from various researches, which have been done on the isolation and diversity of N_2 fixing bacteria in various termite species.

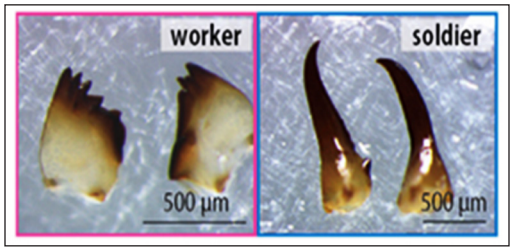
Termite Classification

Termites are classified into a few castes which comprise of queen, soldiers, workers, nymphs, and larvae (Kambhampati & Eggleton, 2000). Soldier class termites have the mandible, which is a jaw like structure on their heads that helps in protecting the colonies as shown in Figure 1 (Watanabe, Gotoh, Miura, & Maekawa, 2014; Watanabe & Maekawa, 2012).

According to the recent data, there are approximately 3106 species of termites. They are classified into 12 families which are further divided into 282 genera. From the 12 families, the lower temites' families are Cratomastotermitidae, Mastotermitidae, Termopsidae, Archotermopsidae, Hodotermitidae, Stolotermitidae, Kalotermitidae, Archeorhinotermitidae, Stylotermitidae, Rhinotermitidae, and Serritermitidae whereas, the higher termites' family is Termitidae (Krishna, Grimaldi, Krishna, & Engle, 2013; Kambhampati & Eggleton, 2000).



(a)



(b)

Figure 1. The morphology of termites (worker and soldier): (a) The illustration of worker and soldier termite; (b) The mandible of soldier (right) and worker (left). Adapted from Watanabe et al. (2014)

Termite's Gut Structure

Termite gut is divided into three parts: foregut, midgut, and hindgut. The internal structure of a termite gut is shown in Figure 2. Termite's hindgut is colonized by diverse microbial symbionts from three different domains which are flagellate protists, bacteria, and archaea (Ohkuma & Brune, 2011). Lower termites' gut is colonized by a diverse species of flagellated protists and prokaryotes whereas the gut of higher termite is colonized only by prokaryotes (Brune, 2006; Peterson & Scharf, 2016).

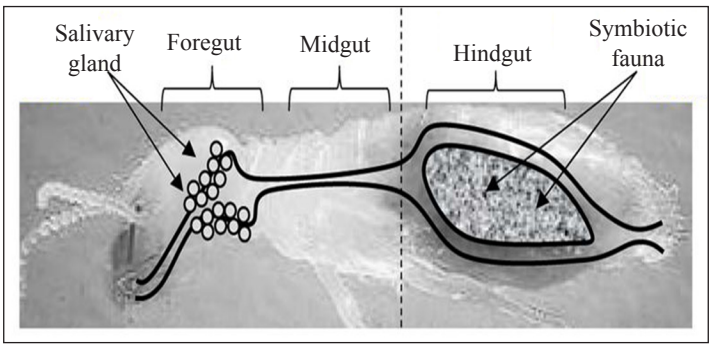


Figure 2. The internal structure of termite guts showing the foregut, midgut, and hindgut. Adapted and modified from Scharf, (2015)

Termite's Diet

Termites act as a decomposer where they feed on decaying and dead plant material with relatively high carbon to N_2 ratio. They also consume fungi and soil-rich organic matter (Engel & Moran, 2013). Such a feeding habit of termites contributes to the balanced ecosystem (Freymann, Buitenwerf, Desouza, & Olff, 2008).

According to Inward, Beccaloni and Eggleton (2007), and Donovan, Eggleton and Bignell (2001), gut contents and morphology were used to classify termites' feeding habits. Lower termites have simple gut and they feed on wood whereas, higher termites with complex gut feed on wood, grass, lichen, litter, epiphytes, and soil.

Microbiota of Termite's Gut

The complexity of termite gut leads to the presence of hundreds of phylotypes. Based on 16S rRNA studies, most of the species isolated from termite guts are novel. Those species are not environmental bacteria and, are specifically classified as termite gut specialists. Termites' gut microbes possess mutualistic relationship with their host in several ways. For instance, gut microbes aids in lignocellulose digestion which leads to the production of acetate, a main carbon source for the termites. Besides, the microbes also supply N_2 sources for the host (Breznak, Brill, Mertins, & Coppel, 1973; Odelson & Breznak, 1983).

About 90% of the termite's hindgut is colonized by flagellates from the phylum Parabasalia that occupy the gut of lower termites (Ohkuma & Brune, 2011). The flagellates found in termites' gut are unique and most of them cannot be found anywhere else (Brugerolle & Radek, 2006). Some of the flagellates such as *Trichonympha sphaerica* and *Trichonympha termosidis* remain uncultured. These two protists were isolated from the hindgut of *Zootermopsis angusticollis* (Tai, James, Perlman, & Keeling, 2013). There are also some protists associated with methanogens in termite gut. For instances, *Microjoenia* and *Dinenympha* are two different protists isolated from the termite *Hodotermopsis sjoestedti*. The flagellate *Spirotrichonympha leidyi* which was isolated from the gut of *Coptotermes formosanus* is associated with endosymbiotic methanogens (Hongoh & Ohkuma, 2011).

The gut of higher termites, especially soil-feeding termites of the family Termitidae, is colonized by a wide range of archaea. However, their presence is minute compared to bacteria (Brauman et al., 2001). At present, methanogens from the genus *Methanobrevibacter* and non-methanogens like *Thermoplasmoles* and *Crenarchaeota* have been isolated from termite gut (Friedrich, Schmitt-Wagner, Lueders, & Brune, 2001; Leadbetter, Crosby, & Breznak, 1998).

The most abundant bacteria found in the gut of wood-feeding termites are *Spirochaetes* whereas, *Bacteroideres* are abundant in the gut of fungus-cultivating termites (Dietrich, Kohler, & Brune, 2014; Hongoh, 2010). Several cellulolytic bacteria like *Cellulomanas, Citrobacter* and *Enterobacter* have also been isolated from the gut of termite (Upadhyaya et al., 2012). Cellulolytic bacteria secrete the enzymes cellulose and hemicellulose to allow them to degrade the cellulose and hemicellulose materials (Lima et al., 2014). *Dysgonomonas termitidis*, a lignocellulose degrading bacteria, was isolated from the gut of the termite *Reticulitermes speratus* (Pramono, Sakamoto, Limo, Hongoh, & Ohkuma, 2015). A study conducted on the termite *Odontotermes formosanus* had successfully isolated nine different isolates with cellulolytic property. All the nine isolates were closely related to *Bacillus cereus, Bacillus thuringiensis, Bacillus pumilus, Pseudomonas aeroginosa, Citrobacter freundii, Serratia marcescens, Salmonella enterica, Staphylococcus gallinaum*, and *Enterococcus casseliflavus* (Kavitha, Vijayarani, & Kumanan, 2014). There are also some acetogenic bacteria in the termite gut. Most of them are from the phylum *Firmicutes* and genera *Clostridium* and *Acetobacterium* (Drake, Gobner, & Daniel, 2008).

N_2 Fixing Bacteria in Termite Gut

Termites have a symbiotic relationship with microbes and play a vital role in N_2 fixation. Microbial fixation in termite gut is the main source of N_2 for the termite colony (Vecherskii, Kostina, Gorlenko, Dobrovol'skaya, & Umarov, 2008). Several studies have proved the presence of N_2 fixing

bacteria in the gut of various termite species. Several bacterial endosymbionts that have the ability to fix N_2 have also been found in the gut of lower termites (Peterson & Scharf, 2006). Since termites grow well in nitrogen-poor diet, there are many types of symbiotic N_2 fixing bacteria, living in the gut of termites (Masepohl et al., 2002).

In the study conducted by Doolittle, Raina, Lax and Boopathy (2008), *Klebsiella pneumoniae* was isolated from *Coptotermes formosanus* which was the Formosan subterranean termites (FST). N_2 fixation assay which was done using basic salt media supplemented with NaMo and $Fe_2(SO4)_3$ revealed that *K. pneumonia* was able to fix N_2 anaerobically which contributes to the N_2 source of FST. Apart from this, another study conducted by Potrikus and Breznak (1977), demonstrated the presence of *Enterobacter agglomerans* in the gut of *C. formosanus* and proved its ability to fix N_2 through acetylene reduction assay. Inhibition of acetylene reduction by oxygen proved that *E. agglomerans* can only fix N_2 anaerobically.

Few studies have demonstrated the presence of N_2 fixing bacteria in various Australian termite species. In order to confirm the presence of N_2 fixation in Australian termite, French, Turner and Bradbury (1976) used three different termite species including *Mastotermes darwiniensis*, *Nasititermes exitiosus*, and *Coptotermes zactues* which were obtained from mounds at Townsville, Seymour, and Canberra, respectively, to isolate and characterize N_2 fixing bacteria from the hindgut. Using acetylene reduction assay (ARA), N_2 fixation activity was discovered in all the isolates obtained from all three species. Based on $^{15}N_2$ incorporation test, isolate from *Mastotermes drawiniensis* showed highest $^{15}N_2$ incorporation. All the isolates obtained from this study were characterized as *Citrobacter freundii*. Another study was conducted by Eutick, O'Brien and Slaytor (1978), to isolate N_2 fixing bacteria from few species of Australian termites including *Coptotermes lacteus*, *Coptotermes acinaciformis*, *Cryptotennes primus* Hill, *Mastotennes darwiniensis*, *Nasutitermes exitiosus*, *Nasutitermes walker*, *Nasutitermes graveolus*, *Heterotermes ferox,* and *Schedorhinotermes intermedius intermedius*. Based on their morphological and biochemical characteristics, all the isolates were identified as *Enterobacter* spp.

Two facultative anaerobes, namely, *Clostridium* sp. and *Klebsiella* sp. with the ability to fix N_2 have been isolated from *Mactotermes* sp., the fungus cultivating termite. In this study, N_2 fixing bacteria were isolated from the gut of queen, soldier and worker termites. These bacteria were isolated using Hill's medium and were characterized using Hino and Wilson medium (Gomathi, Ramalakshmi, & Ramasamy, 2005). This research showed that, compared to others, the amount of bacteria enumerated was highest in worker termites. Previously, Breznak et al. (1973), also discovered similar findings whereby, in worker termites fed with wood, the N_2 fixing activity was higher, compared to the soldier termites. This was demonstrated using ARA.

N_2 Fixation Activity in Termite Guts

The distribution of sulfate reducing bacteria in termite gut was studied using various termite species that includes *Mastotermes darwiniensis*, *Neotermes jouteli*, *Neotermes castaneus*, *Nasutitermes nigriceps*, *Zootermopsis angusticollis*, *Zootermopsis nevadensis*, *Kalotermes flavicollis*, *Heterotermes indocola*, *Reticulitermes santonensis*, and *Odontotermes obesus*. A total of seven pure culture isolates were obtained in this study. Nitrogenase activity of those isolates was tested using ARA. All the isolates had the ability to fix N_2 which eventually provide N_2 source for the termites. Based on biochemical and physiological characteristics, the seven isolates were identified as *Desulfovibrio* sp. (Kuhnigk et al., 1996).

Several other researches were also conducted on the isolation of N_2 fixing bacteria from termites. The isolates with N_2 fixing ability which were isolated from various termite species have been tabulated in Table 1. These researches showed the variety of N_2 fixing bacteria that occupy termites gut and form symbiotic relationship, which benefits both hosts and microorganisms.

Culture independent molecular method was used to study the expression of N_2 fixation genes in the gut microbiota of *Neotermes koshunensis*. As a primary screening, the worker larvae were tested for N_2 fixation activity using ARA. The relationship between N_2 fixation activity and quantity of mRNA was tested by feeding the termite with two different diets; filter paper with added N_2 source and filter paper

Table 1
N_2 fixing bacteria isolated from various termite species

N_2 Fixing Bacteria	Host	Reference
Klebsiella pneumoniae	*Coptotermes formosanus*	Doolittle et al., 2008
Enterobacter agglomerans	*Coptotermes formosanus*	Potrikus & Breznak, 1977
Citrobacter freundii	*Mastotermes darwiniensis* *Nasititermes exitiosus* *Coptotermes zactues*	French et al., 1976
***Clostridium* sp.** ***Klebsiella* sp.**	*Mactotermes* sp	Gomathi et al., 2005
***Enterobacter* sp.**	*Coptotermes lacteus* *Coptotermes acinaciformis* *Cryptotennes primus* Hill *Mastotennes darwiniensis* *Nasutitermes graveolus* *Heterotermes ferox* *Schedorhinotermes intermedius intermedius*	Eutick et al., 1978
***Desulfovibrio* sp.**	*Mastotermes darwiniensis*	Kuhnigk et al., 1996
Desulfovibrio desulfuricans	*Reticulitermes santonensis*	
Desulfovibrio termitidis	*Heterotermes indicola*	
***Desulfovibrio* sp.**	*Odontotermes obesus*	

without N_2 source. The acetylene reduction activity was higher in termites fed with filter paper without any N_2 source. Nitrogenase activity was reduced in termites fed with filter paper containing N_2 source (Noda, Ohkuma, Usami, Horikoshi, & Kudo, 1999). This finding was similar to the experiment performed by another researcher using *Coptotermes formosanus* (Breznak et al., 1973).

The N_2 fixing activity was also studied by Desai and Brune (2012), to identify the presence of diazotrophs in four different termites, *Cryptotermes longicollis*, *Incisitermes marginipennis*, *Neotermes castaneus* and *Kalotermes flavicollis* which belong to genera *Kalotermitidae*. Termites were fed under two different diet conditions (wood and filter paper) followed by ARA to test nitrogenase activity. Although the termites were able to reduce acetylene in both conditions, the acetylene reduction activity was higher when termites were fed with filter paper compared to wood. PCR and RT-PCR were performed using universal primers IGK and YAA for hindgut homogenate of four termite species followed by creation of a clone library. Based on the clone library, it was revealed that the *nifH* homologous are diverse in hindgut of those termite species. Based on the phylogenetic analysis of *nifH* homologs, it was found that the gut of those termites is colonized by *Treponema* sp., *Bacteriodales*, and *Azoarcus* sp. All the *nifH* homologs obtained in this study were clustered into respective groups as referred to in the group nomenclature of Yamada, Inoue, Noda, Hongoh and Ohkuma (2007). The *nifH* homologs from hindgut of *I. marginipennis* and *N. castaneus* were clustered in group I and were closely related to *Azoarcus* strain BH72 and actively expressed. *NifH* homologs in group II were obtained from all three termites species except for *I. marginipennis* and had a close identity with *anfH* gene of *Clostridium pasteurianum*. Some homologs were related to *Spirochaetes* but not expressed. The *nifH* homologs in group III had a close relationship with *A. pseudotrichonymphae* and few homologs clustered together with *Treponema azotonutricium* where the *nifH* gene in both clusters was expressed.

In another study, lower termites of *Reticulitermes speratus* were used to investigate the diversity of *nifH* genes in their intestinal microbiota (Ohkuma et al., 1996). The DNA of mixed microbiota from termites' hindgut was extracted and *nifH* gene was amplified using four primer combinations (KAD-GEM, KAD-YAA, IGK-GEM and IGK-YAA). Four clones, TDG, TDY, TKG and TKY were isolated respectively. A total of 27 *nifH* amino acid sequences were obtained from *R. speratus* and all the sequences were not similar to any of the published sequences in database. Phylogenetic analysis was performed using neighbor joining tree constructed using 25 *nifH* sequences (Figure 3).

Based on the phylogenetic tree, although most of *nifH* amino acid sequences from the termite group are closely related to the sequences of *Clostridium pasterenium* and also *Desulfovibrio gigas* and *Chromatium buderi*, there are no similarities between

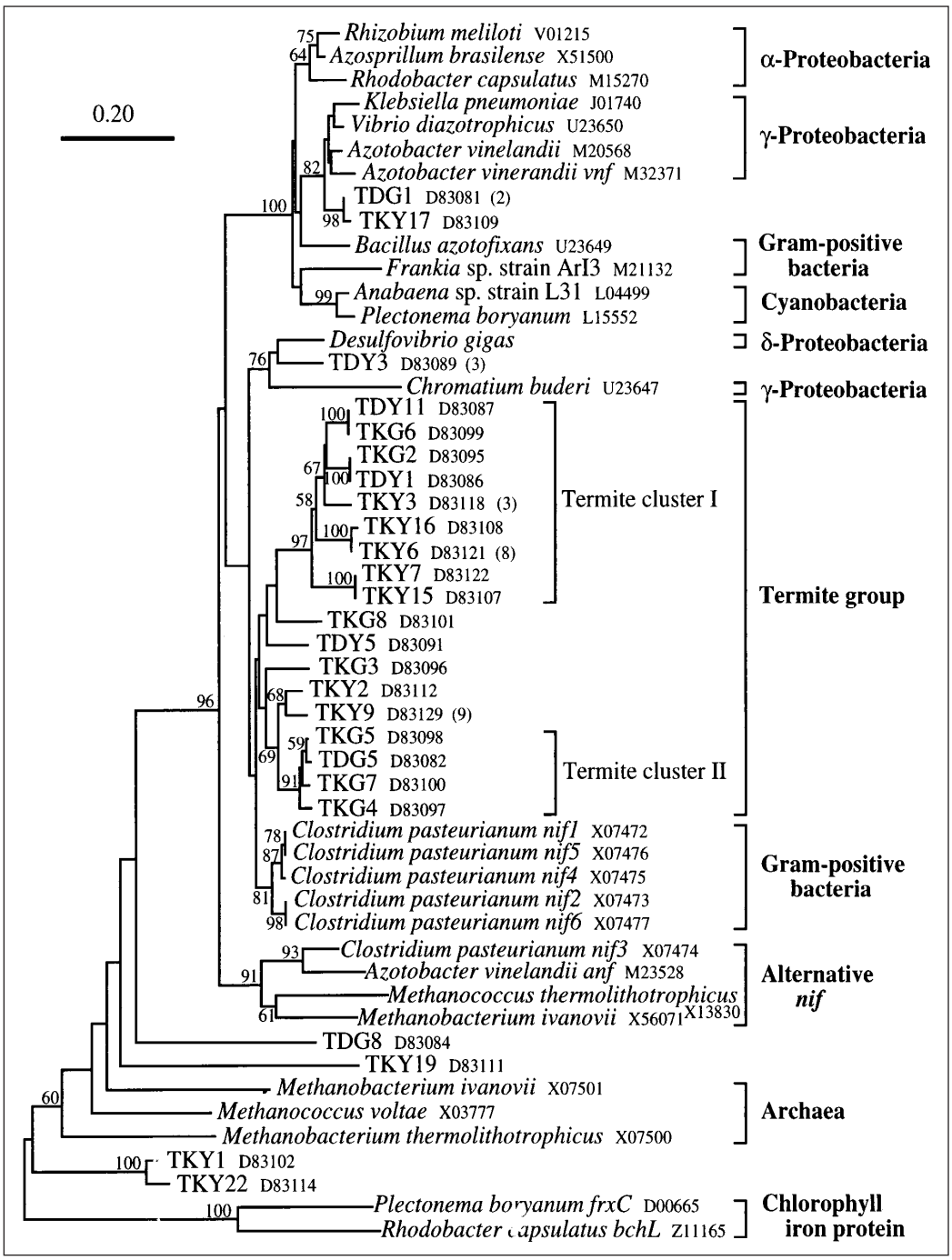


Figure 3. Neighbor-joining relationship constructed using *nifH* amino acid sequences of 24 sequences from database and 25 sequences from termites. Two chlorophyll sequences were used as outgroups. The *nifH* fragments used correspond to 45-153 amino acid residues of *K. pneumoniae* sequence. Scale bar 0.2 denotes substitutions of 20 nucleotides over 100 nucleotides. Numbers shown in the internal branches are the bootstrap values derived from 1000 replications when above 50% is shown at each node. Adapted from Ohkuma et al., 1996

the nucleotide sequences of clones and *C. pasterenium*. The *nifH* sequence of clone TDG1 and TKY17 are grouped together in gamma subclass of proteobacteria which includes *Azotobacter* sp., *Vibrio* sp. and *Klebsiella* sp. The sequence of TDY3 is clustered with *C. buderi* and *D. gigas*. The sequences of other clones are distantly related to the sequences obtained from database and they formed their own clusters (Ohkuma et al., 1996). There are also many copies of *nifH* sequence in one single organism due to the presence of many *nifH* genes like alternative nitrogenase genes. Although a few sequences were obtained from the same organism, there is a unique diversity among them.

Previous study proved that *Reticulitermes speratus* was contributed by N_2 source through N_2 fixation of microbes in its gut (Ohkuma et al., 1996). Likewise, the diversity of N_2 fixing microbes in intestinal microflora of *Reticulitermes chinensis* Synder were studied using the culture independent method. Primary screening was done using *nifH* gene amplification with few primers combinations (IGK–YAA, IGK–GEM, KAD–YAA, and KAD–GEM). From 63 clones, six chimeric sequences, and several similar sequences were eliminated. About 34 sequences were used for phylogenetic analysis. About 20 *nifH* sequences clustered together which were true functional *nifH* and 17 of them had a close relationship with *Clostridium* sp. with the similarity of 75-88%. One sequence had a higher relationship with a spirochetes, *Treponema primitia* ZAS-2, one sequence had 95% similarity with *Candidatus Azobacteroides pseudotrichonymphae genomovar,* CFP2 which is endosymbionts of *Coptotermes formasanus*. One more sequence is related to *D. vulgaris*. Another 14 *nifH* sequences are clustered together into alternative nitrogenases groups where six of them are related to proteobacteria, *Rhodospirillum rubrum*. Five sequences having higher similarity with spirochetes were obtained from *Z. angusticollis*. Besides, another three sequences are related to *Methanosarcina barkeri*. Although nitrogenase activity was not performed for all the isolates obtained from this study, there are diverse groups of N_2 fixer in the intestinal microbiota of *R. chinensis* (Du et al., 2012).

The microaerophilic nature of Termite Associated Verrucomicrobium 2 (TAV2) was studied by Isanapong et al. (2013). Through proteomic and transcriptome method, the genes and proteins expressed in different oxygen, O_2, concentration were identified. This study revealed the presence of peptides corresponding to *nifH* genes in the cells which were grown at 2% O_2 concentration. This indicates that TAV2 is involved in the BNF and contributes to the metabolism of microbial community in the gut of termites. In another study, the N_2 fixation activity of TAV2 was confirmed through genomic analysis. The TIGRFAM and Pfam protein family databases were used to perform protein model comparisons. In addition, Kyoto Encyclopedia (KEGG) and Clusters of Orthologous Groups (COG) databases were also used to justify the

presence of N_2 fixing activity. Based on the results obtained, the presence of *nifHDK* and *anfHDGK* operons in TAV2 was confirmed (Wertz, Kim, Breznak, Schmidt, & Rodrigues, 2012).

Other than that, the ability of symbiotic N_2 fixation in fungus growing termites was tested using ARA. Two termite species, *Mactotermes natalensis* and *Odontotermes badius* were used in this study. The study revealed that there was a positive acetylene reduction activity in live termites of those two termite species and not in the fungus comb. This indicated the presence of N_2 fixer in termite guts. The fixation was higher in worker than in soldier in both the species (Sapountzis et al., 2016).

N_2 Fixing Bacteria and *NifH* Gene

NifH gene is responsible for N_2 fixation and used as identification marker to detect the presence of N_2 fixing microbes (Zehr, Jenkins, Short, & Steward, 2003). The genome of spirochetes strains ZAS-1, ZAS-2, and ZAS-9 were examined in the previous study to check for the presence of *nifH* gene and the ability to fix N_2 (Noda, Ohkuma, & Kudo, 2002). The study showed the presence of two *nifH* homologs in each strain.

Study conducted on *Spirochaeta aurantia, Spirochaeta zuelzerae,* and treponeme ZAS-9 showed their ability to fix N_2 due to the presence of *nifH* gene (Hongoh, Ohkuma, & Kudo, 2003; Lilburn et al., 2001). The ZAS-strain which belongs to *Treponema* sp. isolated from the gut of *Zootermopsis angusticollis* has two homologous *nifH* genes with nitrogenase activity (Hongoh et al., 2003). Apart from this, the *nifH* gene was also found in the bacteria isolated from *Hodotermopsis sjostedti* and *Zootermopsis nevadensis*.

In another study, *Endobacterium proavitum* strain Rsa215 was isolated from *Reticultitermes santonensis* (Rsa) and *Zootermopsis nevadensis* and was tested for the ability to fix N_2. This strain contains a single gene cluster encode for nitrogenase. The phylogenetic analysis of *nifD, nifK,* and *nifH* demonstrated that Rsa215 has group IV nitrogenase. The group IV nitrogenase gene is known as *nif*-like gene with some of these genes involved in N_2 fixation. *Endobacterium proavitum* has a single set of *nifHDK* genes which are functional in N_2 fixation (Zheng, Dietrich, Radek, & Brune, 2016).

According to Noda et al. (2002), a strong N_2 fixation activity had been shown by *C. formosanus* in Japan. The culture independent studies of *nifH* gene show that there are diverse species of microbes such as spirochetes, clostridia, archaea, and proteobacteria which are able to fix N_2 available in the gut of *C. formosanus*.

Besides, the termite *Neotermes koshunensis* has abundant *nifH* genes in its gut and it possesses high N_2 fixation activity (Noda et al., 1999). There is also potential N_2 fixer which has symbiotic interaction in the gut of *Reticulitermes chinensis*. This was demonstrated from the phylogenetic analysis of the clones of *nifH* gene isolated from *R. chinensis* (Du et al., 2012).

Role of Metagenomic Technologies in Studying the N_2 Fixing Bacteria

In termites, gut consortium play direct roles in N_2 fixation, amino acid biosynthesis and lignocellulose digestion. However, a majority of gut microbes are unculturable thus molecular methods such as single-species-targeting metagenomics analysis and other omics approaches are crucial in capturing and revealing the diversity of the termite's gut microbe. Metagenomics studies employing bacterial 16S rRNA sequences have been used to catalog bacteria and archaea in termites (Do et al., 2014; He et al., 2013; Peter & Scharf, 2016; Rajarapu, Shreve, Bhide, Thimmapuram, & Scharf, 2015; Tartar et al., 2009). Large metagenomic dataset will allow in-depth analysis of microbial functions and could offer resources for advancing integrative sociogeomic, digestomic, and termitosphere in order to better understand the intricate symbiotic relationships between termites and their gut microbes. Overall, six main bacterial phyla are represented across higher and lower termites, namely Bacteroides, Firmicutes, Sphirochetes Proteobacteria, Fibrobacteres, and Elusimicrobia (Brune, 2014). Metagenomic study on wood and soil-feeding higher termites revealed that community structure and functional potential of microbes in gut compartments are determined by digestive approach of the host (Rossmaler et al., 2015). Omics science collectively catalog, enumerate, and illustrate biological molecules that transform into organization, function, and life processes of an organism. Omics research has also provided advances in understanding symbiotic roles of individual microbial species. Currently, 82 termite species have been studied using several omics methods, with bigger representation by lower (72%) compared to higher termites (28%).

Recently, studies on metatranscriptome using ribodepletive strategy on lower termite *Reticulitermes flavipes* substantiate earlier discoveries of physiological contribution of bacteria with regard to biosynthesis, catabolism, and transport of major organic molecules and ions (Peterson & Scharf, 2016). The existence of N_2 metabolism genes like nitrogenase, nitroreductases, and ureases for N_2 recycling and fixation in lower termites gut bacteria were also identified through genome sequencing (Hongoh et al., 2008; Inoue et al., 2015).

Metagenomic approach is an important tool to uncover the diversity of uncultured N_2 fixing microbes associated with termites' gut. Currently, metagenomic studies focusing on N_2 fixing ability of guts microbiome utilizing gene encoding nitrogenase enzyme, *nifH* are still lacking as many studies focus more on lignocellulase and xylanase screening from genome sequence compared to N_2 metabolism (Bastien et al., 2013; Liu et al., 2013). Although the nature of N_2 fixation in termites is still in the dark, they deserve attention because of their potential influence on N_2 metabolism in tropical soil. By uncovering the diversity of N_2 fixing bacteria in termite gut through metagenomic

and other omics study, we might uncover a unique and exceptional N_2 fixer that could be incorporated into soil and plant to promote plant growth.

CONCLUSION

The main source of N_2 for termites is from the microbial fixation of N_2. There is an abundance of microbes in the gut of various termite species which help in N_2 fixation. They are detected by looking at the presence of N_2 fixing genes, most commonly nitrogenase encoded by *nifH*. The importance of N_2 fixation in termite should be analyzed further using other housekeeping genes, for instance, other *nif* genes and *fix* genes that are involved in N_2 fixation. The importance of N_2 fixing genes should be emphasized because those genes can be manipulated to provide significant impact on agriculture since N_2 fixing bacteria have the potential to increase plant productivity. The bacteria can be released into plants as free living bacteria. If there is significant improvement in the plants' nutrients, the *nifH* gene from these bacteria can be manipulated and incorporated into non-leguminous crops for better conversion of N_2 gas. At the same time it may reduce contamination caused by chemical fertilizers.

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