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Horizontal gene transfer among nitrogen-fixing bacteria in the guts of termite *Coptotermes gestroi*

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ABSTRACT

Aims: Arthropods guts, such as termite harbor diverse microorganisms including those that are capable of fixing atmospheric nitrogen (N_2). Nitrogen-fixing bacteria can help termite to overcome their shortage of dietary N by providing fixed N_2. Nitrogenase enzyme is responsible for this trait and encoded by *nif* genes which are highly conserved and are primarily used in the identification of N_2-fixing microorganisms. Here, we characterized N_2-fixing bacteria isolated from the hindguts of termite *Coptotermes gestroi*.
Methodology and results: A total of 46 bacterial isolates were obtained after a primary screening based on their ability to grow on Burk's media. Subsequently, the *nifH* gene from two of these isolates, namely S7 and S20, were successfully amplified and sequenced. Molecular phylogenetic analysis of 16S rRNA gene sequence revealed that isolate S7 is closely related to *Ralstonia pickettii* ATCC27511 (99.34% similarity, 1059 bp), whereas isolate S20 is closely related to *Microbacterium* sp. NCCP-451 (LC488936) (99.06% similarity, 948 bp). Besides that, the *recA* gene of isolate S7 is closely related to *Ralstonia pickettii* 12D (CP001644) (100% similarity, 442 bp) and the type strain of *Ralstonia pickettii* (ATCC 27511) (NZ KN050646) (98.97% similarity, 438 bp). Meanwhile, *nifH* gene of isolate S7 showed highest similarity to the uncultured bacterium NR1606 (AF035490) (99.93% similarity, 277 bp). Moreover, the *nifH* gene of isolate S20 is clearly separated from *Azoarcus* sp. and distantly related to *Microbacterium* sp. The incongruence between the partial 16S rRNA and *nifH* gene sequences could indicate the possibility of horizontal transfer of *nif* genes.
Conclusion, significance and impact of study: The phylogenetic incongruence between housekeeping genes (16S rRNA and *RecA*) and *nifH* gene in these bacteria provides new insight on potential horizontal gene transfer (HGT) activity taking place in bacterial communities particularly in the guts of arthropods. The finding of this study on potential HGT can also aid in the prediction of origins and evolution of gene transfer among bacteria.

Keywords: Nitrogenase, nitrogen fixation, 16S rRNA, *Coptotermes gestroi*, *nifH*

INTRODUCTION

Nitrogen (N) is a primary limiting nutrient in the diet of many insects and thus, most insects depend on mutualistic bacteria that have N metabolism to obtain sufficient N (Engel and Moran, 2013; Bala *et al*., 2018). N_2-fixing bacteria were reported to form symbiotic interaction with insects like termites, cockroaches and beetle among others. For instance, it was reported that bacteria in termite guts are capable of fixing N_2 and converting nitrogenous waste excreted by termites into high-value N for utilization (Thong-On *et al*., 2012; Thanganathan and Hasan, 2018).

Termite has a complex and specific microbiome which aids them in many functions (Bourguignon *et al*., 2018). Despite having a poor N diet, termites can grow well due to the presence of symbiotic N_2-fixing bacteria living in their gut (Masepohl *et al*., 2002; Sapountzis *et al*., 2016). For example, their gut microbiomes aid them to thrive by facilitating the digestion of woods especially in the wood dwelling species such as *Cryptotermes secundus*, *Prorhinotermes simplex*, *Zootermopsis nevadensis* and *Hodotermes sjostedti* (Korb *et al*., 2015; Waidele *et al*., 2019). Many studies have demonstrated the presence of these microbes in termite guts especially in lower termites from the phyla *Bacteroides*, *Actinobateria*, *Proteobacteria* and *Elllusimimicrobia* (Ohkuma *et al.*, 1999; Peterson and Scharf, 2016). It has been reported that termites such as *Coptotermes formosanus*, *Neotermes koshunensis*, *Reticulitermes speratus* and *Odontotermes obesus* possess N_2-fixing bacteria in their gut (Upadhyay, 2011).

In the study conducted by Gomathi *et al*. (2005), few N_2-fixing bacteria were isolated from fungus cultivating termite, *Macrotermes* species and these include two free-

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living aerobes (*Azotobacter* spp. and *Beijerinckia* spp.) and two facultative anaerobes (*Klebsiella* spp. and *Clostridium* spp.).

Nasutitermes, the wood feeding termites (Termitidae), have rich *nifH* gene diversity in their hindguts. Acetylene reduction test revealed highest N_2-fixing activity in *Nasutitermes koshunensis* compared to *R. speratus*, *Cryptotermes formosanus*, *Coptotermes domesticus*, *Glyptotermes fuscus* and *Hodotermopsis sjoestedti* (Ohkuma *et al*., 1999). A single cell metagenomics study conducted by Treitli *et al*. (2019) revealed the ability of the protist *Streblomastix strix* to fix N_2 in the gut of termite *Zootermopsis angusticollis*. Also, the presence of nitrogenase operon showed that there are other symbionts, mainly *Bacteroidetes* bacteria present on the surface and inside the cell of *S. strix* that contribute to N_2 fixation.

N_2-fixing bacteria can fix atmospheric N_2 due to the presence of several enzymes including the key enzyme nitrogenase (Ayayee *et al*., 2014). Nitrogenase plays a vital role to fix atmospheric N_2 and enables the conversion of diatomic N into ammonia (Waidele *et al*., 2019). It consists of two conserved metalloproteins (Vecherskii *et al*., 2008; Mustafa *et al*., 2019). Nitrogenase subunits are encoded by *nifH, nifD* and *nifK* genes (Kim and Rees, 1994; Li *et al*., 2016). Being a biomarker, *nifH* has been used extensively in many ecological and evolutionary studies of N_2-fixing bacteria, due to its conserved nature, thus making it a great tool to identify N_2-fixers and study the evolution of N_2 fixation mechanism in bacteria (Dias *et al*., 2012; Gaby and Buckley, 2014; Mahmud *et al.*, 2020).

While there have been numerous studies on the evolution of N_2 fixation especially in lower termites, it is still poorly understood. Since N_2-fixer contributes greatly to the diet and nutrient source of termites, this study aimed to isolate N_2 fixing bacteria from the lower termite gut and molecularly identify them via homology comparison of 16S rRNA and *recA* gene sequences. The presence of nitrogenase enzyme was confirmed by *nifH* gene amplification and sequencing. Overall, this study provides a snapshot of the potential horizontal gene transfer of important genes that takes place between different species of bacteria in termite's guts.

MATERIALS AND METHODS

Sample collection

Soldier and worker termites were obtained from infested woods and trees from four different areas in Ipoh, Perak. The first sampling site was a housing area in Taman Buntong Ria (4°35' 17.36"N 101°02' 58.13"E), the second sampling site was a garden in Taman Silibin (4°36' 17.57"N 101°03' 15.14"E), the third sampling site was a forest area at Buntong (4°36' 34.23"N 101°03' 33.22"E) and the fourth sampling site was a housing area in Pengkalan (4°29' 19.16"N 101°04' 21.96"E). The termites were kept in separate plastic containers provided with some woods. The samples were sprayed with water to maintain moisture.

Identification of termite species

The genus and species of the termites were identified based on the phenotypic characteristics using microscopic images of the soldier class termites. Soldier termites have been considerably used in the identification of species instead of worker termites because they are morphologically indistinguishable (Wang *et al*., 2009). The mandible of soldier termites from each sample was enlarged under stereomicroscope and the images were sent to Vector Control Research Unit (VCRU) in Universiti Sains Malaysia (USM) for species identification.

Isolation of bacteria

The termite gut removal was done based on the protocol described by Ramin *et al*. (2008) with slight modification. The lower termites were surface-sterilized using ethanol (70%) followed by the removal of hind guts. By using aseptic techniques, the guts were grinded with the addition of sterile distilled water. The gut samples were serially diluted and plated on HiMedia's Burk's N free media. The plates were incubated at 37 °C for 48 h in the incubator. Similar method was repeated for the other termite samples collected from different sites. Different colonies were identified from the master plates and were sub-cultured onto Burk's media plates to obtain pure cultures.

Identification of bacteria

The bacterial colonies obtained were initially identified at both microscopic and macroscopic levels. This was done based on the physical, morphological and biochemical characteristics of the colonies. The colonies were examined for their colour, form (punciform, circular, filamentous, irregular and rhizoid), elevation (raised, flat, convex, crateriform and umbonate) and margin (entire, filliform, undulate, curled and lobate) (Akhtar *et al*., 2013; Ali *et al.*, 2016). Three different staining methods including Gram staining, capsule staining and endospores staining were performed.

Biochemical characterization

Six biochemical tests (oxidase test, catalase test, indole test, urease test, MRVP test and mannitol fermentation test) were also conducted. Oxidase test was performed by observing the colour change of the oxidase test stripe to blue colour that indicates oxidase positive. For catalase test, the effervescence that produces gas bubbles indicates catalase positive (Adetunji *et al*., 2012). Indole test was performed using tryptophan broth where colony was inoculated and incubated overnight and the next day Kovac's reagent was added. The formation of cherry-red ring after the addition of Kovac's reagent indicates indole

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positive. Mannitol fermentation test was carried out by streaking a small colony onto mannitol salt agar containing phenol red and incubated at 37 °C for 24 h. After 24 h of incubation, the colour change of the agar from pink to yellow indicates the ability of the bacteria to ferment mannitol (Hemraj *et al*., 2013).

Urease test was conducted by growing bacteria in urea broth overnight and the colour change of broth from yellow to pink indicates urease positive (Hemraj *et al*., 2013). Methyl Red (MR) and Voges-Proskauer (VP) tests were performed based on standard procedures. For MR test, five drops of methyl red were added to bacteria which were grown for 48 h. The production of red colour indicates positive result, whereas the colour of the broth remains yellow for negative result. For VP test, 12 drops of Barritt's A reagent were added into to the bacteria culture and was shaken followed by the addition of four drops of Barritt's B reagent. The solution was gently swirled and left undisturbed for 30 min. Positive result is indicated by the formation of pink red colour, whereas the colour of the solution remains unchanged for negative result (Dinesh *et al*., 2015).

Molecular identification of bacterial isolates

DNA of the isolates was extracted using the boiling lysis method (Junior *et al*., 2016). PCR reaction was performed for the amplification of *nifH*, 16S rRNA and *recA* genes. The *nifH* gene was amplified using the primers, PolF (5'-TGCGAYCCSAARGCBGACTC-3') and PolR (5'-ATSGCCATCATYTCRCCGGA-3') (Poly *et al*., 2001). The 16S rDNA gene was amplified using the universal primers 16S-F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and 16S-R1494 (5'-CTACGGYTACCTTGTTACGAC-3') (Weisburg *et al*., 1991). The primer set recA_41F (5'-TTCGGCAAGGGMTCGRTSATG-3') and recA_640R (5'-ACATSACRCCGATCTTCATGC-3') were used for the amplification of *recA* gene (Vinuesa *et al*., 2005; Behrendt *et al*., 2016).

PCR conditions

PCR was performed using Invitrogen Taq DNA Polymerase kit. Each PCR tube contained 2.5 µL of 10× PCR minus Mg buffer, 0.75 µL of 50 mM $MgCl_2$, 0.5 µL of 10 mM dNTP mix, 10 pmol of each primer, 1 U of Invitrogen Taq polymerase and 2 µL of genomic DNA in a total volume of 25 µL. Negative control was used to ensure that there is no contamination in the reaction mixture. Sterile ultrapure water was used in negative control to replace DNA template. All PCR was performed using ESCO Swift MaxPro thermal cycler.

Amplification of *nifH* gene

The amplification of *nifH* gene was performed according to Poly *et al*. (2001) with slight modifications: template denaturation at 95 °C for 3 min and 35 cycles of denaturation at 94 °C for 1 min, annealing for 30 sec at appropriate temperature that is suitable for the isolate and elongation at 72 °C for 1 min. The PCR tubes were incubated for an additional elongation and the reactions were maintained at 4 °C. Optimization of annealing temperature was carried out, ranging from 50 °C to 61 °C, for all 46 isolates. The specific annealing temperature for isolate S7 was 52 °C and for isolate S20 was 55 °C.

Amplification of 16S rRNA and *recA* genes

Bacterial isolates with the presence of *nifH* gene were subjected to partial 16S rRNA gene and *recA* gene amplification. The reaction was performed by incubating the tubes at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing for 30 sec at 55 °C for 16S rRNA gene and 61°C for *recA* followed by 72 °C for 90 min for elongation. Next, the PCR tubes were incubated for an additional elongation for 10 min at 72 °C and the reaction was maintained at 4 °C. All PCR products were resolved via 1% agarose gel electrophoresis. The gel was then viewed under UV light using UV transilluminator.

Sequence analysis

The PCR products were sent to Apical Scientific Sdn. Bhd for sequencing. The DNA sequence data obtained via Sequence Scanner Version 1.0 (Applied Biosystems) was compared to other sequences from GenBank using the BLASTN program from the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/). Identification of bacteria was determined based on the sequence identity by comparing the bacterial 16S rRNA, *recA* and *nifH* genes obtained with known sequences available in the database. The sequences obtained in this study have been deposited in the GenBank sequence database and their accession numbers (Genbank Acc. No.) are shown in the phylogenetic trees (Figures 5, 6 and 7).

Phylogenetic analyses

Phylogenetic trees were constructed for *nifH*, 16S rRNA and *recA* genes for isolates S7 and S20 with 1000 bootstrap replication with partial deletion using MEGAX software. The dendrogram was constructed using the Maximum-Likelihood algorithm using the model Tamura-Nei parameter for 16S rRNA gene tree of isolate S7, Tamura 3 parameter for 16S rRNA gene tree of isolate S20, *recA* and *nifH* gene trees. Suitable outgroups were added to the trees. Reference sequences from few journals and sequences of relevant type strains from List of Prokaryotic Names with Standing in Nomenclature (LPSN) website were also used in the phylogenetic tree construction. Only bootstrap probability values ≥50 % are shown on the trees.

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RESULTS AND DISCUSSION

Identification of termite sample

The microscopic images of soldier termite from each sample were used to identify the species of termite. The images were taken with scale to estimate the size of the termite and its mandible for identification. Generally, the external morphology of soldier termite was used in termite identification. Soldier termites can be easily differentiated between species using their enlarged head and mandible structure which are absent in worker class termites. The size and shape of head capsule, shape of mandibles and pronotum and number of antennae are few key features in identification of termite species (Sornnuwat *et al*., 2004; Norsyarizan and Wan Nurainie, 2016).

The image of the soldier termites from each sampling sites are shown in Figure 1. VCRU in USM identified all the termites sampled here as *Coptotermes gestroi* species, an Asean Subterranean termite. It was formerly known as *Coptotermes vastator* due to the similar morphological characteristics. However, *C. vastator* was later categorized as the junior synonym of *C. gestroi* (Yeap *et al*., 2007; Hapukotuwa and Grace, 2012). *Coptotermes gestroi* is one of the most destructive termites in Asia which damage structural woods and vegetation (Chouvenc and Foley, 2018). It is a highly competitive species which has the ability to out-compete other subterranean species such as *Coptotermes formosanus and Heterotermes tenuis* (Prasad, 2013).

The gut structure of worker caste is slightly different from the gut structure of soldier caste. The gut of lower termite is colonized by a diverse species of flagellated protists and prokaryotes whereas the gut of higher termite is colonized only by prokaryotes (Peterson and Scharf, 2016). Since the worker class termites also known as lower termites are involved in labor works, they contribute to terrestrial ecosystem by providing fixed N due to the presence of N_2-fixing bacteria in their guts. Therefore, worker class termites were chosen for subsequent work instead of higher-class soldier termites.

Isolation of bacteria

Termite gut microbiota have significant roles in which they aid in cellulose degradation, N_2 fixation and other metabolic activities which are beneficial to both the termites and environment. Few N_2-fixing bacteria have been isolated from the gut of other *Coptotermes* species such as *C. formosanus*, *C lacteus*, *C. acinaciformis* and so on (Thanganathan and Hasan, 2018). In a previous study conducted by Breznak *et al*. (1973), ethylene reduction test indicated the presence of N_2-fixing bacteria in the gut of *C. formosanus.* Engagement of *Klebsiella pneumoniae* in symbiotic associations with *C. formosanus* was also identified in previous study (Doolittle *et al*., 2008). However, no publications are available for the isolation of N_2-fixing bacteria from *C. gestroi*.

In this study, a total of 46 different bacterial colonies were obtained from the samples grown on Burk's media. Although Burk's N free medium is a special growth medium for N_2-fixing bacteria isolation, it is not selective enough to screen for N_2-fixing bacteria. Since this media does not contain any inhibitory agent to prevent the growth of other bacteria, there are possibilities for the growth of other bacteria which only requires salt to grow such as *Escherichia coli*. Therefore, further screening by amplifying *nifH* gene must be conducted.

nifH gene amplification

nifH gene amplification was performed to screen for N_2-fixing bacteria from the termite gut sample. *nifH* gene was successfully amplified from only two isolates; S7 and S20 isolated from termites obtained from the first sampling site (Figure 2), while the other 44 isolates did not yield any *nifH* PCR products. Although the termite samples collected from four different sampling sites are of the same species which is *Coptotermes gestroi*, amplification of *nifH* gene from termites obtained in three other sampling sites was unsuccessful. Bacterial strain H1-2 isolated from the root nodule of *Leucaena leucocephala* by Koh (2019) was used as a positive control.

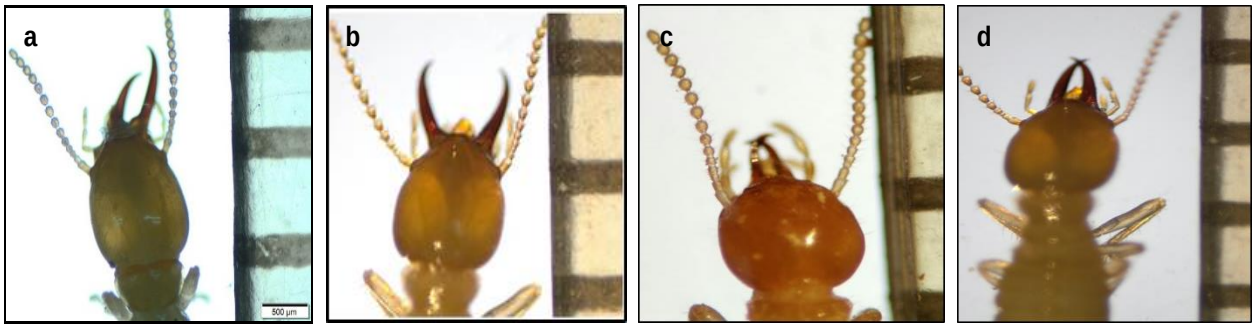

Figure 1: Microscopic image of soldier class termites showing the focused image of their mandibles taken with scale. (a) termite from first sampling site, (b) termite from second sampling site, (c) termite from third sampling site and (d) termite from fourth sampling site. A ruler with 1 mm division was used.

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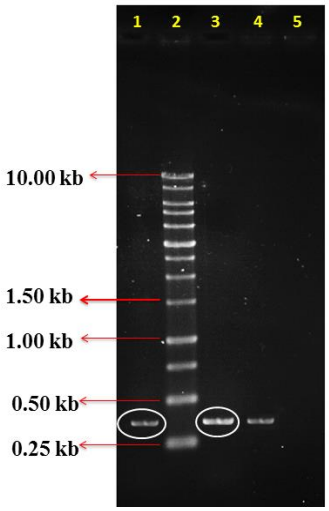

Figure 2: Agarose gel electrophoresis indicates the PCR amplicons of *nifH* gene with the size of approximately 0.35 kb amplified from isolates S7 and S20. Lane 1: Isolate S7, Lane 2: YEA 1 kb DNA ladder marker, Lane 3: Isolate S20, Lane 4: Positive control (genomic DNA extracted from H1-2 strain isolated from *Leucaena leucocephala*), Lane 5: Negative control. The size of relevant bands of the marker is indicated with arrows on the left side.

Morphological and biochemical characterization of isolates S7 and S20

The two isolates with *nifH* were chosen for further analysis and identification. Morphological observation was used for general identification using colony morphology (size, texture, shape and colour), staining and few biochemical tests. The morphology observation was performed to identify the cell shape, presence of endospore or capsule and also the Gram reaction. The summary of colony characteristics, staining and biochemical tests was tabulated in Table 1. Based on the colony morphology, isolate S20 was suspected as an actinomycetes. It appeared as bright yellow colonies on Burk's N free agar media and the colonies were adherent to the agar. Previous studies also mentioned the natural production of pigments by actinomycetes on the agar medium (Udhyakumar *et al*., 2017; Sapkota *et al*., 2020). In addition, isolate S20 is Gram-positive which agrees with the statement of UK standard for microbiology investigation characteristic, where most of the actinomycetes are generally Gram-positive (Public Health England, 2016).

Amplification of 16S ribosomal RNA gene

The 16S ribosomal RNA gene of isolate S7 and S20 was successfully amplified and produced single bands with the size of approximately 1.5 kb (Figure 3). Amplification of 16S rRNA gene fragment from genomic DNA of *Pseudomonas* sp. USM-KH 201 was used as a positive control.

Amplification of *recA* gene

The amplification of *recA* gene was performed to justify that the sequencing results of 16S rRNA gene is coincide with the sequencing results of *recA* gene. Since both 16S rRNA and *recA* genes are housekeeping gene, these can be used to support the occurrence of horizontal gene transfer in this study. The *recA* gene was successfully amplified from the isolate S7 with the size of approximately 600 bp (Figure 4). However, the amplification was unsuccessful for isolate S20. Based on

Table 1: Analysis of colony morphology, staining properties and biochemical test results of isolates S7 and S20 isolated form termites' s gut.

Staining and biochemical tests		Isolate S7	Isolate S20
Colony morphology	Size	Moderate	Moderate
	Texture	Smooth	Smooth
	Shape	Irregular	Circular
	Colour	Milky white	Yellow pigmented
Staining	Gram	-	+
	Shape	Rod	Rod
	Endospore	-	-
	Capsule	-	-
Oxidase test		+	-
Catalase test		+	+
Indole test		-	+
Urease test		+	-
MRVP test	MR	-	-
	VP	-	-
Mannitol fermentation test		-	-
MacConkey agar		+	-
Mannitol salt agar		-	-

(+): Positive; (-): Negative.

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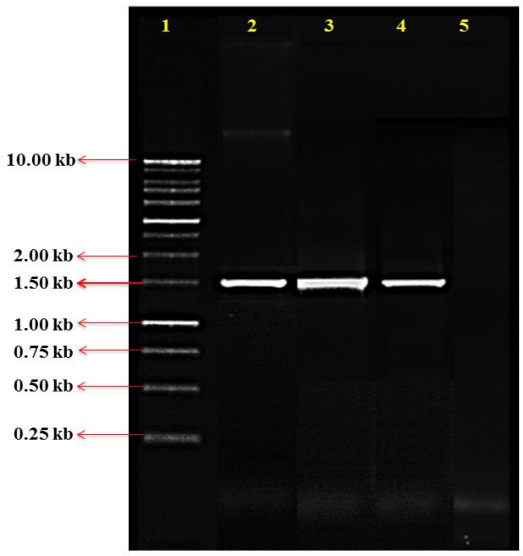

Figure 3: Agarose gel electrophoresis indicates PCR amplicons of 16S rRNA gene with approximately 1.5 kb in size amplified from isolates S7 and S20. Lane 1: YEA 1 kb Ladder DNA marker, Lane 2: Positive control (genomic DNA extracted from *Pseudomonas* sp. USM-KH201), Lane 3: Isolate S7, Lane 4: Isolate S20, Lane 5: Negative control. The size of relevant bands of the marker is indicated with arrows on the left side.

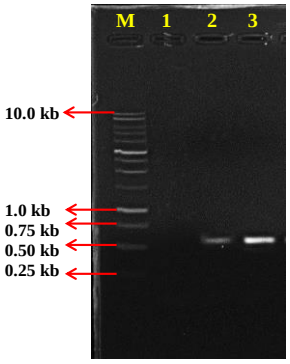

Figure 4: Agarose gel electrophoresis indicates PCR amplicons of *recA* gene with approximately 600 bp in size amplified from isolates S7. Lane M: YEA 1 kb Ladder DNA marker, Lane 1: Negative control, Lane 2: Isolate S7, Lane 3: Positive control (genomic DNA extracted from CT38 strain). The size of relevant bands of the marker is indicated with arrows on the left side.

colony characteristics, staining properties and biochemical test results, isolate S20 is suspected as an actinomycetes. Thus, the primer used may not be suitable or specific enough for the amplification of *recA* gene. Amplification of *recA* gene fragment from genomic DNA of strain CT38, isolated from the root nodule of *Clitoria ternatea* was used as a positive control (Cheng, 2016; Ow, 2018).

Molecular identification

The housekeeping genes 16S rRNA and *recA* of two isolates; S7 and S20 were subjected to gene sequencing for genus identification. The homology search of 16S rRNA gene sequences of isolate S7 against NCBI GenBank database showed 99.64% similarity (1108 bp) with *Ralstonia pickettii* CP12 (KY378754). Isolate S20 was found to show highest similarity with bacterial sequences that belong to the genus *Microbacterium* whereby it showed 99.28% similarity (959 bp) to *Microbaterium* sp. NCCP-451 (LC488936) and 99.27% similarity (958 bp) to *Microbacterium paraoxydans* C12 (MK452785) strains. The BLASTN analysis revealed that the *recA* gene sequence of isolate S7 was identical (486 bp) to that of *Ralstonia pickettii* 12D strain (CP001644).

The *nifH* sequence of isolate S7 does not share high similarity with any available known species in the BLASTN analysis but shows 99.29% similarity (283 bp) with the uncultured bacterium strain 1-2_25 (KM245932) isolated from an environmental sample. Meanwhile, for isolate S20, it was found to show 95% similarity (290 bp) to *Azoarcus olearius* DQS4 strain (CP016210). However, BLASTN results are not sufficient for the isolates identification. Therefore, the isolates were further subjected to phylogenetic analysis.

The phylogenetic analysis of 16S rRNA gene (Figure 5a) showed that isolate S7 clustered closely with *Ralstonia pikettii* type strain (accession no AY741342) isolated from patients undergone tracheotomy (Yabuuchi *et al*., 1995) (99.34% similarity, 1059 bp) and other *Ralstonia* sp. sequences obtained from NCBI database.

The 16S rRNA gene sequence of isolate S20 is closely related to and form a clade with *Microbacterium* spp. sequences obtained from NCBI database (Figure 5b). Isolate S20 clustered together with *Microbacterium* sp. NCCP-451 (LC488936) (99.06% similarity, 948 bp), *Microbacterium paraoxydans* HZLJC2-1 (MT605416) (97.49% similarity, 932 bp), *Microbacterium* sp. H85 (MH669262) (97.49% similarity, 932 bp), *Microbacterium* sp. InS605 (MN315452) (97.49% similarity, 932 bp), *Microbacterium* sp. YPS-003 (MT328062) (97.49% similarity, 932 bp), and clustered together with the type strain of *Microbacterium oxydans* DSM20578 (MH321609) (96.86% similarity, 925 bp). This clearly shows that isolate S20 is a member of genus *Microbacterium*. However, further studies involving various identification tests have to be done for an accurate species identification of the isolate.

RecA sequence analysis for isolate S7 also concurred with that of 16S rRNA gene whereby it shares highest similarity to *Ralsonia pickettii.* The ML tree constructed for *recA* gene sequence (Figure 6) shows that isolate S7 formed a clade with a closely related non type strain of *Ralstonia pickettii* 12D (CP001644) (100% similarity, 442 bp) and clustered together with the type strain of *Ralstonia pickettii* (ATCC 27511) (NZ KN050646) (98.97% similarity, 438 bp).

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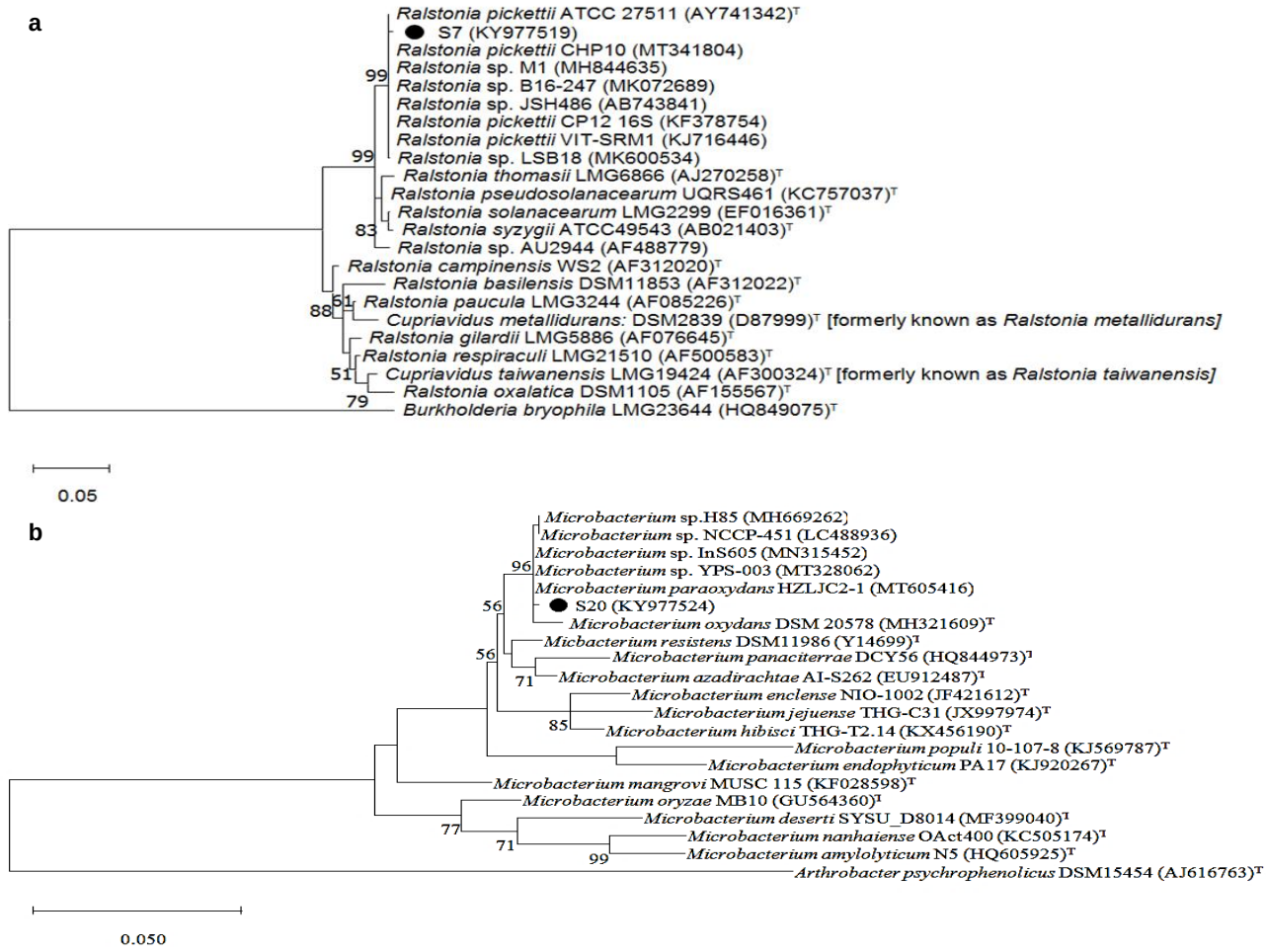

Figure 5: Maximum Likelihood trees of 16S rRNA gene sequences of (a) isolate S7 (ca.1076 bp) and (b) isolate S20 (ca. 975 bp) isolated from termite guts of *Coptotermes gestroi*, selected type strains and closely related strains. *Burkholderia bryophila* LMG23644 and *Arthrobacter psychrophenolicus* DSM15454 were used as outgroups for (a) and (b), respectively. The trees were constructed using MegaX software using the respective models: (a) Tamura Nei parameter (T93), gamma distribution (+G) with invariant sites (+I); (b) Tamura 3 parameter (T92), gamma distribution (+G) with invariant sites (+I). GenBank accession numbers are in parentheses. Numbers shown in the internal branches are the bootstrap values derived from 1,000 replications when above 50% is shown at each node. Scale bar = 50% sequence divergence (5 substitutions per 100 nucleotides). Superscript 'T' indicates the type strains.

The phylogenetic tree of *nifH* gene (Figure 7) showed that isolate S7 is clearly separated and not homologous to the *nifH* gene sequence of *Ralstonia taiwanensis* type strain LMG19424 (AJ505320) and *Ralstonia* sp. S1SM82 (KU527069). It formed a clade with uncultured bacteria strain NR 1628 (AF035512) (98.57% similarity, 276 bp) and strain NR 1606 (AF035490) (99.93% similarity, 277 bp) isolated from surface water sample of Neuse River, North Carolina. The uncultured bacteria strain 1-2C25 (KM245932) isolated from gastrointestinal tract of Amazonian catfish, *Panaque nigrolineats* also grouped together in the same clade (99.93% similarity, 277 bp). Likewise, isolate S20 clustered with, but is clearly separated from, *Azoarcus* sp. and formed a separate clade. Its *nifH* gene sequence is distantly related to *Microbacterium* sp. Zapt11 (DQ164419).

The phylogenetic of 16S rRNA and *nifH* gene sequences of isolate S7 and S20 demonstrated that both isolates are not from the same bacterial species. The 16S rRNA and *recA* gene sequences of isolate S7 are similar to *R. pickettii* whereas its *nifH* sequence is similar to an uncultured bacterial strain and is distantly related to the *nifH* sequences of *R. pickettii* type strain. On the other hand, the 16S rRNA gene sequence of isolate S20 is most closely related to *M. oxydans* whereas its *nifH* gene sequence is distantly related to *Azoarcus* sp. This coincides with the analysis of 16S rRNA and *nifH* genes where, the 16S rRNA and *nifH* gene sequences of the isolates showed similarity to different bacterial genus.

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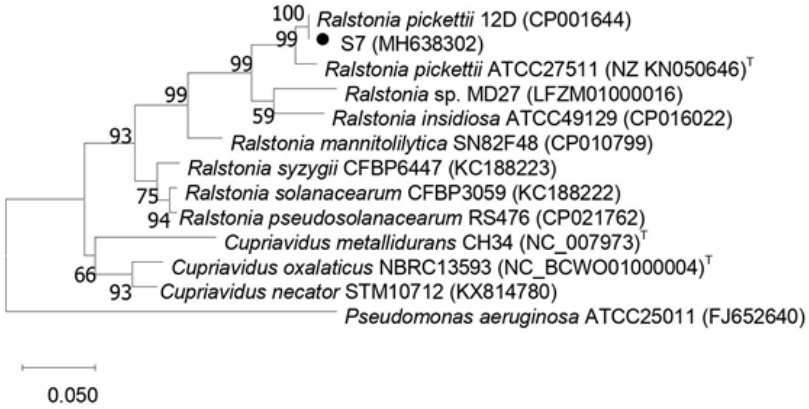

Figure 6: Maximum Likelihood tree of *recA* gene sequences (ca. 442 bp) of isolate S7 isolated from termite gut of *Coptotermes gestroi*, selected type strains and most closely related non type strains. *Pseudomonas aeruginosa* ATCC25011 was used as outgroup. This tree was constructed using MegaX software with the Tamura 3 parameter (T92), gamma distribution (+G) model. GenBank accession numbers are in parentheses. Numbers shown in the internal branches are the bootstrap values derived from 1,000 replications when above 50% is shown at each node. Scale bar = 5% sequence divergence (5 substitutions per 100 nucleotides). Superscript 'T' indicates the type strains. *Cupriavidus* = *Ralstonia*.

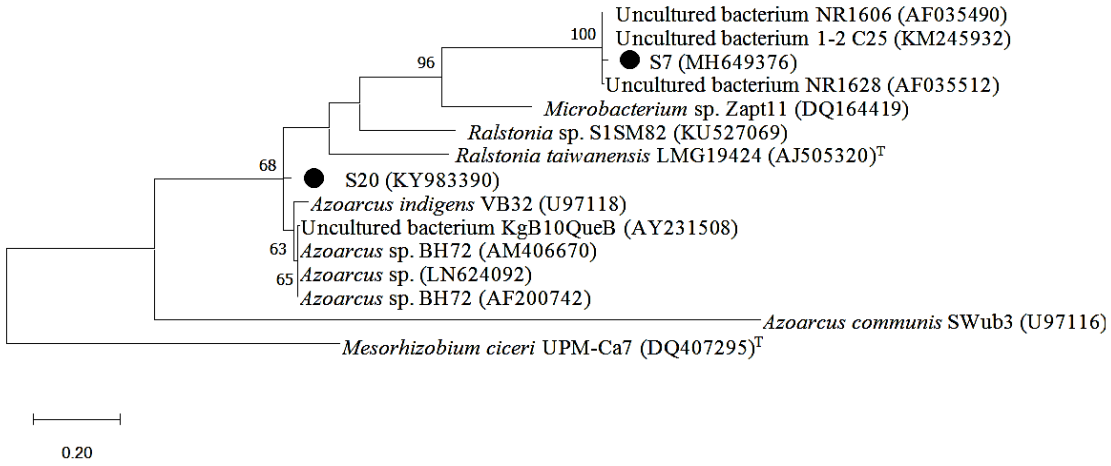

Figure 7: Maximum Likelihood tree of *nifH* gene sequences (ca. 300 bp) of two bacterial strains; S7 and S20 isolated from termite gut of *Coptotermes gestroi* and selected most closely related non type strains. *Mesorhizobium ciceri* UPM-Ca7 was used as outgroup. This tree was constructed using MegaX software with the Tamura 3 parameter (T92), gamma distribution (+G) model. GenBank accession numbers are in parentheses. Numbers shown in the internal branches are the bootstrap values derived from 1,000 replications when above 50% is shown at each node. Scale bar = 20% sequence divergence (20 substitutions per 100 nucleotides). Superscript 'T' indicates the type strains.

In symbiotic bacteria, the *nifH* gene is located on plasmid together with other genes involve in N_2 fixation such as other *nif* and *nod* genes (Okubo *et al*., 2016; Wongdee *et al*., 2016). Since *nifH* gene is in plasmid, it may easily be subjected to horizontal gene transfer (Zehr *et al*. (2003). Therefore, there could be a possibility for horizontal gene transfer where most likely isolate S7 and S20 have acq*uired nifH* gene from other bacterial species.

Specifically, although the 16S rRNA of isolate S7 showed highest similarity to the type strain of *Ralstonia pickettii* (ATCC 27511), the type strain only possesses *fix* genes and does not possess any *nif* genes-based on its full genome sequence (KN050646). While it has genes that may code for *fix* protein, these genes function to regulate and metabolize oxygen and are not known to be involved in nitrogen fixation independently without the presence of *nif* genes (Black *et al*., 2012). The *nif* genes are essential in N_2 fixation as they encode for nitrogenase, a key enzyme that catalyzes N_2 fixation. A functional nitrogenase enzyme complex includes an iron protein dinitrogenase reductase that encoded by *nifH* and iron-molybdenum protein dinitrogenase encoded by *nifD* and *nifK* (Liu *et al*., 2018).

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In addition, there are no reports on the presence of *nif* genes in other *Ralstonia* type strains with the exception of *Ralstonia taiwanensis* which has since been reclassified as *Cupriavidus taiwanensis* (Vandamme and Coenye, 2004). As such these may further support the possibility of horizontal gene transfer of *nifH* gene in isolate S7.

The event of horizontal gene transfer (HGT) has been identified in the gut microbiota of other insects. Brown and Wernegreen (2019) have reported the occurrence of HGT in two Acetobacteraceae strains (ABB2 868 and ABB2 880) isolated from *Camponotus chromaiodes*, a red carpenter ant. Full genome sequence analysis revealed the presence of about 342 and 276 horizontally transferred genes in ABB2 868 and ABB2 880, respectively. Similarly, *Paenibacillus polymyxa* A18, isolated from termite gut has acquired few genes, via HGT. Those genes were absent in the genome of other closely related *P. polymyxa* strains (Pasari *et al*., 2019). The event of HGT was observed in *Burkholderia* sp. Lv-StB genome where about 148 genes were obtained from Gammaproteobacteria and Alphaproteobacteria (Waterworth *et al*., 2020).

There are very limited studies available on the HGT of insect gut microbiota, particularly in relation to the *nif* genes. However, there are findings on the HGT of *nif* genes in soil bacteria. Raymond *et al*. (2004) reported that the *nifH* gene from a set of reference species of α-*Proteobacteria* was used to study the evolutionary history of *Rhodopseudomonas palustris*. The direct comparison of 16S rRNA and *nifH* gene phylogenetic trees showed that the *nifH* gene of *R. palustris* is closely related to *Rhodobacter* sp. and α-*Protobacteria*, the phototropic purple non-sulfur bacteria. However, its 16S rRNA gene showed closed relationship to *Bradyrhizobium japonicum*. In order to support this, comparison was done using few informational and operational genes from *R. palustris* CGA009*, Rhodobacter sphaeroides* and *B. japonicum* USDA 110. Based on the outcome, it was deduced that the genome of *R. palustris* is more closely related to *B. japonicum* as compared to *R. sphaeroides*. In another study, conducted on pure culture of type strain *M. chthonoplastes* PCC7420 does not show any nitrogenase activity. It has been reported that *Microcoleus chthonoplastes* acquired *nif* operon via horizontal transfer from Deltaproteobacteria (Bolhuis *et al*., 2010).

Horizontal gene transfer of symbiosis genes was also observed between few *Bradyrhizobium* spp. The phylogenetic analysis conducted by Chahboune *et al*. (2011) demonstrated that the *Bradyrhizobium* isolated from *Cytisus villosus* in Morocco was found to have diverse housekeeping genes (*glnII* and *recA*) and 16S rRNA gene which is closely related to *Bradyrhizobium* of α-photobacteria. Out of 23 strains obtained, 19 strains grouped into *Bradyrhizobium*, three into *Mesorhizobium* and one into *Rhizobium*/*Agrobacterium* group. The *nifH* and *nodC* gene sequences are mostly closely related to *Bradyrhizobium japanicum* sv. *genistearum*. In another study, the housekeeping gene of *Bradyrhizobium* isolated from *Cytisus scoparius* in United States (US) showed higher similarity to the indigenous *Bradyrhizobium*. However, their symbiosis genes (*nifH, nifD* and *nodC*) are closely related to *Bradyrhizobium* stains from Spain. The study suggested that the *Bradyrhizobium* of US has harboured the genes through horizontal gene transfer (Horn *et al*., 2014).

Similar to previous studies, there could be possibility of HGT in this study. As the isolates S7 and S20 were pure culture, contamination is not possible. Thus, it can be concluded that isolate S7 and S20 has most likely acquired *nifH* gene through horizontal transfer.

CONCLUSION

BLAST analysis and phylogenetic analysis of housekeeping genes have identified isolates S7 and S20 obtained from the guts of *C. gestroi* as *R. pickettii* and *Microbacterium* sp., respectively. This study also revealed that isolate S7 and isolate S20 may have potentially harboured their *nifH* genes via horizontal gene transfer. Further study has to be conducted to identify and validate the origin of their *nifH* gene.

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