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Research article

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# Comparative Gut Microbiomes of Four Species Representing the Higher and the Lower Termites

**LiJuan Su,[1,*] LeLe Yang,[1,*] Shi Huang,[2] XiaoQuan Su,[2] Yan Li,[1] FengQin Wang,[1,3] EnTao Wang,[4] Ning Kang,[2] Jian Xu,[2] and AnDong Song[1,3,5]**

[1]College of Life Sciences, Henan Agricultural University, Zhengzhou, Henan 450002, China, [2]BioEnergy Genome Center, CAS Key Laboratory of Biofuels and Shandong Key Laboratory of Energy Genetics, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao, Shandong 266101, China (xujian@qibebt.ac.cn; songandong@henau.edu.cn), [3]Key Laboratory of Enzyme Engineering of Agricultural Microbiology, Ministry of Agriculture, Zhengzhou, Henan 450002, China, [4]Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, México, DF 11340, México, and [5]Corresponding author, e-mail: songandong@henau.edu.cn

*These authors contributed equally to this work.
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## Abstract

Aiming at learning the association between the gut microbiota and termites with different diet habits and phylogenetic positions, the gut bacteria of three populations for each of the two higher termites (wood-feeding *Mironasutitermes shangchengensis* and fungus-feeding *Odontotermes formosanus*) and two wood-feeding lower termites (*Tsaitermes ampliceps* and *Reticulitermes flaviceps*) were analyzed by high-throughput 454 pyrosequencing of 16S V1–V3 amplicons. As results, 132 bacterial genera and some unidentified operational taxonomic units within 29 phyla in the gut bacteria were detected, with Spirochaetes (11–55%), Firmicutes (7–18%), Bacteroidetes (7–31%), and Proteobacteria (8–14%) as the main phyla, and *Treponema*, TG5, *Dysgonomonas*, *Tannerella*, za29, *Lactococcus*, *Pseudomonas*, and SJA-88 as the common genera in all the four termites. The diversity of gut bacterial communities in the higher termite guts was significantly greater than that in the lower termites; while the gut microbiota in *M. shangchengensis* (wood-feeding higher termite) was more similar to those of the wood-feeding lower termites rather than that of *O. formosanus* (fungus-feeding higher termite), and phylum Spirochaetes and nitrogen-fixing bacteria were super-dominant in the wood-feeding termites, despite of their phylogenetic relations. This study reported for the first time the gut bacterial communities for the termites of *M. shangchengensis* and *T. ampliceps* and the comparative analyses showed that the gut microbial communities varied according to the phylogeny and the diet habits of termites.

**Key words**: termite, comparative study, gut microbiota, bacterial diversity, pyrosequencing

It is well known that the microbial communities associated with termites play crucial roles in digesting lignocelluloses, immunity, reproduction, and other physiological functions of their hosts (Warnecke et al. 2007; Werren et al. 2008; Fraune and Bosch 2010; Brune 2014; Scharf 2015). The so-called higher termites (family Termitidae) missed their eukaryote symbionts and rely primarily on their gut bacteria and fungus outside the intestinal to assist in decomposing lignocelluloses, whereas the gut of lower termites is occupied by a dense community of protist symbionts working in concert with gut bacteria (Cleveland 1923; Brugerolle and Radek 2006; Brune 2014). The lignocellulose degrading capability, diversity, and eusociality of termites are thus inextricably linked with the composition, diversity, and digestive capabilities of their gut symbionts (Warnecke et al. 2007; Berlanga et al. 2011; Scharf et al. 2011a,b; Brune 2014).

Early researches by culture-dependent counting and direct counting revealed that a single *Reticulitermes flavipes* hindgut contained at least $3 \times 10^6$ symbiotic microorganisms. However, these data did not include the protozoans and unculturable bacteria, which now are recognized as the major components of the termite microbiota and play an irreplaceable role in lignocellulose degradation (Dolan 2001; Ohkuma 2003; Stingl et al. 2005). Currently, high-throughput sequencing approaches based on 16S rRNA amplicons have been used to survey the diversity of the termite gut microbiota to overcome the insufficiency of the traditional methods (Berlanga et al. 2011; Huang et al. 2013; Santana et al. 2015). These analyses have shown that the microbial diversity and complexity in the termite gut were far exceeded our previous understanding. Therefore, comparing study on gut microbiota of various termite species is needed for better understanding the co-evolutionary relationship of

the termites and their gut microbiota. In recent years, several studies have highlighted that the structure of microbiota varied for different termite species (Brauman et al. 2001; Otani et al. 2014; Abdul Rahman et al. 2015; Tai et al. 2015) and that the termite phylogeny affected the gut microbial communities more than environment or diets (Hongoh et al. 2005; Abdul Rahman et al. 2015; Tai et al. 2015). Even so, the co-evolutionary relationships between the hosts and the gut bacteria are still not clearly demonstrated.

In China, about 300 species of termites are widely distributed in the tropic and subtropical regions as pests and they are also recorded as traditional medicine in the ancient Chinese literatures. However, the study on microbiota associated with these termites is not enough, especially for the local species *Tsaitermes ampliceps* and *Mironasutitermes shangchengensis*. So, in this study, four termite species representing the lower (*Reticulitermes flaviceps* and *T. ampliceps*) and the higher species (*Odontotermes formosanus* and *M. shangchengensis*) were collected from fields in Henan Province of China, in which the *O. formosanus* was fungus-feeding species and the remaining termites were wood-feeding species. According to the records in "Fauna of China" (Huang et al. 2000), *O. formosanus* and *R. flaviceps* are widely distributed in the tropic and subtropical regions of China, as important pests to trees and woody materials, including tea, bamboo, etc.; *M. shangchengensis* and *T. ampliceps* are species restricted to the region around Henan Province (Huang et al. 2000; Su et al. 2011). The bacterial community composition in each termite species was comparatively analyzed by 454 high-throughput sequencing. The aims of our study were to facilitate a more comprehensive understanding about the termite gut microbiota associated with different termite lineages and explore the effects of diet and phylogeny of termite hosts on the diversity and metabolic capacities of the intestinal microbes.

## Materials and Methods

### The Termite Collection and Identification

In this study, the wood-feeding *R. flaviceps* and *T. ampliceps* representing the lower termites, while the fungus-feeding *O. formosanus* and wood-feeding *M. shangchengensis* representing the higher termites were collected from four regions in Henan Province (Supp Fig. 1 [online only]). The *R. flaviceps* populations were sampled from Mangshan Mountain at the suburb of Zhengzhou City (N 34.4°, E 113.4°), where they mainly feed on the trunks of black locust (*Robinia pseudoacacia* L.) trees. The *T. ampliceps* colonies were sampled from the mountains at Yuzhou City (N 34.2°, E 113.2°) where they feed on black locust, arborvitae [*Platycladus orientalis* (L.) Franco)] and pine (*Pinus massoniana* Lamb.) trees. The colonies of *M. shangchengensis* were sampled from the mountains at Shangcheng County (N 32.3°, E 113.6°), where they mainly eat *Pinus massoniana*. The colonies of *O. formosanus* were sampled from bank of Shangcai Reservoir at Zhumadian City (N 31.8°, E 115.5°). The underground nests of *O. formosanus* were dig out from about 1 m of depth together with the surrounding soil, while the colonies for the other three species were collected from the died trees (beneath the tree park or inside the decomposed trunk). Three colonies for each termite species were collected independently. All the sampling sites were wild fields without any disturb of people, and the four test termite species were not endangered.

In the original nests together with surrounding logs and soils, the sampled termites were separately stored in complete darkness, >70% humidity, at room temperature until used. Termites were identified based on their morphology (Huang et al. 2000). In addition, the identification of *R. flaviceps* and *O. formosanus* was confirmed by the sequence analysis of mitochondrial cytochrome oxidase II gene and 16S RNA gene as reported previously (Austin et al. 2004; Legendre et al. 2008). A approximately 769-bp fragment of the COII gene was amplified with the forward primer fP: 5′-TCT AAT ATG GCA GAT TAG TGC-3′ and the reverse primer rP: 5′-GAG ACC AGT ACT TGC TTT CAG TCA TC-3′ (Legendre et al. 2008); and an 396-bp fragment of the mitochondrial 16S rRNA gene was amplified with the forward primer fP: 5′-TTA CGC TGT TAT CCC TAA-3′ and the reverse primer rP: 5′-CGC CTG TTT ATC AAA AAC AT-3′ (Legendre et al. 2008). Both the obtained amplicons were sequenced directly with the corresponding forward primers in laboratory of Sangon Biotech (Shanghai, China). The obtained sequences were used for searching the related sequences in GenBank database by NCBI blast. In the mitochondrial DNA barcoding, the obtain sequences from *R. flaviceps* colonies showed 100% similarities with the reported sequences of *R. flaviceps* (AF107479 and AY101831 for COII and 16S RNA gene, respectively); while those from the *O. formosanus* colonies were identical with the reported COII (JQ429119) and 16S RNA gene (JQ518437) of *O. formosanus*. Being no reference sequence available, the identification of *M. shangchengensis* and *T. ampliceps* were only based on their morphology (Huang et al. 2000).

### Metagenomic DNA Extraction From Guts

Mature worker-caste termites were used for all experiments within 2 days after sampling. Being washed with 70% ethanol and sterilized water, 200 worker termites for each sample (nest) were dissected in an ice-cold dish and their intestinal tracts were pulled out by a needle from the abdominal tips. The isolated guts were immersed in ice-cold 0.2 M phosphate buffer saline (PBS; pH = 7.4) (Pernthaler et al. 2004) and were homogenized with tissue homogenizer on ice. After centrifugation at 3000 × *g* for 5 min at 4 °C, the supernatant was recovered and used for metagenomic DNA extraction using the E.Z.N.A Tissue DNA Kit (OMEGA, USA), following the manufacturer's instruction. The concentration of DNA extract was measured spectrophotometrically (Qubit Assays, Life Technologies, USA) at 260 nm (Green and Sambrook 2012) and the DNA samples were stored at −20 °C before further processing.

### PCR Amplification of Microbial 16S rRNA Genes

The forward primer 16S-1 (5′-TGG AGA GTT TGA TCC TGG CTC AG-3′) and reverse primer 16S-2 (5′-TAC CGC GGC TGC TGG CAC-3′) (Vliegen et al. 2006) that correspond to *E. coli* nucleotide positions 4–532 containing 10 bp multiplex identifiers and the Roche 454 pyrosequencing adaptors Lib-L were used to amplify the V1−V3 region of the 16S rRNA gene from the metagenomic DNA (Schloss et al. 2011) using a GeneAmp PCR Systems 9700 (Applied Biosystems, USA). The PCR mixture contained 1.25 U of DNA polymerase (Takara, Japan), 5 µl of Pfu reaction buffer, 4 µl of dNTPs (10 mM), and 0.2 µM of each primer, 100 ng of gel-purified genomic DNA, and the total volume was adjusted to 50 µl with double distilled water. The cycling conditions were: initial denaturation at 94 °C for 3 min, 30 cycles at 95 °C for 30 s, 58 °C for 60 s, and 72 °C for 60 s; followed by a final 2-min extension at 72 °C. The reaction was performed in triplicate for each sample. The products were checked by electrophoresis in 1% (w/v) agarose gel. The DNA amplicons were gel-purified using Agencourt AMPure XP beads (Beckman Coulter, USA) following the guidance of manufacturer and the concentrations were determined using an Agilent BioAnalyzer 2100 (Invitrogen, USA) and a NanoVue spectrophotometer (GE, USA). The triplicates

of amplicons for the same sample were pooled together at equimolar ratios before sequencing.

**Pyrosequencing of the 16S rRNA Gene Amplicons**

Pyrosequencing was performed for the 16S rRNA gene amplicons for each sample in triplicates by using 454 Life Sciences Genome Sequencer FLX Titanium (Branford, CT), which produced 400-bp reads on average. The sequences generated by pyrosequencing were analyzed with MOTHUR software package (Schloss et al. 2009) (http://www.mothur.org/) for preprocessing, identifying operational taxonomic units (OTUs), taxonomic assignment, and community structure comparisons. To minimize the effects of random sequencing errors and to avoid overestimates of the phylogenetic diversity, relatively stringent quality-based read trimming was performed (Kunin et al. 2010): the reads <150 bp, with an average quality score of <35 in each 50-bp window rolling along the whole read, with an ambiguous base call (*N*), with any homopolymers of >8 bases, or without the primer sequence were excluded. The remaining reads were then sorted based on the tag sequences. To reduce sequencing noise in the pyrosequencing data, a pre-clustering step using the "pre.cluster" script in MOTHUR was performed and all the chimerical sequences detected by UCHIME (Edgar et al. 2011) were removed. These trimming processes were completed using RDP Initial Process (http://pyro.cme.msu.edu). The 16S rRNA gene reads obtained in this study have been deposited in Sequence Read Archive of NCBI under the accession number of SRP067996 and were further processed by computational pipelines customized for termite gut microbiome analysis.

**Data Analysis**

*Assessment of Microbial Diversity Based on OTUs*. To assign the phylotypes of the tagged sequences, the trimmed reads were clustered using UCLUST program (http://www.drive5.com/uclust/). An in-house Perl script was then used to convert the output from UCLUST into a format recognized by MOTHUR software package for determining the alpha-diversity and all the reads were assigned to OTUs at the 97% identity (species level). Species richness and diversity estimators (ACE, Shannon Index and InvSimpson, etc.) were calculated. The relative abundance was compared for the OTUs defined in different treatments. Rarefaction curves were generated and compared among the samples.

*Taxonomy Assignment*. The obtained reads were used to phylogenetically determining their taxonomic affiliation based on searches against the Greengenes 16S rRNA gene database (http://greengenes.secondgenome.com/) using MOTHUR (DeSantis et al. 2006). The relative abundances of bacterial taxa were calculated at the phylum, class, order, family, and genus levels, as well as unclassified taxa if they were not clearly defined into any level.

*Comparison of the Microbial Community Structures*. Principal coordinates analysis (PCoA) (Lozupone and Knight 2005) was performed based on the matrices of pairwise distances among all the microbiotas. Venn diagram generated with the online program at http://bioinfogp.cnb.csic.es/tools/venny/index.html was used to compare the distribution of OTUs in the four termites. Cladogram was constructed with the METAGENassist tool (http://www.metagenassist.ca/METAGENassist/faces/Upload View.jsp2). One-Way ANOVA was used to detect statistically significant differences, and Tukey HSD was used for multiple comparisons with SPSS 17.0. All the statistical tests for microbiota comparisons based upon the abundance of bacterial taxa (phyla, genera and OUT/species) were two-sided with a significance level of $P < 0.05$ (*P*-value according to *F*-test and degrees freedom, $F =$ between group variability/within group variability), in which number of groups were 4 (termite species, $K = 4$), number of observations in each group was 3 (triplicate, $i = 3$), the overall sample size was 12 (4 species × 3 replicates, $N = 12$).

*Functional Implications*. To probe the microbial metabolic and functional pathways in the microbiota, the OTUs were automatically taxonomy-to-phenotype mapped using approximately 20 different phenotypic categories in METAGENassist (http://www.metagenassist.ca/METAGE Nassist/faces/Home.jsp).

## Results

**Pyrosequencing Data and Diversity Analysis of Sequence Reads**

In this study, a total of 210,628 post-trimming 16S rRNA gene reads (average read length = 436 bp) were generated. After the quality filtration, only 45,459 (about 21.6%) of the reads were used in further study: 8912–14,327 reads corresponding to 967–1603 OTUs at species level (3% genetic distance) for the 4 termite gut samples (Table 1). The lower termite species *R. flaviceps* and *T. ampliceps* exhibited lower diversity with Shannon index of 5.98 and 5.92, respectively; while the higher species *M. shangchengensis* and *O. formosanus* presented 6.37 and 6.29 of Shannon index, respectively (Table 1). High Goods Coverage values between 0.94 and 0.97 (Table 1) were obtained and the results of rarefaction analysis and the Shannon curve (available as Supp Fig. 2 [online only]) also showed that OTU richness was almost saturated. The higher termites harbored more diverse taxa than the lower termites: *M. shangchengensis* > *O. formosanus* > *R. flaviceps* > *T. ampliceps* (Table 1; Supp Fig. 2 [online only]).

**Taxonomic Classification of Sequence Reads From Gut Microbiota**

In total, the gut microbiota covered 29 phyla (Fig. 2, detailed information available in Supp. Tables 1 and 2 [online only]), of which 13 were detected in all the 4 termite species. In addition, the proportion of unclassified reads in the two higher termites (15.6% for *O. formosanus* and 15.1% for *M. shangchengensis*) was apparently greater than that in the lower termites (10.1% for *R. flaviceps* and 4.7% for *T. ampliceps*). The phyla Spirochaetes (46.3, 56.2, 39.3, 11.0% for *R. flaviceps, T. ampliceps, M. shangchengensis* and *O. formsanus*, respectively), Firmicutes (11.2, 6.4, 14.0, 17.7%), Bacteroidetes (8.9, 8.2, 13.5, 30.1%), and Proteobacteria (11.5, 12.6, 8.1, 13.9%) were the main groups in all the four termites (Fig. 2), and the fifth abundant phyla varied according to the termites: Elusimicrobia (4.0 and 4.5%) in the lower termites *R. flaviceps* and *T. ampliceps*, Planctmycetes (3.6%) in *O. formosanus*, and Actinobacteria (2.5%) in *M. shangchengensis* (Fig. 3; Supp Tables 1 and 2 [online only]). Comparatively, the abundances of phyla Spirochaetes, Elusimicrobia, and Verrucomicrobia were significantly greater in the microbiotas of the lower termites than in those of the higher termites, whereas the abundances of phyla Firmicutes, Bacteroidetes, Synergistetes, TM7, and Chlorobi were reverse. Furthermore, the minor phyla TG3, Fibrobacteres, and Deferribacteres were found only in the two higher termites; while Gemmatimonadetes, Thermi, Spam, Tm6, BRC1, and WS3 presented only in the lower termites (Fig. 2; Supp Tables 1 and 2 [online only]).

In total, 132 genera were detected in the gut communities; while high proportions of the reads were unidentified at this level: 43% for *R. flaviceps*, 33% for *T. ampliceps*, 78.8% for *O. formosanus*, and 48.6% for *M. shangchengensis* (details available in Supp Tables 3 and

**Table 1.** The number of sequences after post-trimming of raw reads, the number of identified taxa, the percentage of reads successfully assigned to the phylum and genus levels (based on relative abundances) as well as the estimated richness and diversity indices for the bacterial communities (at 3% dissimilarity threshold)

| Termites species | Number of sequences | OTUs at 3% difference | Classification | | Richness and diversity indices | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Phylum | Genus | Chao1 | Good's coverage | ACE | Shannon | InvSimpson |
| *R. flaviceps* | 14,327 | 1254 | 89.76 | 58.22 | 1647.5 | 0.9724 | 1613.56 | 5.98 | 137.85 |
| *T. ampliceps* | 8912 | 967 | 95.24 | 66.74 | 1344.33 | 0.9582 | 1299.2 | 5.92 | 189.69 |
| *O. formosanus* | 10,749 | 1603 | 84.36 | 21.19 | 1884.1 | 0.9569 | 1829.55 | 6.29 | 297.8 |
| *M. shangchengensis* | 11,471 | 1334 | 84.91 | 51.31 | 2321.29 | 0.9467 | 2559.16 | 6.37 | 287.56 |

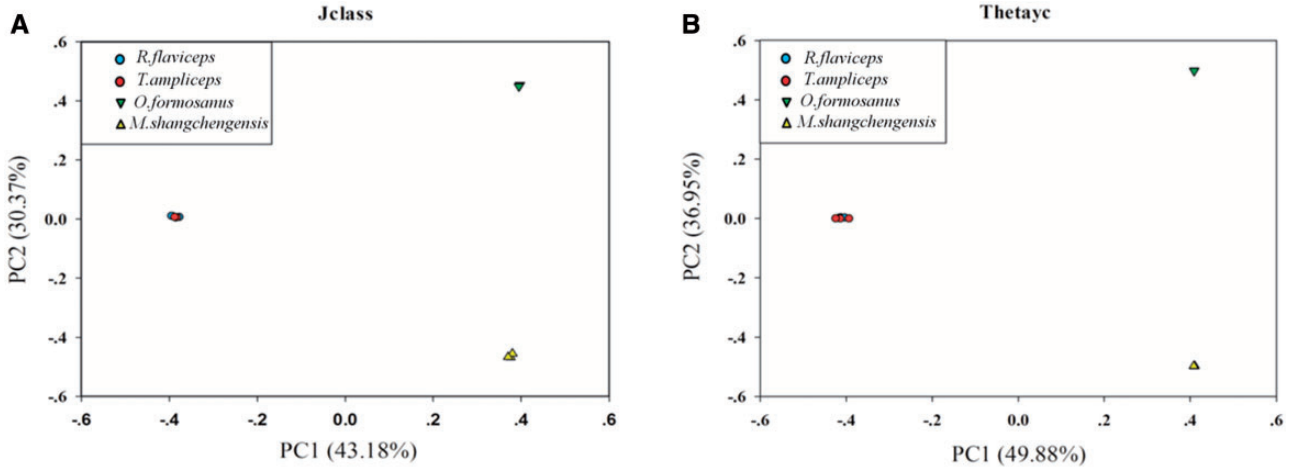


**Fig. 1.** Principal coordinates analysis (PCoA) of gut microbiota in different kinds of termites based on OTU. (A) The community structures were analyzed by PCoA based on jclass distance matrix. (B) The community structures were analyzed by PCoA based on thetayc distance matrix. Each point corresponds to a microbial community where the color indicates its category. PCO1 and PCO2 are shown with the percentage variation explained for each axis.

4 [online only]). The five most abundant genera (>1% of the total reads) *Treponema*, Candidatus *Azobacteroides*, *Dysgonomonas*, *Desulfovibrio*, and TG5 were common for the three wood-feeding termites, but their relative abundances varied (Fig. 3). *Treponema* (10.1%) and *Dysgonomonas* (2.3%) were also dominant in gut of *O. formosanus*; while TG5 (2.1%), *Arcobacter* (0.8%), and *Bacteroides* (0.7%) were the other three most abundant genera for this fungus-feeding termite. In addition, some minor genera (being greater than 1% in relative abundance for one of the termites), like *Tannerella*, za29, *Lactococcus*, and *Clostridium*, were also common in all the four termite species with different relative abundances (>0.2%; Supp Tables 3 and 4 [online only]).

### Comparison of Gut Microbiotas in the Four Termites

Significant differences were observed among the gut microbiotas of the four termites in the present study (Figs. 1–5; also Supp Tables 1–10 [online only]). In the jclass PCoA and thetayc analyses based on the OTU (species) level (Fig. 1), the gut microbiotas of the two lower termites were clustered together; while those of the two higher termites were separated from the gut microbiotas of lower termites at both axes, and from each other at the PC2 axis. The PCA (available as Supp Fig. 3 [online only]) produced consistent results, except that the microbiota of *M. shangchenensis* was closer to those of the lower termites than to that of *O. formosanum*, although the diversity indexes (Shannon index) in microbiotas of the higher termites were similar and greater than those in the lower termites (Table 1), The four most abundant bacteria phyla were Spirochaetes (51.3% mean), Proteobacteria (12.0% mean), Firmicutes (8.7% mean), and Bacteroidetes (8.6% mean) in wood-feeding lower termites; Spirochaetes (39.3%), Firmicutes (13.9%), Bacteroidetes (13.5%), and Proteobacteria (8.1%) in wood-feeding higher termite *M. shangchengensis*; and Bacteroidetes (30.7%), Firmicutes (17.7%), Proteobacteria (13.9%), and Spirochaetes (11.0%) in fungus-feeding higher termites *O. formosanus* (Fig. 2). In fungus-feeding higher termite, the relative abundance of the phyla Spirochaetes, Bacteroidetes, Firmicutes, Fibrobacteres, and TG3 were significantly different comparing to those in the other three termites (Fig. 3; Supp Table 4 [online only]). Elusimicrobia was the fifth abundant bacteria phyla in lower termites, while it was very rare or was undetectable in higher termites.

At the genus level, the bacterial community structures of *R. flaviceps* and *T. ampliceps* were rather similar, characterized by the greater abundances of *Treponema* (45.3–54.9%), Candidatus *Azobacteroides* (2.4–3.1%), and *Desulfovibrio* (1.6–1.8%) compared with those of the two higher species. In addition, the absence of *Fibrobacteres*-2, *Arcobacter*, and *Bacteroides* also distinguished the two lower species from the higher ones. The bacterial biota of *O. formosanus* was the most divergent in those of the four termites and was differentiated from those in the other three termites by its low abundance of *Treponema* (10.0%), high abundances of unidentified reads (78.4%), TG5 (2.1%), *Dysgonomonas* (2.3%), *Arcobacter* (0.9%), and *Bacteriodes* (0.6%), as well as the absence of Candidatus *Azobacteroides* and the presence of Pei061, etc. (Supp Tables 3 and 4 [online only]).

When comparing the OTUs (Fig. 4), only 4 OTUs are shared by all the 4 termites, while 76 and 836 OTUs were shared by the 2 higher termites and by the 2 lower ones, respectively. In addition,

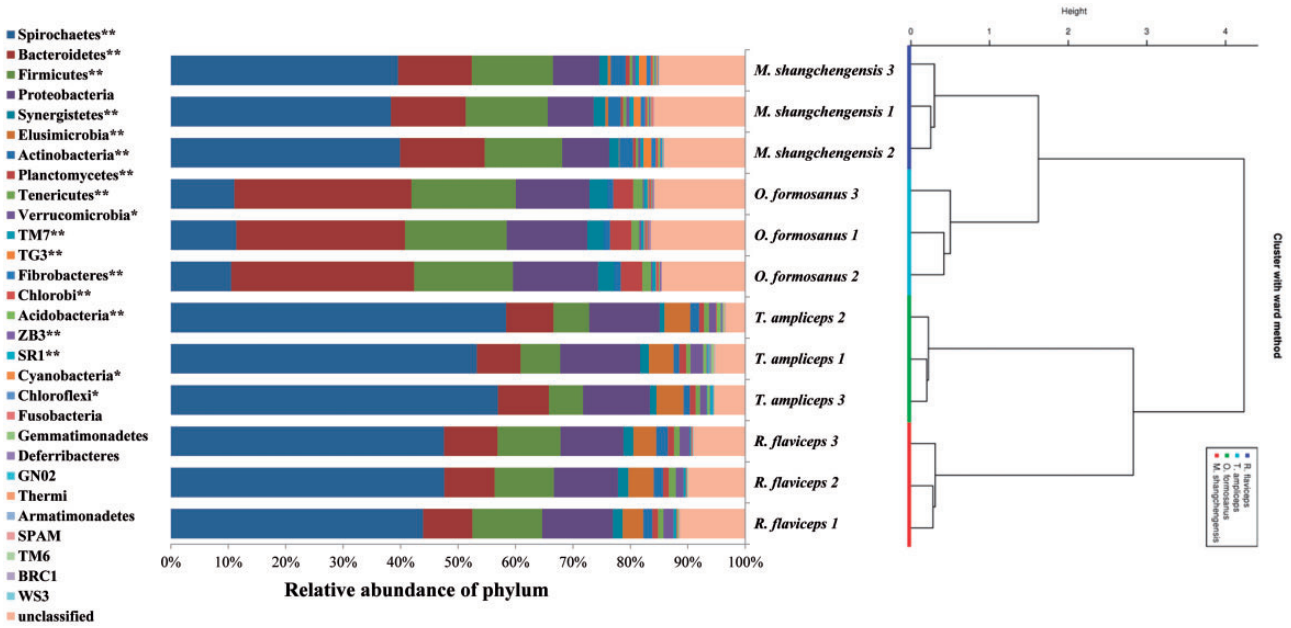


**Fig. 2.** Distribution of the phylum in the gut bacterial community composition estimated from the triplicate samples of the four termite species. *Significant difference ($P < 0.05$); **extremely significant difference ($P < 0.01$) among samples using *F*-test. Cladogram was constructed with the METAGENassist tool (http://www.metagenassist.ca/METAGENassist/faces/UploadView.jsp2.).

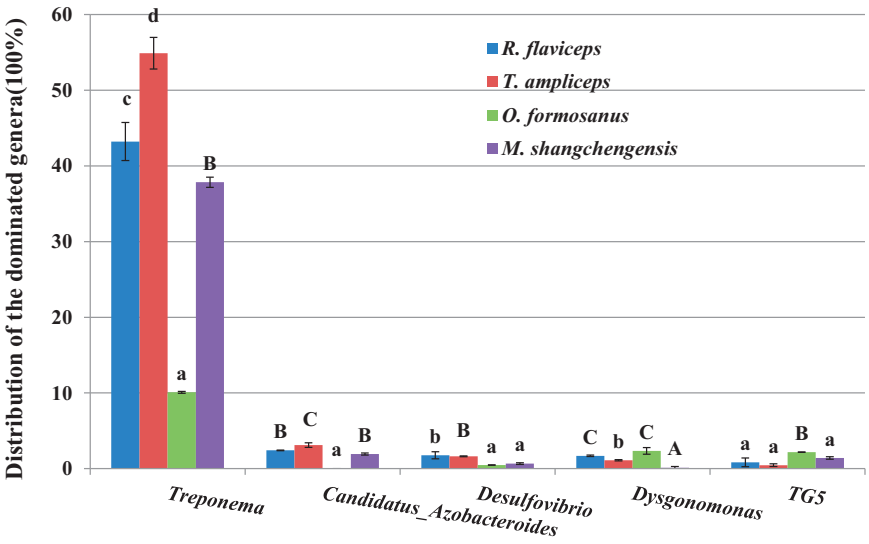


**Fig. 3.** Distribution of the five most abundant genera in the gut bacterial community composition estimated from the triplicate samples of the four termite species. The measure of error was represented by the error bar. Different small letters above bars mean significant difference ($P < 0.05$) and different capital letters above bars mean extremely significant difference ($P < 0.01$) among samples using *F*-test.

*M. shangchengensis* shared 43 and 68 OTUs with *T. ampliceps* and *R. flaviceps* (85 in total), respectively; and *O. formosanus* shared 31 and 47 OTUs with *T. ampliceps* and *R. flaviceps* (57 in total), respectively.

### Functional Implications of Different Microbiota

Based upon the automatically taxonomy-to-phenotype mapping, 27 types of bacterial metabolic activities (Fig. 5) were identified, including nitrogen fixation, ammonia oxidizer, dehalogenation, sulfate reducer, nitrite reducer, sulfide oxidizer, aromatic hydrocarbons degrader, xylan degrader, chitin degrader, sulfur oxidizer, cellulose degrader, selenate reducer, lignin degrader, and sulfur reducer. The stability and abundance of the gut microbes appeared to vary for different metabolic types (Fig. 5). For example, the relative abundance for each group of nitrogen fixers, ammonia oxidizers, dehalogenation bacteria, and sulfate reducers was >10%, whereas those of denitrifying bacteria and streptomycin producers were about 0.1% for each (Fig. 5). Furthermore, several metabolic types related to the lignocellulose degradation, particularly the aromatic hydrocarbons, xylan, cellulose, and lignin degradations, were identified (Fig. 5).

## Discussion

Although the investigation on termite gut microbiota has been increased in the last decade (Warnecke et al. 2007; Otani et al. 2014; Abdul Rahman et al. 2015; Santana et al. 2015; Tai et al.

2015), the diversity of termite associated microorganisms and their interaction with the host phylogeny and dietary habits still remains unclear in many cases. In the present study, the community structures of gut bacteria of *T. ampliceps* and *M. shangchengensis* were revealed by pyrosequencing of 16S rRNA genes for the first time, while several studies have been performed to investigate the diversity and functions of gut microbes of *O. formosanus* (Shinzato et al. 2007; Huang et al. 2012; Hayashi et al. 2013; Li et al. 2016) and of *R. flavipes* with cloning techniques or 16S rRNA gene and metagenomic pyrosequencing (Berlanga et al. 2011; He et al. 2013; Arango et al. 2014; Mikaelyan et al. 2015; Benjamino and Graf 2016). The high Goods coverage values (0.94–0.97; Table 1; also Supp Fig. 2 [online only]) evidenced that the 967–1603 OTUs detected in the gut samples in the present study (Table 1) covered most of the gut bacteria associated with the 4 sampled termite species and was comparable with the results reported in previous studies (Warnecke et al. 2007; Boucias et al. 2013; Huang et al. 2013).

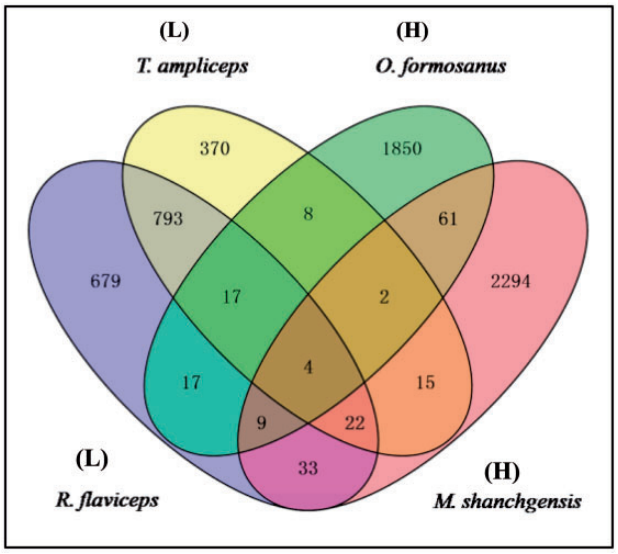


**Fig. 4.** Venn diagram showing distribution of common OTUs among different termite's species. Consensus classification is shown for OTUs found only in 2 lower termites (for the 836 OTUs) and different number represents the number of OTUs.

The results of the present study demonstrated that the gut bacterial community associated with different colonies of the same termite species in a certain ecosystem is stable as evidenced in Fig. 1. Meanwhile, the diversity and composition of gut microbiota might be impacted by the phylogeny and the diet habits of termites (Fig. 1). For instance, the super dominance of phylum Spirochaetes, following by Proteobacteria, Firmicutes, and Bacteroidetes, was a common features for the wood-feeding termites (*R. flavipes*, *T. ampliceps*, and *M. shangchengensis*) despite their phylogenetic relationships; and the super dominant phylum Bacteriodetes, following by Firmicutes, Proteobacteria, and Spirochaetes, was unique for the fungus-feeding *O. formosanus*. In addition, the lower diversity indexes (Shannon and InvSimpson) of gut microbiotas associated with the lower termites comparing with those of the higher termites is a common revealed phenomenon as observed in the present study (Table 1; also Supp Fig. 2 [online only]) and in several previous studies (Warnecke et al. 2007; Boucias et al. 2013; Huang et al. 2013; Dietrich et al. 2014; Otani et al. 2014). The greater diversity of gut microbiota in the higher termites might be related to the absence of a dense community of protist symbionts in their gut (Ohkuma 2008; Otani et al. 2014) and they depend more on the symbiotic fungi and/or bacteria for lignocellulose degradation (Ohkuma 2008).

In the present study, core microbiomes might be estimated for all the four species, for the two lower termites, for the two higher termites, as well as for the three wood-feeding species. In all the 4 tested termite species, 11 phyla (Spirochaetes, Bacteroidetes, Firmicutes, Proteobacteria, Synergistetes, Actinobacteria, Planctomycetes, Tenericutes, Verrucomicrobia, TM7, and Chlorobi) and 10 genera (*Treponema*, TG5, *Dysgonomonas*, *Tannerella*, *Desulfovibrio*, Candidatus *Tammella*, Za9, *Lactococcs*, *Pseudomonas*, and SJA88) were detected at different abundances. When the three wood-feeding termites were considered, phylum Elusimicrobia and genera

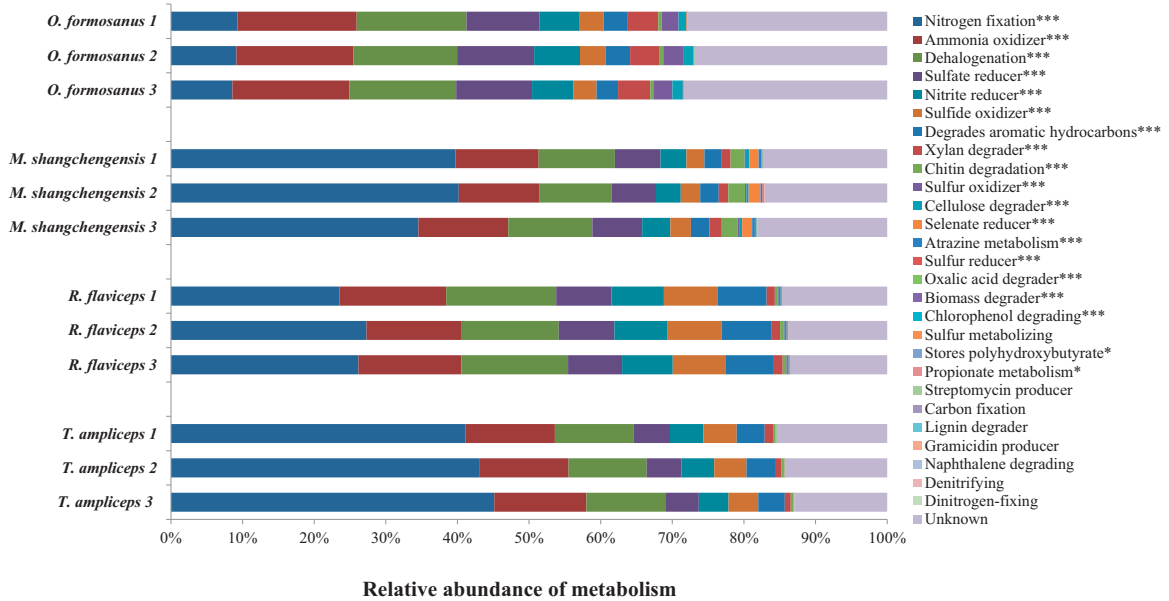


**Fig. 5.** Microbial functions encoded in termite gut microbiota from different termite species. The comparison was conducted at phylum level (*$P<0.05$, **$P<0.01$, ***$P<0.001$). The function estimation was performed as reported previously (Schäfer et al. 1996; Kuhnigk and König 1997; Nakashima et al. 2002; Arakawa et al. 2009; Bugg et al. 2011).

Candidatus *Azobacteroides, Bacillus* could be included as the core microbiome. For the two lower termites, phylum Elusimicrobia and genera *Bacillus, Rodocyclus, Entrococcus*, and *Clostridium* could be added as the core microbiome. For the two higher termites, the phyla TG3, Fibrobaxteres, and Cyanobacteria and genera *Fibrobacteres-2, Clostridium, Acetivibrio, Oxalobacter, Leclercia, Acinetobacter*, and LE30 were added as common taxa. These differences in core microbiomes might be related to the phylogeny of the termite and to their diet habits. For example, it could be estimated that Elusimicrobia and Candidatus *Azobacteroides* is essential for the wood-feeding termite, but not for the fungus-feeding termite; while the presence of phyla TG3 and Fibrobaxteresn as well as the absence of protozoan (Cleveland 1923; Brugerolle and Radek 2006; Brune 2014) might be microbial markers for the higher termites.

In addition to the core microbiomes, many OTUs or taxa were host specific in the gut microbiota of tested termite species (Fig. 4). The community composition of gut bacteria detected from *R. flavipes* in the present study was similar to that reported previously, but Spirochaetes was the super dominant phylum in our study (Fig. 3) while Bacteroidetes and Spirochaetes were the most abundant phyla in the report of Arango et al. (2014). These variations may be related to the differences of the diets and environmental factors between the involved termite populations. In the present study, the higher relative abundance of Spirochaetes (*Treponema*) and lower abundance of Firmicutes, as well as the presence of *Clostridium* and *Paenibacillus* in *T. ampliceps* differentiated this species from another lower termite *R. flavipes* (Figs. 1 and 5).

The difference between the gut microbiota of wood-feeding *M. shangchengensis* and fungi-feeding *O. formosanus* was greater than that between the two lower termites (Figs. 2–5), as evidenced by the lower proportion of common taxa at various levels between the two termites. Only 76 OTUs (1.72%) among the total OTUs, *Treponema* among the most abundant genus, and Bacteroidetes, Firmicutes, and Spirochaetes among the five most abundant phyla were common for these two higher termites. And even for *Treponema*, the relative abundance was 27% more in *M. shangchengensis* than that in *O. formosanus*. Therefore, we could conclude that both the diet habit and phylogeny of the termites affected the gut microbiota.

As endosymbionts, the gut bacteria are associated with some metabolic functions, especially polymer degradation and nitrogen fixation. It is well known that the gut microbes of the termites are of importance in the food digestion of the hosts, especially in the supply of nitrogen source (review of Davila et al. 2013) and degradation of the polymers of carbohydrates (review of Brune 2014).

In relation to the low nitrogen content and poor nutritional value of the wood diet, the nitrogen fixing, and ammonia oxidizing microorganisms would be an important component in the microbiota of the wood-feeding termites (Breznak 2000). In the present research, the nitrogen fixation microorganism possesses 25.6–43.1% of the total bacteria (reads) in the three wood-feeding termites; while it only occupied 8.9% of the total reads in *O. formosanus* (Fig. 5), suggesting that this fungus-feeding termite mainly obtained their nitrogen nutrient from its food. The dominance of Spirochaetes and Bacteroidetes in the wood-feeding termites might be related to the nitrogen fixation based the previous reports (Lilburn et al. 2001; Warnecke et al. 2007; Yamada et al. 2007; Hongoh et al. 2008). In addition, the detection of bacteria related to dehalogenation, sulfate reduction, nitrite reduction, sulfide oxidization, aromatic hydrocarbons degradation, xylan degradation, chitin degradation, cellulose degradation, selenate reduction, and lignin degradation in the present study (Fig. 5) and in several previous studies (Schäfer et al. 1996; Kuhnigk and König 1997; Nakashima et al. 2002; Arakawa et al. 2009; Bugg et al. 2011) implies that the gut microbiota may play multiple function for the host termites, such as nutrient (polymer degradation, nitrogen fixation) supplement and recycling of nutrients, detoxification (nitrite reduction, sulfide oxidization, selenate reduction), and resistance to pathogens (chitin degradation).

In summary, this study identified the gut microbiotas in four termite species (two species of higher termites and two species of lower termites), in which the gut bacteria of *T. ampliceps* and *M. shangchengensis* were the first time to be investigated by the 16S rRNA gene pyrosequencing. *Treponema*, *Desulfovibrio*, *Dysgonomonas* and TG5 in genus-level, and Spirochaetes, Firmicutes, Bacteroidetes, and Proteobacteria in phylum-level were identified as the common taxa for all the four termites; while Candidatus_*Azobacteroides* was detected only in the wood-feeding termite. The higher termites harbored more diverse gut bacteria than the lower termites. Comparing with that in *O. formosanus* (fungus-feeding higher termite), the gut microbiota in *M. shangchengensis* (wood-feeding higher termite) shared more common OTUs with those of the wood-feeding lower termites. The super dominant phylum was Spirochaetes in three wood-feeding termites, while was Bacteroidetes in *O. formosanus*. Both the nitrogen-fixing and the lignocellulose-degrading bacteria were more abundant in the wood-feeding termites than in the fungus-feeding termites. Therefore, the gut bacterial community may be regulated by both the phylogeny and the diet habitat of termite. Future work to elucidate the link between gut bacterial composition, the phylogeny, and the dietary habitat under controlled conditions is needed.

## Supplementary data

Supplementary data are available at *Journal of Insect Science* online.

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We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the position presented in the manuscript entitled, "Variation in the gut microbiota and sensitivity to dietary changes in termite hosts".

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