



Linking lignocellulosic dietary patterns with gut microbial Enterotypes of *Tsaitermes ampliceps* and comparison with *Mironasutitermes shangchengensis*

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ABSTRACT. *Tsaitermes ampliceps* (lower termites) and *Mironasutitermes shangchengensis* (higher termites) are highly eusocial insects that thrive on recalcitrant lignocellulosic diets through nutritional symbioses with gut dwelling prokaryotes and eukaryotes. We used denaturing gradient gel electrophoresis and a 16S rRNA clone library to investigate i) how microbial communities adapt to lignocellulosic diets with different cellulose and lignin content, ii) the differences in the dominant gut microbial communities of the 2 types of termites. The results indicated that gut microbiota composition in *T. ampliceps* was profoundly affected by 2-week diet shifts. Comparison of these changes indicated that Bacteroidetes and Spirochaetes act in cellulose degradation, while Firmicutes were responsible for lignin degradation. Additionally, Proteobacteria consistently participated in energy production and balanced the gut environment. Bacteroidetes may

function without hindgut protozoans in higher termites. The diversity of enteric microorganisms in *M. shangchengensis* was higher than that in *T. ampliceps*, possibly because of the more complicated survival mechanisms of higher termites.

Key words: Denaturing gradient gel electrophoresis; Gut microbiota; Lignocellulosic diets; *Mironasutitermes shangchengensis*; *Tsaitermes ampliceps*

INTRODUCTION

Colonies of termites are canonical examples of highly eusocial insects, and their success, adaptability, diversity, and eusociality are thus inextricably linked with the composition, diversity, and digestive capabilities of their gut symbionts (Scharf et al., 2011; Brune, 2014). The foregut of lower termite is relatively simple, while the expansion portion or paunch stomach of the hindgut is filled with a large number of flagellates (protozoa). However, higher termites have a highly partitioned gut containing a very small amount of intestinal protozoa. Several different morphological types and densities of bacteria and archaea settle in the digestive tract of both types of termites; particularly, the number of bacteria in the hindgut can reach up to 10^5 - 10^{11} /mL. It was estimated that a single *Reticulitermes flavipes* hindgut contained at least 3×10^6 heterotrophs according to anaerobic cultivation and direct counting methods (Schultz and Breznak, 1978). These symbiotic microorganisms can break down plant fiber and ferment the products to acetate and variable amounts of methane, with hydrogen as a central intermediate; termites thus rely on the biosynthetic capacities of their gut microbiota as a nutritional resource (Yang et al., 2005; Matson et al., 2010; Brune, 2014).

Termites play an important role in the degradation of dead plant materials in nature. Over the last century, numerous studies have investigated the mechanisms of their lignocellulose-digesting system. Several previous studies examining the intestinal microbial community were based mainly on the use of anaerobic culture techniques, which are typically very labor-intensive and time-consuming. However, this estimate did not include the unculturable bacteria associated with the protozoan community, which now is recognized as a major component of the termite intestinal microbial community (Iida et al., 2000; Dolan, 2001; Stingl et al., 2005; Ohkuma et al., 2007). The diversity and complexity of the termite gut microbiota have been evaluated using molecular technology techniques based on 16S rRNA (Warnecke et al., 2007; Berlanga et al., 2011; Mathew et al., 2012). Recent studies have indicated that different dietary habits shape the structure of microbiota in the termite gut (Boucias et al., 2013; Huang et al., 2013; Raychoudhury et al., 2013). Dietary manipulation induces measurable differences among gut bacterial communities, and the recalcitrance of plant substrates may be the major cause of this variation in the diversity and richness of gut bacterial communities (Boucias et al., 2013; Huang et al., 2013; Raychoudhury et al., 2013). However, influences on the abundance and diversity of the intestinal microbial of termite fed with different substrates containing various lignin and cellulose or hemicellulose remain unclear.

Tsaitermes ampliceps is a canonical example of lower termites of Rhinotermitidae, while *Mironasutitermes shangchengensis* is a type of higher termite and are only widely distributed

in the Henan Province in China (Su et al., 2011). For the insects that cause significant damage to forests and released by the Chinese Ministry of Forestry, the taxonomic composition of the gut prokaryotic microbiota and its relative role(s) in lignocellulose degradation in the 2 types of termites have not been determined. To comprehensively understand the distinction between the 2 types of termites and changes in the dominant gut microbial communities in the lower termites *T. ampliceps* fed with 3 types of food (wood, corn stalk, and filter paper) with different cellulose, hemicellulose, and lignin content, we used 2 different molecular techniques, including denaturing gradient gel electrophoresis (DGGE) analysis and a 16S rRNA clone library to explore these issues.

MATERIAL AND METHODS

Termite collection and experimental design

T. ampliceps was collected in May 2012 from the town of Jiushan in Xuchang, located at N34.22°E113.18°, while *M. shangchengensis* was collected in October 2011 from the town of Jingangtai in Xinyang, located at N31.8°E115.5°. They were fed in complete darkness at 25°C and >70% humidity until they were used in the experiment.

T. ampliceps collected from the same colony was divided into 3 groups and fed with *Robinia pseudoacacia*, corn stalk, and filter paper, with the following compositions: i) *R. pseudoacacia* (W) containing approximately 25-30% lignin and 50-60% cellulose and hemicellulose; ii) corn stalk (C) containing approximately 10-15% lignin and 55-65% cellulose and hemicellulose; iii) filter paper (F) containing only cellulose. All processing conditions were the same among groups except for the 2 weeks of different diets. Diet materials were cut into approximately 0.5-cm pieces and oven-dried before use. All feeding boxes were 10 x 10 x 10 cm and prepared using 500 mL 1% agarose as basal and moderate one of diet assays on the basal. Boxes were sealed with breathable cotton gauze and placed in a dark incubator at 25°C for 2 weeks; each diet treatment was rehydrated with 100 mL water after every 48 h.

Extraction of DNA from gut microbes and polymerase chain reaction (PCR) amplification

Fifty workers were dissected in an ice-cold dish with their intestinal tracts removed using a sterile needle from the abdominal tips. Tracts were placed in 800 µL ice-cold 0.2 M phosphate-buffered saline, pH 7.4. The intestinal tracts were disrupted using Bullet Blender™ (Next Advance, Averill Park, NY, USA). After centrifugation at 200 g at 4°C for 3 min, the supernatant was removed. The total genomic DNA of gut microbes was isolated from the samples using the Soil DNA Kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer protocol.

Meta-genomic DNA was used as a template for amplification after proper dilution in a LabCycler Standard PCR (SensoQuest, Göttingen, Germany). The V3 region of the 16S rRNA gene was amplified using the eubacteria-specific primers F341-GC and R518 (F341-GC, 5'-CGC CCG CCG CGC GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3';

R518, 5'-ATT ACC GCG GCT GCT GG-3'). A GC-clamp (CGC CCG CCG CGC GCG GGC GGG GCG GGG GCA CGG GGG GCC) was applied to the 5'-end of the forward primer to increase the sensitivity of DGGE analysis. PCR amplification was performed using a hot start and touchdown PCR. The following conditions were used: hot start of 3 min at 94°C; 20 cycles consisting of 94°C for 30 s, 65°C for 30 s, decreasing the temperature by 0.5°C per cycle (touchdown PCR), 72°C for 30 s; 15 cycles consisting of 94°C for 30 s, 53°C for 30 s, 72°C for 30 s; a final step of 7 min at 72°C. To construct library of 16S rRNA genes, nearly full-length 16S rRNA products were amplified using the P1 and P2 universal primers of 16S rRNA genes (P1, 5'-AGA GTT TGA TCC TGG CTC AGA ACG AAC GCT-3'; P2, 5'-TAC GGC TAC CTT GTT ACG ACT TCA CCC C-3') (Brosius et al., 1978). The reaction program was as follows: 3 min at 95°C, followed by 25 cycles for 30 s min at 95°C, 30 s at 60°C, and 2 min at 72°C, with a final extension for 10 min at 72°C. All amplification products were separated by 1% agarose gel electrophoresis and stained with ethidium bromide. The DNA bands of approximately 180 bp for the V3 region and 1500 bp for 16S were excised and purified using the SanPrep Gel Extraction Mini Kit (Sangon Biotech, Shanghai, China).

DGGE

The PCR products of the V3 region for the 16S rRNA genes were electrophoretically separated by DGGE using the Bio-Rad Dcode mutation detection system (Hercules, CA, USA) according to the manufacturer instructions. Next, 40 µL of each PCR product was loaded on 10% (w/v) polyacrylamide gels with a denaturing gradient from 30-60% and containing 12.6-25.2% (w/v) urea and 12-24% (v/v) deionized formamide (Sangon Biotech). Electrophoresis was performed in 1X TAE buffer for 10 min at a constant voltage 200 V at 60°C, and then at 85 V for 16 h. The gel was stained with 0.5 µL/mL ethidium bromide for 20 min, rinsed with deionized water for 20 min, and photographed. Characteristic bands were removed from the gel. The small gel pieces were rinsed with 100 µL 70% precooled ethanol 3-4 times, and then 50 µL double-distilled H₂O was added to the gel pieces after they were air-dried and soaked for approximately 10 h at 4°C. PCR was conducted for the recovered DNA fragments under the same conditions as were used for the V3 region, and detected and purified by DGGE. Next, the recovered DNA fragments were amplified in the V3 region-specific primers F341 (5'-ACG GGG GGC CTA CGG GAG GCA G-3') and R518 (5'-ATT ACC GCG GCT GCT GG-3') without a GC-clamp. The PCR products were sequenced by Sangon Biotech.

Clone library construction

Purified PCR products of nearly full-length 16S rRNA were ligated into the pMDTM19-T vector according to the manufacturer instructions (TaKaRa, Shiga, Japan) and transformed into *Escherichia coli* DH5α cells. A total of 100-µL transformation reaction was plated on an LB plate containing ampicillin, isopropyl β-D-1-thiogalactopyranoside, and X-Gal and the plates were incubated overnight at 37°C. White clones were picked and amplified using the universal primer P1/P2 according to the method described by Zoetendal et al. (1998). Inserts of the correct

size were considered to have come from positive clones. Several positive clones were selected randomly and amplified using the primers F341-GC and R518, and grouped into operational taxonomic units (OTUs) by DGGE. For OTUs with more than 30 clones, 5 representative clones were randomly selected and sequenced by Sangon Biotech. For OTUs with fewer than 6 clones, all clones were sent for sequencing. For OTUs with more than 6 clones, 3 or 4 clones were randomly selected for sequencing.

Contrastive analysis of images and sequences

All DGGE images were exported as uncompressed TIF files and analyzed using the Quantity One software (Bio-Rad). Sorenson's similarity index and unweighted pair group method with arithmetic mean were determined by the Quantity One software, while the Shannon-Wiener index, Jaccard index, and Cody index were determined from the data exported from Quantity One. In addition, we determined the relative Q-value of each band in each lane and obtained the relative abundance. Additionally, sequencing results were compared with those available in GenBank to identify the closest relatives using the BLAST algorithm. Three phylogenetic trees were constructed using the neighbor-joining method with the TREECONW software packages. The partial 16S rRNA gene sequences from the clone library were deposited in the GenBank database and assigned accession numbers KF145967-KF145981.

RESULTS

DGGE profiles and their sequencing

In order to understand the gut microbial community composition in *M. shangchengensis* and 3 groups of *T. ampliceps*, we conducted DGGE analysis of the V3 regions of 16S rRNA amplified from total DNA extracted from gut samples (Figure 1). Approximately 20 DGGE bands were observed on the DGGE profile of *T. ampliceps* (Figure 1A) and *M. shangchengensis* (Figure 1B). Each sample profile displayed a unique banding pattern. The results showed that the gut microbial communities' diversity and relative abundance in *T. ampliceps* were influenced by the diet materials containing different components. Furthermore, an unweighted pair group method with arithmetic mean dendrogram (Figure 1C) determined by cluster analysis using the Quantity One software revealed that the 2 groups fed with corn stalk and filter paper were very similar to each other, and 1 clade associated with the 2 groups with a coefficient of 0.73, which is more than 0.53.

Eighteen and 16 sequences obtained from the samples were compared with other sequences available in the NCBI database; relative abundance was analyzed using the Quantity One software (Tables 1 and 2) to identify differences between samples from the 2 types of termites and changes caused by feeding of different substrates. Sequence analysis showed that the intestine of termites was dominated by Proteobacterium, Firmicutes, Actinobacteria, Fibrobacteres, Spirochaetes, Bacteroidetes, Deferribacteres, and some protozoans or protozoan symbionts. Protozoan symbionts were only observed in the samples from lower termites.

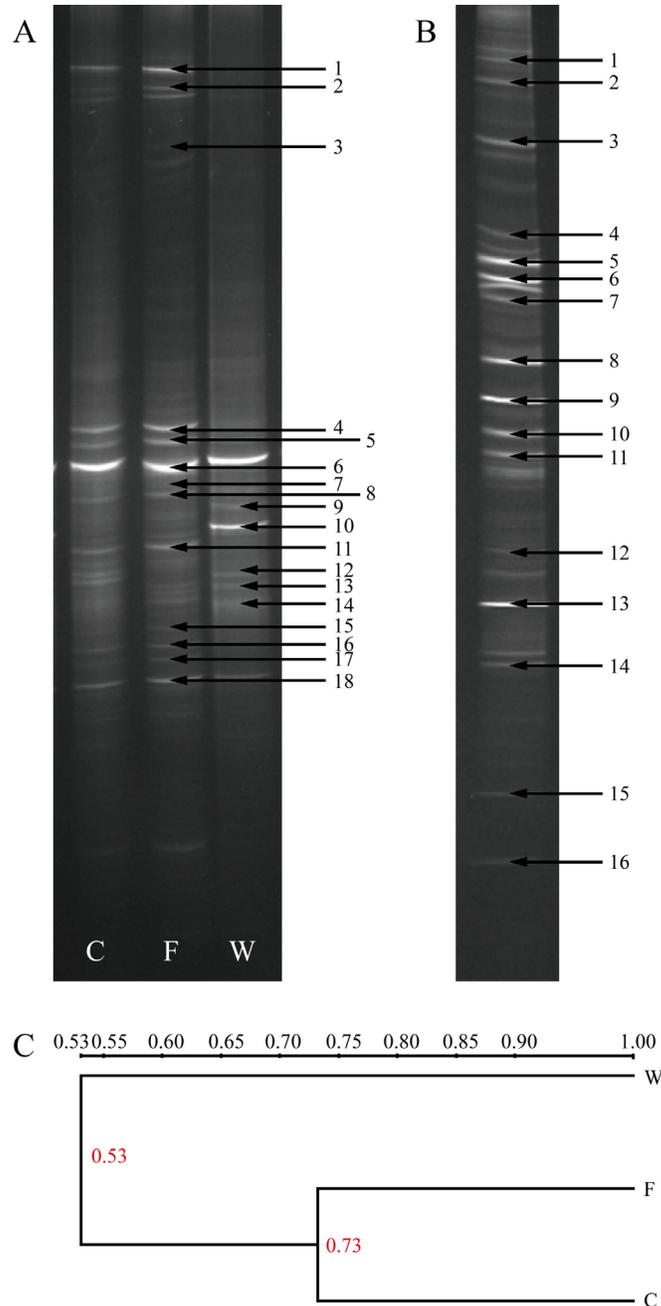


Figure 1. Denaturing gradient gel electrophoresis (DGGE) profiles. **A.** DGGE profiles of the V3 region amplified by PCR from DNA extracted from the gut samples of *Tsaitermes ampliceps* fed with different materials (C: corn stalk; F: filter paper; W: *Robinia pseudoacacia*); **B.** DGGE profiles of the V3 region amplified by PCR from the DNA extracted from the gut samples of *Mironasutitermes shangchengensis*; **C.** Hierarchical cluster analysis results of Figure 1A demonstrated graphically as an unweighted pair group with arithmetic mean dendrogram.

Table 1. Homologous sequence, identity, accession number in GenBank, and relative abundance of the major bands in *Tsaitermes ampliceps* from the 16S rRNA V3 DGGE shown as in Figure 1A.

Band No.	Homologous sequence in GenBank	Identity (%)	Relative abundance (%)		
			W	C	F
	Bacteroidetes		0	20.63	23.03
T.a 1	Uncultured Bacteroidetes bacterium clone Cf2-21(GQ502490.1)	93	0 ^A	10.51 ^B	8.79 ^B
T.a 2	Uncultured Bacteroidetes bacterium clone: RsStar413(AB522116.1)	93	0 ^A	5.45 ^B	4.24 ^B
T.a 3	Uncultured Bacteroidales bacterium clone: RsTu1-23(AB192206.1)	95	0 ^A	0 ^A	4.85 ^B
T.a 15	Uncultured Bacteroidetes bacterium clone CTF2-117(GU959229.1)	91	0 ^A	4.67 ^B	5.15 ^B
	Proteobacterium		52.54	35.8	44.55
T.a 4	Uncultured epsilon proteobacterium clone Rc238 (JQ617848.1)	94	0 ^A	11.28 ^B	8.48 ^B
T.a 5	Uncultured <i>Sulfurimonas</i> sp clone BFB083 (KC545729.1)	96	10.17 ^A	10.51 ^A	7.88 ^B
T.a 7	Uncultured <i>Escherichia</i> sp clone 7d14221 (GU132148.2)	95	0 ^A	0 ^A	5.76 ^B
T.a 8	Uncultured <i>Escherichia</i> sp (AM712054.1)	98	10.17 ^A	14.01 ^B	9.70 ^A
T.a 9	Uncultured alpha proteobacterium clone Rc282 (JQ617836.1)	95	11.44 ^A	0 ^B	9.09 ^A
T.a 10	Uncultured <i>Escherichia</i> sp (AM748793.1)	100	20.76 ^A	0 ^B	3.64 ^C
	Protozoa				
T.a 11	<i>Amblyospora</i> sp (U68474.1)	88	8.47 ^A	8.56 ^A	6.97 ^A
	Firmicutes		20.76	12.06	7.27
T.a 12	Uncultured Eubacteriaceae bacterium clone: Rs-N82 (AB088960.2)	93	8.05 ^A	4.28 ^B	3.03 ^B
T.a 14	<i>Bacillus</i> sp C271 (AB741803.1)	80	12.71 ^A	7.78 ^B	4.24 ^C
	Deferribacteres				
T.a 13	Uncultured Deferribacteraceae bacterium clone SL70 (JN680689.1)	88	9.32 ^A	0 ^B	5.45 ^A
	Spirochaetes		8.91	22.96	12.73
T.a 16	Uncultured Spirochaetes bacterium clone Rc342 (JQ617773.1)	100	0 ^A	5.06 ^B	5.15 ^B
T.a 17	Uncultured <i>Treponema</i> sp clone 290cost002-P3L-1298 (EF453910.2)	97	0 ^A	3.89 ^A	0 ^A
T.a 18	Uncultured <i>Treponema</i> sp clone rG16 (HQ187677.1)	96	8.91 ^A	14.01 ^B	7.58 ^A
T.a 6*	<i>Incisitermes snyderi</i> (GQ337717.1)	97	20 ^A	24.41 ^B	13.61 ^C

*The sequence belonged to *T. ampliceps*, so the relative abundance of each operational taxonomic unit was calculated without the data of T.a 6. The letters marked on the data indicate significance levels, where lowercase letters represent significance with $P < 0.05$ and uppercase letters represent very significant values with $P < 0.01$. W = *Robinia pseudoacacia*; C = corn stalk; F = filter paper.

Table 2. Homologous sequence, identity, accession number in GenBank, and relative abundance for the major bands in *Mironasutitermes shangchengensis* from the 16S rRNA V3 DGGE shown as in Figure 1B.

Band No.	Homologous sequence in GenBank	Identity (%)	Relative abundance (%)
	Proteobacterium		51.73
M.s 1	<i>Enterobacter</i> sp SBS1 (KC758848.1)	98	5.2
M.s 2	<i>Klebsiella oxytoca</i> strain A11n (KC513855.1)	98	4.46
M.s 4	<i>Enterobacter</i> sp NCCP-242 (AB665216.1)	98	4.21
M.s 5	<i>Enterobacter</i> sp Hb-0512 (GQ487560.1)	99	7.9
M.s 6	<i>Enterobacter</i> sp clone CD01013C12 (HM122394.1)	99	9.41
M.s 7	<i>Comamonas</i> sp UVS1 (AM503546.1)	98	6.44
M.s 8	Uncultured delta proteobacterium clone RsStar232 (AB522152.1)	95	8.91
M.s 14	Uncultured beta proteobacterium clone RsW02-065 (AB198538.1)	91	5.2
	Firmicutes		13.86
M.s 3	<i>Anoxybacillus flavithermus</i> strain CM (KC508690.1)	91	4.7
M.s 11	Uncultured Lachnospiraceae bacterium clone FS_46.41-1 (GU939256.1)	85	5.69
M.s 15	Uncultured <i>Lactococcus</i> sp clone RsStar323 (AB522148.1)	86	3.47
	Actinobacteria		
M.s 9	Uncultured Actinomycete clone MgMjR-011 (AB234517.1)	99	8.42
	Fibrobacteres		14.11
M.s 10	Uncultured Fibrobacteres bacterium Nd-Fib-2 (AB255951.1)	95	7.43
M.s 12	Uncultured Fibrobacteres clone Nt2-023 (AB255932.1)	93	6.68
	Spirochaetes		
M.s 13	Uncultured <i>Treponema</i> sp clone 290cost002-P3L-1298 (EF453910.2)	96	8.17
	Bacteroidetes		
M.s 16	Uncultured Bacteroidales clone RsW01-089 (AB198512.1)	94	3.71

16S rRNA gene clone library

A nearly full-length of 16S rRNA gene clone library was constructed from *T. ampliceps* guts consisting of 507 positive clones, which were randomly selected and tested by colony PCR. The positive rate was approximately 74.6%. All 378 positive clones were separated into 15 different hypotypes by DGGE, and almost the full-length of the 16S rRNA gene was sequenced and analyzed (Table 3). All sequences showed sequence identities of more than 92% with sequences that had been submitted to NCBI and GenBank accession numbers were assigned (Table 3). The sequences revealed a large number of microbial communities and that the clones of the sequences mainly belonged to Spirochaetes (28.07%), Firmicutes (26.46%), Proteobacteria (22.22%), and Protozoan symbionts (23.28%).

Table 3. Sequence alignment of 16S rRNA gene clone library of *T. ampliceps*.

Accession No.	Homologous sequence in Genbank	Identity (%)	Numbers of Clones	Relative abundance (%)
	Spirochaetes			28.04
KF145967	Uncultured Spirochaetes bacterium clone Rc342 (JQ617773.1) JQ617773.1	99	34	8.99
KF145972	Uncultured <i>Spirochaeta</i> sp gene clone: Rs-H09 (AB088910.1)	99	32	8.47
KF145977	Uncultured Spirochaetes bacterium clone Rc12 (JQ617747.1)	99	28	7.41
KF145981	Uncultured <i>Treponema</i> sp gene clone: Rs-P25 (AB088882.2)	97	12	3.17
	Proteobacteria			22.22
KF145968	Uncultured alpha Proteobacterium gene clone: RsStar117 (AB522149.1)	97	20	5.29
KF145969	Uncultured alpha Proteobacterium gene clone: RPK-49 (AB192282.1)	98	22	5.82
KF145973	Uncultured delta Proteobacterium gene clone: HsTph-09 (AB444077.1)	99	16	4.23
KF145978	Uncultured delta Proteobacterium clone Rc328 (JQ617846.1)	95	26	6.88
	Firmicutes			26.46
KF145970	Uncultured Clostridiales bacterium clone: MFeClo012 (AB702886.1)	90	34	8.99
KF145974	Uncultured Clostridiales bacterium clone: RsW01-035 (AB198470.1)	99	21	5.56
KF145975	Uncultured Eubacteriaceae bacterium clone: RPK-55 (AB192278.1)	99	16	4.23
KF145979	Uncultured Clostridiaceae bacterium clone MTG-3 (DQ307710.1)	92	21	5.56
KF145976	<i>Lactococcus</i> sp TSB11 (JX291542.1)	98	8	2.12
	Endosymbiont of Protozoa			23.28
KF145971	Uncultured endosymbiont of <i>Pyrrsonympha vertens</i> clone RfPv16 (AY572024.1)	95	46	12.17
KF145980	Uncultured endosymbiont of <i>Pyrrsonympha vertens</i> clone RfPv15 (AY572023.1)	95	42	11.11

Linking dietary patterns with gut microbial of termites

The food effect was examined by comparing the DGGE lanes from the 3 diet groups (Figure 1A). Table 1 shows that large changes occurred in the relative abundance of the 3 groups (W, C, and F), and statistical analyses showed that 2-week diet shifts significantly impacted microbiota composition. The results of Student *t*-tests in Table 1 showed that most of the differences in species between the gut microflora of termite feeding with wood diet and feeding with corn stalk diet reached very significant levels ($P < 0.01$), particularly within Bacteroidetes, Spirochaetes, and Firmicutes. In addition, we also calculated the diversity indices including Shannon-Wiener index and 3 β -diversity indices, including Sorenson's similarity index, Jaccard index, and Cody index (Table 4). Although the rate of community species change was low ($C_j \leq 4.5$), the similarity indices between 2 of the 3 groups were also low (51.5-61.8, 43.8-66.7). Moreover, the Shannon-Wiener index increased progressively (2.15, 2.40, and 2.72) with the 3 groups. Wood-fed workers showed 40% lower community diversity than cellulose-fed workers of *Centrocestus formosanus* fed for 30 days (Tanaka et al., 2006), while our results suggest that this value is 43.75%. Additionally, in the case of worker termites feeding on cellulose, the number of some protozoa decreased when compared to those feeding on wood and wood powder. According to Table 1, the protozoan

symbionts also reduced. The results of the indexes ($P < 0.01$) suggested consistently differences in gut bacterial community composition as a result of diet.

The sequence of T.a 6 (Table 1) belonged to *T. ampliceps*. As shown in Figure 2, we determined that the surface of the peaks of T.a 6 (Table 1) for the 3 groups were approximately the same size. This indicates that the total number of microorganisms was the lowest in the gut of group fed with corn stalk with T.a 6 at 24.41%, while group fed with filter paper showed the highest numbers of microorganism with band 6 at 13.61%.

Table 4. Diversity indices of the 3 groups of *Tsaitermes ampliceps* fed with different diets.

		Numbers of phylotypes	Shannon-Wiener index	Sorenson's			Jaccard index (%)			Cody index		
				W	C	F	W	C	F	W	C	F
<i>T. ampliceps</i>	W	9	2.15	100.0			100.0					
	C	12	2.40	61.8	100.0		43.8	100.0		4.5	0	
	F	16	2.72	51.5	61.1	100.0	58.8	66.7	100.0	3.5	3	0

These indexes were calculated using the following equation: Shannon - Wiener index (H') = $-\sum_{i=1}^s (P_i)(\ln P_i)$ where s is the number of species in the sample and P_i is the proportion of species i in the sample. P_i could be calculated by the relative surface of the peak in the DGGE profile ($P_i = N_i/N$, where N_i is the surface of the peak, and N is the sum of the surfaces for all peaks within the lane), Sorenson's similarity index (SI) = $2(na \ nb)/(na + nb)$; Jaccard index (C_j) = $(na \ nb)/(na + nb)$; Cody index (β_c) = $(na + nb - 2 \ na \ nb)/2$, where na and nb are numbers of bands in different lanes. W = *Robinia pseudoacacia*; C = corn stalk; F = filter paper.

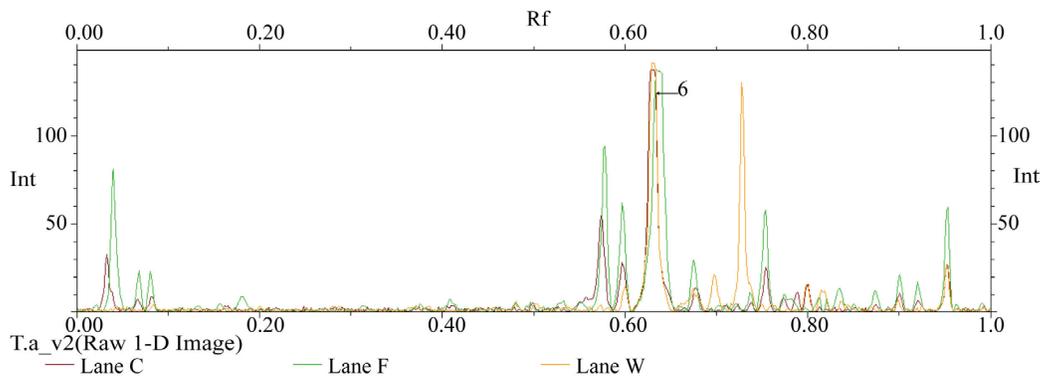


Figure 2. Lanes compared using the Quantity One software (Bio-Rad) from Figure 1A. Lane C with the red line represents the group fed with corn stalk; lane F with the green line represents the group fed with filter paper, and lane W is the group fed with *Robinia pseudoacacia*.

Comparing diversity of termite gut microbiota

Table 5 shows the phylotypes within the 3 major phyla (Spirochaetes, Firmicutes, and Proteobacteria) and others detected in the termite gut. The number and relative abundance of phylotypes within Proteobacteria was the largest among the 6 phyla in *M. shangchengensis*, while the number of phylotypes within Firmicutes and Proteobacteria in *T. ampliceps* were higher than in others. Higher termites not only shared the same 3 major phyla with *T. ampliceps*, but also showed the same phylotypes within Bacteroidetes, Actinobacteria, and Fibrobacteres. However, the phylum of protozoan symbionts was observed in large numbers in lower termites. In addition, the Shannon-

Wiener index showed the highest diversity of gut microbial communities in *M. shangchengensis*, with an index value of 2.72. These data indicate remarkably abundant protozoan symbionts in the gut of lower termites, and higher termites showed more complex and diversity for the intestinal symbiont environment. The phylotypes of the sequences showed only a few differences between the 2 sets of *T. ampliceps*, but the number and the relative abundance of the phylotypes and the diversity index were not similar. The number of phylotypes in the clone library was much larger than that of sequences determined by DGGE; the relative abundance of Spirochaetes and Proteobacteria of the 3 major phyla showed large changes. This may be attributable because of the method used for testing: both methods inevitably lost a few of phylotypes; however, DGGE had the advantages of reflecting diversity of the intestinal flora, while 16S rRNA library analyses were used to obtain full-length sequences of the 16S rRNA gene to identify the species present.

Table 5. Phylotypes and relative abundance of gut microbe in termite colonies.

	<i>M. shangchengensis</i>		<i>T. ampliceps</i>		<i>T. ampliceps</i>	
	No. of phylotypes	Relative abundance (%)	No. of phylotypes	Relative abundance (%)	No. of phylotypes	Relative abundance (%)
Firmicutes	3	13.86	2	20.76	5	26.46
Proteobacteria	8	51.73	4	52.54	4	22.22
Spirochaetes	1	8.17	1	8.90	4	28.04
Bacteroidetes	1	3.71				
Actinobacteria	1	8.42				
Fibrobacteres	2	14.11				
Deferribacteres			1	9.32		
Protozoa			1	8.48	2	23.28
Total	6/6/16	100.00	4/5/11	100.00	3/4/15	100.00
Shannon-Wiener index	2.72		2.15		2.62	

**T. ampliceps*: *Tsaitermes ampliceps*; *M. shangchengensis*: *Mironasutitermes shangchengensis*.

DISCUSSION

In this study, we found that higher termites *M. shangchengensis* had a more complex and diversity intestinal environment than did lower termites *T. ampliceps*. The 2-week diet shifts significantly impacted microbiota composition, including both the number and the relative abundance of the phylotypes.

Analyzed sequences were used to construct a phylogenetic tree (Figure 3). Most sequences were very close to each other in the tree and were gathered into groups, which respectively represented Spirochaetes, Bacteroidetes, Proteobacteria, and others. Members of 7 bacterial phyla were detected in the microhabitat of the 2 types of termite hindgut lumen, with more than 76% belonging to Spirochaetes, Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Fibrobacteres, and Deferribacteres. For some protozoa found in *T. ampliceps*, the sequence similarity of results obtained by DGGE and the clone library were lower, which may have resulted from limitations of the method itself.

Previous studies identified Spirochaetes as a major component of the lower termite hindgut microbiome (Hongoh, 2003; Warnecke et al., 2007; Husseneder et al., 2010). They exist in the hindgut and are recognized as host-specific ectosymbionts of various protozoans inhabiting the termite hindgut (Iida et al., 2000; Ohkuma, 2008). These organisms play an important role in encoding for an array of hydrolytic enzymes involved in cellulose digestion (Warnecke et al., 2007) and have CO₂-reducing acetogenesis activity (Leadbetter and Breznak, 1996). Studies also have

shown that select ectosymbiotic spirochetes associated with the protist *Mixotricha paradoxa* provide motility (Cleveland and Grimstone, 1964). This conclusion has been used as a strong evidence of the endosymbiosis hypothesis. In this study, we not only found both *Treponema*-related and *Spirochetes*-related phlotypes, but also obtained a sequence marked as T.a 17, which showed 90% sequence similarity to the existing rod-shaped symbiont of *Mixotricha paradoxa* (AJ488195.1) in GenBank. This organism was present in both *T. ampliceps* and *M. shangchengensis*, and increased to 22.96% in *T. ampliceps* after the diet change. Thus, Spirochaetes play an important role in degrading lignocellulose, particularly cellulose.

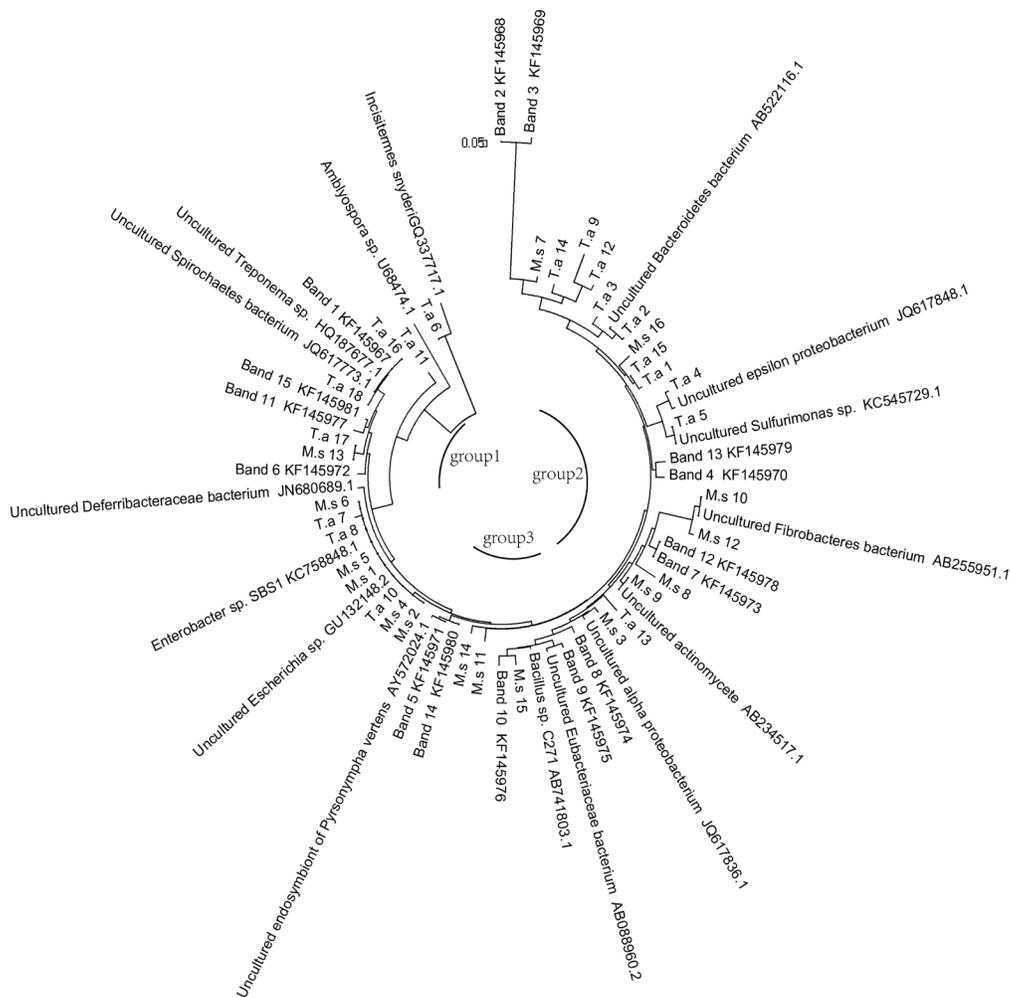


Figure 3. Dendrogram of 16S rRNA gene or V3 region of 16S rRNA gene sequences showing the phylogenetic affiliation of intestinal bacteria in *Tsaiterms ampliceps* and *Mironasutiterms shangchengensis*. A neighbor-joining tree was constructed from all gene sequences obtained in this study of each phylotype and from sequences retrieved from the GenBank database. T.a represents the sequence acquired from *T. ampliceps* by DGGE, while M.s represents the sequence from *M. shangchengensis*. Groups 1, 2, and 3 represent Spirochaetes, Bacteroidetes, and Proteobacteria, respectively. Other details are the same as in the tables above.

The phylum Firmicutes contained members that could be annotated to the orders Clostridiales and Bacillales. Several phylotypes within the Clostridiales group were annotated to the *Lachnospiraceae*, *Clostridiales*, and *Eubacteriaceae* species, known for their ability to digest cellulose. *Clostridiales* have been reported to be affiliated with the intestinal lining of the hindgut (Yang et al., 2005), and thus may participate in digestion on the intestinal lining of the termite hindgut. Members of Bacillales belonged to *Lactococcus*, *Bacillus*, and *Anoxybacillus flavithermus*. *Lactococcus* produces α -galactosidases and likely plays a role in digesting both galactose-containing oligosaccharides and polymeric galacto(gluco)mannans in hemicelluloses (Boucher et al., 2003). *A. flavithermus* was able to utilize a wide range of carbon sources, such as glucose, xylose, arabinose, starch, maltose, and sorbitol, and anaerobically fermented ethanol as a minor product (Peng et al., 2008).

Bacteroidetes are both ecto- and endosymbionts of hindgut protozoans in lower termites (Noda et al., 2006). However, we discovered Bacteroidetes in higher termites of *M. shangchengensis*. Bacteroidetes may function without hindgut protozoans in higher termites. Moreover, in the lower termites of *T. ampliceps* fed with filter paper and corn stalk, more Bacteroidetes were observed (>23%) than in termites fed on wood. Thus, celluloses rather than lignin was the substrate on which Bacteroidetes acted.

In this study, the Fibrobacteres was separated phylogenetically from only *M. shangchengensis*. This poorly defined phylum includes species involved in cellulose digestion in ruminant animals (Brumm et al., 2011). Fibrobacteres associated with *R. flavipes* represent novel members affiliated with subphylum 2 (Hongoh et al., 2005, 2006). Warnecke et al. (2007) and Engelbrektson et al. (2010) supported these findings by showing that Fibrobacteres was the second-most abundant bacterial group in *Nasutitermes*.

Proteobacteria contained members annotated to the α , β , γ , δ , and ϵ sections, which were ubiquitous inhabitants of insect digestive tracts with the relative abundances of 52.54, 35.8, 44.55, 22.22, and 51.73%. Jiao et al. (2006) revealed a Proteobacteria rhodopsin, which converted light energy into a chemical potential gradient of protons inside and outside the cell membrane for the synthesis of ATP. Eight groups of Proteobacteria with nitrogenase activity were isolated from *Oryza rufipogon* (Tan et al., 2009). Furthermore, the bacterial nitrification gene (*hao*) was obtained from the α , β , γ -Proteobacteria. Thus, Proteobacteria has a wide range of functions in photosynthesis, bacteriolysis, nitrification, and azotification, which may be significant for balancing energy and the milieu interne in the gut of termites.

Previous studies found that a large number of symbiotic protozoa, such as flagellates, settled in the posterior intestine in some lower termites. A study on *Reticulitermes chinensis* Snyder found 70% more symbiotic Oxymonad flagellates (Chen et al., 2011). An American study listed the intestinal symbiotic types of giardia in 205 kinds of wood-feeding lower termites and found 430 types of symbiotic protozoa that play an important role in fiber depolymerization and fermentation. We also found a high abundance of protozoan symbionts in the intestinal tract of *T. ampliceps*, indicating that protozoa plays important roles in the decomposition, digestion, and metabolism of diets in *T. ampliceps*.

By comparing microbiota composition after the 2-week feeding of diets containing different lignocellulose compositions, we found that changes in food composition affected the relative abundance and diversity of enteric microorganisms. When fed with wood with a higher content of lignin, Bacteroidetes and Spirochaetes showed the lowest number and relative abundance, indicating that these phyla consume celluloses rather than lignin; Deferribacteres, Firmicutes, and Protozoa were most abundant in the group fed with wood, suggesting that they mainly act on lignin

rather than celluloses. Proteobacteria retained the dominant position because of its wide range of functions in energy production and gut environment balance.

Furthermore, Spirochaetes and protozoa were the main groups that digest lignocellulose in *T. ampliceps*, and they decompose and ferment cellulose into acetic acid, CO₂, and H₂; next, the *Treponema*-related and *Spirochetes*-related phylotypes deoxidate CO₂ into acetic acid. Hence, termites are provided with the nutrition and energy to survive (Warnecke et al., 2007; Brune, 2014). Proteobacteria also play vital function in supporting termite survival and metabolism. There were nearly no protozoa in *M. shangchengensis* that decomposed cellulose. The main sets of microbiological compositions in the gut included Spirochaetes, Bacteroidetes, Proteobacteria, Fibrobacteres, and Actinobacteria. *M. shangchengensis*, which show intricate anatomical features, mainly depend on its own digestive juice secreted to decompose lignocellulose. The diets were chewed up into granules and delivered into the P3 region during preliminary digestion. The *Spirochete* and *Fibrobacteres* species in the P3 region decomposed non-starch polysaccharides, and then produced intermediates. Next, O₂, H₂, and N₂ can be transformed into acetic acid and microbial proteins by H₂ metabolism, CO₂-reductive acetogenesis, and N₂ fixation (Warnecke et al., 2007). This indicates that the mechanisms of lignocellulose degradation in the 2 types of termites were quite different.

In conclusion, the results of this study showed that the higher termite *M. shangchengensis* constituted 25% of the phylotypes, which was higher than the lower termite *T. ampliceps*. Without symbiotic protozoa, higher termites depended on a more complicated survival mechanism of lignocellulose degradation than did *T. ampliceps*. Significant impacts on the microbiota composition in the groups of *T. ampliceps* following 2-week diet shifts were observed. Our results showed that Bacteroidetes and Spirochaetes acted on celluloses, while Firmicutes was responsible for lignin degradation. Additionally, Proteobacteria was consistently involved in energy production and balancing of the gut environment. Bacteroidetes may function without hindgut protozoans in higher termites.

Conflicts of interest

The authors declare no conflict of interest.

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