

## Phylogenetic Diversity of Bacterial Symbionts in the Guts of Wood-Feeding Termites

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### ABSTRACT

The diversity of bacterial symbionts in termite guts was investigated by 16S rDNA analyses and terminal restriction fragment length polymorphism (T-RFLP). Bacterial DNAs from guts of two wood-feeding termites, consisting of a lower termite, *Reticulitermes speratus* and a higher termite, *Microcerotermes* sp. were extracted and 16S rRNA genes were amplified by PCR using several sets of bacterial specific primers. PCR products were then cloned, sequenced and analyzed for T-RFLP. Two thousands one hundred and eighty four clones from *R. speratus* and 288 clones from *Microcerotermes* sp. were analyzed and sorted into 320 and 141 phylotypes, respectively. Most of them were spirochetes, which were found more than half of the sequenced clones and detected T-RFs, while the second-dominant groups were Clostridia and Bacteroides. Interestingly, over 90% of the phylotypes obtained in this study were found at the first time, and several termite-specific lineages, including a novel bacterial division, Termite Group I, which are as-yet unculturable bacteria, were revealed. These results indicated that termite gut is really a great reservoir of new bacterial species, and that the termite gut is still a new frontier to microbiologists.

**Key words:** diversity, gut bacterial symbionts, *Reticulitermes speratus*, *Microcerotermes*

### INTRODUCTION

Termites harbor an abundance and diversity of symbiotic gut bacteria. To date, a number of bacterial strains have been isolated from termite guts, and a part of them were characterized as decomposers of ligno-cellulose, uric acid, and other aromatic compounds, and/or nitrogen-fixers, and/or H<sub>2</sub>/CO<sub>2</sub>-acetogens (Breznak, 2000). However, the direct microscopic counting indicated that only 10% of bacterial population can be cultured from termite guts, meaning that most of the constituents are as-yet unculturable and we do not even know which kinds of bacteria are

contained. The basic information of bacteria from gut such as diversity, community structure, phylogenetic affiliation is needed not only for further understanding of the termite-gut microbe symbiosis, but also for applied studies of the bacteria inside the termite gut.

In this study, we conducted culture-independent studies using termites from Japan and Thailand, to obtain those basic information. We reported on an enormous diversity and novel phylotypes of bacteria by molecular taxonomy using 16S rDNA sequences analysis and terminal restriction length polymorphism (T-RFLP).

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## MATERIALS AND METHODS

### Sample collection and DNA extraction

Wood-feeding lower termites, *Reticulitermes speratus* (family Rhinotermitidae), were collected at Ogose, Saitama prefecture, Japan. The higher termites, *Microcerotermes* sp. (family Termitidae, subfamily Termitinae), were collected from various places in Thailand. The whole gut of 20 termites from each collected sample were drawn out, and DNAs were extracted using the combination of ISOPLANT II (Nippon Gene) and DNeasy Tissue Kit (QIAGEN).

### PCR and preparation of clone libraries

16S rDNAs were amplified from the extracted DNA by PCR using *Ex-Taq* polymerase (Takara) and bacterial specific primers, 27F, 39F, 41F, 63F, 64F, 1389R and 1492R (Table 1). PCR was performed using a PTC-200 Thermal Cycler (MJ Research) and the program: 95°C for 2 min, followed by 12, 18 or 25 cycles of denaturation at 95°C for 30 sec, annealing (at 45, 50 or 55°C for 1 min) and extension at 72°C for 4 min with a final extension at 72°C for 10 min. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), followed by ethanol precipitation. The products were cloned into pCR-4 TOPO TA

cloning vectors (Invitrogen) and plated out onto LB agar medium containing 50 µg/ml ampicillin.

### 16S rDNA sequencing and phylogenetic analysis

Ninety-six clones were randomly chosen from each clone library for analysis. The 16S rDNA clones were sequenced with the primers 533F or 908R (Table 1) using the Big-Dye terminator cycle sequencing kit (Applied-Biosystems) and ABI 310 or 377 genetic analyzers (Applied-Biosystems). The phylogenetic position of the sequences were, first, approximately inferred using BLAST 2.0. Those with more than 98% identity were grouped into the same phylotypes. In case of *R. speratus*, full length of 16S rDNA of the representative clones of all the phylotypes were sequenced and sorted again with the criterion of 97% sequence identity. All of the sequences were subjected to Check-Chimera Program on the Ribosomal Database Project (RDP) web site, for elimination of chimeric sequences. The sequences were aligned with reference sequences retrieved from databases, using Clustal X 1.8 (Thompson *et al.*, 1994). Neighbor-joining trees were constructed using the software MEGA V 2.0 (Kumar *et al.*, 2001) based on the distance calculated with Juke-Cantor Model. Sites containing gaps were excluded from the analysis.

**Table 1** Bacteria-universal primers for PCR and sequencing.

Primer	Sequence
27F	5'-AGAGTTTGATCMTGGCTCAG-3'
39F	5'-TGGCTCAGRWYGAACGCTRG-3'
41F	5'-GCTCAGATTGAACGCTGGCG-3'
63F	5'-CAGGCCTAACACATGCAAGTC-3'
64F	5'-BGYCTWANRCATGCAAGTYG-3'
533F	5'-TGCCAGCAGCCGCGGTAA-3'
908R	5'-CGTCAATTCMTTTGAGTT-3'
1389R	5'-ACGGGCGGTGTGTACAAG-3'
1492R	5'-GGTTACCTTGTTACGACTT-3'

### T-RFLP analysis

PCR was performed using fluorescent dye-labeled primers, 6-FAM-27F and HEX-1389R, under the same conditions as described above with 50°C for annealing and 25 cycles. The PCR products were purified using QIAquick Gel Extraction Kit (Qiagen), and digested using *Hha* I (Takara). Digested DNA fragments were electrophoresed with formamide and the ROX-500 internal size standard (Applied-Biosystems) using ABI 377 or ABI 310 genetic analyzer. Data were analyzed using GeneScan Analysis 3.1.2 software (Applied-Biosystems).

## RESULTS AND DISCUSSIONS

### Diversity and phylogeny of gut bacteria

Two thousands one hundred eighty four clones from *R. speratus* and 288 clones from *Microcerotermes* sp. were analyzed and sorted into 320 and 141 phylotypes, respectively (Figure 1). The most diverse group in *R. speratus* gut was *Clostridia*, consisted of the genera *Clostridium* and *Eubacterium*. Spirochetes were also diverse, mainly comprised of the genera *Treponema*. The third-most diverse group was bacteroides-related bacteria in the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group. In *Microcerotermes* sp. these bacteria were also found with diversity, but the most diverse group was spirochetes, consisted mainly of *Treponema*. *Treponema* have been recovered from the other various termite species, and most of them are reported to form two phylogenetic clusters, ‘Termite *Treponema* cluster I and II’ (Lilburn *et al.*, 1999; Iida *et al.*, 2000). In this study, the *Treponema* phylotypes found from *R. speratus* were also affiliated with the two clusters, whereas those from *Microcerotermes* sp. were affiliated only to the cluster I.

It is noteworthy that we found some deep-branched lineages only distantly related to known sequences in databases. The “unknown cluster A” (Figure 1A) were only ambiguously related to the

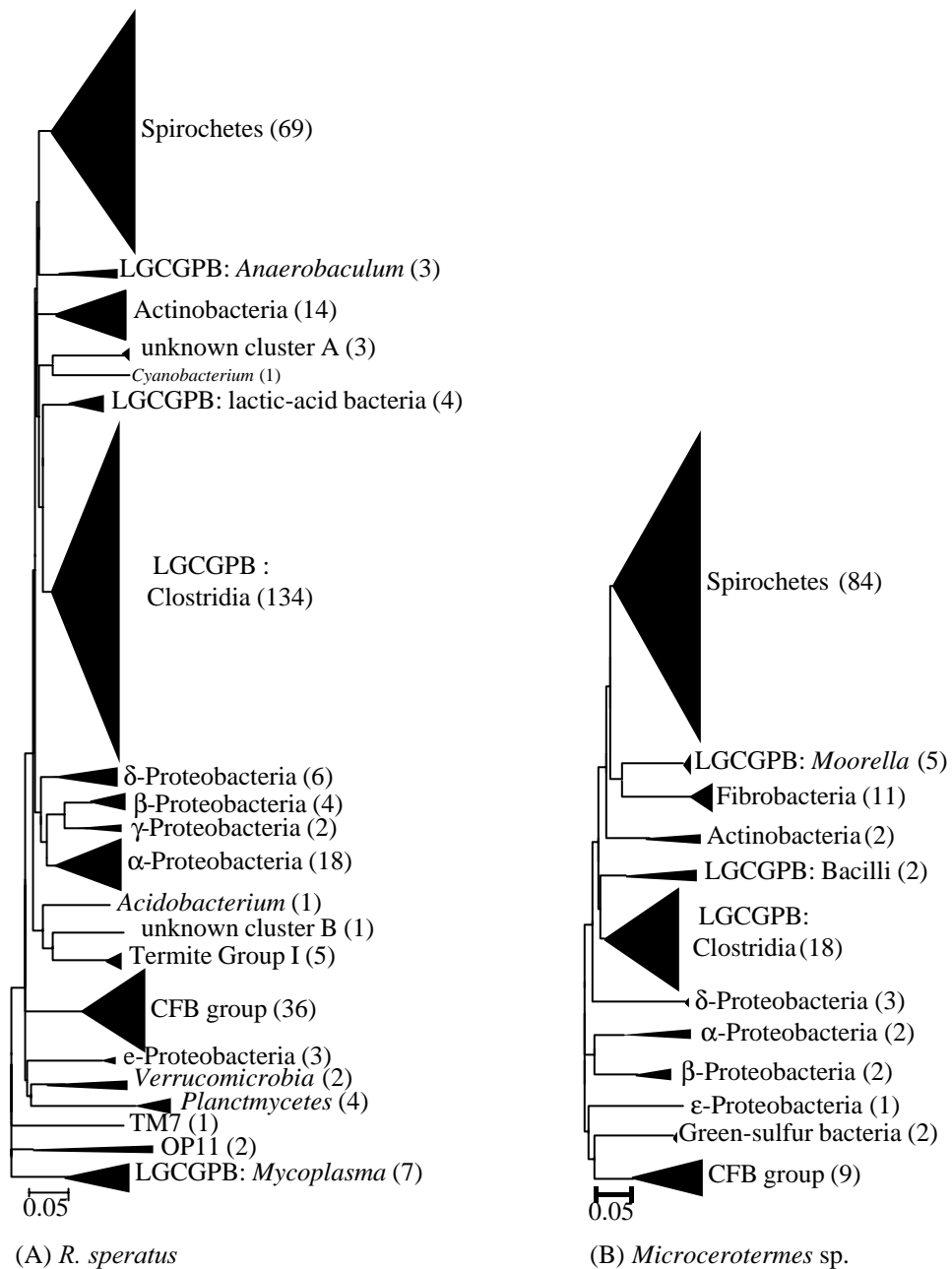
low GC Gram-positives, and showed not more than 82% sequence identity to any known sequences. The “unknown cluster B” (Figure 1A) was found only one clone, and less related to another newly found cluster, “Termite Group I”. This candidate new division Termite Group I was consisted of the termite-derived cluster and other 4 sequences from cow rumen, sludge, and wine vinasse. Whereas the monophyly of this group was significantly supported by the bootstrap confidence value more than 97, the sequence identity between the termite-derived cluster and the sequences from other environments was below 82%.

Since sequences almost identical to the Termite Group I sequences found in this study were also amplified from the gut of *Reticulitermes* sp., a new termite species found in Thailand (data not shown), it is possible that this group is not allochthonous, but closely related to the gut environment of, at least, *Reticulitermes* termites. The “termite-specific” lineages were also found within the other known bacterial groups, like Spirochetes, Clostridia, Bacteroides, and *Mycoplasma*. However, since there are only a small amount of sequence data from the other termite species, it is impossible to examine co-evolutionary relationship between termites and their gut bacteria at this point.

It should also be noted that more than 90% of obtained phylotypes in this study were found at the first time. Only several phylotypes shared more than 97% sequence identity to known described species. It indicates that termite gut is a great reservoir of new species of bacteria, and we expected that the information revealed in this study prompt further attempts for culture and characterization of these as-yet unculturable bacteria.

### Bacterial community structure in termite guts

To compare the community structure of bacteria from the guts of both termite species, T-RFLP analyses were conducted (Figure 2).



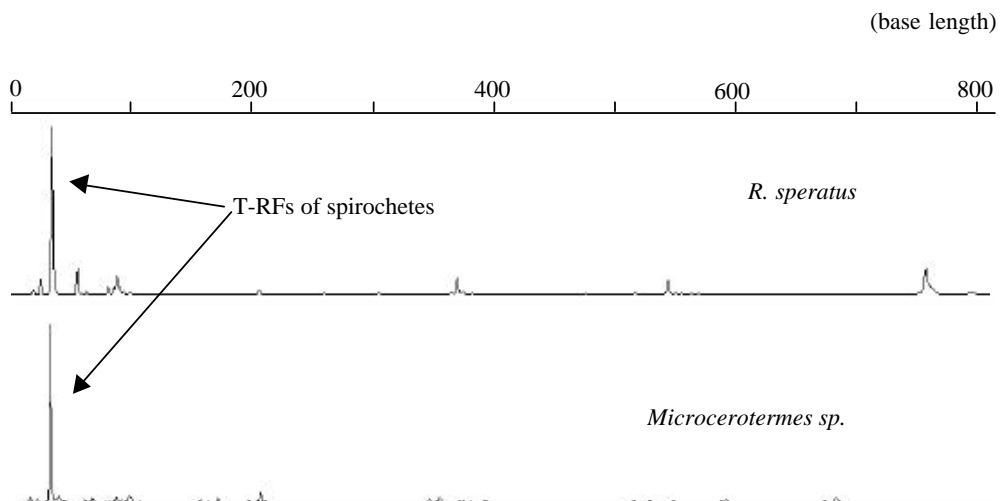
**Figure 1** 16S rDNA phylotypes found in this study. (A) 320 phylotypes from the gut of *R. speratus*. (B) 141 phylotypes from the gut of *Microcerotermes* sp. The region 64-1388 and 228-732 corresponding to the position in *E. coli* (J01695) were used for the reconstruction of tree (A) and (B), respectively. Numbers in parentheses indicate the numbers of phylotypes. Note the “Termite Group I” in (A) is the candidate novel division of as-yet unculturable bacteria. LGCGPB: Low G+C Gram positive bacteria; CFB group: *Cytophaga-Flavobacterium-Bacteroides* group.

Interestingly, the T-RFLP profiles were very similar between the two distantly related termite species, with the prominent peak at the calculated size of T-RFs derived exclusively from most of the spirochetal sequences.

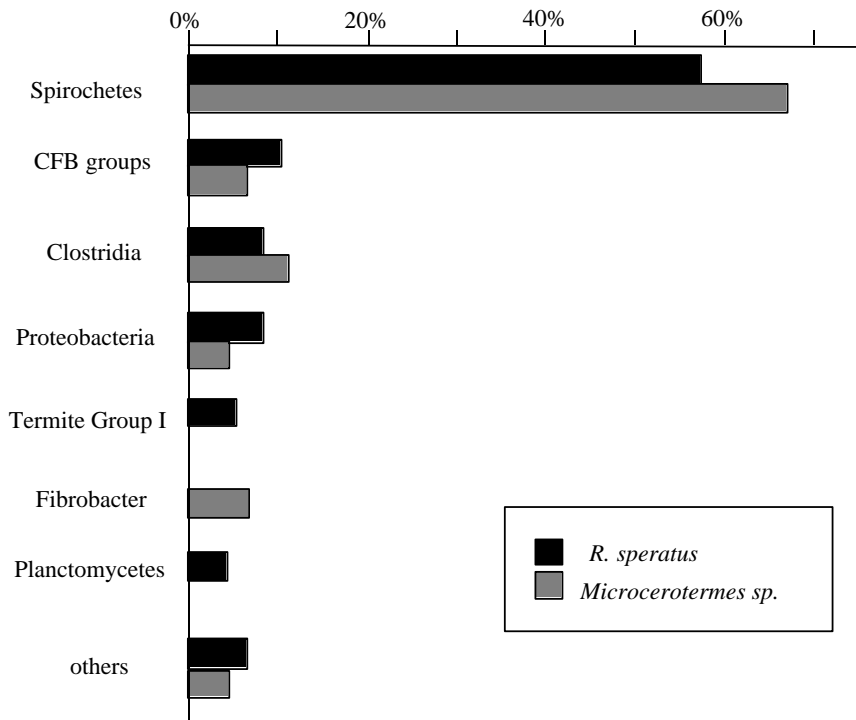
The predominance of spirochetes suggested by the T-RFLP analysis was also supported by the analysis of clone libraries (Figure 3). When the clone libraries prepared under the same PCR condition were compared between *R. speratus* and *Microcerotermes* sp., it was found that spirochetes predominated the clone libraries, accounting for 57% and 67%, respectively. The proportion of clostridial and bacteroides clones were also similar between the two termite species. The Termite Group I were only found from *R. speratus*, and accounted for 5-10% of sequenced clones. On the other hand, fibrobacterial clones were only found from *Microcerotermes* sp.

The ecological and physiological meanings of the resemblance of the gut bacterial community structure between these very distantly related termites, are difficult to be discussed only from these data. However, the importance of spirochetes

in the host termite nutrition has been suggested from the mortality of the wood-feeding termites *Nasutitermes exitiosus* (Termitidae, Nasutitermitinae) and *Coptotermes lacteus* (Rhinotermitidae) (Eutick *et al.*, 1978) and also from the characterization of *Treponema* sp. ZAS-1 and ZAS-2 from the gut of the wood-feeding termites *Zootermopsis angusticollis* (Hodotermopsidae) (Leadbetter *et al.*, 1999; Lilburn *et al.*, 2001). The *Treponema* isolates can fix atmospheric nitrogen and produce acetic acid from hydrogen and carbon dioxide. These two microbial properties are thought to be essential to the nutrition of wood-feeding termites, because wood materials generally contain relatively small amount of nitrogenous compounds. Considerably proportion of cellulosic materials ingested by termites are anaerobically fermented, finally, into hydrogen and carbon dioxide in the hindgut, which are harmful to termite and cause energy wasting if they are not recycled efficiently. Therefore, the predominance of spirochetes might be attributed to the wood-feeding habit of the two termite species.



**Figure 2** T-RFLP profiles based on *Hha* I digestion of 16S rDNA. *Hha* I digestion produce T-RFs at the identical size (38 base length) exclusively from most of spirochetal sequences. There was no peak detected over 800 base length. The reproducibility was confirmed from replicated analyses.



**Figure 3** The bacterial community structure in the gut of *R. speratus* and *Microcerotermes sp.* Ninety-six clones for *R. speratus* and 288 clones for *Microcerotermes sp.* were used for comparison. Those clones were randomly picked up from the clone libraries prepared from PCR using the primer set 27F-1389R with 50°C for annealing and only 12 cycles.

#### ACKNOWLEDGEMENTS

We thank Dr. Tetsushi Inoue for helpful suggestions on termite ecology, and thank Dr. Shigeharu Moriya, Dr. Toru Johjima, and Dr. Moriya Ohkuma for advice on ecology of termite gut microbes. This work was partially supported by grant from Public-Private Technology Development and Transfer Center, Kasetsart University.

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