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Full Length Research Paper

Phylogenetic analysis of the gut bacterial microflora of the fungus-growing termite *Macrotermes barneyi*

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Termites are an extremely successful group of wood-degrading organisms and are therefore important both for their roles in carbon turnover in the environment and as potential sources of biochemical catalysts for efforts aimed at converting wood into biofuels. To contribute to the evolutional study of termite digestive symbiosis, a bacterial 16S rRNA gene clone library from the gut microbial community of the fungus-growing termite *Macrotermes barneyi* was constructed. After screening by restriction fragment length polymorphism (RFLP) analysis, 25 out of 105 clones with unique RFLP patters were sequenced and phylogenetically analyzed. Many of the clones (95%) were derived from three phyla within the domain bacteria: *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. In addition, a few clones derived from *Deferribacteres*, *Actinobacteria* and *Planctomycetes* were also found. No one clone affiliated with the phylum *Spirochaetes* was identified, in contrast to the case of wood-feeding termites. The phylogenetic analysis revealed that nearly half of the representative clones (11 phylotypes) formed monophyletic clusters with clones obtained from other termite species, especially with the sequences retrieved from fungus-growing termites. These results indicate that the presence of termite-specific bacterial lineages implies a coevolutional relationship of gut microbes and host termites. The remaining 14 clones formed a cluster, and there was very low sequence similarity (30 to 40%) to known 16S rRNA sequences. The 16S rRNA gene sequence data showed that the majority of the intestinal microflora of *M. barneyi* consisted of new, uncultured species previously unknown to microbiologists.

Key words: Termite, bacteria, symbiosis, restriction, fragment, length, polymorphism.

INTRODUCTION

Termites are predominant terrestrial social insects living from temperate to tropical regions in great enrichment. They are major decomposers which are able to degrade plant matter efficiently, and greatly contribute to the global carbon cycle (Freymann et al., 2008; DeSouza et al., 2009). There are three feeding groups of termites; wood-feeders, fungus-growers and soil-feeders (Donovan et al., 2001), each with its own degradation process, (Kudo, 2009).

Termites harbor diverse and unique microbial populations (protozoa, fungi, bacteria and archaea) in their hindgut, most of which are unique to the termite gut ecosystem. These microorganisms form a complex community, with densities reaching up to 10^{11} cells/mL (Ohkuma, 2003; Ohkuma and Brune, 2011). Termites largely depend on the gut microbial symbionts for the digestion and utilization of their food, especially highly recalcitrant lignocellulose (Ohkuma and Brune, 2011). The microbial symbionts in termite guts play an important role in lignocellulose digestion, termite nutrition and gas production, and this mutualism is considered as a model

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of symbiotic association between animals and microorganisms (Breznak and Brune, 1994).

Nearly 3,000 species of termites have been described (http://vsites.unb.br/ib/zoo/catalog.html) and they are conventionally classified into lower and higher termites. Termites possess a dual cellulolytic system: in lower termites the cellulases are contributed by both the insect and its gut flagellates, whereas in higher termites, host cellulases and hindgut bacteria participate in fiber digestion (Kudo, 2009). Some higher termites have been observed to secrete their own cellulases and ligninase, including endo-1,4-β-glucanase,β-glucosidase and laccase, in their salivary glands or gut to degrade recalcitrant lignocellulose (Tokuda and Watanabe, 2007; Watanabe and Tokuda, 2010; Coy et al., 2010). Although termites secrete their own digestive enzymes, the digestion of recalcitrant foods largely depends on the diversity of their gut microorganisms. Hence, to elucidate the mechanism of how the termites can survive on such recalcitrant and poor-quality foods, detailed investigation of the gut microbial ecosystem is essential. The gut microbiota (microbial community) comprises all the three domains of life, eukaryotes (protists), bacteria, and Archaea. In lower termites, an abundance of flagellated protists fill up the dilated portion, or paunch, of the hindgut, while most of the higher termites harbor only a small number of gut protists. Bacteria and archaea reside in the gut of both lower and higher termites. In general, termites harbor several hundred or more bacterial species, most of which are found exclusively in termite guts (Hongoh, 2011). An obstacle in the study of these gut microorganisms is that the majority are as yet unculturable. Besides, the microbiota is too complex to manipulate experimentally. Therefore, conventional microbiological methods are less effective at clarifying the detailed symbiotic mechanism in the termite gut ecosystem.

In the past decade, culture-independent molecular approaches using small-subunit rRNA genes have enhanced our ability to assess naturally occurring microbial diversity. Such approaches have been applied to the analysis of the termite-gut microbial community, and have demonstrated that the majority of the gut community consists of phylogenetically various species that are yet-uncharacterized and thus unknown to microbiologists (Kudo et al., 1998; Ohkuma, 2002). Most previous studies of the bacterial community in the gut of termites focused on the family Rhinotermitidae of lower termites (Hongoh, 2003; Shinzato et al., 2005; Fisher et al., 2007), Recently microbial communities of higher termite have been investigated by rRNA gene-based molecular techniques, the knowledge of this symbiosis has been expanded considerably (Hongoh et al., 2006b; Shinzato et al., 2007).

Although the gut symbiosis of termites has long intriguing researchers of both basic and applied sciences, the complexity and formidable unculturability of the gut microbiota has hampered the clarification of the molecular mechanism of this symbiotic system. In the effort to overcome the difficulty, recent advances in omics, such as metagenomics, metatranscriptomics, and metaproteomics have gradually unveiled the black box of this symbiotic system. Metagenome analysis (Warnecke et al., 2007; Mattéotti et al., 2011; Chandrasekharaiah et al., 2011; Liu et al., 2011) of the bacterial gut microbiota of a wood-feeding higher termite and metatranscriptome analyses (Warnecke et al., 2007; Yuki et al., 2008; Tartar et al., 2009; Burnum et al., 2011) of the protistan gut microbiota have revealed the presence of diverse glycoside hydrolase genes in both the bacterial and protistan microbiota. In the previous analysis, bacterial genes required for fermentation, reductive acetogenesis, and nitrogen fixation were also identified. These functions have been recognized as essential bacterial activities in this symbiotic system, by the long-term efforts in cultivation of the fastidious microorganisms and in ecological, physiological, and biochemical studies of the whole insects and cultured gut symbionts. Furthermore, genomics targeting an unculturable, single bacterial species has succeeded by using isothermal whole genome amplification from only several hundred cells. The functional analysis of the complete genome sequences acquired from intracellular symbionts of gut protists revealed that the endosymbionts play crucial roles in the nitrogen metabolism, that is, nitrogen fixation, recycling, and upgrade (Hongoh et al., 2008a, b). However, such detailed investigations have been performed only for a limited number of termite species, and more information from other termite species is needed to understand better the mechanism and evolution of digestive symbiosis in termite guts.

Termites of the subfamily Macrotermitinae, broadly known as termites that grow fungi, are assumed to be one of the most abundant and influential insects in tropical and subtropical ecosystems in Asia and Africa (Yamada et al., 2005). They consume more than 90% of dry wood in some arid tropical areas and directly mineralize up to 20% of the net primary production in wetter savannas (Abe et al., 2000). The higher fungus-growing termite *Macrotermes barneyi* Light is spread over a wide range of the division in China, especially south of the Yangtze River. It is a notorious insect pest and an economically important termite species, because it damages more than 100 main economic forest tree species, agronomic crops, and wooden structures. Furthermore its nesting behavior underground has endangered earthen dikes and dams (http://termite.sppchina.com). Therefore, investigating the microbial community structure and phylogenetic relationships of the constituents in the gut of *M. barneyi* could be meaningful not only for evolutional study of the digestive symbiosis of termites, but also for termite control.

Termites harbor diverse symbiotic gut microorganisms, the majority of which are as yet uncultivable and their interrelationships are unclear. In addition to evolving eusocial lifestyles, two equally fascinating aspects of

termite biology are their mutualistic relationships with gut symbionts and their use of lignocellulose as a primary nutrition source. Although termites are worldwide pests, they are considered excellent model systems for studying the production of bioethanol and renewable bioenergy from 2nd generation (non-food) feedstocks. In the present study, *M. barneyi* is one of the main harmful termites which damages garden trees in Hunan province, in order to contribute to the evolutional study of digestive symbiosis, a 16S rRNA gene clone library was constructed by culture-independent molecular approaches, from which a total of 105 clones were analyzed by restriction fragment length polymorphism (RFLP). Twenty-five clones with unique RFLP patters were sequenced and phylogenetically analyzed. The phylogenetic analysis revealed that half of the representative clones formed monophyletic clusters with clones obtained from other fungus-growing termites.

MATERIALS AND METHODS

Termite collection

Specimens of *M. barneyi* were collected from the forest farm of Hunan Forestry Academy, Changsha, China. 2 colonies were collected and 100 individuals (young to old, workers and soldiers) were collected in each colony. The termite specimens were brought back to the laboratory in polypropylene containers, and were maintained at 25°C until use. The containers were periodically moistened with water.

DNA extraction from termite gut

The surface of the termites was sterilized with 70% ethanol and briefly rinsed in sterilized water. Their guts were drawn out using sterilized forceps. The isolated guts of termites were then placed in phosphate-buffered solution (pH 7.2) and gently crushed using a sterilized pestle. Total DNA was prepared with the PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction.

PCR amplification of bacterial 16SrRNA genes

The PCR primers used to selectively amplify the bacterial 16S rRNA gene were 41F (5'-GCTCAGATTGAACGCTGGCG-3') and 1389R (5'-ACGGGCGGTGTGTACAAG-3') (Hongoh, 2003). The amplification reaction mixture (30 µL) contained 2.4 ng of total DNA, 3.0 µL of 10×PCR buffer, 2.4 µL of dNTP mixture (2.5 mmol/L), 0.15 µL of Taq DNA polymerase (Takara, Dalian, China), 0.3 µL of each primer, and sterilized distilled water. The reaction was performed as follows: initial denaturation at 94°C for 5 min, 26 cycles of 94°C for 30 s, 50°C for 1 min, and 72°C for 1.5 min, and a final extension at 72°C for 10 min. PCR products were purified using a quick Midi Purification Kit (Tiangen, Beijing, China), and cloned with a TA cloning Kit (Takara, Dalian, China) according to the manufacturer's instructions for construction of the clone library.

RFLP analysis

16S rRNA gene clones were randomly selected from the library, and inserted fragments were amplified by polymerase chain reaction (PCR). PCR was performed as follows: initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 90 s, and a final extension at 72°C for 10 min. The PCR products were digested with two four-base-specific restriction enzymes (MspI, AfaI) (Takara, Dalian, China) at 37°C for 2 h. The products of digestion were electrophoresed on 2% agarose gel and stained with ethidium bromide. A 100 bp ladder (Takara, Dalian, China) was used for a Deoxyribonucleic acid (DNA) marker. Each clone was named with prefix BMb (Bacterial rRNA gene clones derived from *M. barneyi*) followed by numerals indicating the set of restriction fragment length polymorphism (RFLP) analysis and the number of clones in each analysis.

Sequencing and sequence analysis

Near full-length 16S rRNA gene sequences (approximately 1.4 kb) of all representative clones with unique RFLP patterns were sequenced by the company (BGI, Beijing Genomics Institute, China) using the universal M13 primers (forward and reverse). The sequences of all clones were compared with those in the Genbank database by BLAST search at the the website of National Center for Biotechnology Information (NCBI). The taxonomic assignment was confirmed at an 80% confidence level using the naive Bayesian rRNA Classifier program on the Ribosomal Database Project Web site (Wang et al., 2007) at the same time. All clonal sequences and the reference sequences from the Genbank database were aligned using a Clustal X1.83 multiple sequence alignment program. After the sequence alignment, phylogenetic trees were constructed by the neighbor-joining distance matrix method, and 1,000 bootstrap replicates were performed using MEGA V5.0 (Tamura et al., 2011).

RESULTS

Bacterial community structure in the gut of M. *barneyi*

To investigate bacterial diversity in the digestive tract of *M. barneyi*, a bacterial 16S rRNA gene clone library was constructed by PCR with the whole DNA extracted from the gut of the termite. A total of 105 clones were randomly selected, and their partial sequences (about 1.4 kb) were determined after RFLP typing. As a result, 25 fragment patterns were identified. Each clone was named with prefix BMb, and the phylotypes were also represented by the names of the the representative clones. The phylotypes and the number of clones belonging to the respective phylotypes are summarized in Table 1. The most abundant sequence obtained was BMb0-03, which comprised 23.8% of the analyzed clones. In addition, 11 out of the 25 phylotypes in this analysis occurred only once. On the basis of the naive Bayesian rRNA Classifier program on the Ribosomal Database Project Web site, all of the sequences could be classified into known divisions in domain *Bacteria*. They were spread over a wide range of the division. The distribution (phylum) of the 105 clones retrieved from *M. barneyi* was as follows: *Bacteroidetes*, 47.6%; *Firmicutes*, 28.6%; *Proteobacteria*, 14.3%; *Deferribacteres*, 4.8%; *Actinobacteria*, 2.8%; *Planctomycetes*, 1.9% (Table 1).

Phylogenetic affiliation of 16S rRNA gene clone

All of the representative phylotypes obtained from this

Table 1. Sequence identities to the closest relatives and RFLP Types of 16S rRNA Gene Sequences.

Category (frequency %)	Phylotype	No. of clones	Length (bp)	Blast match organism	Accession No. of related sequence	Identity/similarity (%)
Bacteroidetes	BMb0-03	25	1382	clone MgMjD-032	AB234409	1311/1361(96/96.3)
(47.6)	BMb0-09	5	1382	clone MgMjD-055	AB234413	1288/1363(94/94.4)
	BMb1-48	1	1433	clone MgMjR-049	AB234377	1310/1360(96/45.2)
	BMb1-58	8	1379	clone MgMjD-087	AB234404	1335/1352(99/98.6)
	BMb1-62	3	1383	Clone RsStar140	AB522128	1264/1393(91/90.6)
	BMb1-87	2	1383	clone BOf1-02	AB288875	1269/1364(93/92.9)
	BMb3-19	2	1432	clone BOf1-02	AB288875	1307/1364(96/40.3)
	BMb3-25	1	1383	clone SWADLP6-16	FJ535561	1203/1316(91/43.4)
	BMb3-29	1	1374	clone MgMjR-003	AB234427	1341/1347(99/45.3)
	BMb3-57	2	1380	clone MgMjD-032	AB234409	1316/1359(97/96.5)
Firmicutes	BMb0-17	2	1108	clone MgMjW-02	AB234503	1070/1090(98/44.5)
(28.6)	BMb1-01	14	1377	clone MgMjD-096	AB234468	1343/1351(99/99.3)
	BMb1-35	1	1422	clone Rs-M23	AB089028	1199/1274(94/40.8)
	BMb1-37	1	1508	clone MgMjD-068	AB234464	1269/1286(99/42.5)
	BMb3-06	11	1370	clone MgMjR-090	AB234497	1278/1354(95/94.4)
	BMb3-43	1	1363	clone PeH17	AJ576334	1307/1370(95/44.6)
Proteobacteria	BMb3-23	1	1380	clone MgMjR-019	AB234523	1339/1356(99/45.7)
(14.3)	BMb3-26	10	1386	clone BOf5-16	AB288903	1349/1367(99/42.3)
	BMb3-32	1	1448	clone A2A5	EU885093	1319/1398(94/42.4
	BMb3-35	1	1367	clone BOf3-11	AB288895	1329/1342(99/99.0)
	BMb3-44	1	1367	clone MgMjD-024	AB234539	1323/1342(99/43.2)
	BMb3-58	1	1386	clone RP-3aaa01d10	EU778501	1382/1385(99/99.7)
Deferribacteres(4.8)	BMb1-80	5	1390	clone MgMjD-062	AB234550	1250/1375(91/90.6)
Actinobacteria(2.8)	BMb3-07	3	1352	clone MgMjR-011	AB234517	1314/1326(99/33.4)
Planctomycetes(1.9)	BMb3-04	2	1404	clone RsStar237	AB522154	1316/1403(94/39.1)

study are presented in phylum specific trees in Figure 1. The neighbors of these sequences found by BLAST search and representative bacteria of each phylum were included in the phylogenetic analysis. As shown in Figure 1, nearly half of the representative clones (11 phylotypes) were close to some known sequences obtained from other termite species, and formed monophyletic clusters with clones, especially with the sequenes retrived from fungus-growing termites. Interestingly, the majority of the clones (14 clones) were not gathered together with 11 clones, but separately formed a cluster without being contained in any known sequence, for there were very low sequence similarity (30 to 40%) to any known 16S rRNA sequences (Table 1). These results indicated that the gut of the termite *M. barneyi* existed in some particular bacteria groups.

The largest phylogenetic clade in the clone library constructed here was the phylum of *Bacteroidetes*. Ten phylotypes were affiliated with the order *Bacteroidales*, 4 phylotypes were unclassified by using the rRNA Classifier on RDP Database (Table 2), only 6 phylotypes formed clusters with clones obtained from other termite species (Figure 1). The most frequently identified phylotype, BMb0-03, formed a cluster together with BMb0-09, BMb3-57, and sequences retrieved from termite (*M. gilvus*). The tree also shows that over half of the phylotypes assigned *Bacteroidales* (60%, 6 out of 10 phylotypes) were found to be located in the six monophyletic clusters comprised only of termite related clones, in which the sequences retrieved from various termite genera, such as *Reticulitermes*, *Macrotermes*, *Odontotermes*, were included. While four phylotypes (BMb0-03, BMb0-09, BMb1-58 and BMb3-57) out of 6 were closely related to the sequences originating in other fungus-growing termite (MgMiD clones from *M. gilvus*), the other two phylotypes (BMb1-62、BMb1-87) had high sequence similarity to the sequence from *Reticulitermes speratus* and *Odontotermes formosanus*, respectively.

The second most abundant phylogenetic group was the *Clostridiales* group, which included 6 phylotypes and 30 clones accounting for 28.6% of total clones (Table 1). One third of the phylotypes (2 phylotypes and 25 clones) formed two clusters with the sequences reported from fungus-growing termite gut. 4 phylotypes were unclassified by using the rRNA Classifier on RDP Database (Table 2).

In Table 2, the 6 phylotypes comprised of 15 clones were affiliated with the β-, γ-, δ- and ε- subdivisions of the

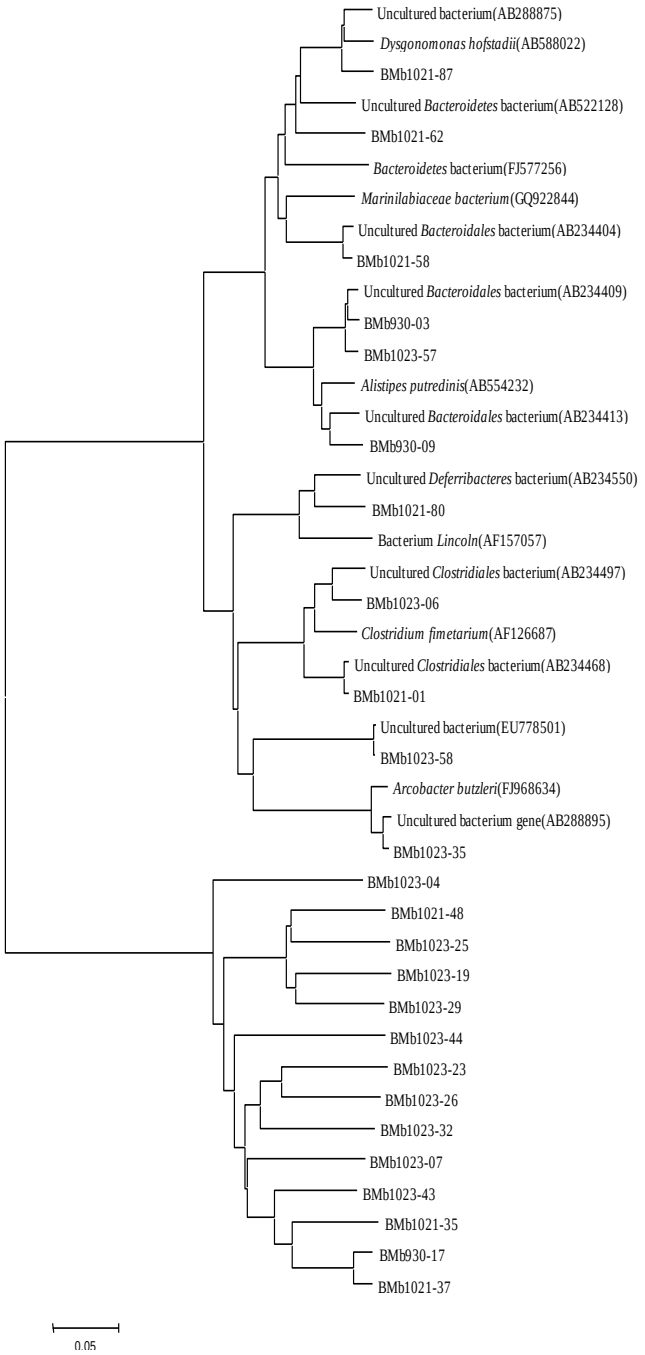

Figure 1. Phylogenetic tree of 16S rRNA gene sequences of *M. barneyi* gut bacteria (bootstrap values: 1000).

phylum *Proteobacteria*. Only one out of 6 phylotypes formed cluster with the sequence from termite gut, the other formed a cluster without associating with termite related sequences.

One phylotype, representing of a total of 5 clones belonging to the order *Deferribacterales*, was closely related to clone MgMiD-062 derived from *M. gilvus* young workers. In addition, 2 phylotypes obtained from our clone library of the *Actinobacteria* and *Planctomycetes* bacteria were unclassified by using the rRNA Classifier on RDP Database (Table 2) were also included.

DISCUSSION

Termites harbor a symbiotic gut microbial community that is responsible for their ability to thrive on recalcitrant plant matter. The community comprises diverse micro-organisms, most of which are as yet uncultivable and the detailed symbiotic mechanism remains unclear. In order to acquire an accurate description of the phylogenetic relationships of termite gut microbes clones, a bacterial 16S rRNA gene clone library from the gut microbial community of the fungus-growing termite *M. barneyi* was constructed. After screening by restriction fragment length polymorphism (RFLP) analysis, 25 out of 105 clones with unique RFLP patters were sequenced and phylo-genetically analyzed. Phylogenetic analysis showed that the clones corresponded to a diverse range of members of the domain Bacteria. All sequences grouped into one of six major bacterial phyla: *Bacteroidetes*, *Firmicutes*, *Proteobacteria, Deferribacteres*, *Actinobacteria*, and the *Planctomycetes*. This demon- strates that the gut bacteria of *M. barneyi* encompasses many diverse species, and perhaps this can help in carbon turnover in the environment and as potential sources of biochemical catalysis for efforts aimed at converting wood into biofuels (Warnecke et al., 2007).

The phylogenetic ananlysis showed that many representative clones found in our study tend to form some clusters with sequences reported to have been cloned from several termite guts. Ten phylotypes out of 11 clustered with the clones originated from termite guts, of which more than half (9 phylotypes) were closely related to clones originating from fungus-growing termites (*M. gilvus* and *O. formosanus*). This trend was mentioned in studies of the gut bacterial community of various termites species (Hongoh et al., 2003; Shinzato et al., 2005; Hongoh et al., 2006b; Schmitt-Wagner et al., 2003a; Yang et al., 2005), and suggests the existence of termite-specific bacterial lineages. The remaining 14 clones formed a cluster, which had a very low sequence similarity (30 to 40%) to known 16S rRNA sequences. The 16S rRNA gene sequence data showed that there was significant microbial diversity within the gut of a single termite species, and the majority of the intestinal microflora of *M. barneyi* consisted of new, uncultured species previously unknown to microbiologists.

Recently, based on 16S rRNA gene phylogenetic diversity of gut bacteria obtained from various termite species was constructed at the phylum level, and 24 divisions of bacterial rRNA genes were reported (Hongoh, 2010). Although the abundant population of bacteria: *Proteobacteria*, *Bacteroidetes*, *Spirochaetes* and *Firmicutes* was consistent with the results of various termites studied (Hongoh et al., 2003; Shinzato et al.,

Table 2. The rRNA classifier on RDP database of 16S rRNA gene sequences from the Clone Library.

Phylotype	Domain	Phylum	Class	Order	Family	Genus
BMb0-03	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Alistipes
BMb0-09	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Alistipes
BMb1-48	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	unclassified
BMb1-58	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomonas
BMb1-62	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomonas
BMb1-87	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomonas
BMb3-19	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	unclassified
BMb3-25	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	unclassified
BMb3-29	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	unclassified	
BMb3-57	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Alistipes
BMb0-17	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified
BMb1-01	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Robinsoniella
BMb1-35	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified
BMb1-37	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified
BMb3-06	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Lactonifactor
BMb3-43	Bacteria	Firmicutes	Clostridia	Clostridiales	unclassified	
BMb3-23	Bacteria	Proteobacteria	β-proteobacteria	Rhodocyclales	Rhodocyclaceae	unclassified
BMb3-26	Bacteria	Proteobacteria	γ-proteobacteria	Enterobacteriales	Enterobacteriaceae	unclassified
BMb3-32	Bacteria	Proteobacteria	δ-proteobacteria	unclassified		
BMb3-35	Bacteria	Proteobacteria	ε-proteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter
BMb3-44	Bacteria	Proteobacteria	ε-proteobacteria	Campylobacterales	Campylobacteraceae	unclassified
BMb3-58	Bacteria	Proteobacteria	δ-proteobacteria	Enterobacteriales	Enterobacteriaceae	Shigella
BMb1-80	Bacteria	Deferribacteres	Deferribacteres	Deferribacterales	Deferribacteraceae	Mucispirillum
BMb3-07	Bacteria	Actinobacteria	Actinobacteria	Coriobacteriales	Coriobacteriaceae	unclassified
BMb3-04	Bacteria	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	unclassified

2005; Schmitt-Wagner et al., 2003a; Yang et al., 2005), one of the characteristic features of the clone library for *M. barneyi* is that no clone affiliated with the phylum *Spirochaetes* was identified in our clone analysis. The precise reason for failure to detect spirochetal clones might be due to our relatively small screening size (105 clones). In addition, *Spirochetes* are also infrequent in the fungus-growing genera *Macrotermes* and *Odontotermes* (Hongoh et al., 2006b; Shinzato et al., 2007) and in the soil-feeding genus *Cubitermes* (Schmitt-Wagner et al., 2003a, b). These differences are attributed to complex diets consisting of different plant components caused by the diversity of microbes between different termite species.

The microbial community, whose structure and spatial distribution seems to be characteristic for a termite species (but may differ between genera), consists of mostly novel lineages that seem to have co-evolved or converged with their particular host. In the fungus growers, *Bacteroidetes* and *Firmicutes* (particularly *Clostridiales*) are the most abundant, and in the soil feeders, *Clostridiales* are the most abundant. In our study, the clone of *Bacteroidetes* and *Firmicutes* are also the most abundant. These observations indicate that the feeding habits of the termite host affect the bacterial composition. However, more comprehensive investigations with diverse termite species are necessary to understand the exact relationships.

Most gut bacteria have not yet been cultivated. In most cases, more than 90% of the phylotypes are novel, having no close relatives represented in the database sequences. Although the properties of the uncultured bacteria are unknown, the predominance of fermenting bacteria, the Bacteroidales and Clostridiales, is concordant with the substantial amount of acetate, a typical end product of microbial fermentation of carbohydrates, found in the gut of *M. subhyalinus* (Anklin-Mühlemann et al., 1995). Thus, these bacteria could contribute to the termite host in the dissimilation of plant-derived materials together with the Termitomyces fungi.

A few clones derived from *Deferribacteres* were identified in our clone analysis. Additionally, minor clones affiliated with the phylum *Deferribacteres* were also detected in *M. gilvus* young workers (Hongoh et al., 2006b). In a previous report, the workers changed their food with age after the final moult, from newly moulted to young and to old, while major and minor workers of the same age ingested basically the same food type (Hinze et al., 2002). The clustering of the old workers (MjD and mnD) and the soldiers (MjS and mnS) may be also due to

their similar food source, that is the aged part of the fungus combs, although the principal food of minor soldiers of *M. gilvus* is uncertain. In addition, since young workers (MjR and mnR) mainly ingest more intact dead-plant matter, chiefly fallen leaves (Johjima et al., 2003), the difference in collected plant matter between the two colonies may affect the gut bacterial community structure.

The efficiency of biorecycling of lignocellulose by termites is attributed to symbioses with microbes expressing a variety of function that termites do not possess. Recent application of novel technology and molecular methods has greatly enhanced our knowledge of these symbioses. However, detailed knowledge is lacking, because the relationships between termites and microbes as well as among microbes probably include a variety of functional interactions, which the symbiotic systems have accumulated and optimized during their evolution. Therefore, it is necessary to understand the mechanisms of these interactions at both the cellular and the molecular level. Indeed, several symbiotic systems of termites should be studied and compared so as to understand their evolution. Since many manufacturing techniques are simulations of a natural processes, these studies will help us not only to manipulate an existing system, consisting of multiple processes, but also to create new combinations of different organisms having desired functions.

In conclusion, the results of this study represent first and important insights into microbial community structure in the intestinal tract of *M. barneyi*. Our study indicates the gut of the termite *M. barneyi* existed in some particular bacteria groups. Although the molecular and phylogenetic data collected in this study cannot help in inferring an ecological role for these microorganisms in the environment, these findings are of fundamental value for understanding the complexity of *M. barneyi* gut ecosystems. The fungus-growing termites of the subfamily Macrotermitinae employ the most complex polyethism, organized by different castes and ages. The gut microbial community varies among castes and ages, and is clearly more related to a difference in age than in caste (Hongoh et al., 2006a). This suggests that the variations are crucially affected by their food, which can comprise dead grass and leaves, composted forage, small dead wood, and the conidia and mycelia of the symbiotic fungus Termitomyces.

Although the termite gut provides only a tiny, microliter-scale habitat, it is a reservoir of novel and complex microbial diversity. Termites have evolved a sophisticated, multilayered symbiotic system by harboring a complex gut microbiota. In this system, termites ingest woody materials and masticate and degrade them into fine particles by means of their mandibles, gizzard, and endogenous endoglucanase and β-glucosidase, which are secreted from the salivary gland and or midgut. The discovery of these endogenous cellulases has raised a question: do termites really need the gut microbiota for their survival? Yes, they do. The necessity of the gut microbiota for the digestion of woody materials has been demonstrated in numerous studies. However, the complexity and formidable unculturability of the gut microbiota have hampered the clarification of the molecular mechanism of this symbiotic system. Recently, innovative technologies in omics sciences have been applied. The metagenome analysis of the bacterial gut microbiota of a wood-feeding higher termite and metatranscriptome analyses of the protistan gut microbiota have revealed the presence of diverse glycoside hydrolase genes in both the bacterial and protistan microbiota. Furthermore, genomics targeting an unculturable, single bacterial species has succeeded by using isothermal whole genome amplification from only several hundred cells. The functional analysis of the complete genome sequences acquired from intracellular symbionts of gut protists revealed that the endosymbionts play crucial roles in the nitrogen metabolism. Further investigations using both meta- and single-species- targeting genomics, transcriptomics, and proteomics will greatly promote the understanding of this highly evolved, complex symbiotic system.

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