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ORIGINAL ARTICLE

Comparison of gut-associated and nest-associated microbial communities of a fungus-growing termite (*Odontotermes yunnanensis*)

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Abstract The digestion of cellulose by fungus-growing termites involves a complex of different organisms, such as the termites themselves, fungi and bacteria. To further investigate the symbiotic relationships of fungus-growing termites, the microbial communities of the termite gut and fungus combs of *Odontotermes yunnanensis* were examined. The major fungus species was identified as *Termitomyces* sp. To compare the micro-organism diversity between the digestive tract of termites and fungus combs, four polymerase chain reaction clone libraries were created (two fungus-targeted internal transcribed spacer [ITS] – ribosomal DNA [rDNA] libraries and two bacteria-targeted 16S rDNA libraries), and one library of each type was produced for the host termite gut and the symbiotic fungus comb. Results of the fungal clone libraries revealed that only *Termitomyces* sp. was detected on the fungus comb; no non-*Termitomyces* fungi were detected. Meanwhile, the same fungus was also found in the termite gut. The bacterial clone libraries showed higher numbers and greater diversity of bacteria in the termite gut than in the fungus comb. Both bacterial clone libraries from the insect gut included Firmicutes, Bacteroidetes, Proteobacteria, Spirochaetes, Nitrospira, Deferribacteres, and Fibrobacteres, whereas the bacterial clone libraries from the fungal comb only contained Firmicutes, Bacteroidetes, Proteobacteria, and Acidobacteris.

Key words fungus comb, gut, ITS, phylotype, termite, *Termitomyces*

Introduction

Termites are the major contributors to the biodegradation of lignocellulose and hemicellulose in tropical areas (Lee & Wood, 1971). Therefore, these insects are important not only for their roles in carbon turnover in the environment, but also as potential sources of biochemical catalysts for converting wood into biofuels (Warnecke *et al.*, 2007). Termites are a group of social insects usually classified at the taxonomic rank of the order Isoptera. This group contains five families of lower termites and one family of higher termites (Noirot, 1992). The special subfamily of higher termites, Macrotermitinae, which can digest plant litter with high efficiency, is also referred to as fungus-growing termites. These termites have gained the widespread interest of researchers due to their symbiotic relationship with basidiomycete fungi of the genus *Termitomyces* (Agaricales: Tricholomataceae) (Abe & Matsumoto, 1979; Wood & Sands, 1978).

Fungus-growing termites are distributed throughout tropical and subtropical areas in Africa and Asia. They have evolved into approximately 330 extant species, belonging to approximately 12 genera (Kambhampati & Eggleton, 2000). Inside the nests of these termites, *Termitomyces* is cultured by termites on a special

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structure called the fungus comb. The comb is constructed from undigested dead plant material and termite primary feces; it is then inoculated with asexual spores of the fungal symbiont (Leuthold *et al.*, 1989). A few weeks after the inoculation, innumerable conidia rich in nitrogen form many white nodules on the fungus comb. The host termites feed on the mature parts of the fungus comb; the mycelium then develops on the surface of the comb and on the fungal nodules for nutrition (Lefèvre & Bignell, 2002). Fecal pellets (primary feces) produced by workers (a caste of the termite) are added continuously to the top of the comb, and fungal mycelium rapidly develops in the newly added substrate.

Termites and gut bacteria, together with *Termitomyces* fungi, form a symbiotic complex (Bignell, 2000); however, the relationship within the complex and the mechanism of cellulose digestion remain unclear. Several hypotheses concerning the main role of *Termitomyces* fungi have been proposed. First, the fungi are presumed to possess the ability to degrade lignin to make cellulose more susceptible to attack by the termites' own cellulase (Hyodo *et al.*, 2000; Johjima *et al*., 2003b; Taprab *et al.*, 2005). Veivers *et al.* (1991) supported this view and found that symbiotic fungi in association with *Macrotermes michaelseni* and *M. subhyalinus* could not completely degrade cellulose. Ohkuma (2003) also reviewed evidence that higher termites relied primarily on endogenous cellulases for cellulose digestion. Second, Martin & Martin (1978) and Matoub & Rouland (1995) proposed that symbiotic fungi provide termites with cellulase and xylanases. In an experiment on enzyme activity detection, Deng *et al.* (2008) demonstrated that the symbiotic fungus had a synergistic effect on the cellulase activity of *Odontotermes formosanus*. Other studies (Sands, 1969; Darlington, 1994; Hyodo *et al.*, 2000) have identified a number of fungal cellulases, further supporting the results of Deng *et al.* (2008). Third, the fungi are thought to serve as nitrogen fixers, providing termites with nitrogen-rich food (Matsumoto, 1976). Hyodo (2003) further explained that the main role of the symbiotic fungus is to degrade lignin in *Macrotermes* spp., but it does not serve as a food source in *Odontotermes* spp., *Hypotermes makbamensis*, *Ancistrotermes pakistanicus*, or *Pseudacantbotermes militaris*.

Odontotermes yunnanensis, a species of fungus-growing termite, is widely distributed throughout Yunnan Province, China, particularly in the Xishuang Banna area. These termites play an important role in carbon turnover and the ecology of this area. To characterize the relationship between the fungus-growing termites, their symbiotic fungi, and bacteria within the comb and termite gut, we investigated the diversity of microorganisms under symbiotic conditions within colonies of *O. yunnanensis.*

Materials and methods

Termites and fungi

The workers, fungus nodules, and fungus combs of *O. yunnanensis* (Y3) were directly collected under aseptic conditions from a nest in Xishuang Banna, Yunnan Province, China, in March 2008. All samples were quickly frozen in liquid nitrogen in the field and then stored at −80°C until use.

Isolation and cultivation of fungi

One fungus nodule was carefully grasped with sterile forceps and gently washed three times with sterile distilled water. The nodule was then aseptically inoculated onto an improved potato dextrose agar plate (IPDA: 20% (w/v) potato extract, 2% (w/v) glucose, 0.001% (w/v) VB1, 0.0003% (w/v) VB2, 0.0003% (w/v) VB6, 0.00002% (w/v) biotin, 1.5% (w/v) agar, pH 4.5 regulated by citric acid) at 28°C. When white mycelium developed from a nodule, it was further streaked and sub-cultured on a new plate. This purification step was replicated at least three times. The pure fungi were then inoculated into 100 mL of improved potato dextrose liquid medium (IPD, the same components with IPDA except no agar). The flasks were incubated at 28°C for 5 days under constant shaking at 150 cycles per minute. The fragmented mycelial culture (1%, v/v) was used as the inoculum for successive transfer cultures.

DNA extraction

A FastDNA® SPIN for Soil KIT (Qbiogene, Carlsbad, CA, USA) was used to extract DNA of nodules and combs. Five hundred (± 5) mg of nodules and combs of Y3 were each prepared in 2-mL Lysing Matrix E tubes (supplied by the Kit mentioned above) together with 978 μL of sodium phosphate buffer and 122 μL of MT buffer. The samples were treated with bead beating using a FastPrep® Instrument (MP Biomedicals, Solon, OH, USA) for 30 s at a speed setting of 5.5 m/s. The suspension was centrifuged at 14 000 *g* for 30 s, and then the supernatant fluid was continued according to the instructions provided with the extraction kit.

The complete guts of five individual termites were dissected and placed into phosphate buffer solution

(137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, 2 mmol/L KH_2PO_4). For gut dissection, the head of the termite was removed using a sterile pin, and then the entire gut was drawn out from the end of the abdomen using sterile forceps. Total DNA was extracted using a QIAamp DNA Stool Mini Kit (QIAGEN, Hamburg, Germany) according to the manufacturer's instructions.

The DNA of Y3 liquid-cultured mycelium was extracted and purified following Wang *et al.* (2005), except for the cell lysing procedure. The lyophilized mycelium was entirely crushed in liquid nitrogen using a mortar instead of a spatula, and the powders were carefully transferred into a 1.5-mL Eppendorf tube with 1 mL of washing buffer (1% [w/v] polyvinyl pyrrolidone, 0.05 mol/L ascorbic acid, 0.1 mol/L Tris-HCl [pH 8.0], 2% (w/v) β-mercaptoethanol). The suspension was centrifuged at 20 000 *g* for 3 min. The pellet was resuspended with 700 μL 2 $\times$ hexadecyl trimethyl ammonium bromide (CTAB) (100 mmol/L Tris-HCl, pH 9.0; 40 mmol/L edetic acid (EDTA), pH 8.0, 100 mmol/L sodium phosphate buffer, pH 8.0; 2% (w/v) sodium dodecyl sulfate (SDS); 30% (w/v) benzylchloride), which differed from the extraction buffer used by Wang *et al.* (2005). Subsequent steps were performed as described in Wang *et al.* (2005). The concentration and quality of the purified DNA were evaluated by both 1% (w/v) agarose (Biowest, Madrid, Spain) gel electrophoresis and spectrophotometry.

PCR amplification of the ITS region and identification of the strain

Polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) gene fragment was performed using a Flexigene thermal cycler (Flexigene TECHNE, Abingdon, UK) with forward primer ITS1 (TCC GTA GGT GAA CCT GCG G) and reverse primer ITS4 (TCC TCC GCT TAT TGA TAT GC) (Mackay *et al.*, 2001). The different genomic DNA from host gut and fungus comb was used as template. Amplifications (50 μL) were performed using 10 ng of genomic DNA, 5 μL of 10 $\times$ PCR buffer (200 mmol/L Tris-HCl, pH 8.4; 2.5 mmol/L $MgCl_2$; 500 mmol/L KCl), 0.25 mmol/L each of deoxyribonucleotide triphosphates (dNTPs), 0.2 mmol/L of each primer, and 1 U of *Taq* DNA polymerase (TaKaRa, Dalian, China). The following reaction program was used: initial denaturation cycle, 3 min at 94°C; 30 cycles of 45 s at 94°C, 45 s at 58°C, and 1 min at 72°C; and 1 cycle of 10 min at 72°C. Aliquots of each PCR product from these amplifications were purified using a QIAquick PCR Purification Kit protocol (QIAGEN, Hamburg, Germany) and electrophoresed on a 1% (w/v) agarose gel. The purified products were sequenced by SUNNYBIO (Shanghai, China) on a 3730XL DNA Sequencer. Resulting sequences and other homologous sequences were aligned using Clustal X (Thompson *et al.*, 1997) and were adjusted by eye using MacClade 4.0 (Maddison & Maddison, 2000). All sequences were used for the construction of phylogenetic trees to determine the taxonomy of the strain.

The construction of the ITS-rDNA and 16S-rDNA libraries

Bacterial 16S rDNA genes were amplified using the universal primers 27F (5′-TAG AGT TTG ATC CTG GCT CAG-3′) and 1392R (5′-GAC GGG CGG TGT GTA CA-3′) (Lane, 1991). The genomic DNA from host gut and fungus comb was also used as a template. PCR reactions began with an initial denaturation at 95°C for 3 min and were followed by five cycles consisting of denaturation (30 s at 94°C), annealing (30 s at 60°C), and extension (4 min at 72°C); five cycles consisting of denaturation (30 s at 94°C), annealing (30 s at 55°C), and extension (4 min at 72°C); 10 cycles consisting of denaturation (30 s at 94°C), annealing (30 s at 50°C), and extension (4 min at 72°C), and a final extension at 72°C for 10 min. The PCR product was visualized on a 1% (w/v) agarose gel stained with ethidium bromide. ITS region genes of the rDNA were amplified as described in the previous paragraph.

The purified products were ligated into pMD18 T-Vectors (TaKaRa, Dalian, China) by incubating at 16°C overnight, in accordance with the manufacturer's instructions. The prepared TOP10 cells were transformed using a heat shock protocol; plated onto Luria-Bertani (LB) agar containing ampicillin (50 μg/mL), isopropyl β-D-1-thiogalactopyranoside (IPTG) (20% w/v), and X-gal (2% w/v) solution; and then incubated at 37°C overnight. The selected white transformants were examined using PCR with the universal primers M13F (GTA AAA CGA CGG CCA G) and M13R (CAG GAA ACA GCT ATG AC). All positive transformants were selected for sequencing and analysis.

Phylogenetic analysis

Inspection of sequence chromatograms and assembly of bi-directional sequences were conducted using a Sequence Scanner v1.0 (Applied Biosystems, Foster City, CA, USA) and CodonCode Aligner software (Codon Code, Dedham, MA, USA). The alignments were performed with BioEdit (Hall, 1999) using the ClustalW

method. The aligned sequences were corrected manually, and DNA sequence data were analyzed to provide pair-wise percentage sequence divergence. Diversity coverage by the clone library was analyzed using Analytical Rarefaction software (version 1.4; S. M. Holland, University of Georgia, Athens, GA, USA). Molecular evolutionary analyses were conducted according to the neighbor-joining method after 1 000 bootstrap replicates using MEGA version 4 (Tamura *et al.*, 2007). Using the PAUP 4.0 b10 (Swofford, 2000) program package, the maximum parsimony (MP) method was employed to construct phylogenetic trees based on the sequences of the 16S rDNA gene. Bootstrap values for individual branches were also estimated by bootstrapping with 1 000 replications. Rooted trees were generated using TREEVIEW version 1.6.6 (Page, 1996).

Results

Identification of cultures

Using micromorphology alone, the *Termitomyces* nodules were difficult to identify. Therefore, two sequences for the ITS regions of a wild nodule and cultivated mycelium (FJ769409, FJ769410) obtained through PCR were aligned and molecular information was used for identification. The length of ITS1-5.8S-ITS2 was moderately large (~670 bp), which was in accordance with the value of 600–800 bp generally reported for fungi (Gardes & Bruns, 1996). Consensus was observed between the two sequences with almost entirely uniform sequences. These results indicated that the wild nodule and cultivated mycelium were the same species of fungus. We were also able to concomitantly cultivate this strain of *Termitomyces* in the laboratory.

To determine the taxonomy of an isolated strain from a Y3 nest, a sequence of the ITS region from the wild nodule (FJ769410) was aligned with other submitted sequences downloaded from GenBank. To help distinguish the strain, a phylogenetic tree was constructed using the sequencing of ITS (Fig. 1). Comparison of the sequence similarity showed that 21 strains were significantly related, and these were grouped into two clusters. The Y3_nodule sample, together with 15 other sequences, formed a larger cluster with high support (100%). The clade composed of Y3_nodule_ITS and *Termitomyces* sp. Group 8 was also well supported (99%). *Termitomyces* sp. Group 8 was not given an exact species name because of the imperfection of the classification of the genus *Termitomyces*. Thus, the strain isolated from the Y3 nest was simply identified as *Termitomyces* sp.

Abundance of fungi in the comb and gut of Y3

The ITS-rDNA libraries of the fungus comb and gut of Y3 were constructed separately. With the exception of some inferior sequences, 87 and 79 available sequences for the fungus comb and Y3 gut libraries, respectively, were obtained for subsequent analysis. For the fungus comb library, all sequences were aligned after the primer segments were removed. The sequences generated were identical to FJ769409/FJ769410 mentioned earlier. Using PCR of the ITS region, results indicated that only *Termitomyces* sp. fungi were cultivated by termites on the fungus comb. The pattern of the Y3 gut library was nearly identical to that of the fungus comb. Interestingly, two sequences similar to *Aspergillus penicillioides* (AY373862) and *Chlamydomonas allensworthii* (AF326852) were found; support for these identities was 95% and 82%, respectively. As far as we know, no previous studies have found these two types of fungi in the gut of *Odontotermes* termites.

Abundance of bacteria in the comb and gut of Y3

To compare bacterial diversity between the termite digestive tract and the fungus comb, two bacterial 16S rDNA gene clone libraries were constructed with PCR using the complete DNA extracted from the gut of the termite and the fungus comb. After alignment and tests for chimera, 83 and 66 available sequences ($\approx$ 1.4 kb) of the fungus comb and gut libraries, respectively, were obtained from the web (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi). Suspect sequences were omitted from subsequent analysis, resulting in 40 and 66 phylotypes identified based on an arbitrarily defined criterion of > 97% sequence identity in the fungus comb and gut library, respectively. Of the total 106 phylotypes in the analysis, 86 occurred only once. The representative sequences of all phylotypes were submitted to the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp) for classification and sequence matching (data not shown). Figure 2 presents rarefaction curves for the phylotypes; the number of samples for the gut library was not adequate for analysis. However, the sample numbers for the phylotypes of the fungus comb library were considered saturated.

Figure 3 presents the relative clone frequencies of the 16S rDNA libraries of the fungus comb and Y3 gut. The abundance of bacteria in the termite gut was much higher than in the fungus comb. The basal bacterial composition of the two libraries was very similar, but the

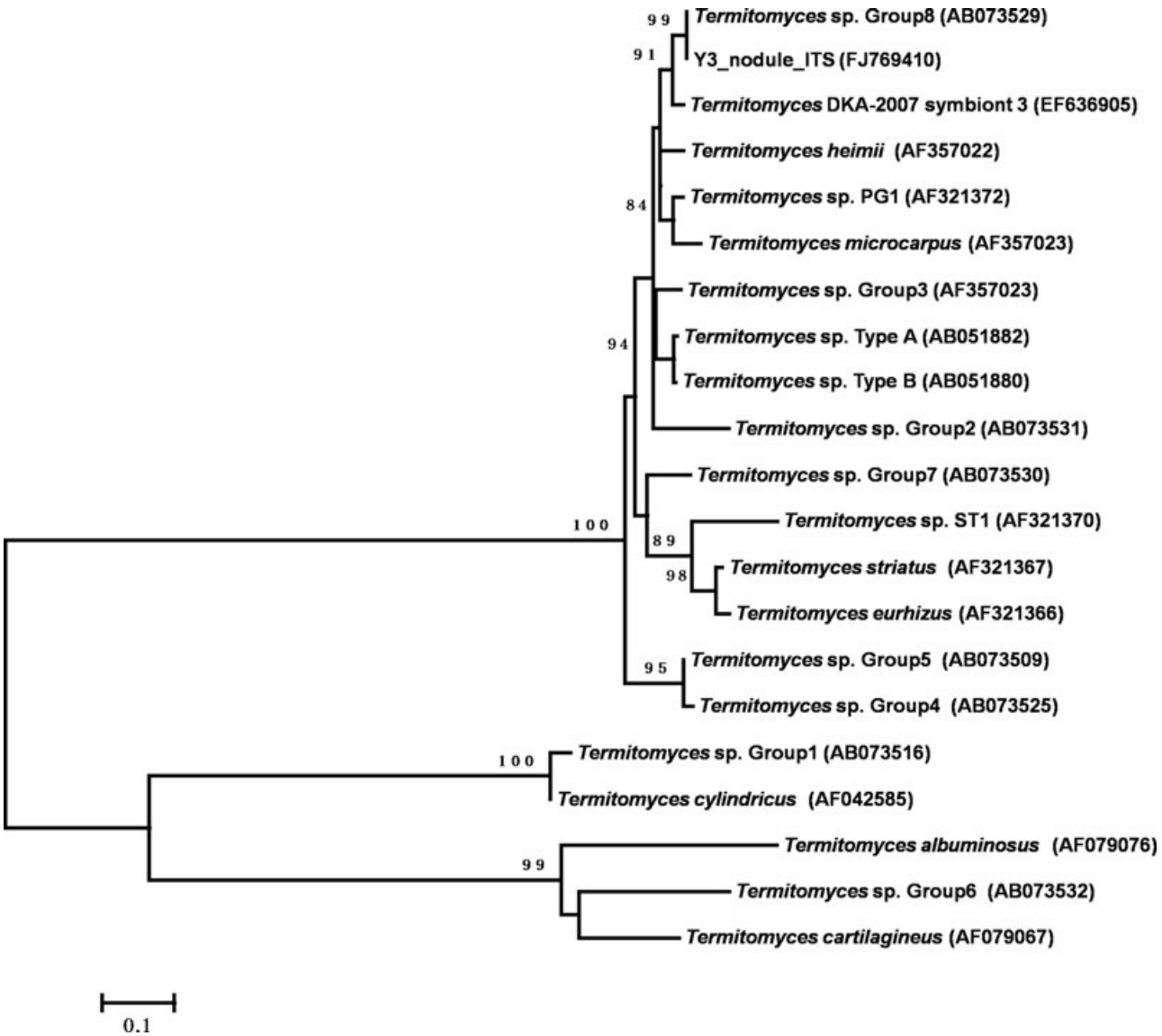

Fig. 1 Phylogenetic tree of *Termitomyces* based on ITS1-5.8S-ITS2 complete sequence. Numbers above the branches indicate bootstrap values of parsimony analysis from 1 000 replicates. The numbers that follow species names are the accession numbers for public databases. The scale bar indicates 10% estimated sequence divergence.

proportions of major phyla were distinguishable. The bacterial community structure of the fungus comb was strongly dominated (77.5% of phylotypes) by bacterial sequences affiliated with the Firmicutes phylum. However, clones of this phylum also represented the largest component of the gut library, comprising approximately 38% of all assigned clones. Bacteroidetes (26.7%) were identified as the second major constituent in the gut library, and this group only comprised 2.5% of the fungus comb library. The sequences of Proteobacteria in the fungus comb (15%) were slightly more abundant than in the gut library (11.8%). Differences between the two libraries were tested using *P*-test pair-wise comparisons (data not shown). At the level of phylum, the two libraries exhibited significant differences for Firmicutes, Bacteroidetes, Proteobacteria, and Spirochaetes. Additionally, our analysis of the fungus comb library revealed the presence of Actinobacteria clones (2.5%), which were not found in the termite gut library. Moreover, no clones affiliated with the phylum Spirochaetes were identified in the fungus comb

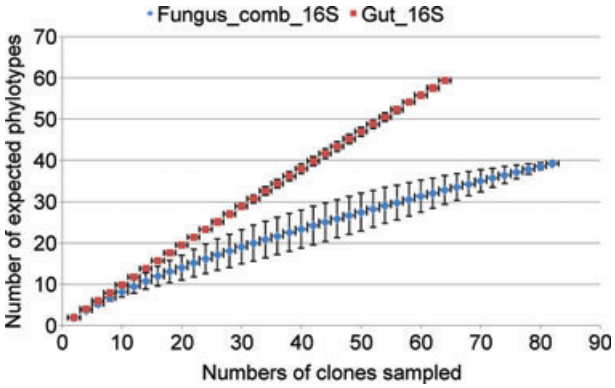

Fig. 2 Rarefaction curves for phylotypes of 16S rDNA gene clones of different samples. The expected number of phylotypes was calculated from the number of clones, with inclusion in the same species based on 97% sequence similarity.

library, whereas this group comprised 8% of phylotypes in the gut library.

Because the fungus comb was dominated by the Firmicutes phylum, we used the MP calculation method (incorporating 50 phylotypes of the two libraries) to construct a dendrogram and to map bootstrap scores (Fig. 4). All sequences of Firmicutes derived from the fungus comb or gut formed four distinct groups. Groups I and III were primarily composed of clones from the fungus comb, whereas groups II and IV were composed of clones from the gut, with the exception of two clones. Clones derived from the same origin clearly clustered together. Groups I, II and III were considered Clostridiidiales, but Group IV remains unidentified. In Group I, the origin of all clones was determined as *Clostridium*, except for comb_2_08. This clone was clustered with L35515 (*Acetivibrio*), which had only 61% support. Nearly half of the Firmicutes clones from the fungus comb were derived from *Clostridium* genera closely affiliated with two sequences from the termite gut in Group I or possibly with all sequences of Group II.

Discussion

The entire ITS region of fungi often ranges between 600 and 800 bp and can be readily amplified using "universal primers" that are complementary to sequences within rRNA genes (White *et al.*, 1990). Several studies have reported potential disadvantages when PCR-amplifying DNA templates from a broad range of organisms, including fungi, plants, protists and animals (White *et al.*, 1990; Gardes *et al.*, 1991; Gardes & Bruns, 1991; Baura *et al.*, 1992; Chen *et al.*, 1992; Lee & Taylor, 1992); despite this, the method is still widely used in studies of fungal identification and phylogeny (Moriya *et al.*, 2005; Shinzato

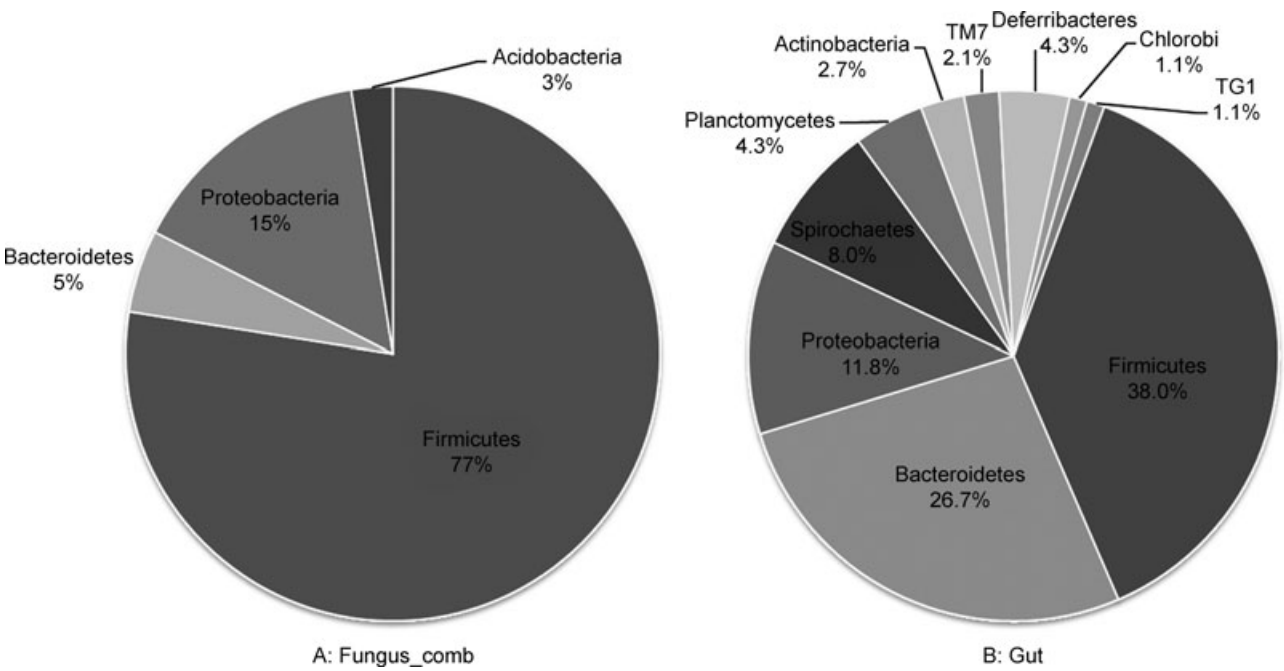

Fig. 3 Relative clone frequencies of 16S rDNA libraries. A: fungus comb of Y3. B: gut of Y3.

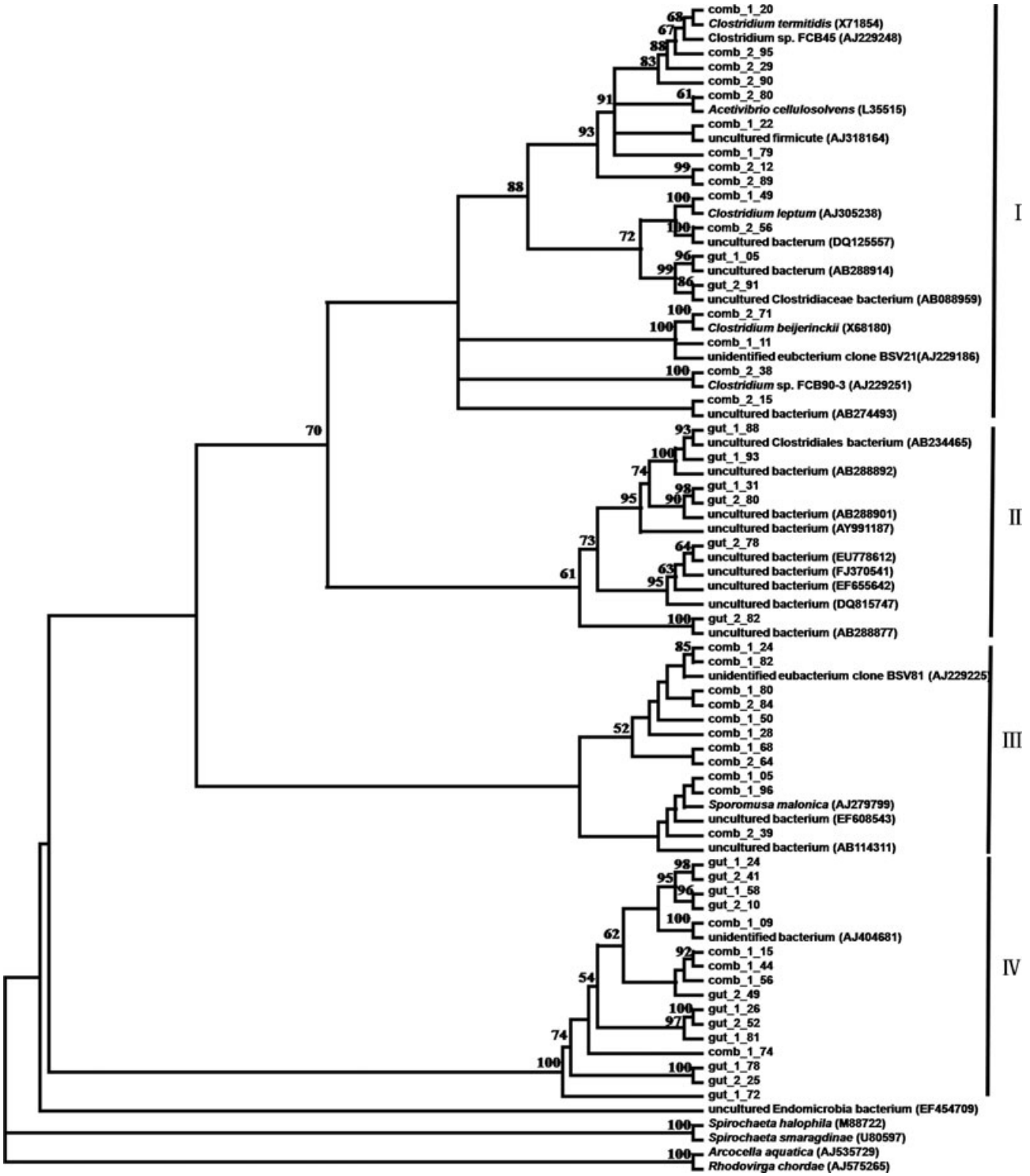

Fig. 4 Molecular phylogenetic tree of 16S ribosomal RNA partial gene sequences associated with Firmicutes phylum using maximum-pasimony analysis. Numbers above the branches indicate bootstrap values of parsimony analysis from 1 000 replicates. The numbers that follow species names are the accession numbers for public databases.

et al., 2005; Lefèvre *et al.*, 2002; Aanen *et al.*, 2007). In the present study, the Y3_nodule_ITS sequence (FJ769410) and other *Termitomyces* sp. sequences from fungus nodules or fruit (downloaded from the National Center for Biotechnology Information [NCBI], which were reported in papers examining symbiotic fungi of termites) were significantly related. Meanwhile, FJ769410 exhibited nearly the same sequence as *Termitomyces* sp. Group 8 (Katoh *et al.*, 2002; Taprab *et al.*, 2002), which had not yet been given an exact species name. The two sequences also clustered together with 99% support in the phylogenetic tree. Additionally, due to the imperfection of classification information concerning the genus *Termitomyces*, we could only identify this strain as *Termitomyces* sp. based on uniform experience of molecular identification using PCR with available primers. Remarkably, the host termites of these two strains are not the same. The host of *Termitomyces* sp. Group 8 is *M. annandalei* (Katoh *et al.*, 2002; Taprab *et al.*, 2002), whereas the cultures obtained in this study were associated with another genus, *Odontotermes*. A similar phenomenon was also demonstrated by Aanen *et al.* (2007); because they found either similar or higher diversity in the symbiont, the fungal symbiont clearly does not always follow the general pattern of the endosymbiont.

One important result was that no other fungi except *Termitomyces* could be detected in the fungus comb using the method of library construction of ITS. Similar patterns have been observed by other researchers. For example, using the method of terminal restriction fragment length polymorphism (T-RFLP), Moriya *et al.* (2005) concluded that the growth pattern of *Termitomyces* was a monoculture, whereas non-*Termitomyces* inhabitants were only minor components in fungus gardens. Shinzato *et al.* (2005) examined the growth state of fungi using laboratory-scale fungus combs with or without termites under laboratory conditions. They found that non-*Termitomyces* species grew vigorously in combs without termites, although no significant differences were observed in combs with termites. The monoculture pattern of *Termitomyces* in combs with termites was also confirmed using molecular-based analyses of the microbial communities in the combs (Shinzato *et al.*, 2005). Recently, Guedegbe *et al.* (2009) demonstrated that active combs were dominated by bands of *Termitomyces* fungi, which were isolated using direct polymerase endonuclease restriction–denaturing gradient gel electrophoresis (PCR-DGGE). Taken together, these previous studies confirm the credibility of our present results.

However, in the studies mentioned above, non-*Termitomyces* fungi, such as some filamentous fungi and yeasts, were detected in laboratory culture (Shinzato *et al.*, 2005) and by using the suicide polymerase endonuclease restriction (SuPER) PCR-DGGE method (Guedegbe *et al.*, 2009). During our experiment, we also found that some filamentous fungi developed within the combs (without termites) when they were removed from the nests, whereas the growth of these non-*Termitomyces* fungi was inhibited within the nests (with termites). Nevertheless, no sequences of these filamentous fungi were obtained from the ITS-rDNA gene clone library. The absence of such sequences may have occurred for several reasons. The first reason is related to the methodology of sample collection. In the field, the combs were quickly removed from nests and placed into liquid nitrogen. Thus, the samples used to construct libraries originated from the most natural conditions possible, perhaps avoiding contamination. Another possible reason could be due to the relatively small screening size (87 clones). The lack of filamentous fungal contamination may also be related to why only the genus *Termitomyces* thrives on the fungus comb with termites. For example, Macrotermitinae excrete several fungicides (Bulmer & Crozier, 2004; Lamberty *et al.*, 2001), although the range of sensitivity of these fungicides has not yet been determined. Such fungicides may have prevented contamination in the cultivation of fungus gardens and helped to maintain the *Termitomyces* monocultures (Moriya *et al.*, 2005). Additionally, antimicrobial substances derived from a *Streptomyces* bacterium may have played a role in preserving uncontaminated conditions in colonies of the attine ant (Lamberty *et al.*, 2001; Mueller & Gerardo, 2002; Shinzato *et al.*, 2005). Further studies are necessary to determine whether special bacteria with similar functions are present in the intestinal tract of termites. Finally, high concentrations of CO_2 in the nests may provide a competitive edge for *Termitomyces* relative to non-*Termitomyces* species, as *Termitomyces* may be more tolerant of high CO_2 concentrations compared to other fungi (Batra & Batra, 1966).

In the comparison of bacterial microbiota in the termite gut and fungus comb, the phyla of Firmicutes were significantly dominant, followed by phyla of Bacteroidetes and Proteobacteria in the gut and comb, respectively. The diversity of bacterial flora of the gut was clearly higher than that of the fungus comb (Fig. 3). Yet, the similar pattern of floral structure could indicate strong proof that the fungus comb was composed of termite feces. In the gut, the abundant population of Clostridiales (Firmicutes) was consistent with the results of studies of other fungus-growing termites, such as *Macrotermes gilvus* (Hongoh *et al.*, 2006), *Cubitermes niokoloensis* (Fall *et al.*, 2007), and *O. formosanus* (Shinzato *et al.*, 2007). Furthermore, the cluster analysis of the Firmicutes clones from the two libraries (Fig. 4) unequivocally indicated that clones from

the same sample clustered together even though they all belonged to the same phylum. More than half of the Firmicutes clones from the fungus comb were Clostridiales genera, which were closely affiliated with sequences from the termite gut. Although most clones blasted as uncultured bacteria that also originated from the termite gut or environmental samples, the predominance of Clostridiales, together with the *Termitomyces* fungi, likely aids the termite host in the dissimilation of plant-derived materials (Anklin-Mühlemann *et al.*, 1995; Hongoh *et al.*, 2006). The gut symbionts of various fungus-growing termite species, especially those of the genus *Odontotermes*, are often comprised of similar bacterial communities. This phenomenon can be explained by the theory that gut microbes, which co-evolved with termites, were transferred via vertical transmission (Shinzato *et al.*, 2007). Additionally, spirochetes are often a major constituent of the bacterial flora of the guts of wood-feeding termites but not of fungus-growing termites (Leadbetter *et al.*, 1999; Lilburn *et al.*, 1999). Similarly, the population of spirochetes (8%) observed in the gut of our termites also comprised a small fraction compared to that observed in wood-feeding termites. A similar result has also been reported for *O. formosanus* (Shinzato *et al.*, 2007). The existence of such a relatively minor component of this phylum in fungus-growing termite guts was likely of minimal physiological significance. In the analysis of the two libraries, 2.5% of phylotypes of the fungus comb were identified as a type of acidophilic bacteria (Acidobacteria), which play an important role in ecological systems, yet remain understudied. The presence of these bacteria suggests that the pH of the fungus comb should be further examined to learn the exact association between the comb and the gut. However, no clones of this phylum were found in the termite gut, perhaps due to the fact that fewer phylotypes were obtained during the construction of the 16S rDNA library of the termite gut.

The observed bacterial community of the fungus comb distinctly differed from those reported previously. For example, Fall *et al.* (2007) found that Actinobacteria phylotypes dominated in the termite mound. In contrast, we found that Firmicutes were dominant in the combs. This discrepancy may have resulted from the use of different genera of termites (*Cubitermes* and *Odontotermes*), the limited size of screened clones, or differences in sample origin (ours was the fungus comb composed of feces and lignin materials, whereas the other was an internal mound wall consisting of soil). In fact, Fall *et al.* (2007) did observe a shift in the bacterial community structure between the termite gut and mound; this change was obvious even in samples of fresh feces sampled < 1 h after being deposited in the mound. The nature of such rapid bacterial community shifts deserves further investigation.

No gene clones associated with the degradation of lignocelluloses were found in either the gut or fungus comb library because the current research did not focus on lignocellulase identification, whereas genes obtained through the 16S-rDNA library were strongly related to metabolism. Within such a complex symbiotic system composed of a termite, bacterium and fungus, the fungus could possibly play a crucial role in the degradation of lignocelluloses. There is evidence in the literature of lignocellulases in *Termitomyces* fungi. For example, several genes or enzymes have been cloned or purified from the *Termitomyces* fungus, such as laccase (Taprab *et al.*, 2005; Bose *et al.*, 2007), phenol oxidase (Johjima *et al*., 2003a), cellobiase (Mukherjee & Khowala, 2002; Mukherjee *et al.*, 2006), amyloglucosidase (Ghosh *et al.*, 1997), and xylanase (Matoub & Rouland, 1995). The current study provides a step in the process of identifying novel fungal lignocellulases. Thus, future studies should seek to identify lignocellulase-coding genes from fungal symbionts.

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