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ORIGINAL ARTICLE

Bacterial microbiome of *Coptotermes curvignathus* (Isoptera: Rhinotermitidae) reflects the coevolution of species and dietary pattern

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Abstract *Coptotermes curvignathus* Holmgren is capable of feeding on living trees. This ability is attributed to their effective digestive system that is furnished by the termite's own cellulolytic enzymes and cooperative enzymes produced by their gut microbes. In this study, the identity of an array of diverse microbes residing in the gut of *C. curvignathus* was revealed by sequencing the near-full-length 16S rRNA genes. A total of 154 bacterial phylotypes were found. The *Bacteroidetes* was the most abundant phylum and accounted for about 65% of the gut microbial profile. This is followed by *Firmicutes*, *Actinobacteria*, *Spirochetes*, *Proteobacteria*, TM7, *Deferribacteres*, *Planctomycetes*, *Verrucomicrobia*, and Termite Group 1. Based on the phylogenetic study, this symbiosis can be a result of long coevolution of gut enterotypes with the phylogenic distribution, strong selection pressure in the gut, and other speculative pressures that determine bacterial biome to follow. The phylogenetic distribution of cloned rRNA genes in the bacterial domain that was considerably different from other termite reflects the strong selection pressures in the gut where a proportional composition of gut microbiome of *C. curvignathus* has established. The selection pressures could be linked to the unique diet preference of *C. curvignathus* that profoundly feeds on living trees. The delicate gut microbiome composition may provide available nutrients to the host as well as potential protection against opportunistic pathogen.

Key words *Coptotermes curvignathus*, diet preference, gut microbiome, selection

Introduction

In contrast to the most studied termite *Reticulitermes speratus* Kolbe, diversity and community structure of microbiota in the gut of *Coptotermes curvignathus* Holmgren is largely unknown. The role of the gut microbiota of *C. curvignathus* in the survival of its host is still obscure. This termite species is found in the Indo-Malaysia region, and display several unique features that have attracted the attention of science communities to explore its potential as gold mine for robust industrial enzymes.

Unlike most of the termite species that feed on semi or partially degraded wood residues, *C. curvignathus* has the ability to attack and consume tissues of healthy living trees. This indicates the unique digestive feature of

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C. curvignathus that can overcome plant defense mechanism and achieve effective lignocellulosic biomass conversion. Discerning understanding on the gut microbial community is very important in order to comprehend the digestion mechanism of *C. curvignathus.*

Studies have shown that the alteration of the gut microbiota could affect the metabolic versatility of termites (Nazarczuk *et al*., 1981). The dense and diverse intestinal microbiota assemblages in the gut of termites help capacitate various metabolic reactions to be performed. As a result of this, an intriguing yet delicate physiochemical balance environment is created in this tiny termite gut, approximately 1–10 μL of volume.

The multifarious metabolic activities also provide and sustain the necessary aerobic and anaerobic condition unto this tiny environment. Many of these microbes are believed to be unique to termites and perhaps are the direct descendants of those inhabited termites millions of years ago (Nalepa *et al*., 2001). Molecular analysis of bacterial microbiota in the gut of *R. speratus* revealed that more than 90% of the phylotypes were found for the first time. Some phylotypes also constituted monophyletic clusters with sequences recovered from the gut of other termite species. This implies the existence of termite-specific bacterial lineages (Hongoh *et al*., 2003).

The research of termites' gut is often complicated by their tiny gut structures and diversiform physicochemical niches in the gut. These made *in vitro* duplication of gut fluid and condition challenging and hardly possible. Thus a serious obstacle in studying the gut microbes is in isolating and cultivating microbes *in vitro*. Therefore, most studies of termite gut microbiota diversity are culture-independent. In this study, the microbial diversity in the gut of *C. curvignathus* was probed based on both culture-independent and cultivation approaches in order to better comprehend the interaction between the host digestive as well as innate immune systems and microbes residing in the termites' guts.

Materials and methods

Sample collection and extraction of DNA

Wood-feeding lower termites, *C. curvignathus* (Family Rhinotermitidae) that were actively feeding on the oil palm, were collected from an oil palm plantation in Bintulu, Sarawak, Malaysia. A total of 13 different species of termites were found within the plantation vicinity (Bong *et al*., 2012), and *C. curvignathus* was the dominance species (Bong, personal communication). Termites were washed in sterilized water, and their guts were drawn out using sterilized fine tip forceps. The isolated guts of 60 individuals were torn to release their gut fluid in ATL solution (Qiagen DNeasy Tissue Kit, Qiagen Inc., Valencia, CA, USA). Proteinase was added and incubated overnight at 55 °C. DNA was extracted using the Qiagen DNeasy Tissue Kit.

PCR amplification and preparation of clone libraries

The 16S rRNA genes were amplified from the gut sample by PCR, using Bacteria-universal primers, 63F (5′CAGGCCTAACACATGCAAGTC3′)–1389R (5′ACGGGCGGTGTGTACAAG3′) (Marchesi *et al*., 1998) and 27F (5′AGAGTTTGATCMTGGCTCAG 3′)–1429R (5′GGTTACCTTGTTACGACTT 3′) (Suzuki & Giovannoni, 1996). PCR was performed using a PTC-225, Peltier Thermal Cycler (MJ Research Inc., Waltham, MA, USA), Go-Taq (Promega Corporation, Madison, WI, USA), and the following program: 2 min of initial denaturation at 95 °C, followed by 24 cycles of denaturation (30 s at 95 °C), annealing (60 s at 55 °C), and extension (2 min at 72 °C), with a final extension at 72 °C for 10 min. The concentrations of the template and primers were 35 ng/μL and 20 pmol, respectively. The PCR product was purified from 200 μL of reaction mixture, using the QIAquick PCR Purification kit (Qiagen Inc., Valencia, CA, USA). The products were cloned into pJET1/blunt cloning vectors (GeneJET PCR cloning Kit, Fermentas International Inc., Burlington, ON, Canada) and a clone library was established on LB agar plates.

Plasmid extraction and purification

A total of 576 clones from each library were cultured in 2× Luria-Bertani (Miller)-ampicilin broth at 37 °C at 320 r/m for 20 h. Cultures were centrifuged and pellets were resuspended in Solution 1 (Millipore, Billerica, MA, USA). Plasmids were extracted and purified using MultiScreen Plasmid 96-well plates according to Plasmid Miniprep Millipore protocol.

Sequencing and phylogenetic affiliation of clones

Purified plasmids were sequenced with pJET1 forward sequencing primer (5′GCCTGAACACCATATCCATCC3′)/reverse sequencing primer (5′GCAGCTGAGAATATTGTAGGAGATC3′) (GeneJET PCR Cloning Kit, Fermentas International Inc., Burlington, ON, Canada), using ABI 3710XL analyzer. All sequences were subjected to Pregap4 base calling, vector masking before

aligning using Gap4 program. These near-full-length 16S rRNA gene sequences (approximately 1 400 bases) were analyzed by BLAST 2.0 (National Center for Biotechnology Information [NCBI]) to find the closest phylogenetic neighbors. A maximum parsimony tree of all sequences was constructed using MEGA V2.1. The tree was tested statistically using bootstrap resampling of 500 times.

Rarefraction analysis

The coverage of the biodiversity assessment in this study was evaluated with the Analytic Rarefraction software (version 1.2; M Holland, University of Georgia, Athens, GA; http://www.uga.edu/strata/Software.html).

Results

Bacterial community in the termite gut

A total of 1 152 clones were sequenced and 154 phylotypes were identified. Phylogenetic analysis revealed that the clones represented 10 bacterial phyla. Almost 65% of the clones belonged to *Bacteroidetes* (41 phylotype), and followed by *Firmicutes* that were about 15% of total analyzed clones (57 phylotypes). About 82 clones of the *Firmicutes* were *Lactobacillales*, 73 clones were *Clostridia*, and the rest fell within the class *Mollicutes*. *Actinobacteria* were the third most abundant bacteria in the gut of *C. curvignathus*, approximately 6% of the total analyzed clones (21 phylotypes). A total of 10 phylotypes were found to belong to *Proteobacteria*, 7 phylotypes clustered among *Spirochetes*, 5 phylotypes were members to candidate phylum Termite Group 1 (TG1), 4 phylotypes were clustered among the candidate division TM7, 2 each among *Planctomycetes* and *Verrucomicrobia*, and a single phylotype among *Deferribacteres*. There were 4 phylotypes assigned as uncultured bacteria with unknown phylum, and 1 phylotype (MgKI3d002D04) had no significant hit against NCBI database. Rarefaction analysis indicated that the number of analyzed clones was sufficient to give reasonable coverage at a sequence similarity threshold of 95% (Fig. 1).

Phylogenetic affiliation of 16S rDNA phylotypes

Bacteroidetes were the most abundant microbes in the gut of *C. curvignathus*. A total of 744 clones were assigned to *Bacteroidetes* and the most abundant clones were represented by phylotypes MgKI3d011C08 (649 clones) and 3d011e08 (8 clones) (Fig. 2). These phylotypes shared 98%–99% sequence similarity with clones representing endosymbionts of gut cellulolytic protist *Pseudotrichonymphae*. Another abundant group (100 clones) represented by 32 phylotypes were closely related to bacteria in the family *Porphyromonadaceae*, and majority of them affiliated with the genera *Dysgonomonas* and *Tannerella*. The clone libraries also contained 2 phylotypes each from family *Rikenellaceae* and *Bacteroidaceae.* Phylotypes 1c001b04 (6 clones) and 3d005d09 (2 clones) formed a minor cluster affiliated with the order cytophagales which forms the CFB group with *Bacteroidetes* and *Flavobacterium* (Woese, 1987).

A total of 166 clones that belonged to *Firmicutes* fell almost equally within the class *Clostridia* (44%) and *Lactobacillales* (49%), whereas another 7% in class *Mollicutes* (Fig. 3). The most abundant group was represented by phylotype MgKI1c001D09 (61 clones), which had 99% sequence similarity with *Lactococcus garvieae.* Phylotypes 3d011f09 and 3d002g11 were distantly related to *L. garvieae* (90%–94% sequence similarity). Within the class *Lactobacillales*, 4 phylotypes were clustered with *Enterococcus* (93%–98% sequence similarity), 1 phylotype each clustered with *Leuconostoc citreum* (99% sequence similarity), *Streptococcus* (94% sequence similarity), *Oscillospira guilliermondii*, and *Pilibacter termitis* (90% sequence similarity). Another abundant group, represented by 3d005h02, was affiliated with uncultured members of *Clostridiaceae* (92% sequence similarity) from termites, mammalian, and ruminant guts. Others members of class *Clostridia* were closely related to *Clostridium saccharolyticum, Clostridium amygdalinum*, and *Acetanaerobacterium.* Clones assigned to Class *Mollicutes* were minority (11 clones) and mostly fell within family *Spiroplasmas* and *Mycoplasmas* (2 phylotypes).

Actinobacteria constituted the third largest phylum in the intestinal tract of *C. curvignathus* (66 clones) (Fig. 4). The majority of clones (32 clones) were represented by phylotype MgKI 3d003h07 that had 92% sequence similarity with *Actinobacterium* (NCBI Sequence ID: GQ502466.1). There were several phylotypes representing clusters affiliated with the family *Cellulomonadaceae* (9 clones), *Microbacteriaceae* (43 clones), *Micrococcaceae* (3 clones), *Coriobacteriaceae* (10 clones), and *Mycobactericeae* (1 clone) (Fig. 4). Almost all the clones had the highest sequence similarity hit with clones that were extracted from the gut of *Coptotermes formosanus* Shiraki as deposited in NCBI.

The majority of the remaining clones (1.3% of the library) belonged to the phylum *Proteobacteria*. Most of the clones were Gammaproteobacteria of the family *Enterobacteriaceae.* Phylotypes MgKI3d002c04 (6 clones) shared 99% sequence similarity with *Serratia marcescens*

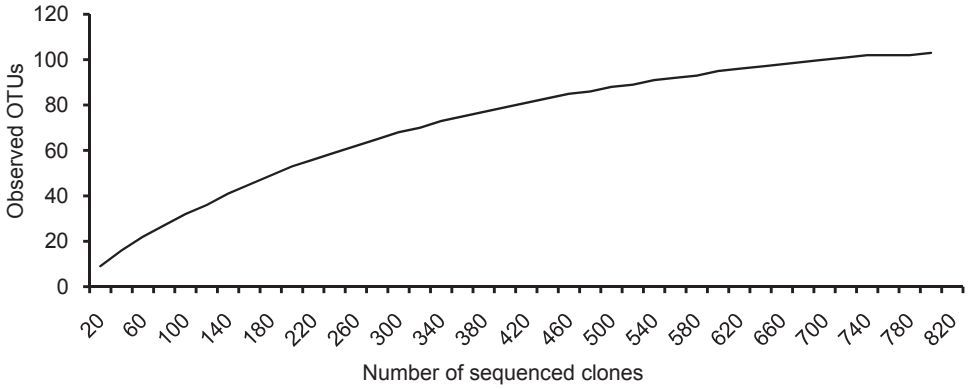

Fig. 1 Rarefaction analysis of all bacterial 16S rRNA gene clones recovered from the digestive trait of *Coptotermes curvignathus*. The expected number of clones was calculated from the number of clones analyzed at a sequence similarity level of 95%.

isolated (Fig. 5). There were 1 phylotype each assigned to Class *Alphaproteobacteria*, *Betaproteobacteria*, and *Deltaproteobacteria*.

Phyla *Spirochetes*, *Deferribacteres*, Candidate TG1, *Verrucomicrobia*, *Planctomycetes*, and Candidate Division TM7 were only scarcely represented. Only 2% of total analyzed clones belonged to spirochetal sequences (Fig. 6). All the spirochetal clones clustered together among a group exclusively of termite. The phylotypes MgKI1c3g09, MgKI1c2b06, and MgKI3e02 formed a monophyletic clade that was distantly related to other spirochetes of other termite species.

Two phylotypes in the libraries belonged to phylum *Planctomyctes*. Phylotype MgKI1c001a06 was closely affiliated (99% sequence similarity) with an uncultured *Planctomycetes* from the gut of *C. formosanus* (NCBI Sequence ID: GQ502587.1) but only shared about 95% sequence similarity with the uncultured *Planctomyctes* from *Reticulitermes chinensis* Snyder (NCBI Sequence ID: JQ617849.1). Phylotype MgKI1c002e07 was neighbored by *Planctomyctes* sequences from anaerobic digesters (Fig. 7). Four clones represented by phylotypes MgKI1c001f09 and 3d011g07 were assigned to uncultured *Verrucomicrobia* from termite guts (97% sequence similarity with sequences from *C. formosanus*). Five clones represented by Phylotypes MgKI3d005a03, MgKI3d005h11, MgKI1c003e08, and MgKI1c001h12 formed a single clade with members of the candidate division TM7. Phlyotype MgKI3d002e04 belonged to the phylum *Deferribacteres*, with the next closest uncultivated *Deferribacteres* from the gut of termite *Macrotermes gilvus* Hagen. Phylotypes MgKI3d002e09, MgKI3d003g11, and MgKI1c001h07 formed a large cluster with TG1 that consists exclusively of clones from termite. Phylotypes MgKI3d002e09 and MgKI1c001h07 were more closely related than phylotype MgKI3d003g11, which was close neighbored with the uncultured endomicrobia in termite *Cryptotermes* spp. gut (99% sequence similarity).

The Blast-n result showed that higher percentage of similarity in gut microbiome assemblage was observed when compared to *C. formosanus*. About 25% of the phylotypes in *C. curvignathus* library had highest similarity (95%–99% sequence similarity) to sequences from *C. formosanus*. This is relatively higher than the percentage of phylotypes in *C. curvignathus* library with the highest sequence similarity to sequences from *Reticulitermes* spp. (16%).

Discussion

The predominance of spirochetes observed in the gut of *R. speratus* (Isoptera: Rhinotermitidae) (Nakajima *et al*., 2005) was an obvious distinguishing factor of gut microbiome between 2 members of family Rhinotermitidae: *Reticulitermes* and *Coptotermes*. In the gut of *C. curvignthaus*, *Spirochetes* only constituted about 2% in the clone libraries, whereas *Spirochetal* clones accounted for approximately half of the analyzed clones in *R. speratus* (Hongoh *et al*., 2003). Interestingly, the differences are not limited to *Spirochetes*; *Bacteriodetes* were more represented in the gut of *C. curvignathus* (65% of analyzed clones) than *R. speratus* (Hongoh *et al*., 2003). However, when compared to *R. speratus* (Hongoh *et al*., 2003), depletion of bacterial members belonged to TG1 was observed in *C. curvignathus* microbiome (approximately 0.3%). In a library of 1 344 clones, microbiome of the *R. speratus* gut contains 42%–63% of *Spirochetal* clones, whereas clones related to *Bacteroides* and the candidate division TG1 each accounted for about 5%–15% of the sequenced clones (Hongoh *et al*., 2003).

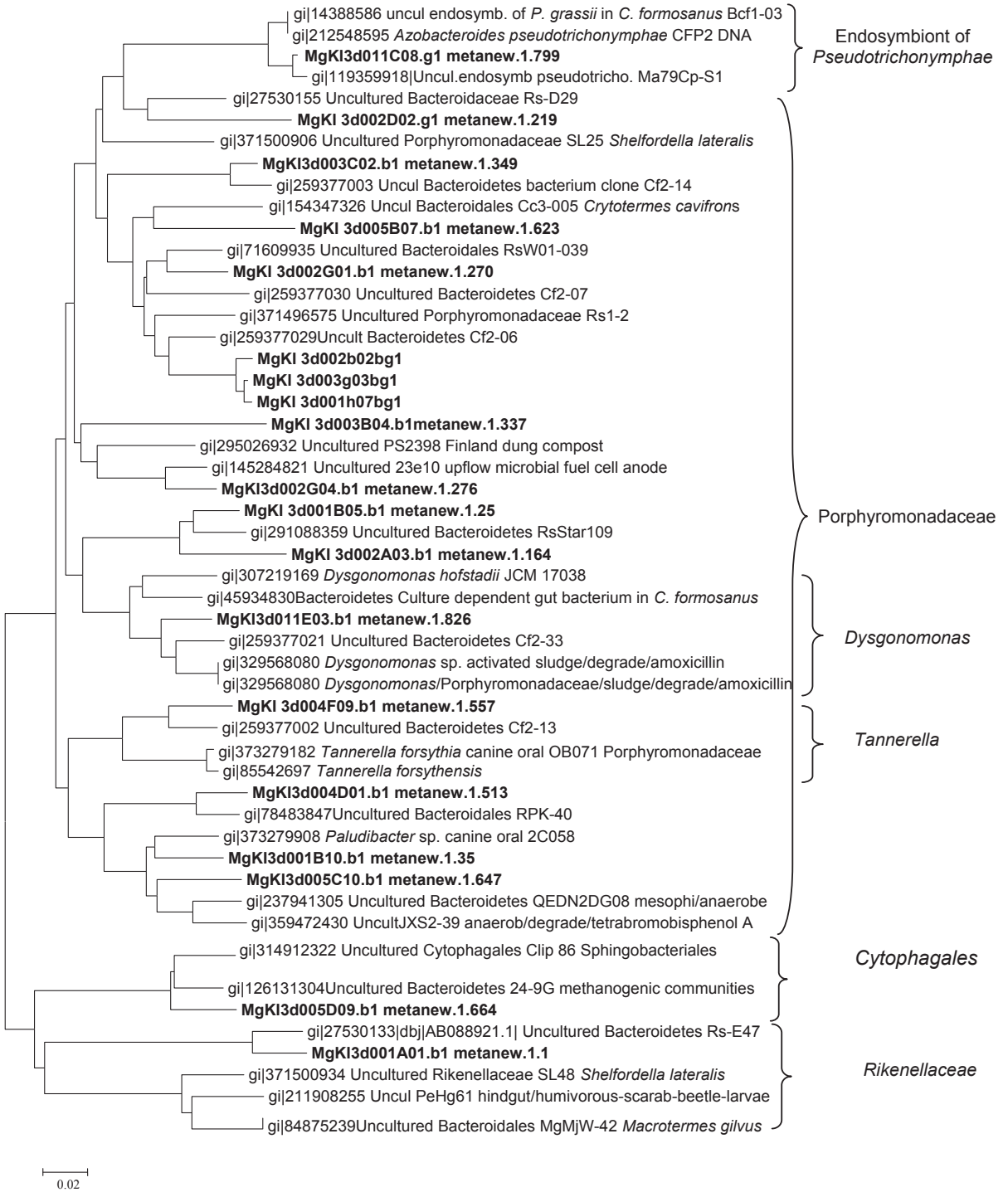

Fig. 2 Phylogenetic relationships of the phylotypes in *Bacteroidetes*. The tree was inferred by the neighbor joining with 1 000 Bootstrap replications with 1 241 unambiguously aligned nucleotide positions (MEGA 5.10). Phylotypes from this study are shown in bold. The scale bar represents a 2% estimated sequence divergence.

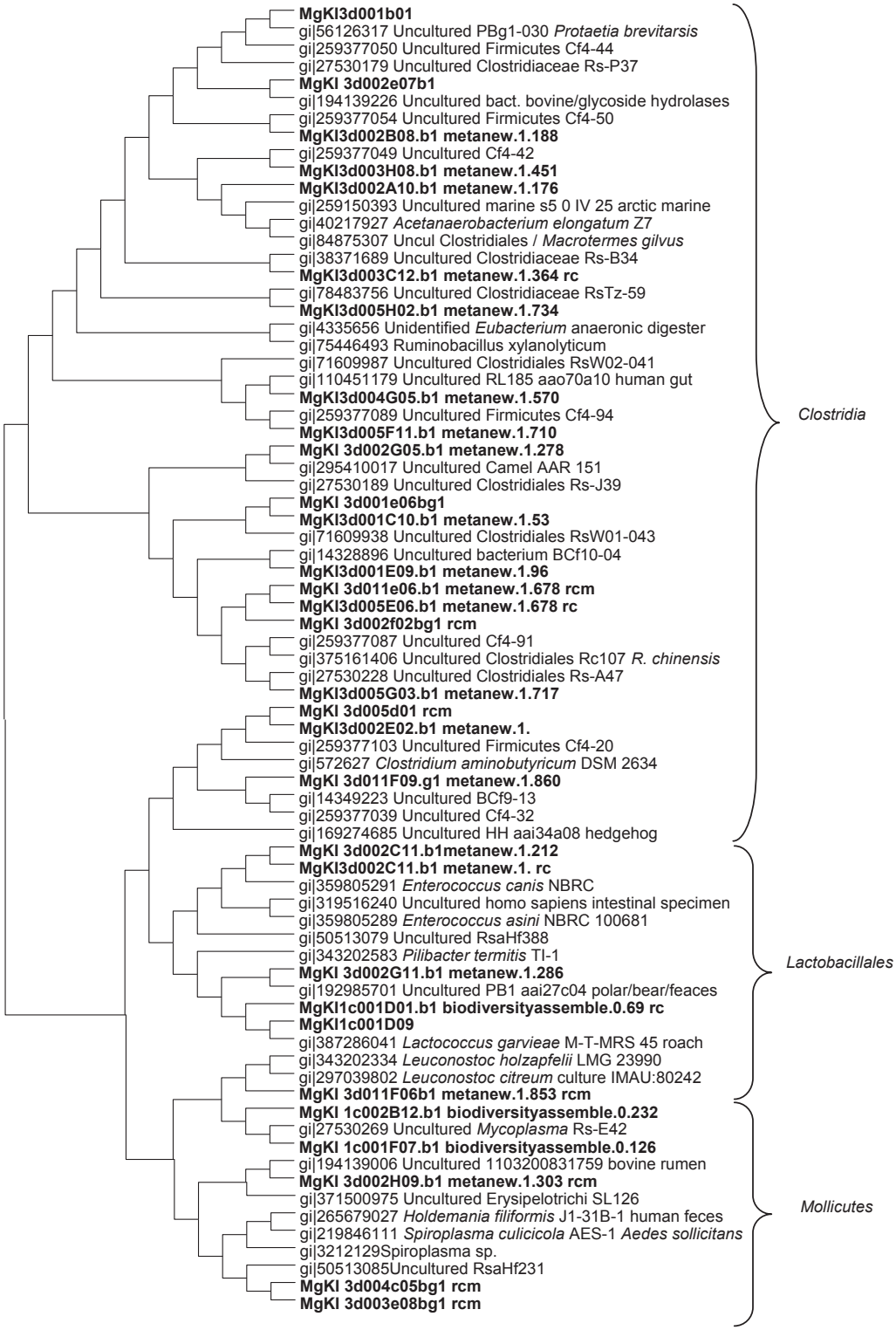

Fig. 3 Phylogenetic relationships of the phylotypes in *Firmicutes*. The tree was inferred by the neighbor joining with 1 000 Bootstrap replications with 1 344 unambiguously aligned nucleotide positions (MEGA 5.10). Phylotypes from this study are shown in bold.

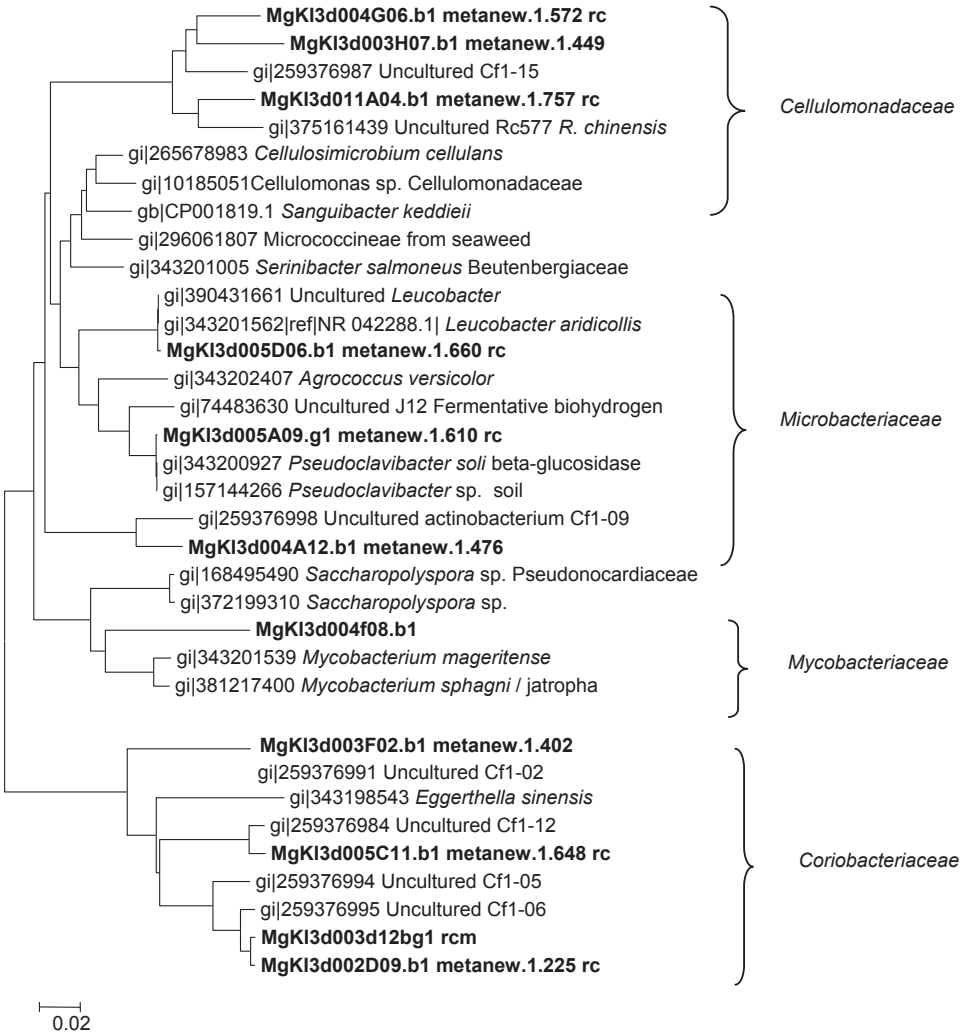

Fig. 4 Phylogenetic relationships of the phylotypes in actinobacteria. The tree was inferred by the neighbor-joining methods with 1 271 unambiguously aligned nucleotide positions. Phylotypes determined in this study are indicated in bold. The scale bar represents a 2% estimated sequence divergence.

Results from the Blast-n indicated that *C. curvignthus* shared more enterotypes with *C. formosanus* than *Reticulitermes* spp. These observations suggest codivergence of intestinal microbes with genusiation their host. The phylogenetic distances among *C. curvignathus*, *C. formosanus*, and *Reticulitermes* spp. were to some extent reflected among their microbial gut communities. Codivergence of gut microorganisms with host termites is probably established through strict vertical transmissions along the evolutionary lineages via proctodeal trophallaxis.

Besides the phylogenetic distance between genera *Coptotermes* and *Reticulitermes*, the differences in their gut microbial assemblages could also be the result of their diet preference. Although both *Coptotermes* spp. and *Reticulitermes* spp. feed on wood, *Coptotermes* spp. are evidently more obvious in feeding on living trees and consume more solid wood in the field than *Reticulitermes* spp. (Lenz *et al*. 1991). Feeding preference between these 2 genera also has been reported by Cornelius *et al*. (2003) as well as Morales-Ramos and Rojas (2003) where the wood consumption of *Reticulitermes* spp. is generally negatively correlated with wood hardness, whereas *Coptotermes* are more related to the nutritional components of the wood. In general, species of *Coptotermes* are known to have greater capacity for destruction than *Reticulitermes*. Modulation of gut microbiome composition associated with dietary pattern has been established in many studies

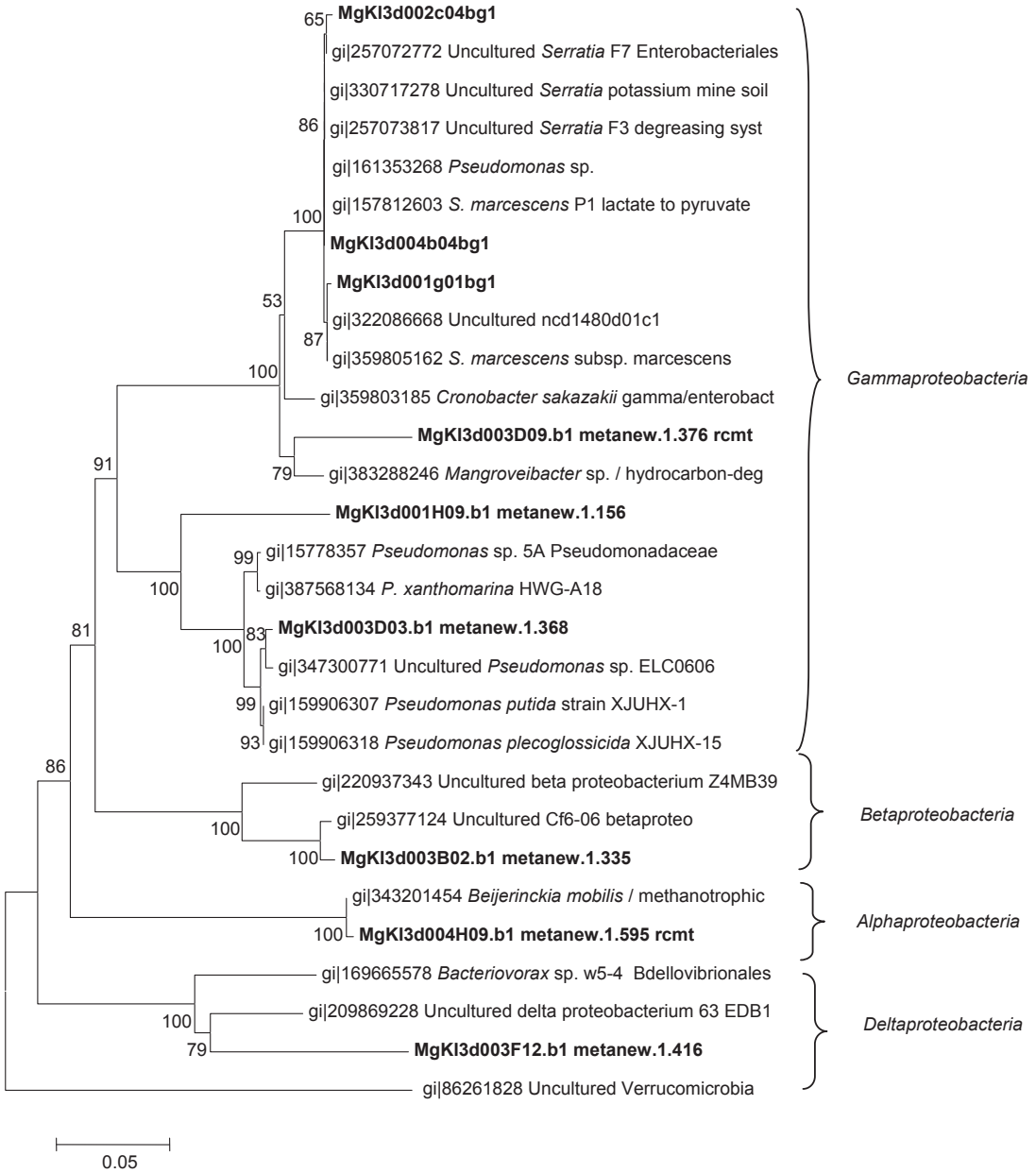

Fig. 5 Phylogenetic relationships of the phylotypes in *Proteobacteria*. The tree was inferred by the neighbor-joining methods with 1 274 unambiguously aligned nucleotide positions. Phylotypes determined in this study are indicated in bold. Bootstrap values are indicated at the nodes. The scale bar represents a 5% estimated sequence divergence.

especially in humans (De Filippo *et al*., 2010; Martin *et al*., 2010; Wu *et al*., 2011; Neyrinck *et al*., 2012), however the microbial dimension in insect nutritional ecology is gaining recognition (Douglas, 2009). The study of gut microbiome modulation linked with dietary pattern could help explain the variation in microbial diversity and distribution among closely related termite species.

The diversity of *Bacteroidetes* in the gut of *C. curvignathus* was very diverse, ranging from family *Porphyromonadaceae*, *Cytophagales*, and *Rikenellaceae* to the endosymbiont of flagellate *Pseudotrichonymphae*. *Bacteroidetes* were known to exhibit a variety of cellular morphologies and were extremely heterogeneous in their biochemical and physiological attributes (Skerman *et al*.,

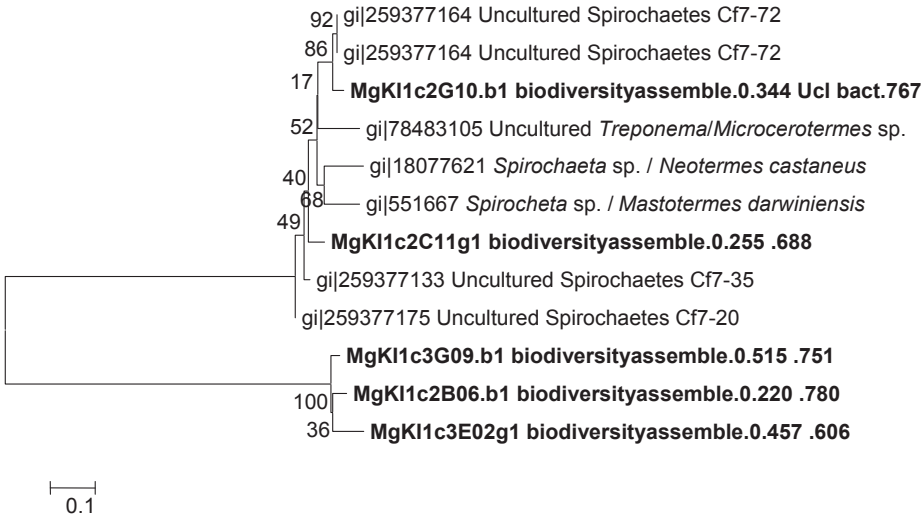

Fig. 6 Phylogenetic relationships of the phylotypes in *Spirochetes*. The tree was inferred by the neighbor-joining methods with 600 unambiguously aligned nucleotide positions. Phylotypes determined in this study are indicated in bold. Bootstrap values are indicated at the nodes. The scale bar represents a 10% estimated sequence divergence.

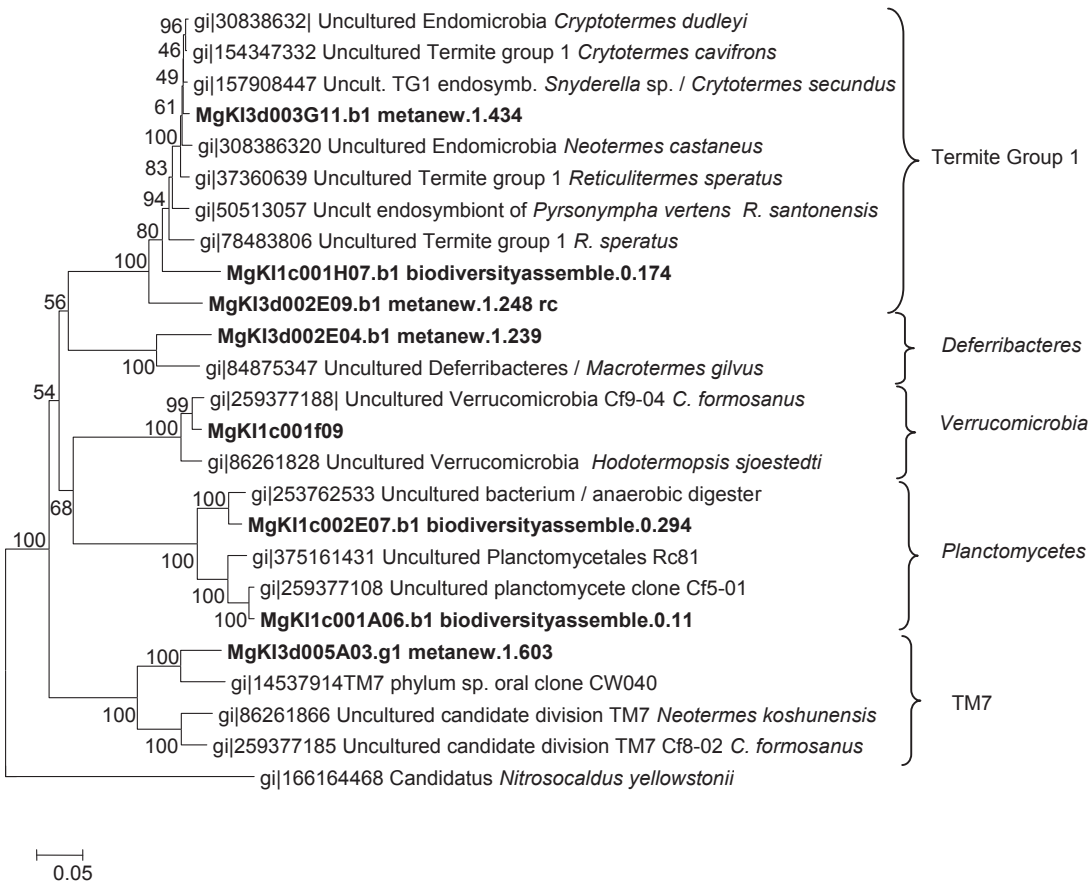

Fig. 7 Phylogenetic relationships of the phylotypes in other phyla. The tree was inferred by neighbor-joining methods with 1 200 unambiguously aligned nucleotide positions. Phylotypes determined in this study are indicated in bold. Bootstrap values are indicated at the nodes. The scale bar represents a 5% estimated sequence divergence.

1989). Their versatility helps them establish at different niches in the termite gut. The percentage of *Bacteroidetes* present in the gut of *C. curvignathus* (65% of clones library) is one of the highest among all other studied termite species. They are the most abundant microbes in the gut of *C. curvignathus* and probably play an active role in the degradation of polysaccharides from plant fibers, such as cellulose, xylan, arabinogalactan, and pectin at an anoxic condition in the termite gut. De Filippo *et al*. (2010) reported a positive correlation of *Bacteroidetes* enrichment in subjects with a high-fiber diet.

Bacteroidetes are associated with a wide variety of gut protist species as either intracellular endosymbionts or surface-attached ectosymbionts (Noda *et al*., 2009). The most abundant clones represented by phylotype MgKI3d011C08 were endosymbionts of flagellate *Pseudotrichonymphae*. *Pseudotrichonymphae grasii* is an important enterophyte in termites' gut that aids in lignocellulosic digestion. In a controlled feeding study, *P. grasii* that was found in great abundance in the gut microbiome of *C. formosanus* (Noda *et al*., 2005) was completely lost when fed on a low-molecular weight carbohydrate diet (Tanaka *et al*., 2006). This is a marked case of linking lignocellulosic diet with protist *P. grasii*.

Phylotype MgKI3d011C08 shared 98%–99% sequence similarity with phylotype Bcf1–03 that constitutes 70% of the 261 analyzed clones from *C. formosanus* (Shinzato *et al*., 2005). Both phylotypes were found in great abundance (more than 60%) in *C. curvignathus* and *C. formosanus*, respectively. Phylotypes MgKI3d011C08 and Bcf1–03 were similar, but there were more than 29 single nucleotide polymorphism (SNP) within their 16S rRNA genes. This may be another evidence of cospeciation of bacteria symbiont with host termite.

The phylotype MgKI3d011C08 outnumbered others by several orders of magnitude. This indicates not only the importance of its metabolic contribution to the nutritional ecology of *C. curvignathus* and *Pseudotrichonymphae* but also the presence of *Pseudotrichonymphae* in *C. curvignathus*. However, this does not imply the abundance of *Pseudotrichonymphae* in *C. curvignathus* as it has been established that a single flagelate protist *P. grasii* can accommodate $1.08 \times 10^5 \pm 0.04 \times 10^5$ bacteroidetes endosymbiont (Noda *et al*., 2005).

About 15% of analyzed clones belonged to *Firmicutes* and 49% of these were *Lactobacillales*, and 44% were *Clostridia*. Phylotype 3d011c05 was the most abundantly found *Firmicutes* with 99% sequence similarity with *L. garvieae.* Other lactic acid bacteria found in *C. curvignathus* were the *L. citreum, Enterococcus* spp., *Streptococcus* spp., *O. guilliermondii*, and *P. termitis.* Lactic acid bacteria are known to be the producer of dextransucrase, an extracellular enzyme involved in the synthesis reaction of dextran from sucrose (Barker & Ajongwen, 1991). Lactic acid bacteria also contribute to effective oxygen reduction in termite gut that is vital in maintaining microoxic zones for the strictly anaerobic symbiont (Tholen *et al*., 1997; Bauer *et al*., 2000; Higashiguchi *et al*., 2006). Other than that, *P. termitis* may be involved in sugar fermentation and pH regulation of the gut (Hutkins & Nannen, 1993). Several lactic acid bacteria that are involved in carbon and nitrogen recycling by metabolizing uric acid have also been reported (Potrikus & Breznak, 1981). This is important as termites consume wood having low-nitrogen content (approximately 0.03%–0.15%) of wood diet (Potrikus & Breznak, 1981).

Clostridia spp. were major genus of *Firmicutes* in *C. curvignathus* (44% of *Firmicutes* clones). They are distinguished from the *Bacilli* by lacking aerobic respiration. Members of *Clostridiales* in the clone library include *C. saccharolyticum*, *C. amygdalinum*, and *Acetanaerobacterium. Acetanaerobacterium* generate hydrogen from the glucose during their anaerobic respiration (Miyake *et al*., 1984; Taguchi *et al*., 1992; Chen & Dong, 2004). *Clostridia* are believed to be the major contributor to the hydrogen available for other metabolisms in the termite gut (Cao *et al*., 2010). Without *Clostridia*, important process such as H_2-dependent acetogenesis and methanogenesis may be adversely affected (Breznak & Switzer, 1986; Brauman *et al*., 1992). Based on culture independent studies, acetogenic clostridial species are one of the major groups in termites (Hongoh *et al*., 2003, 2006; Shinzato *et al*., 2005, 2007; Yang *et al*., 2005). Many *Clostridia* degrade polysaccharides to produce acetone, alcohol, acetate, lactate, carbon dioxide, and hydrogen (Johnston & Goldfine, 1985; Chen, 1995), and some *Clostridia* involve in nitrogenous or lipid compounds fermentation (Elsden & Hilton, 1979). *Clostridia* produce end products such as short-chain fatty acids, for example, butyric acid, acetic acid, butanol, and acetone, which are important physiological energy source for colonic cells and play a vital role in the maintenance of hindgut health (Roediger *et al*., 1989; Basson & Sgambati, 1998). There are some members of the *Clostridia* that produce cellulosomes and noncellulosomal (hemi)cellulolytic enzymes to degrade plant cell walls (Han *et al*., 2004) and some exhibit high xylanase or pectate lyase expression such as *C. cellulovorans*. This explains the importance of *Clostridia* to the versatile metabolism in the gut of *C. curvignathus*.

In *Reticulitermes* spp., spirochetes constituted 42%–63% of the total gut bacteria (Hongoh *et al*., 2003), whereas in this study, they comprise only 2% of total analyzed clones from the gut of *C. curvignathus*. The depletion of *Spirochetes* (11.3% of 1 876 analyzed clones)

in gut microbiome assemblage was also observed in *C. formosanus* (Husseneder *et al*., 2010). *Spirochetes* are undoubtedly an important group of the natural termite microbiota, which contribute to termite nutrition via acetonegenesis from H_2 plus CO_2 (Leadbetter *et al*., 1999). However, the reduction in *Spirochetal* composition in the gut of *Coptotermes* spp. reflects a decline in host termite dependency on *Spirochetes*. On the other hand, a sharp increase in the abundance of *Bacteroidetes* endosymbiont of *Pseudotrichonymphae* was observed in *Coptotermes* gut microbiome. The key factor attributed to this modulation of gut microbiome composition is still obscure at this time. However, it may be due to its dietary preference. Most of the wood feeder termites feed on dry dead wood or semi to partially degraded wood residues; however, unlike these termites, *C. curvignathus* preferentially consumes living plant (Bong *et al*., 2012). In peat area where *C. curvignathus* is vastly found, the decomposition rate was slow. *C. curvignathus* has evolved and adapt to feed on living plant instead of decomposed wood residue. Therefore, *C. curvignathus* is considered a natural living plant pest, whereas its closest phylogeny, *C. formosanus*, is an occasional pest that feeds mostly on dead wood, and *Reticulitermes* feed on tree stump or dead wood. Generally, the diet adaptation comes naturally with gut microbiome modulation. Feeding on living trees means the diet of *C. curvignathus* has much higher water content than other wood feeder species. Higher water content diet is important for the free living prostist *Pseudotrichonymphae* to flourish in the gut of *C. curvignathus*. From the phylogentic study (Fig. 6), 60% of the spirochetal phylotypes in this study form a monophyletic cluster exclusively composed of *C. curvignathus* gut-derived clones. This could be an indication of cospeciation of *Spirochetes* with *C. curvignathus*.

In summary, the reported phylogenetic distribution of cloned rRNA genes in the bacterial domain was considerably different from other termite species. The difference in phylogenetic composition of the gut microbes among *R. speratus*, *C. formosanus*, and *C. curvignathus* reflects the strong selection pressures inherent in such tiny (1 μL of volume), yet physiological delicate, environment in the intestine where natural gut microbes have to adapt themselves to the specific diet of their host. Single and specific diet such as a living tree or sound wood would favor some microbial species to flourish and change the composition of the gut bacterial community that reshapes the physicochemistry of the gut. The physiococchemistry of the gut will constraint and conserve the gut microbial assemblage, and *vice versa*, and the firmly established gut microbiome will maintain the physiocochemical condition of the gut and provide available nutrients to the host as well as protection against opportunistic pathogen. The mutual symbiosis relation of the gut microbiome with the host termite is established through coevolution that can be observed in the phylogenetic studies on termites' enterotypes. The capability of *C. curvignathus* to feed on healthy living tree tissues is indeed remarkable digestion adaptability, linking termite nutritional ecology with its gut microbial enterotypes.

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Disclosure

There are no conflicts of interest among authors involved in this manuscript.

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