APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2005, p. 6590–6599 Vol. 71, No. 11
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.11.6590–6599.2005

Intra- and Interspecific Comparisons of Bacterial Diversity and Community Structure Support Coevolution of Gut Microbiota and Termite Host†

Yuichi Hongoh,[1,2]* Pinsurang Deevong,[3] Tetsushi Inoue,[1] Shigeharu Moriya,[1,2,4] Savitr Trakulnaleamsai,[3] Moriya Ohkuma,[2,5] Charunee Vongkaluang,[6] Napavarn Noparatnaraporn,[3] and Toshiaki Kudo[1,2,4]

International Cooperative Research Project, Japan Science and Technology Agency (JST-ICORP), Saitama 351-0198, Japan[1]; Environmental Molecular Biology Laboratory, RIKEN, Saitama 351-0198, Japan[2]; Department of Microbiology, Kasetsart University, Bangkok 10900, Thailand[3]; Graduate School of Integrated Science, Yokohama City University, Kanagawa 230-0045, Japan[4]; Precursor Research for Embryonic Science and Technology, JST (JST-PRESTO), Saitama 351-0198, Japan[5]; and Royal Forest Department, Bangkok 10900, Thailand[6]

Received 17 December 2004/Accepted 1 June 2005

We investigated the bacterial gut microbiota from 32 colonies of wood-feeding termites, comprising four *Microcerotermes* species (Termitidae) and four *Reticulitermes* species (Rhinotermitidae), using terminal restriction fragment length polymorphism analysis and clonal analysis of 16S rRNA. The obtained molecular community profiles were compared statistically between individuals, colonies, locations, and species of termites. Both analyses revealed that the bacterial community structure was remarkably similar within each termite genus, with small but significant differences between sampling sites and/or termite species. In contrast, considerable differences were found between the two termite genera. Only one bacterial phylotype (defined with 97% sequence identity) was shared between the two termite genera, while 18% and 50% of the phylotypes were shared between two congeneric species in the genera *Microcerotermes* and *Reticulitermes*, respectively. Nevertheless, a phylogenetic analysis of 228 phylotypes from *Microcerotermes* spp. and 367 phylotypes from *Reticulitermes* spp. with other termite gut clones available in public databases demonstrated the monophyly of many phylotypes from distantly related termites. The monophyletic "termite clusters" comprised of phylotypes from more than one termite species were distributed among 15 bacterial phyla, including the novel candidate phyla TG2 and TG3. These termite clusters accounted for 95% of the 960 clones analyzed in this study. Moreover, the clusters in 12 phyla comprised phylotypes from more than one termite (sub)family, accounting for 75% of the analyzed clones. Our results suggest that the majority of gut bacteria are not allochthonous but are specific symbionts that have coevolved with termites and that their community structure is basically consistent within a genus of termites.

Termites harbor an abundance and diversity of gut bacteria, which are thought to play essential roles in the carbon and nitrogen metabolism of their host termites. While these characteristics of gut bacteria have been extensively studied by tracing the flow of carbon and nitrogen or characterizing isolated strains of bacteria (5, 25), the bacterial microbiota has remained a black box due to difficulties in cultivation of most of these bacteria. This has been an obstacle to a comprehensive understanding of symbiosis between gut bacteria and their host termites. Recently, we conducted a detailed census of the bacterial community in the gut of the termite *Reticulitermes speratus* by analyzing clones of 16S rRNA (18, 19). We found 314 phylotypes (defined with 97.0% sequence identity) of 16S rRNA from 1,923 analyzed clones. The majority of the clones were affiliated with groups of anaerobic bacteria such as the genus *Treponema* and the orders *Clostridiales* and *Bacteroidales*, and most of the phylotypes were found for the first time. Many of them constituted novel lineages in several bacterial phyla, including the candidate phylum termite group I (TG1), which was one of the dominant groups in *R. speratus* (18, 34). For the African soil-feeding termite *Cubitermes orthognathus*, a culture-independent analysis of gut bacteria was also conducted. It was revealed that distinct bacteria inhabited each of the distinct gut compartments, and most of the bacteria were considered novel species (42). Although the novelty of these bacterial lineages prompts us to assume that they are not allochthonous but rather autochthonous symbionts that are specifically associated with termites, only a few studies have examined the phylogenetic relationship of gut bacteria among different termite samples. Besides, since these studies focused only on the genus *Treponema* (27, 33) and the order *Bacteroidales* (35), they were insufficient to evaluate the overall relationship of the diverse termite gut bacteria, which are affiliated with more than 10 phyla (18). In addition, little is known about the variation of bacterial gut microbiota between individuals, colonies, locations, and species of termites.

* Corresponding author. Mailing address: Environmental Molecular Biology Laboratory, RIKEN, Saitama 351-0198, Japan. Phone: 81-48-467-9648. Fax: 81-48-462-4672. E-mail: yhongo@postman.riken.go.jp.

† Supplemental material for this article may be found at http://aem.asm.org/.

For this study, we collected 32 termite colonies from various locations to investigate and compare their bacterial gut microbiota, using a combination of terminal restriction fragment length polymorphism (T-RFLP) analysis and clonal analysis of 16S rRNA. We used four *Reticulitermes* species as representatives of the lower termites, which have a simply structured gut and harbor gut protistan symbionts, and four *Microcerotermes* species as representatives of the higher termites, which have a highly compartmentalized gut and lack protistan symbionts. The phylogenetic relationship of gut bacteria and the variation of their diversity and community structure within and between host termite species are discussed.

MATERIALS AND METHODS

Collection of termites. We collected 25 colonies comprising four species of the wood-feeding higher termites belonging to the genus *Microcerotermes* (order Isoptera, family Termitidae, subfamily Termitinae) and 7 colonies comprising four species of the lower termites belonging to the genus *Reticulitermes* (family Rhinotermitidae). The locations of the sampling sites and the abbreviations of the samples used in this study are shown in Fig. 1. The termites were identified based on their morphology and the DNA sequence of mitochondrial cytochrome oxidase II. Detailed information on the classification of the genus *Microcerotermes*, including two undescribed species, *Microcerotermes* species M1 and M2, has been described elsewhere, using the same colony sample names (22). Another undescribed species, a *Reticulitermes* sp. from Nan province in Thailand, is designated species R1 in this study. The termites with their nest were carefully transported to our laboratories in Thailand or Japan without heating or sunlight exposure.

DNA extraction and PCR amplification. The collected termites were immediately subjected to DNA extraction, except for *Reticulitermes* samples collected in Tanzawa and Amami Island in Japan, which were kept in the laboratory with their nest log for 7 months before being processed. Whole guts were isolated from 20 randomly chosen workers per colony by using sterilized forceps. DNAs were extracted from the gut homogenates using an Isoplant II kit (Nippon Gene), which chemically lyses bacterial cell walls and membranes with benzyl chloride. The extracts were further purified using a DNeasy tissue kit (QIAGEN) as described previously (48). To test for differences in gut bacterial community structure between individual termites, DNAs were also extracted from the guts of five randomly chosen workers from colony M1NP1, and the samples were named M1NP1a to -e. The DNA sample from *R. speratus* collected at Ogose in Japan (RsOg, Fig. 1) was prepared for our previous study using a QIAGEN RNA/DNA kit (18).

PCR was performed with the *Bacteria*-specific primer pair 27F and 1389R (18) to amplify the near-full-length 16S rRNA gene. For T-RFLP analysis, the 27F primer was labeled with 6-carboxyfluorescein (FAM). To minimize PCR bias (38, 45) and other artifacts (11, 39, 46), the PCR cycle number was limited to 12 and 20 for clonal and T-RFLP analyses, respectively. The PCR mixture contained 1.0 μM of the primer set, 0.025 U/μl of Ex*Taq* polymerase (Takara), its attached buffer, a deoxynucleoside triphosphate mixture, and the DNA template adjusted to 0.1 ng/μl. The concentration of the DNA template was further adjusted to produce approximately the same amount of final PCR product between samples in order to diminish the effect of PCR bias. The reaction tubes were placed into the preheated (95°C) block of a PTC-200 thermal cycler (MJ Research), and the reaction was run with the following program: an initial 2 min of denaturation at 95°C, 12 or 20 cycles of denaturation (30 s at 95°C), annealing (1 min at 50°C), and extension (4 min at 72°C), and a final 10-min extension at 72°C.

T-RFLP analysis. The PCR products (50 μl) were loaded into a 1% agarose gel (Seakem GTG; BMA) and electrophoresed to separate the products. The products at the target size (1.3 to 1.5 kb) were excised from the gel and purified using a MinElute gel extraction kit (QIAGEN). A 5-μl aliquot of the purified sample was digested with the restriction enzyme HhaI or HaeIII (Takara). From this reaction mix, 2 μl was mixed with formamide and a GS-1000ROX internal size standard (Applied Biosystems) and loaded into an ABI377 genetic analyzer. Electrophoresis and data extraction, using the software GeneScan Analysis 3.1.2 (Applied Biosystems), were conducted according to the manufacturer's instructions. The whole procedure was replicated in order to check the reproducibility, using freshly diluted DNA templates for PCRs.

Although peaks were basically identifiable in increments of one base, only a larger peak height was taken into account for all the samples when adjacent peaks were indistinguishable. The peak height was standardized before comparison, basically according to the method of Dunbar et al. (10), using 50 fluorescence units as the threshold of the baseline noise. After the fourth iteration procedure, the standardized data were analyzed with the software BioCLUST v. 1.0 (24), using a dissimilarity index, calculated as follows: $D = 1/2 \times \Sigma|x_{ki} - x_{kj}|$, where x_{ki} and x_{kj} are the ratios (%) of the kth peak heights in the samples i and j, respectively. When the two samples were identical, $D = 0$, and when they were absolutely different, $D = 100$ (16). Dendrograms were constructed based on this dissimilarity index, using the neighbor-joining (NJ) algorithm followed by the tree bisection-reconnection procedure in PAUP* (version 4.0b10; D. Swofford, Sinauer Associates, Sunderland, MA). To test for a differentiation of clusters, P values for a fixation index, F_{ST} (see below), were calculated by permuting samples 10,000 times, using the software ARLEQUIN (version 2.001; S. Schneider, D. Roessli, and L. Excoffier, University of Geneva, Switzerland [http://lgb.unige.ch/arlequin/]).

Clone libraries and sequencing. Nine colony samples (M1NP1, M1NP2, M1PL1, M1PT4, M2PT2, M2PB4, RsTz, RsTu1, and RPK) were also subjected to clonal analysis. Four 50-μl reaction mixtures containing DNA templates from the same colony sample were combined after PCR to decrease the effects of PCR

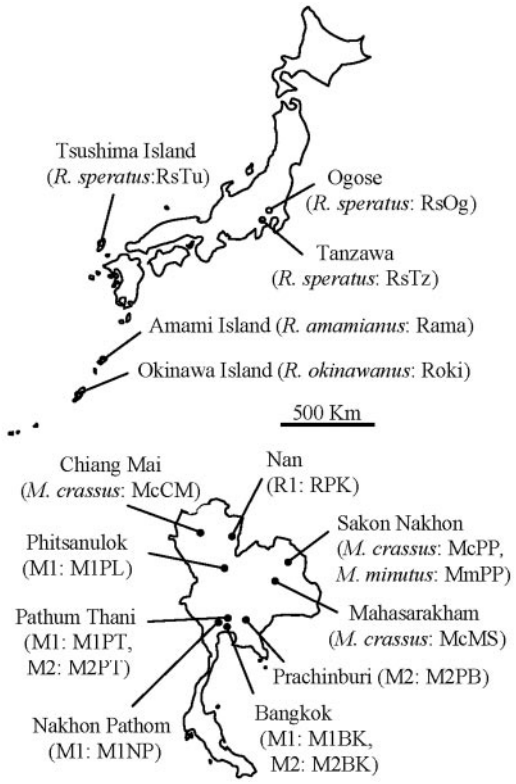

FIG. 1. Sampling sites of termites and abbreviations of colony samples used for this study. Four *Reticulitermes* species (*R. speratus*, *R. okinawanus*, *R. amamianus*, and *Reticulitermes* species R1) were collected in Japan (top) or Thailand (bottom). Four *Microcerotermes* species (*M. crassus*, *M. minutus*, and *Microcerotermes* species M1 and M2) were collected in Thailand. One (M1BK, M2BK, MmPP, RsOg, RsTz, Rama, Roki, and RPK), two (M1PT, M2PT, and RsTu), or three (M1NP, M1PL, M2PB, McCM, McMS, and McPP) colonies were collected from each sampling area for one species. These colony samples were named, e.g., M1NP1, M1NP2, and M1NP3. The three species *Reticulitermes* species R1 and *Microcerotermes* species M1 and M2 are undescribed species. Both species M1 (colonies M1PT1 and M1PT4) and species M2 (colonies M2PT2 and M2PT3) were collected at the same orchard in Pathum Thani, and colonies M1BK1 and M2BK2 were collected at the same university campus in Bangkok. Both *M. minutus* (colony MmPP5) and *M. crassus* (colonies McPP2 to -4) were collected at Phu Phan in Sakon Nakhon province, but from distinct forests.

drift (38). The products were purified using a MinElute PCR purification kit (QIAGEN), and TA cloning was performed using a TOPO TA cloning kit for sequencing (Invitrogen). Ninety-six clones were randomly chosen for sequencing from each clone library. Sequencing was performed using a Big Dye Terminator cycle sequencing kit (Perkin-Elmer) and an ABI377XL or 3700 genetic analyzer, as described previously (18). The sequence data for 96 clones from clone library A in our previous study were used as the data for RsOg (Fig. 1), since the PCR conditions were identical to those of the present study (18). The clones were classified into phylotypes (defined with the criterion of 97.0% sequence identity) as described previously (18). A representative sequence of a phylotype was subjected to identification of a chimeric sequence using the online programs RDP II Chimera Check (30) and Bellerophon server (20). For the analysis using the Bellerophon server, we uploaded the alignment of all of the near-full-length sequences obtained in this and previous studies (18, 19). Candidate chimeras were checked visually by comparing sequences in ARB software, version 030822 (29). If a sequence was suspected of being a chimera, a different clone was chosen as the representative for the phylotype. The name of the representative clone for a phylotype was also used as the name of the phylotype. In the case that a phylotype obtained from *Reticulitermes* spp. in this study was identical to one obtained from *R. speratus* in our previous studies (18, 19), the previous name was used for the phylotype.

Statistical comparison of clone libraries. The sequences for 96 clones from each library were aligned with ARB software, and 527 nucleotide sites from unambiguously aligned regions, corresponding to positions 563 to 1114 in *Escherichia coli* (J01695), were used for the following analyses. This alignment, comprising 960 sequences, is available upon request. The Libshuff test (40, 43), F_{ST} test, and P test (31) were employed to test for differences between clone libraries. The Libshuff test is based on the difference between the homologous and heterologous coverage of two clone libraries. Coverage was calculated by the formula $[1 - (n/N)]$ (14), where n is the number of phylotypes represented by only one clone and N is the total number of clones. The P value was determined by 100,000 permutations of clones between the compared libraries, using the program ∫-LIBSHUFF v1.1 (40). The distance matrix analyzed by ∫-LIBSHUFF was generated with the Jukes-Cantor (JC) model, using the DNADIST program in the PHYLIP package (version 3.573; J. Felsenstein, Department of Genome Science, University of Washington [http://evolution.gs.washington.edu/phylip.html]). The F_{ST} test is based on the difference between the average genetic distances within and between clone libraries, as follows: $F_{ST} = (\theta_T - \theta_W)/\theta_T$, where θ_T is the genetic diversity between communities and θ_W is the genetic diversity within each community averaged over all the communities being compared (31). Statistical significance was evaluated by using 10,000 permutations of the clones, using the software ARLEQUIN. The P test assesses the significance of covariation of a community and the phylogeny of its members. NJ trees constructed with the JC model in PAUP* were used to estimate the minimum number of changes from one community to the other (31). In the case that the phylogeny was contradictory with regard to the monophyly of a bacterial phylum, NJ trees constructed with the Tamura-Nei model with a gamma distribution, optimized by the tree bisection-reconnection procedure in PAUP*, were used. The obtained minimum number of changes was compared with those for 100,000 random trees generated using MacClade (version 4.06; W. P. Maddison and D. R. Maddison, Sinauer Associates, Sunderland, MA). To compare multiple samples based on these pairwise comparisons, P values were adjusted with Holm's modified Bonferroni procedure (17).

Phylogenetic analysis. Alignments and determinations of the preliminary phylogenetic affiliations of the 16S rRNA phylotypes were performed using the ARB software with the database ssujun02. Representative sequences of the phylotypes obtained in this study were incorporated into the database using the Fast Aligner program implemented in ARB software, and the alignment was corrected manually. Closely related sequences found by a BLAST search (1) and termite gut clones available in the public databases DDBJ (DNA Data Bank of Japan), GenBank, and EMBL were also added to the ARB database. Ambiguously aligned regions were excluded, and the remaining 1,220 nucleotide sites, corresponding to positions 28 to 1388 in *E. coli* (J01695), were used for the reconstruction of a phylogenetic tree. MEGA V2.1 (26) was used to generate an NJ tree with the JC model. The robustness of the inferred topology was tested by 2,000 bootstrap resamplings. Each of the phylum-level clusters was further analyzed with the same method, using unambiguously aligned sequence regions for the respective phyla, in order to analyze them with a higher resolution. For some bacterial groups, maximum likelihood (ML) trees were constructed, using the fastDNAml program (37) implemented in the ARB software and/or the PHYML v2.4.4 program (15). A 100-bootstrap resampling test was performed when using the PHYML program. When a cluster was constituted exclusively of clones from termite guts with a bootstrap confidence value of >70%, it was defined as a "termite cluster" in this study.

Estimation of diversity. The coverage of clones in each library was assessed by rarefaction analysis using the program Analytic Rarefaction (version 1.3; S. M. Holland, University of Georgia [http://www.uga.edu/strata/software/]). The estimation of the number of bacterial phylotypes in termite guts was performed using the Chao1 nonparametric richness estimator (7) implemented in the software EstimateS (version 6.0b; R. K. Colwell, University of Connecticut [http://viceroy.eeb.uconn.edu/EstimateS]). Parametric estimation was also conducted using the extrapolation of Curtis et al. (8), assuming a log normal distribution of the bacterial community. In this method, extrapolation is conducted by estimating the area under the species abundance curve for the environment. Equations 10 and 11 in their original paper were solved by assuming that N_{min} (the number of individuals in the least abundant species) = 1, using the program diversity calculator v53, which is distributed online by the authors (http://people.civil.gla.ac.uk/~sloan/). This method provides the maximum possible diversity of microorganisms in a community, in contrast to the Chao1 estimator, which provides the minimum possible diversity (4, 8).

Microscopic observation. The number of prokaryotic cells stained with 4′,6′-diamidino-2-phenylindole (DAPI) was counted for five individual workers chosen randomly from a colony of *Microcerotermes* species M1 and a colony of *Reticulitermes* species R1. The number of spirochete-form cells was also counted to evaluate the results of the molecular analyses. The observation was conducted using epifluorescence microscopy for cells immobilized on a black 0.2-μm filter membrane (Millipore). Before counting of the DAPI-stained cells, cells emitting F_{420} epifluorescence were counted in order to subtract the number of methanogenic archaea from the total number of prokaryotic cells. Data are expressed throughout this paper as means ± standard deviations unless otherwise stated.

Nucleotide sequence accession numbers. The 16S rRNA sequences generated in this study have been deposited with DDBJ under accession numbers AB191790 to AB192133 for bacterial sequences from *Microcerotermes* spp., AB192134 to AB192291 for those from *Reticulitermes* spp., and AB193191 to AB193243 for mitochondrial cytochrome oxidase II gene sequences of termites.

RESULTS

T-RFLP analysis. T-RFLP analysis of the bacterial gut microbiota was performed for the 32 termite colonies and five individual termites. Representative electropherograms are shown in Fig. 2. The T-RFLP patterns were highly concordant with the prediction from the analysis of each corresponding clone library, and the taxonomic origins of approximately 60 and 70% of the detected peaks, including almost all of the prominent peaks, in the HhaI and HaeIII restriction profiles, respectively, were assignable (see Fig. S1 in the supplemental material). As predicted by the clonal analysis, HhaI digestion produced only one T-RF peak from the *Treponema* clones and also one or a few peaks from other dominant groups, while HaeIII digestion generated many T-RF peaks from clones of *Treponema* and other dominant groups.

Both the HhaI and HaeIII profiles exhibited surprisingly high similarity within each termite genus, irrespective of the individual, colony, sampling site, and termite species. In the HaeIII analyses, small but significant differences were also further demonstrated among colony samples within each termite genus. Figure 3 shows the relationship of the HaeIII profiles based on the dissimilarity index (D). The profiles robustly clustered for each termite genus; the average dissimilarity indices were 22.4 ± 6.2, 24.4 ± 5.9, and 81.0 ± 2.7, within genus *Microcerotermes*, within genus *Reticulitermes*, and between the two genera, respectively. This differentiation was statistically supported ($F_{ST} = 0.6322$; $P < 0.0001$). Within each termite genus, the profiles were further clustered by termite species and/or sampling site. In the genus *Reticulitermes*, four profiles from *R. speratus* were clustered and differentiated from the other *Reticulitermes* samples ($F_{ST} = 0.3200$; $P = 0.0289$;

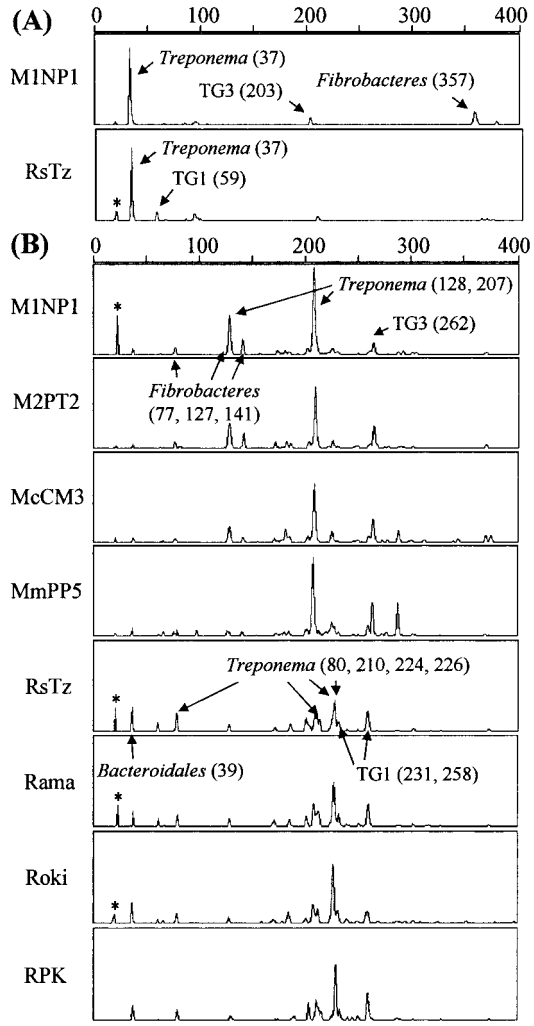

FIG. 2. T-RFLP profiles of 16S rRNAs, amplified by PCR from the guts of *Microcerotermes* and *Reticulitermes* termites. (A) HhaI profiles; (B) HaeIII profiles. The horizontal axis indicates the size (nucleotide length) of the T-RFs, and the vertical axis indicates the relative intensity of fluorescence. Only minor peaks of T-RFs were found above a length of 400 nucleotides (approximately 5 and 1% of the total intensity in the HhaI and HaeIII profiles, respectively). The origins of the peaks were predicted from the sequences and frequencies of the clones (see Fig. S1 in the supplemental material). The reproducibility was quite high for both the HhaI and HaeIII analyses. The replicates of only two samples were not the nearest neighbors in the cluster analysis of the HhaI profiles, but they were the second closest. In the HaeIII analysis, all of the replicates were the nearest neighbors. Therefore, we used the average peak height ratios of the replicates for the following analyses. Asterisks show the PCR primers. Only a representative profile is presented for each termite species. Since the HhaI analyses provided no obvious differences within congeneric termites, only one for each termite genus is presented. See Fig. 1 for the abbreviations of colony samples.

$\alpha = 0.05$). In the genus *Microcerotermes*, all of the profiles for *Microcerotermes crassus* clustered together, except for that of colony McCM2. The differentiation of the *M. crassus* profiles from those for *Microcerotermes* species M1 and M2, respectively, was statistically supported (for H_0 of *M. crassus* = M1, $F_{ST} = 0.3012$ and $P < 0.0001$; for H_0 of *M. crassus* = M2, $F_{ST} = 0.2801$ and $P = 0.0002$). However, the profiles for species M1 and M2 intermingled and formed clusters by sampling site rather than termite species. The four colony samples M1PT3, M1PT4, M2PT1, and M2PT2 from the same sampling site, Pathum Thani, clustered together, and the two colony samples M1BK2 and M2BK1 from Bangkok also showed a close relationship. This was consistent with the acceptance of the null hypothesis that profiles for species M1 are not significantly different from those for species M2 ($F_{ST} = 0.01268$; $P = 0.2859$). The other *Microcerotermes* samples from the same sampling areas also tended to cluster together, as shown by the circles in Fig. 3. For colony M1NP1, a similarity in the gut bacterial communities of the individual termites within a single colony was also demonstrated (Fig. 3).

Estimation of gut bacterial diversity. For the genus *Microcerotermes*, 228 bacterial phylotypes were found from 576 sequenced clones. For the genus *Reticulitermes*, 144 phylotypes were found from 384 clones analyzed, including 53 novel phylotypes not found among the 314 phylotypes obtained for *R. speratus* in our previous studies (18, 19). The rarefaction analysis of the 10 clone libraries (M1NP1, M1NP2, M1PL1, M1PT4, M2PT2, and M2PB4 from genus *Microcerotermes* and RsOg, RsTz, RsTu1, and RPK from genus *Reticulitermes*) showed similar curves within each termite genus and suggested that our sequencing effort was not enough to represent the diversity of bacteria in the gut (data not shown). Therefore, the number of phylotypes was estimated using both parametric and nonparametric analyses (Table 1). The data from the clone libraries that were not significantly different (see below) were combined and used for estimation, in order to improve the reliability of the estimations, by increasing the number of sampled clones. Thus, we estimated the bacterial diversity in each termite species from one or more colonies. To calculate Curtis's parametric estimator, the number of bacterial cells per gut of *Microcerotermes* species M1 ($6.24 \times 10^6 \pm 2.40 \times 10^6$) or *Reticulitermes* species R1 ($1.24 \times 10^6 \pm 0.22 \times 10^6$) was used as the total number of individuals (N_T) in each bacterial community for the *Microcerotermes* and *Reticulitermes* samples, respectively. The most abundant phylotypes in the sequenced clones were *Treponema* phylotypes M2PT2-56 (8.3%), M2PT2-87 (8.3%), Rs-G65 (8.0%), and Rs-A48 (10.4%), for termite species M1, M2, *R. speratus*, and R1, respectively. These values were used as the reciprocal of the ratio N_T/N_{max}, where N_{max} is the number of individuals in the most abundant species (8). The estimated numbers were approximately five to six times larger by Curtis's parametric method than by the Chao1 method, except for *R. speratus*, for which the difference was relatively small (Table 1). Since these methods estimate the maximum and minimum possible diversity, respectively (4, 7, 8), the diversity of gut bacteria is thought to be within this range.

Phylogenetic affiliation and abundance of gut bacteria. Most of the 16S rRNA phylotypes, 227 of 228 from genus *Microcerotermes* and 362 of 367 from genus *Reticulitermes*, showed <97.0% sequence similarity to any clones from other environments and any cultured strains in the public databases. Since only one phylotype, Rs-A44, was shared between these termite genera, it is implied that, in total, 99% of the 594 phylotypes represent novel and as yet uncultured bacteria. These phylotypes were affiliated with 17 phyla (15 from genus *Microcerotermes* and 15 from genus *Reticulitermes*), including the novel

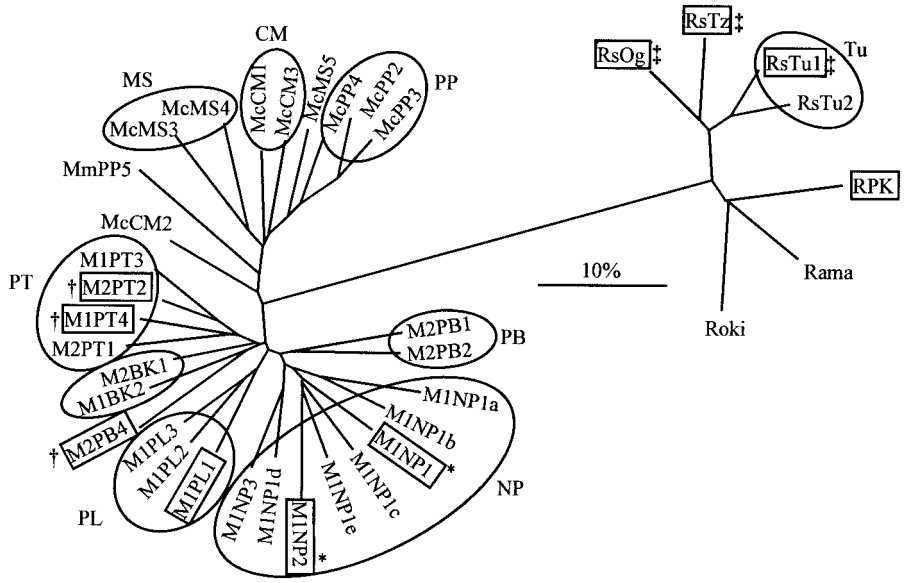

FIG. 3. Dendrogram showing the relationship of HaeIII T-RFLP profiles based on a dissimilarity index. T-RFLP profiles of 16S rRNAs amplified by PCR from termite guts were analyzed for 32 termite colonies and five individual termites from colony M1NP1 (named M1NP1a to -e). Neighbored profiles from the colony samples collected in the same areas are indicated by circles. Samples also used in clonal analysis are indicated by squares. The symbols *, †, and ‡ indicate groups of clone libraries that were not significantly different at a significance level of 0.05. See Fig. 1 for the abbreviations of samples.

candidate phyla TG2 and TG3, according to the phylogenetic analysis (Fig. 4). In both *Microcerotermes* and *Reticulitermes* termites, phylotypes affiliated with the genus *Treponema* (phylum *Spirochaetes*) were predominant, and those affiliated with the orders *Clostridiales* (phylum *Firmicutes*) and *Bacteroidales* (phylum *Bacteroidetes*) were also dominant in the analyzed clones (Fig. 5). The predominance of the genus *Treponema* in the guts of these termites was supported by the result that 50.8% ± 2.8% and 40.6% ± 3.2% of bacterial cells were spirochete-like in the genera *Microcerotermes* and *Reticulitermes*, respectively, upon microscopic observation. Conversely, clones affiliated with the phylum *Fibrobacteres* and the novel candidate phylum TG3 were dominant specifically in the genus *Microcerotermes*, and TG1 clones were dominant specifically in the genus *Reticulitermes*.

The novel candidate phylum TG3 was comprised of six phylotypes from *Microcerotermes* species M1 and M2 and clustered with five uncultured clones from other environments (Fig. 6). This phylum can be divided into subphyla I and II. Subphylum I includes three phylotypes that were dominantly and consistently found in *Microcerotermes* colony samples and related to three short sequences from other environments. These three phylotypes, M1PT4-09, M1PT4-86, and M1PT4-23, accounted for approximately 6% of the sequenced clones from *Microcerotermes* spp. The other three phylotypes from *Microcerotermes* spp. in subphylum II were rarely found and formed a monophyletic cluster with clone R76-150B, amplified from the vent worm *Riftia pachyptila* (28). The sequence similarity between the termite gut clones and R76-150B was only 80.2 to 82.6%, and the sequence similarity within this phylum was 77.1 to 96.5%. Another novel candidate phylum, TG2, was rarely found in *R. speratus* and *Microcerotermes* species M2 (Fig. 6). Five phylotypes from termite guts were clustered with five uncultured clones from other environments. The sequence similarity within this phylum was 80.1 to 96.4% and to any other sequences was <78%.

TABLE 1. Estimated number of 16S rRNA phylotypes of gut bacteria in termites

Termite species	*n*	No. of 16S rRNA phylotypes by indicated method[a]	
		Chao1	Curtis (/gut)
Microcerotermes species M1 (M1NP1 + M1NP2)	192	236 ± 66 (224–245)	1,200
Microcerotermes species M2 (M2PT2 + M2PB4)	192	301 ± 68 (291–312)	1,200
Reticulitermes speratus (RsTz + RsTu1 [18])[b]	1,248	458 ± 52 (449–468)	740
Reticulitermes species R1 (RPK)	96	113 ± 37 (106–121)	540

[a] Data are means ± SD, with 95% confidence intervals determined using log transformation.
[b] Eleven clone libraries from *R. speratus* (18).

Specificity and monophyly of gut bacteria. Within each termite genus, some phylotypes were shared among distinct colony samples and species of termites. For the genus *Microcerotermes*, 68 of 228 phylotypes (29.8%) were shared by more than one termite colony, and 42 of 228 (18.4%) were shared by species M1 and M2. The latter corresponds to 49.7 and 53.6% of the sequenced clones from species M1 and M2, respectively. For the genus *Reticulitermes*, 25 of 50 phylotypes (50.0%) from species R1 were shared by *R. speratus*, corresponding to 72.9% of the sequenced clones from species R1. For *R. speratus*, 39 of 55 (70.9%) and 40 of 53 (75.5%) phylotypes from colonies RsTz and RsTu1, respectively, had already been found in our previous studies (18, 19). These correspond to 77.0 and 80.2%

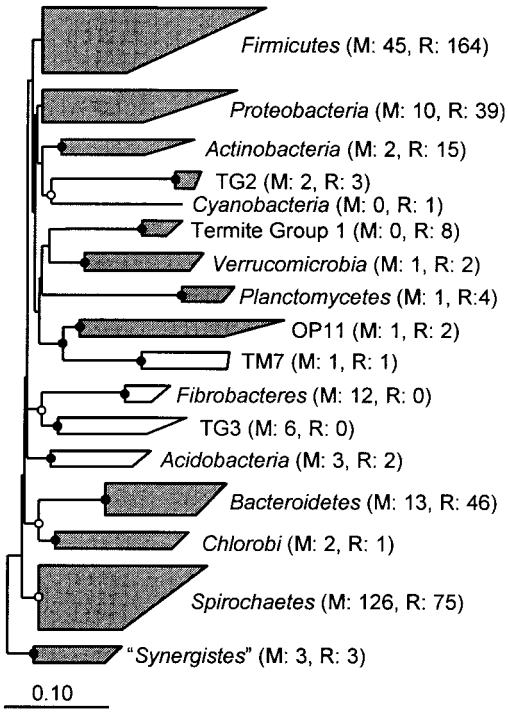

FIG. 4. Phylogenetic tree showing the phylum-level diversity of gut bacteria from *Microcerotermes* and *Reticulitermes* termites. The neighbor-joining tree was reconstructed based on 916 sequences of 16S rRNA, including available termite gut clones and reference sequences retrieved from the public databases. Among them, all but the phylotypes from the genera *Microcerotermes* and *Reticulitermes* obtained in this and previous studies (18, 19) were removed from the phylogeny. The phylum-level clusters, comprising 228 phylotypes from the genus *Microcerotermes* and 367 phylotypes from the genus *Reticulitermes*, are shown in a compressed form, depicted using ARB software. All of the sequences used are shown in the trees for each phylum (Fig. 6; see Fig. S2 to S9 in the supplemental material), except for *Aquifex pyrophilus* (M83548) and *Thermotoga maritima* (AE001703), which were used as the outgroup. The numbers of phylotypes from the genera *Microcerotermes* (M) and *Reticulitermes* (R) are shown in parentheses. The phyla containing termite clusters that were constituted by phylotypes from more than one termite (sub)family are indicated by filled trapezoids. Bootstrap values of >75% (filled circles) and 50 to 74% (open circles) are shown.

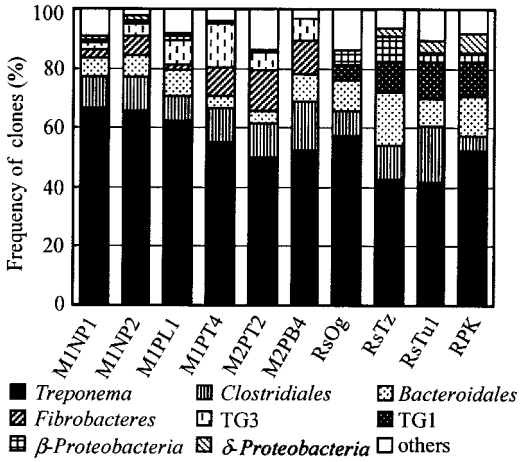

FIG. 5. Taxonomic composition of 16S rRNA clones in libraries prepared from the guts of *Microcerotermes* and *Reticulitermes* termites. See Fig. 1 for the abbreviations of samples.

of the sequenced clones from colonies RsTz and RsTu1, respectively. These data indicate that a substantial portion of bacterial phylotypes are shared among congeneric termites, while only one phylotype was shared between the two termite genera. Nevertheless, many phylotypes from these termites constituted monophyletic clusters (termite clusters) together with clones from other termites, as shown in Fig. S2 to S9 in the supplemental material. Of the 960 clones analyzed in this study, 95% were contained in the termite clusters constituted by those from more than one termite species. These clusters were distributed among 15 phyla. Moreover, the termite clusters constituted by phylotypes from even more diverse termites, i.e., more than one termite (sub)family, occupied 75% of the clones analyzed in this study (see Table S1 in the supplemental material). These termite clusters were distributed among 12 phyla (Fig. 4).

Among the termite clusters, the largest was *Treponema* termite cluster I, which was designated previously (21, 27). This cluster contains phylotypes from 14 termite species in five families (2, 27, 32, 33), including 120 and 68 phylotypes from the genera *Microcerotermes* and *Reticulitermes*, respectively. Phylogenetic analysis of this cluster revealed that the phylotypes from congeneric termites tend to form subclusters, although these genus-specific subclusters are paraphyletic and some phylotypes from distinct termite genera together formed subclusters outside the genus-specific subclusters, as shown in Fig. 7.

Statistical comparison of clone libraries. As shown in Fig. 5, the taxonomic composition of clones was remarkably similar within each genus of the termites, whereas great differences existed between the two termite genera. Pairwise statistical comparisons using the Libshuff-test, F_{ST} test, and P test were conducted, and the results are published online as Table S2 in the supplemental material; the results of multiple comparisons based on these pairwise comparisons are summarized in Table 2. For multiple comparison, the null hypothesis that the samples were drawn from the same population was rejected when compared within each termite genus, although the *P* values for pairwise comparisons between congeneric termites were generally much higher than those between the two termite genera, where all of the *P* values were <0.0001 (see Table S2 in the supplemental material). However, when samples were compared within each termite species, no significant difference was found using any of the three statistical tests, except for *Microcerotermes* species M1 (Table 2). Through these multiple comparisons, three sets of clone libraries were found to be not significantly different in any of the three statistical tests. These were the sets M1NP1 and M1NP2; M2PT2, M2PB4, and M1PT4; and RsOg, RsTz, and RsTu1. These results indicate that (i) there was a considerable difference in clone libraries between *Microcerotermes* and *Reticulitermes* samples; (ii) only samples collected from the same sampling site were not significantly different in *Microcerotermes* species M1; (iii) for *Microcerotermes* species M2, the two clone libraries were not significantly different from each other or from library M1PT4 from species M1; and (iv) the three clone libraries from *R. speratus*

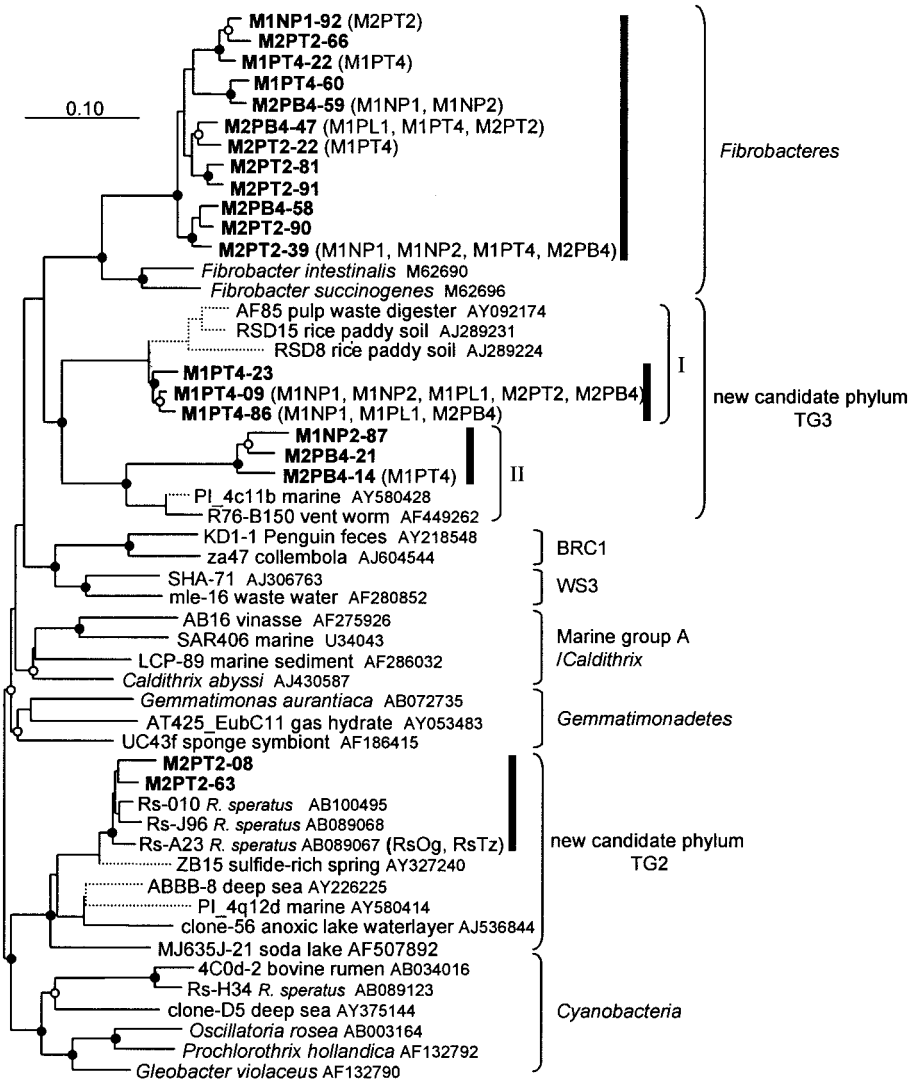

FIG. 6. Phylogenetic tree showing the relationships of 16S rRNA phylotypes affiliated with the novel candidate phyla TG2 and TG3. The sequences of related phyla were used as references to demonstrate the monophyly and distinctness of each novel candidate phylum. A maximum likelihood (ML) tree as the framework tree was reconstructed with the Olsen model using ARB software, based on unambiguously aligned regions (1,227 nucleotide sites) of the alignment of near-full-length sequences (>1,300 bp). The clones connected by dotted lines were short (362 to 841 bp) and were added later to the framework tree by means of the ARB parsimony tool, without changing the overall topology. Novel phylotypes obtained in this study are shown in bold. Termite clusters are shown by thick lines. When a phylotype was found from multiple colony samples, the names of the colony samples are shown in parentheses. When bootstrap values obtained using the PHYML program to construct ML trees under the HKY model are >95% or 70 to 94%, they are shown with filled or open circles, respectively. The "Rs-" clones were obtained from *Reticulitermes speratus* collected at Ogose in our previous studies (18, 19). See Fig. 1 for the abbreviations of samples.

were not significantly different from each other but were different from the R1 sample (colony RPK). These results were exactly concordant with the results of the T-RFLP analysis (Fig. 3).

DISCUSSION

In this study, we aimed to examine whether gut bacteria of termites are allochthonous or autochthonous and also whether they have coevolved with their termite hosts by evaluating the consistency of bacterial gut microbiota with the phylogeny of host termites. Our results clearly demonstrated that congeneric termites harbored very similar bacterial gut microbiota, irrespective of the individual, colony, location, and host species. The similarity in bacterial gut microbiota among congeneric termites was also recently demonstrated with the African soil-feeding termites *Cubitermes* spp. (family Termitidae; subfamily Termitinae), where moderately or considerably high similarity was shown by T-RFLP analysis in a comparison of each gut compartment between three *Cubitermes* species (41). These data suggest that the high similarity of bacterial gut microbiota within a termite genus may be a general trait for termites and that the symbiotic relationship between gut bacteria and their host termites is very stable and strong. Since the majority of gut bacteria from distantly related termites, including the genera *Microcerotermes*, *Reticulitermes*, and *Cubitermes*, constituted

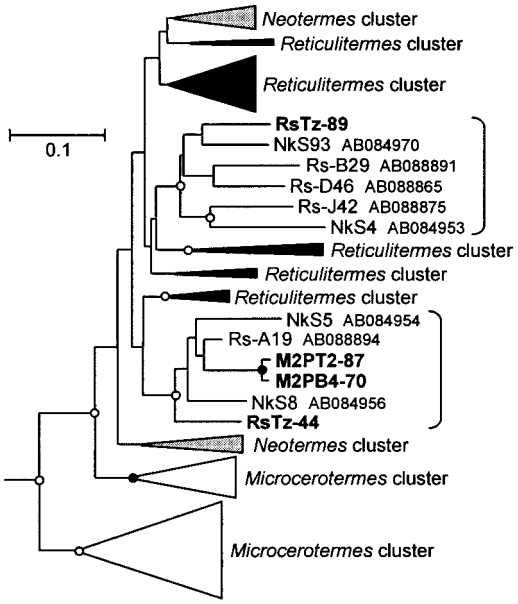

FIG. 7. Phylogenetic tree showing the relationships of 16S rRNA phylotypes affiliated with *Treponema* termite cluster I obtained from three termite genera, i.e., *Microcerotermes*, *Reticulitermes*, and *Neotermes*. A maximum likelihood (ML) tree was reconstructed based on 1,288 unambiguously aligned nucleotides (positions 64 to 1388 in *E. coli*) of 111 sequences, comprising 49 phylotypes from *Microcerotermes* species M1 and M2, 38 from *Reticulitermes speratus* (Rs) (18) and *Reticulitermes* species R1 (RPK), 18 from *Neotermes koshunensis* (NkS) (32), and the outgroup sequences *Spirochaeta halophila* (M34262), *Spirochaeta asiatica* (X93926), *Spirochaeta smaragdinae* (U80597), *Treponema pallidum* (M88726), *Treponema bryantii* (M57737), and *Treponema brennaborense* (Y16568). Only phylotypes representing more than one clone were used. Novel phylotypes obtained in this study are shown in bold. The tree was inferred with the HKY + G (gamma distribution) model, using PHYML v2.4.4. The parameters were optimized for each of 100 bootstrap resamplings. Bootstrap values of >75% (filled circles) and 50 to 74% (open circles) are shown. The monophyletic clusters consisting of phylotypes from one termite genus were compressed. Clusters containing phylotypes from different termite genera are shown with brackets. The full tree is published online as Fig. S10 in the supplemental material.

TABLE 2. *P* values for multiple comparisons of 16S rRNA clone libraries prepared from the guts of *Microcerotermes* and *Reticulitermes* termites

Compared libraries[b]	n[c]	*P* value[a]		
		Libshuff test	F_{ST} test	P test
All	10	**<0.00043**	**<0.00022**	**0.00018**
All M	6	**0.0060**	**0.0015**	**0.00015**
All M1	4	**0.0036**	**0.037**	**0.00012**
All M2	2	0.57	0.79	0.29
All Rs	3	0.75	0.079	0.27
All R	4	**0.021**	0.15	**<0.00006**

[a] If $P < 0.05$ (shown in bold), there was at least one pair of significantly different clone libraries within each group.
[b] M, *Microcerotermes* spp.; M1, *Microcerotermes* species M1; M2, *Microcerotermes* species M2; R, *Reticulitermes* spp.; Rs, *R. speratus*.
[c] Number of compared libraries.

monophyletic clusters that are distinct from other bacterial lineages (see Fig. S2 to S9 and Table S1 in the supplemental material), it is most likely that the majority of gut bacteria are not allochthonous but are autochthonous symbionts which are unique to termites.

It should be noted that the bacterial gut microbiota is greatly different between the host termite genera *Microcerotermes* and *Reticulitermes*, in contrast to the high similarity within each termite genus. This implies that gut bacteria have differentiated after acquisition by the ancestors of these termites. Interestingly, the differentiation from common ancestors occurred not only in dominant bacterial groups such as the genus *Treponema* and the orders *Clostridiales* and *Bacteroidales*, but also in minor ones such as the phyla *Actinobacteria*, *Proteobacteria*, "*Synergistes*," *Planctomycetes*, and others (Fig. 4; see Fig. S2 to S9 in the supplemental material). This suggests that very diverse gut bacteria have coevolved as a community with their host termites and have formed a stable symbiotic complex specific to a genus of termites. Although it is difficult to discuss the extent of cospeciation of gut bacteria in termites here due to the lack of ample sequence data from various termite species, it appears that the phylotypes from one termite genus tend to cluster together, as shown in *Treponema* termite cluster I (Fig. 7). Nevertheless, it also seems obvious that the cospeciation is not as strict as that of intracellular symbionts such as *Buchnera aphidicola* in aphids (13) considering that the complex phylogeny of gut bacteria is not readily comparable to the phylogeny of the host termites (see Fig. S2 to S9 in the supplemental material), as exemplified by the paraphyly of the genus-specific subclusters in *Treponema* termite cluster I (Fig. 7).

Although termite gut bacteria are considered to be transmitted vertically from generation to generation basically via proctodeal trophallaxis, as known for the gut symbiotic protists in lower termites (23), the horizontal transfer of gut bacteria among at least congeneric termites is also inferred from the clustering pattern of the bacterial community profiles from *Microcerotermes* species M1 and M2, which were clustered by sampling site rather than by host termite species. Considering that species M1 and M2 are sympatric in some localities, it is conceivable for the transmission of gut bacteria to occur between these congenerics in the same niche via feces excreted into the immediate environment. However, there are other possible factors affecting the variation in bacterial gut microbiota within congeneric termites, such as ambient temperature, food quality, and humidity. In any case, this clustering pattern suggests a great impact of environmental factors comparable to the host species barrier within a termite genus. Unfortunately, it was impossible to confirm this trait for other pairs of congeneric termites, because other species inhabit different localities. Therefore, the significant differentiation in bacterial community profiles for *M. crassus* and *R. speratus* from those of other congeneric termites might also be due to the difference in sampling sites.

The difference in gut bacterial lineages between the genera *Microcerotermes* and *Reticulitermes* may be partly related to the difference in gut structure and the presence or absence of gut protists. For example, seven phylotypes from *Microcerotermes* species M1 and M2 were affiliated with a termite P1 cluster in the family *Eubacteriaceae* found in our recent study (47), while no phylotypes from lower termites were affiliated with this

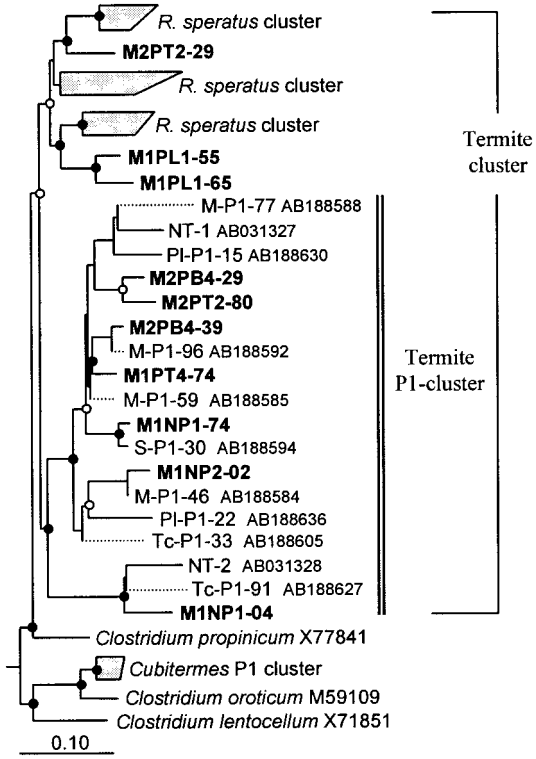

FIG. 8. Phylogenetic tree showing the relationships of 16S rRNA phylotypes affiliated with the family *Eubacteriaceae* in the phylum *Firmicutes*. A maximum likelihood tree with bootstrap values was constructed as described in the legend to Fig. 6, using 1,232 unambiguously aligned nucleotides (positions 64 to 1388 in *E. coli*). A subset of the sequences shown in Fig. S5 in the supplemental material were used. Novel phylotypes obtained in this study are shown in bold. M1- and M2- clones were obtained from *Microcerotermes* species M1 and M2, respectively. Several phylotypes from species M1 and M2 were clustered with clones obtained from the highly alkaline gut regions of higher termites. NT-1 and NT-2 were obtained from the mixed gut segment of *Nasutitermes takasagoensis* (49). Clones M-P1-, Pl-P1-, Tc-P1-, and S-P1- were obtained from the P1 gut segment of *Microcerotermes* sp., *Pericapritermes latignathus*, *Termes comis*, and *Speculitermes* sp., respectively (47). Bootstrap values of >95% (filled circles) and 70 to 94% (open circles) are shown. The outgroup sequences were *Acidaminobacter hydrogenoformans* (AF016691), *Clostridium purinolyticum* (M60491), and *Peptostreptococcus anaerobius* (L04168).

cluster (Fig. 8). This cluster comprises phylotypes from the highly alkaline gut compartments (pH 10 to 12), called the mixed segment and the P1 section, both of which are unique to higher termites (3, 6). Since several "P1 clusters" like this have been found in higher termites (42, 47) and since it was revealed that distinct gut compartments harbor distinct bacterial communities (42), some part of the gut bacteria may have differentiated as symbionts specifically associated with gut structures and physiological conditions unique to higher termites. Conversely, the absence of TG1 clones from *Microcerotermes* spp. may be due to the absence of gut flagellates found only in lower termites, which are thought to be the sole habitats of the termite-associated TG1 bacteria (36, 44).

The coevolution of gut microbiota with the termite host has also been suggested for protistan symbionts in lower termites and some archaeal symbionts. The majority of the protistan gut microbiota is unique to lower termites and wood-feeding cockroaches belonging to the genus *Cryptocercus* (23). These symbiotic protists are basically species specific to their host termites and are stably harbored, although the coevolutionary process cannot be explained by a purely vertical transmission (23). Some archaeal lineages, e.g., a cluster in the order *Methanomicrobiales* and clusters in the genus *Methanobrevibacter*, are also considered unique to termites (9, 12, 50). Combined with the results from the present study, it seems most likely that the majority of gut microbiota, comprising bacteria, archaea, and protists, are autochthonous symbionts that have coevolved with their host termites.

ACKNOWLEDGMENTS

We thank T. Takematsu and O. Kitade for helpful advice on the identification of termites. We also thank C. Disyen, K. Sirihongsuwan, L. Ekpornprasit, C. Boontong, and H. Yuzawa for assisting with experiments. We thank A. Hiraishi for kindly providing the software BioCLUST.

Y.H. is a recipient of a special postdoctoral research fellowship from RIKEN. This work was partially supported by grants for the Bioarchitect Research Program and the Eco Molecular Science Research Program from RIKEN.

REFERENCES

1. **Altschul, S. F., T. L. Madssen, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Berchtold, M., and H. König.** 1996. Phylogenetic analysis and in situ identification of uncultivated spirochetes from the hindgut of the termite *Mastotermes darwiniensis*. Syst. Appl. Microbiol. **19:**66–73.
3. **Bignell, D. E., and P. Eggleton.** 1995. On the elevated intestinal pH of higher termites (Isoptera: Termitidae). Insectes Soc. **42:**57–69.
4. **Bohannan, B. J., and J. Hughes.** 2003. New approaches to analyzing microbial biodiversity data. Curr. Opin. Microbiol. **6:**282–287.
5. **Breznak, J. A.** 2000. Ecology of prokaryotic microbes in the guts of wood- and litter-feeding termites, p. 209–232. *In* T. Abe, D. E. Bignell, and M. Higashi (ed.), Termites: evolution, sociality, symbioses, ecology. Kluwer Academic Publishers, Dordrecht, The Netherlands.
6. **Brune, A., D. Emerson, and J. A. Breznak.** 1995. The termite gut microflora as an oxygen sink: microelectrode determination of oxygen and pH gradients in guts of lower and higher termites. Appl. Environ. Microbiol. **61:**2681–2687.
7. **Chao, A.** 1987. Estimating the population size for capture-recapture data with unequal catchability. Biometrics **43:**783–791.
8. **Curtis, T. P., W. T. Sloan, and J. W. Scannell.** 2002. Estimating prokaryotic diversity and its limits. Proc. Natl. Acad. Sci. USA **99:**10494–10499.
9. **Donovan, S. E., K. J. Purdy, M. D. Kane, and P. Eggleton.** 2004. Comparison of *Euryarchaea* strains in the guts and food-soil of the soil-feeding termite *Cubitermes fungifaber* across different soil types. Appl. Environ. Microbiol. **70:**3884–3892.
10. **Dunbar, J., L. O. Ticknor, and C. R. Kuske.** 2001. Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. Appl. Environ. Microbiol. **67:**190–197.
11. **Egert, M., and M. W. Friedrich.** 2003. Formation of pseudo-terminal restriction fragments, a PCR-related bias affecting terminal restriction fragment length polymorphism analysis of microbial community structure. Appl. Environ. Microbiol. **69:**2555–2562.
12. **Friedrich, M. W., D. Schmitt-Wagner, T. Lueders, and A. Brune.** 2001. Axial differences in community structure of *Crenarchaeota* and *Euryarchaeota* in the highly compartmentalized gut of the soil-feeding termite *Cubitermes orthognathus*. Appl. Environ. Microbiol. **67:**4880–4890.
13. **Funk, D. J., L. Helbling, J. J. Wernegreen, and N. A. Moran.** 2000. Intraspecific phylogenetic congruence among multiple symbiont genomes. Proc. R. Soc. Lond. B **267:**2517–2521.
14. **Good, I. J.** 1953. The population frequencies of species and the estimation of population parameters. Biometrika **40:**237–264.
15. **Guindon, S., and O. Gascuel.** 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. **52:**696–704.
16. **Hiraishi, A., M. Iwasaki, and H. Shinjo.** 2000. Terminal restriction pattern analysis of 16S rRNA genes for the characterization of bacterial communities of activated sludge. J. Biosci. Bioeng. **90:**148–156.
17. **Holland, B. S., and M. D. Copenhaver.** 1988. Improved Bonferroni-type multiple testing procedures. Psychol. Bull. **104:**145–149.

18. **Hongoh, Y., M. Ohkuma, and T. Kudo.** 2003. Molecular analysis of bacterial microbiota in the gut of the termite *Reticulitermes speratus* (Isoptera; Rhinotermitidae). FEMS Microbiol. Ecol. **44:**231–242.
19. **Hongoh, Y., H. Yuzawa, M. Ohkuma, and T. Kudo.** 2003. Evaluation of primers and PCR conditions for the analysis of 16S rRNA genes from a natural environment. FEMS Microbiol. Lett. **221:**299–304.
20. **Huber, T., G. Faulkner, and P. Hugenholtz.** 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics **20:**2317–2319.
21. **Iida, T., M. Ohkuma, K. Ohtoko, and T. Kudo.** 2000. Symbiotic spirochetes in the termite hindgut: phylogenetic identification of ectosymbiotic spirochetes of oxymonad protists. FEMS Microbiol. Ecol. **34:**17–26.
22. **Inoue, T., Y. Hongoh, C. Klangkaew, Y. Takematsu, C. Vongkaluang, N. Noparatnaraporn, M. Ohkuma, and T. Kudo.** 2005. Plasticity and specificity of termite nest structure (Isoptera). Sociobiology **45:**671–678.
23. **Inoue, T., O. Kitade, T. Yoshimura, and I. Yamaoka.** 2000. Symbiotic associations with protists, p. 275–288. *In* T. Abe, D. E. Bignell, and M. Higashi (ed.), Termites: evolution, sociality, symbioses, ecology. Kluwer Academic Publishers, Dordrecht, The Netherlands.
24. **Iwasaki, M., and A. Hiraishi.** 1998. A new approach to numerical analyses of microbial quinone profiles in the environment. Microbes Environ. **13:**67–76.
25. **König, H., J. Fröhlich, M. Berchtold, and M. Wenzel.** 2002. Diversity and microhabitats of the hindgut flora of termites. Recent Res. Dev. Microbiol. **6:**125–156.
26. **Kumar, S., K. Tamura, I. B. Jakobson, and M. Nei.** 2001. MEGA2: Molecular Evolutionary Genetics Analysis software. Bioinformatics **17:**1244–1245.
27. **Lilburn, T. G., T. M. Schmidt, and J. A. Breznak.** 1999. Phylogenetic diversity of termite gut spirochaetes. Environ. Microbiol. **1:**331–345.
28. **Lopez-Garcia, P., F. Gaill, and D. Moreira.** 2002. Wide bacterial diversity associated with tubes of the vent worm *Riftia pachyptila*. Environ. Microbiol. **4:**204–215.
29. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Forster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. Konig, T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K. H. Schleifer.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32:**1363–1371.
30. **Maidak, B. L., J. R. Cole, T. G. Lilburn, C. T. J. Parker, P. R. Saxman, R. J. Farris, G. M. Garrity, G. J. Olsen, T. M. Schmidt, and J. M. Tiedje.** 2001. The RDP-II (Ribosomal Database Project). Nucleic Acids Res. **29:**173–174.
31. **Martin, A. P.** 2002. Phylogenetic approaches for describing and comparing the diversity of microbial communities. Appl. Environ. Microbiol. **68:**3673–3682.
32. **Noda, S., M. Ohkuma, A. Yamada, Y. Hongoh, and T. Kudo.** 2003. Phylogenetic position and in situ identification of ectosymbiotic spirochetes on protists in the termite gut. Appl. Environ. Microbiol. **69:**625–633.
33. **Ohkuma, M., T. Iida, and T. Kudo.** 1999. Phylogenetic relationships of symbiotic spirochetes in the gut of diverse termites. FEMS Microbiol. Lett. **181:**123–129.
34. **Ohkuma, M., and T. Kudo.** 1996. Phylogenetic diversity of the intestinal bacterial community in the termite *Reticulitermes speratus*. Appl. Environ. Microbiol. **62:**461–468.
35. **Ohkuma, M., H. Noda, Y. Hongoh, and T. Kubo.** 2002. Diverse bacteria related to the bacteroides subgroup of the CFB phylum within the gut symbiotic communities of various termites. Biosci. Biotechnol. Biochem. **66:**78–84.
36. **Ohkuma, M., S. Noda, T. Iida, and T. Kudo.** 2001. Presented at The 9th International Symposium on Microbial Ecology (ISME-9), Amsterdam, The Netherlands, 26 to 31 August 2001.
37. **Olsen, G. J., H. Matsuda, R. Hagstrom, and R. Overbeek.** 1994. fastDNAmL: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. Comput. Appl. Biosci. **10:**41–48.
38. **Polz, M. F., and C. M. Cavanaugh.** 1998. Bias in template-to-product ratios in multitemplate PCR. Appl. Environ. Microbiol. **64:**3724–3730.
39. **Qiu, X., L. Wu, H. Huang, P. E. McDonel, A. V. Palumbo, J. M. Tiedje, and J. Zhou.** 2001. Evaluation of PCR-generated chimeras, mutations, and heteroduplexes with 16S rRNA gene-based cloning. Appl. Environ. Microbiol. **67:**880–887.
40. **Schloss, P. D., B. R. Larget, and J. Handelsman.** 2004. Integration of microbial ecology and statistics: a test to compare gene libraries. Appl. Environ. Microbiol. **70:**5485–5492.
41. **Schmitt-Wagner, D., M. W. Friedrich, B. Wagner, and A. Brune.** 2003. Axial dynamics, stability, and interspecies similarity of bacterial community structure in the highly compartmentalized gut of soil-feeding termites (*Cubitermes* spp.). Appl. Environ. Microbiol. **69:**6018–6024.
42. **Schmitt-Wagner, D., M. W. Friedrich, B. Wagner, and A. Brune.** 2003. Phylogenetic diversity, abundance, and axial distribution of bacteria in the intestinal tract of two soil-feeding termites (*Cubitermes* spp.). Appl. Environ. Microbiol. **69:**6007–6017.
43. **Singleton, D. R., M. A. Furlong, S. L. Rathbun, and W. B. Whitman.** 2001. Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. Appl. Environ. Microbiol. **67:**4374–4376.
44. **Stingl, U., R. Radek, H. Yang, and A. Brune.** 2005. "*Endomicrobia*": cytoplasmic symbionts of termite gut protozoa form a separate phylum of prokaryotes. Appl. Environ. Microbiol. **71:**1473–1479.
45. **Suzuki, M. T., and S. J. Giovannoni.** 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. **62:**625–630.
46. **Thompson, J. R., L. A. Marcelino, and M. F. Polz.** 2002. Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by "reconditioning PCR." Nucleic Acids Res. **30:**2083–2088.
47. **Thongaram, T., Y. Hongoh, S. Kosono, M. Ohkuma, S. Trakulnaleamsai, N. Noparatnaraporn, and T. Kudo.** 2005. Comparison of bacterial communities in the alkaline gut segment among various species of higher termites. Extremophiles **9:**229–238.
48. **Thongaram, T., S. Kosono, M. Ohkuma, Y. Hongoh, M. Kitada, T. Yoshinaka, S. Trakulnaleamsai, N. Noparatnaraporn, and T. Kudo.** 2003. Gut of higher termites as a niche for alkaliphiles as shown by culture-based and culture-independent studies. Microbes Environ. **18:**152–159.
49. **Tokuda, G., I. Yamaoka, and H. Noda.** 2000. Localization of symbiotic clostridia in the mixed segment of the termite *Nasutitermes takasagoensis* (Shiraki). Appl. Environ. Microbiol. **66:**2199–2207.
50. **Tokura, M., M. Ohkuma, and T. Kudo.** 2000. Molecular phylogeny of methanogens associated with flagellated protists in the gut and with the gut epithelium of termites. FEMS Microbiol. Ecol. **33:**233–240.