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Short Communication

Identification of Endosymbiotic Methanogen and Ectosymbiotic Spirochetes of Gut Protists of the Termite *Coptotermes formosanus*

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Prokaryotic associations with gut protists of the termite *Coptotermes formosanus* were investigated based on 16S rRNA gene sequences. An endosymbiotic methanogen of *Spirotrichonympha leidy* phylogenetically grouped with endosymbionts of other gut protists in the genus *Methanobrevibacter*, seemed to be unrelated to the host protist phylogeny. Three different lineages of ectosymbiotic spirochetes in the genus *Treponema* were identified in single cells of *Holomastigotoides mirabile*, indicating their simultaneous occurrence. Although these symbionts represented mere minor populations in the gut, their phylogenetic assignments suggest a common symbiotic relationship involving H₂ metabolism.

Key words: symbiosis, protist, methanogen, spirochete, termite

Termites harbor a dense and diverse microbial community in their gut, which is essential for the efficient decomposition of lignocellulose¹⁴. The gut community comprises both flagellated protists (single cell eukaryotes) and prokaryotes. One unique characteristic of the termite-gut community is its highly structured nature. Specific associations of various prokaryotes such as methanogenic archaea^{3,20}, spirochetes^{6,13,21}, *Bacteroidales* members^{10–12,21}, and the candidate phylum Termite Group 1 of bacteria¹⁷, with the cells of gut protists are frequently observed.

The termite *Coptotermes formosanus*, a globally distributed, economically important pest in wooden houses, harbors only three species of gut protists, all of which belong to the phylum *Parabasalia*¹⁶. The presence of diverse bacterial species and archaea has been reported in the gut community based on 16S rRNA gene sequences^{18,19}. A recent observation has revealed that the intracellular endosymbionts of the largest protist species *Pseudotrichonympha grassii* (order *Trichonymphida*) are bacteria of a member of the order *Bacteroidales* and that the endosymbionts constitute the predominant bacteria accounting for more than two-thirds of gut bacteria¹⁰. In this study, we investigated associated prokaryotes of the other two protist species *Spirotrichonympha leidy* and *Holomastigotoides mirabile*, both belonging to the order *Spirotrichonymphida*. The goals of this study are to clarify the community structure in the gut, to know whether there is a common mechanism for the symbiotic relationships, and to understand the evolutionary aspects of the associations. *S. leidy* and *H. mirabile* are of especial importance because, among the protist orders found in termite guts, *Spirotrichonymphida* has been poorly studied with respect to the associations of prokaryotes.

Associations of prokaryote cells were microscopically sur-

veyed in these protist species. The termite *C. formosanus* was collected, maintained in the laboratory, and fed on a cellulose diet for seven days before experimental use⁷. The gut content of the worker termites was prepared as described⁷. Methanogenic archaea were visualized by F₄₂₀ and F₃₅₀ epifluorescence microscopy²⁰, and associated spirochetes were detected by 16S rRNA-targeted fluorescent in situ hybridization (FISH) using probes for spirochetes in the termite *Treponema* cluster I as well as for general bacteria¹³. The methanogen-specific fluorescence was observed in intracellular rods of *S. leidy* (Fig. 1A, B), but not in *H. mirabile* and *P. grassii*. In the posterior portion of the *H. mirabile* cells, the probe for the termite *Treponema* cluster I gave dense FISH signals (Fig. 1C–E), which indicated a typical spirochete-like morphology (Fig. 1F). FISH using the general bacterial probe and staining with DAPI (4',6'-diamino-2-phenylindole HCl) showed no other stable association of prokaryotes in *S. leidy* and *H. mirabile*.

The identity of the endosymbiotic methanogens of *S. leidy* and the ectosymbiotic spirochetes of *H. mirabile* was examined based on their 16S rRNA gene sequences. In order to compare the symbionts within a protist cell and between different protist cells, a single cell of each protist species was manually isolated with a micromanipulator as described previously^{6,13,16} and used for isothermal whole genome amplification (WGA) with a GenomiPhi HY amplification kit (GE Healthcare, Buckinghamshire, England) as described previously⁵. The cell was suspended in the buffer supplied by the kit, and directly used for the amplification without DNA extraction. After heating at 95°C for 3 min, the amplification was performed for 4 hr according to the manufacturer's instructions. A cell harboring spirochete-like appendages was selected in *H. mirabile* and used. The amplified DNA was used as the template for PCR of the 16S rRNA gene with the *Archaea*-specific primer pair A25F (5'-CYGKTTGATCCTGSCRG-3') and A1385R (5'-GGTGT-

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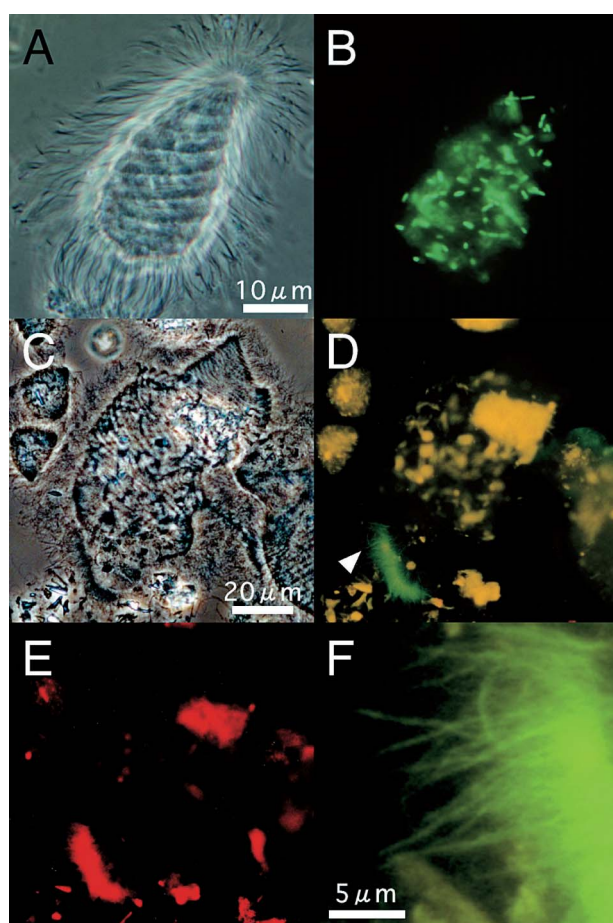


Fig. 1. Detection of prokaryotic associations with gut protists of *C. formosanus*. (A, B) Phase-contrast (A) and F₄₂₀ epifluorescence (B) images of an *S. leidy* cell. Rod-shaped endosymbiotic methanogens are visible with their blue-white signals in (B). (C–F) FISH-based detection of ectosymbiotic spirochetes belonging to the termite *Treponema* cluster I in *H. mirabile*. The probe for cluster I labeled with 6-carboxyfluorescein (D; green) and the general bacterial probe labeled with Texas-Red (E; red) were used simultaneously for FISH and detected separately. (C) Phase-contrast image of the same sample. (F) Magnification of the ectosymbionts at the point indicated by an arrowhead in (D). The amorphous green or yellow in (B) and (D) is probably derived from ingested wood particles.

GTGCAARGAGCA-3'), the bacterial universal primer pair 27F and 1390R⁵), and a primer pair specific for spirochetes, S58F and S1400R¹³). The PCR conditions for the former two primer pairs were 20 cycles of 94°C for 30 s, 50°C for 45 s, and 72°C for 2 min, with the final extension at 72°C for 5 min. The PCR conditions for the latter primer pair were described previously¹³). The cloning and sequencing of the PCR products were performed as described previously¹). The clone sequences were sorted using DOTUR v1.5 (<http://www.plantpath.wisc.edu/fac/joh/dotur.html>), and entire DNA sequences of representative clones were determined. Chimeric sequences were identified using the Bellerophon server (<http://foo.maths.uq.edu.au/~huber/bellerophon.pl>) with an uploaded alignment of the spirochete sequences obtained in this and previous studies^{9,19}). Near full-length sequences were determined and aligned with database sequences using the ARB (<http://www.arb-home.de>), and the alignments were adjusted manually. PHYML ver. 2.4.4

(<http://atgc.lirmm.fr/phyml>) was used for the inference of the maximum likelihood (ML) tree using the best-fitted model chosen by Modeltest 3.7 (<http://darwin.uvigo.es/software/modeltest.html>). The phylotype sequences reported in this study have been deposited in the DDBJ database under the accession numbers AB 360373–AB 360376.

Two independent clone libraries of archaeal 16S rRNA genes, each from a single *S. leidy* cell, were established and 24 clones in each library were examined by partial sequencing. Another clone library from a pool of 20 cells of *S. leidy* with WGA was also examined. An almost unique sequence (represented by clone SIMeN10) was obtained from a total 72 clones, showing more than 99% identity to one another. This strongly suggested that only one unique methanogen species was shared among the *S. leidy* cells. The endosymbiotic methanogen associated with *S. leidy* was affiliated with the genus *Methanobrevibacter*, a methanogen genus distributed widely and abundantly in the gut of diverse termites^{3,18,20}).

In the phylogenetic tree (Fig. 2A), the *S. leidy* endosymbiont formed a robust monophyletic group together with the sequences LRSD3 and LRSM1 identified from the protists *Dinenympha parva* (order *Oxymonadida*, phylum *Preaxostyla*) and *Microjoenia* sp. (*Spirotrichonymphida*) in the termite *Reticulitermes speratus*^{3,20}), respectively, and the clone sequence Cd30 from the whole gut community of the termite *Cryptotermes domesticus*¹⁵). The previously identified sequence from the protist *Pentatrachomonoides scroa* (order *Trichomonadida*; phylum *Parabasalia*) in the termite *Mastotermes darwiniensis*²), although only a 292-bp sequence was available, showed the greatest homology, 99.7% identity, to the sequence of the *S. leidy* endosymbionts. This monophyletic group was a sister group to the sequences identified from the protists *Dinenympha* sp. and *Microjoenia* sp. in the termite *Hodotermopsis sjoestedti*²⁰), suggesting that these sister groups formed a lineage of endosymbiotic methanogens of termite-gut protists. It is noted that, although *Microjoenia* sp. and *S. leidy* belong to the same protist order *Spirotrichonymphida*, the methanogen symbionts of *Microjoenia* spp. were more closely related to those in the distantly related *Dinenympha* spp. than that in *S. leidy*. The endosymbiotic methanogens may be affected not by the host protists but by the termite taxonomy or their locality as discussed previously^{18,20}).

In each of the two independent single-cell samples of *H. mirabile*, two clone libraries of the 16S rRNA gene amplified with the general bacterial primers and the spirochete-specific primers were examined (Table 1). In a total of four libraries, three phylotypes, CfHm03, CfHm15, and CfHm47, were repeatedly and abundantly identified and analyzed further. This result suggested that three distinct spirochete species simultaneously occurred in single *H. mirabile* cells and that they were shared among the *H. mirabile* cells. The identification was conducted using the *H. mirabile* cells in a single termite gut. Since gut community members are significantly conserved among individuals in a colony and among colonies within a termite species⁴), endo- and ecto-symbionts are also considered to be identical. The sequences in each of the clone groups represented by CfHm15 and CfHm47 showed >99% identity, whereas clones in the CfHm03 group exhib-

ited some sequence variation of >97% identity. All the three ectosymbionts of *H. mirabile* were affiliated with the termite *Treponema* cluster I (Fig. 2B), which was congruent with the results of FISH using the cluster-specific probe and previous clonal analyses of the gut community^{9,19}.

One of the three phylotypes of the *H. mirabile* ectosymbionts, CfHm15, was almost identical to the previously reported sequence BCf9-14 in the whole gut community of *C. formosanus*¹⁹, while the other CfHm47 and CfHm03 sequences did not overlap with any sequence identified from

termite guts. CfHm15 and CfHm47 formed a robust cluster, and they were included in a monophyletic group (supported by a bootstrap value of 73%) comprised of sequences from *C. formosanus* and its close relative *Reticulitermes speratus*. These two sequences were distantly related to known ectosymbiont sequences^{6,13,21}, suggesting an independent origin for the association, probably the free-swimming population in the gut fluid. The sequence CfHm03 was distantly related to CfHm15 and CfHm47, and located next to the NkS-Oxy1 sequence identified from isolated cells of the protist *Oxymonas* sp.¹³, although this was not supported by the bootstrap analysis.

Enumeration of cells of these protist-associated methanogens and spirochetes revealed that they represented relatively minor populations in the gut. The number of cells of endosymbiotic methanogens in *S. leidy* was 79±52 per protist (n=19), and almost all the *S. leidy* harbored endosymbiotic methanogens. The proportion of the cells of *H. mirabile* associated with spirochetes was only 5.2%, indicating that

Table 1. Clone numbers of 16S rRNA gene phylotypes detected in *H. mirabile*

Phylotype	Library ^a			
	1B	1S	2B	2S
CfHm03	24	15	25	12
CfHm15	9	5	10	6
CfHm47	4	4	10	5
Others ^b	11	0	3	1
Total	48	24	48	24

^a Libraries 1B and 1S were established from the same single cell of *H. mirabile*, and libraries 2B and 2S were from another single cell. The 16S rRNA gene sequences amplified with the general bacterial primer pair were cloned for libraries 1B and 2B, and those with the spirochete-specific primer pair were cloned for libraries 1S and 2S.

^b In library 1B, "Others" comprised eight phylotypes (one was from a *Mycoplasma*-like organism and four were presumably chimeric artifacts). The three phylotypes in "Others" in library 1B did not overlap with those of library 2B, but did with that of library 2S.

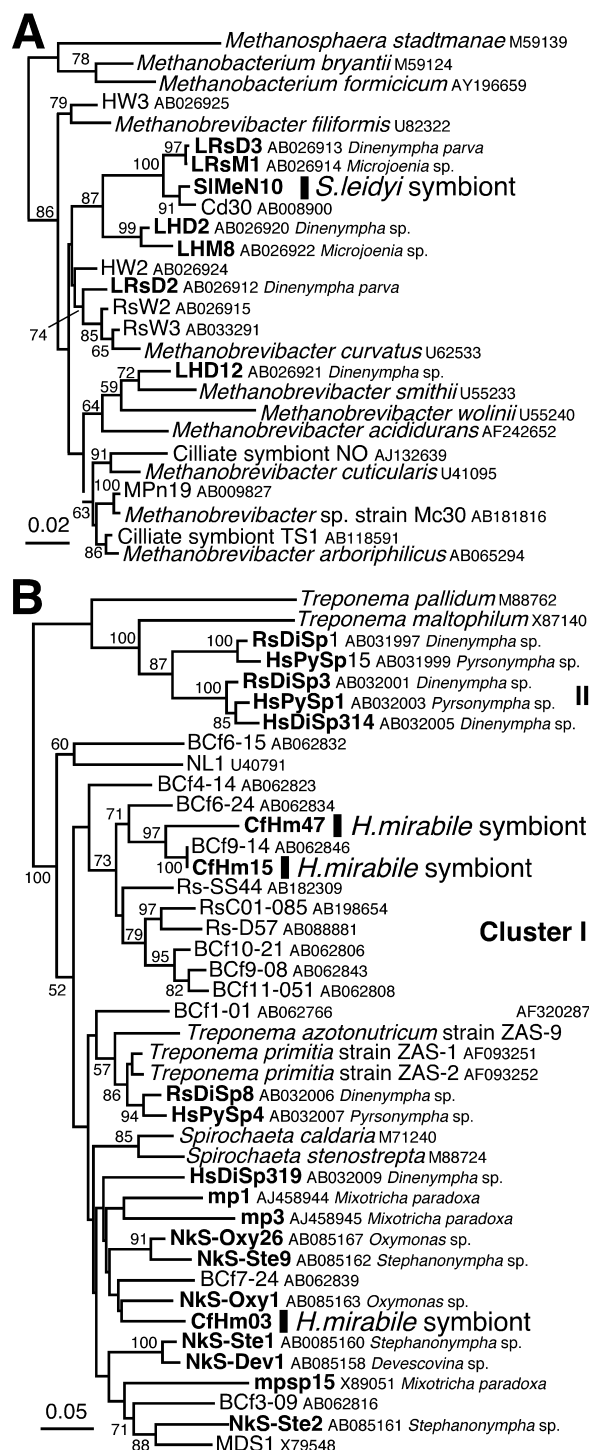


Fig. 2.

Fig. 2. Phylogenetic positions of endosymbiotic methanogens of *S. leidy* in the genus *Methanobrevibacter* (A) and of ectosymbiotic spirochetes of *H. mirabile* in the termite *Treponema* cluster I (B). Each of the ML trees was inferred under a general time-reversible model with gamma distributed rate variation and a proportion of invariable sites, using 938 and 1,289 unambiguously aligned nucleotide positions in (A) and (B), respectively. Numbers at nodes indicate bootstrap values above 50%. The sequence accession number is indicated after the name of each taxon. Phylotype sequences in bold have been identified in symbionts of termite-gut protists, and the host protist is indicated after the name of each taxon. Phylotypes named with LRs and Rs or LHD and Hs have been identified from *R. speratus* or *H. sjoestedti*, respectively, and those with NkS, from *Neotermes koshunensis*. Phylotypes named with mp have been identified from *Mixotricha paradoxa* in *Mastoterms darwiniensis*. In (A), phylotypes named with RsW and HW have been identified from gut walls of *R. speratus* and *H. sjoestedti*, respectively, and Cd30 and MPn19, from whole gut of *C. domesticus* and *Pericaprtermes nitobei*, respectively. In (B), phylotypes named with BCf and Rs have been identified from whole gut of *C. formosanus* and *R. speratus*, respectively, and the phylotypes NL1 and MDS1, from *Nasutitermes lujae* and *M. darwiniensis*, respectively. Species in *Methanospaera* and *Methanobacterium* were used as outgroups in (A). *T. pallidum*, *T. maltophilum*, and phylotypes in the termite *Treponema* cluster II (shown in vertical bar) were used as outgroups in (B).

the ectosymbionts were not obligate for the host *H. mirabile*. The cells of spirochete-like ectosymbionts stained with DAPI were enumerated to be 134 ± 39 cells per protist ($n=9$). Average numbers of the cells of *S. leidy* and *H. mirabile* are 4,800 and 2,700 per gut, respectively²², and the total prokaryotic count is 1.30×10^8 cells per gut¹⁰. Therefore, the endosymbiotic methanogens of *S. leidy* and the ectosymbiotic spirochetes of *H. mirabile* accounted for 0.28% and 0.014% of all prokaryotes in the gut, respectively.

The phylogenetic identification of these symbionts allows us to infer their functional interactions with the host protists. The host protists in the phylum *Parabasalia* are typified by anaerobic-energy producing and H_2 -evolving organelles, hydrogenosomes, in the cells, and indeed as shown in hydrogenosomal hydrogenases of *P. grassii*, they have vigorous H_2 -producing activity⁷. Since members of the genus *Methanobrevibacter* generally assimilate only a limited number of substrates such as H_2 plus CO_2 , the endosymbiotic methanogen of *S. leidy* probably utilizes H_2 produced by the host protist. The ectosymbiotic spirochetes of gut protists are also suggested to utilize H_2 by reducing CO_2 to acetate as postulated from the physiological properties of cultured strains from a termite gut⁸ that belong to cluster I of *Treponema*. The endosymbiont of *P. grassii*, which is assigned to a member of the order *Bacteroidales*, has been demonstrated to have strong H_2 -utilizing ability⁷, although the electron acceptor for the utilization remains unknown. The symbionts of the gut protists identified in this study represented mere minor populations in the gut. Nevertheless, their phylogenetic assignments suggest a common symbiotic relationship involving the inter-species H_2 transfer from the host protists to their symbionts, and thus molecular H_2 may be a key metabolic intermediate in the gut microbial community of termites.

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References

- Deevong, P., Y. Hongoh, T. Inoue, S. Trakulnaleamsai, T. Kudo, N. Noparatnaraporn, and M. Ohkuma. 2006. Effect of temporal sample preservation on the molecular study of a complex microbial community in the gut of the termite *Microcerotermes* sp. *Microbes Environ.* **21**:78–85.
- Fröhlich, J., and H. König. 1999. Rapid isolation of single microbial cells from natural and laboratory populations with the aid of a micro-manipulator. *System. Appl. Microbiol.* **22**:249–257.
- Hara, K., N. Shinzato, T. Oshima, and A. Yamagishi. 2004. Endosymbiotic *Methanobrevibacter* species living in symbiotic protists of the termite *Reticulitermes speratus* detected by fluorescent in situ hybridization. *Microbes Environ.* **19**:120–127.
- Hongoh, Y., P. Deevong, T. Inoue, S. Moriya, S. Trakulnaleamsai, M. Ohkuma, C. Vongkhaluang, N. Noparatnaraporn, and T. Kudo. 2005. Intra- and interspecific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host. *Appl. Environ. Microbiol.* **71**:6590–6599.
- Hongoh, Y., T. Sato, M.F. Dolan, S. Noda, S. Ui, T. Kudo, and M. Ohkuma. 2007. The motility symbiont of the termite-gut flagellate *Caduceia versatilis* is a member of the “*Synergistes*” group. *Appl. Environ. Microbiol.* **73**:6270–6276.
- 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.
- Inoue, J.-I., K. Saita, T. Kudo, S. Ui, and M. Ohkuma. 2007. Hydrogen production by termite-gut protists: characterization of iron hydrogenases of parabasalian symbionts of the termite *Coptotermes formosanus*. *Eukaryot. Cell* **6**:1925–1932.
- Leadbetter, J.R., T.M. Schmidt, J.R. Graber, and J.A. Breznak. 1999. Acetogenesis from H_2 plus CO_2 by spirochetes from termite guts. *Science* **283**:686–689.
- Lilburn, T.G., T.M. Schmidt, and J.A. Breznak. 1999. Phylogenetic diversity of termite gut spirochaetes. *Environ. Microbiol.* **4**:331–345.
- Noda, S., T. Iida, O. Kitade, H. Nakajima, T. Kudo, and M. Ohkuma. 2005. Endosymbiotic *Bacteroidales* bacteria of the flagellated protist *Pseudotrichonympha grassii* in the gut of the termite *Coptotermes formosanus*. *Appl. Environ. Microbiol.* **71**:8811–8817.
- Noda, S., T. Inoue, Y. Hongoh, M. Kawai, C.A. Nalepa, C. Vongkhaluang, T. Kudo, and M. Ohkuma. 2006. Identification and characterization of ectosymbionts of distinct lineages in *Bacteroidales* attached to flagellated protists in the gut of termites and a wood-feeding cockroach. *Environ. Microbiol.* **8**:11–20.
- Noda, S., M. Kawai, H. Nakajima, T. Kudo, and M. Ohkuma. 2006. Identification and *in situ* detection of two lineages of *Bacteroidales* ectosymbionts associated with a termite gut protist, *Oxymonas* sp. *Microbes Environ.* **21**:16–22.
- 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.
- Ohkuma, M. 2003. Termite symbiotic systems: efficient bio-recycling of lignocellulose. *Appl. Microbiol. Biotechnol.* **61**:1–9.
- Ohkuma, M., and T. Kudo. 1998. Phylogenetic analysis of the symbiotic intestinal microflora of the termite *Cryptotermes domesticus*. *FEMS Microbiol. Lett.* **164**:389–395.
- Ohkuma, M., K. Saita, T. Inoue, and T. Kudo. 2007. Comparison of four protein phylogeny of parabasalian symbionts in termite guts. *Mol. Phylogenet. Evol.* **42**:847–853.
- Ohkuma, M., T. Sato, S. Noda, S. Ui, T. Kudo, and Y. Hongoh. 2007. The candidate phylum “Termite Group 1” of bacteria: phylogenetic diversity, distribution, and endosymbiont members of various gut flagellated protists. *FEMS Microbiol. Ecol.* **60**:467–476.
- Shinzato, N., T. Matsumoto, I. Yamaoka, T. Oshima, and A. Yamagishi. 2001. Methanogenic symbionts and the locality of their host lower termites. *Microbes Environ.* **16**:43–47.
- Shinzato, N., M. Muramatsu, T. Matsui, and Y. Watanabe. 2005. Molecular phylogenetic diversity of the bacterial community in the gut of the termite *Coptotermes formosanus*. *Biosci. Biotechnol. Biochem.* **69**:1145–1155.
- 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.
- Wenzel, M., R. Radek, G. Brugerolle, and H. König. 2003. Identification of the ectosymbiotic bacteria of *Mixotricha paradoxa* involved in movement symbiosis. *Europ. J. Protistol.* **39**:11–23.
- Yoshimura, T., T. Watanabe, K. Tsunoda, and M. Takahashi. 1992. Distribution of the cellulolytic activities in the lower termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). *Mater. Org.* **27**:273–284.