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Coevolution of symbiotic spirochete diversity in lower termites

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Summary. The phylogenetic relationships of symbiotic spirochetes from five dry-wood feeding lower termites (*Cryptotermes cavifrons*, *Heterotermes tenuis*, *Kalotermes flavicollis*, *Neotermes mona*, and *Reticulitermes grassei*) was compared to those described in previous reports. The 16S rDNA bacterial genes were PCR-amplified from DNA isolated from intestinal samples using a spirochete-selective primer, and the 16S amplicons were cloned into *Escherichia coli*. Sequences of the cloned inserts were then used to determine closest relatives by comparison with published sequences. Clones sharing more than 97% sequence identity were grouped into the same phylotype. Forty-three new phylotypes were identified. These termite whole-gut-spirochetes fell into two previous defined clusters, designated as *Treponema* Clusters I and II, and one new Cluster III. Thirty-seven phylotypes were grouped in Cluster I. Cluster II comprised three phylotypes, two from *Reticulitermes grassei* (LJ029 and LJ012) and one from *Heterotermes tenuis* (LQ016). Three phylotypes, LK057, LK050 and LK028, were affiliated to Cluster III. Members of Cluster I showed the following characteristics: (i) spirochete phylotypes from a particular species of termite were more closely related to each other than to phylotypes of other termite species; (ii) spirochetes obtained from different genera of the same family, such as *Cryptotermes* sp., *Kalotermes* sp., and *Neotermes* sp., all from the family Kalotermitidae, were also related to each other. It was therefore concluded that spirochetes are specific symbionts that have coevolved with their respective species of termites, are stably harbored, and are closely related to members of the same termite family. [**Int Microbiol** 2007; 10(2):133-139]

Key words: symbiotic spirochetes · termite gut spirochetes · ectosymbionts · symbiotic protists · lower termites · coevolution

Introduction

Six families of termites (Isoptera, Dictyoptera) share with the wood-feeding cockroaches (family Cryptocercidae; Blattaria, Dictyoptera) the unusual ability to degrade lignocellulosic plant material. Depending on the species, food preferences range from wood to leaves, humus, detritus, and herbivore dung. Termites are constituted by the aforementioned "lower" six families of wood-feeders (Mastotermitidae, Kalotermitidae, Hodotermitidae, Termopsidae, Rhinotermitidae and Serritermitidae), and the much larger "higher" family Termitidae, which members are soil-eating and fungi-farming. The intestine of all wood-feeding lower termites harbors a diverse population of prokaryotes and flagellated protists that degrade lignin, cellulose and hemicelluloses to fermentable carbohydrates, and are thus indispensable to their termite hosts. By contrast, Termitidae typically lack protists, and their cellulolytic activity may be due either to acquisition of cellulases from food or cultivated fungi, or it may be endogenous in termite intestinal tissue [23,29].

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Eusociality is polyphyletic in termites, which are only distantly related to other social insects from the Order Hymenoptera (ants, bees, and wasps). The microbiota of termite gut are considered to be transmitted from generation to generation, basically via proctodeal trophallaxis [20].

The symbiotic protists found exclusively in association with the digestive tract of lower termites belong to the orders Trichomonadida, Cristamonadida, Hypermastigida and Oxymonadida [5]. The diversity of *Bacteria* from termite hindguts detected by 16S rRNA analysis is also extensive. "Termite clusters", containing phylotypes from more than one termite species, thus far consist of 15 *Bacteria* phyla, including the novel candidate phyla of termite groups 1 (TG1), TG2, and TG3 [12]. While the relative abundance of the representatives (phylotypes) of these phyla found along the intestinal tract (i.e., midgut, hindgut, etc.) is variable [33], spirochetes have been reported to account for up to 50% of all prokaryotes in the hindguts of some species of lower termites [11,17]. According to the results of 16S rRNA analysis, the major spirochetes from termites can be assigned to termite *Treponema* Clusters I and II [14,17,21,22]. Cluster I termite spirochetes have been found in all termites examined so far and include both ectosymbionts attached to protists and free-swimming gut spirochetes, whereas those of Cluster II have been identified as ectosymbiotic espirochetes of oxymonad protists in *Reticulitermes speratus* and *Hodotermopsis sjoestedti* [14,17,21]. Large symbiotic spirochetes (≥ 0.5 µm in diameter), which are limited to dictyopterids, were classified on the basis of their size and ultrastructure, and included *Clevelandina*, *Diplocalyx*, *Hollandina*, *Pillotina*, and *Canaleparolina darwiniensis* [18,31]. However, no correlated sequence information is available that allows their relationships to other spirochetes to be determined.

Little is known about the differences in bacterial gut microbiota with respect to colonies, geographical locations, and species of termites. Here, we report the spirochetal phylotypes obtained from five wood-feeding lower termites from different geographical locations. Our results show that spirochetal symbionts have coevolved within a specific termite species, and that symbionts of the same termite family are closely related.

Materials and methods

Termites. Five species of lower termites were used in this study. The five species were: *Cryptotermes cavifrons* (family Kalotermitidae), that was collected in Florida, USA; *Neotermes mona* (Kalotermitidae), from St. John Island (US Virgin Islands); *Heterotermes tenuis* (Rhinotermitidae), from the Napo River valley, Tiputini Biodiversity Station, Ecuador; *Kalotermes flavicollis* (Kalotermitidae), from the island of Crete, Greece; and *Reticulitermes grassei* (Rhinotermitidae), from Córdoba, Spain. Infested wood samples were kept in boxes at room temperature. Only termite worker-castes were used in this study.

Isolation of bacterial DNA. The entire hindgut of the insect was removed. Tissue was homogenized using a Mini-beadbeater (BioSpec Products, Inc., Bartlesville, OK) with 0.1-mm glass beads, and bulk DNA was extracted by several washings with phenol–chloroform.

PCR and cloning. A spirochete-selective reverse primer (1483–1503) 5′ GTTACGACTTCACCCTCCT 3′ was used with a universal forward primer (7–27) 5′ GAGAGTTTGATYMTGGCTCAG 3′ to selectively amplify spirochetal 16S rDNA [25]. PCR amplification was carried out in a 50-µl final volume of a reaction mix containing 1 µl DNA template, 20 pmol of each primer, 40 nmol dNTPs, 1.5 mM $MgCl_2$ and 1 U Taq platinum polymerase (Invitrogen, San Diego, CA). Samples were preheated at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and elongation at 72°C for 1.5 min, and finally an elongation step at 72°C for 15 min. The results of PCR amplification were examined by electrophoresis in a 1% agarose gel. DNA was stained with ethidium bromide and visualized under short-wavelength UV light. PCR products were purified by a QIAquick Gel extraction kit (Qiagen, Valencia, CA) and cloned with the TOPO TA cloning kit (Invitrogen) according to the manufacturer's recommendations.

Sequencing and phylogenetic analysis. Purified PCR products were sequenced using an ABI prism cycle-sequencing kit (BigDye® Terminator Cycle Sequencing kit with AmpliTaq DNA polymerase FS, Perkin-Elmer, Boston, MA). The primers used for 16S rRNA sequencing were as previously described [25]. Half-dye or quarter-dye chemistry was used with 3.2 mM primers, and 3 µl PCR product in a final volume of 20 µl. Cycle sequencing was done using an ABI 9700, with 25 cycles of denaturation at 96ºC for 10 s, and annealing and extension at 60°C for 4 min. Sequencing reactions were run on an ABI 3100 DNA sequencer. Partial 16S rRNA sequences were compared to known sequences in GenBank with the advanced gapped BLAST (basic local alignment search tool) algorithm. Phylogenetic analyses were carried out with MEGA version 2.1. The dendrogram was constructed using the neighbor-joining algorithm and the Kimura 2-parameter distance estimation method. One thousand bootstrap trees were generated, and bootstrap confidence levels were determined using the MEGA 2.1 program. Chimeric sequences were identified according to the Chimera check program in the Ribosomal Database Project II.

Nucleotide sequence accession numbers. Partial 16S rRNA gene sequences of clones representing novel phylotypes defined in this study and published sequences are available for electronic retrieval from the EMBL, GenBank, and DDBJ nucleotide sequence databases (AY739123–AY739166).

Results

Twenty, 30, 25, 15, and 20 clones (16S rDNA cloning inserts) were analyzed from the whole gut communities of, respectively, *Cryptotermes cavifrons* (LP phylotypes), *Kalotermes flavicollis* (LK phylotypes), *Heterotermes tenuis* (LQ phylotypes), *Neotermes mona* (LR phylotypes) and *Reticulitermes grassei* (LJ phylotypes). The number of clones attributable to the 16S rDNA of spirochetes was 11, 20, 12, 8, and 13, respectively. Several phylotypes were found repeatedly (number in parentheses after the phylotype): LP008 (2), LP011 (3),

Table 1. Source of spirochetal 16S rDNA sequences used in the phylogenetic analysis

Host	Location	Phylotype/strain	Habitat or host	Reference
Kalotermitidae (termites)				
Cryptotermes cavifrons	Florida, USA	LP-clones	Whole hindgut	This work
Kalotermes flavicollis	Crete, Greece	LK-clones	Whole hindgut	This work
	Germany	Kf428	Whole hindgut	(GenBank)
Neotermes mona	Virgin Islands, USA	LR-clones	Whole hindgut	This work
N. koshunensis	Okinawa, Japan	NkS5; NkS8; NkS97; NkS34	Whole hindgut	[21]
		NkS-Oxy26; NkS-Oxy70; NkS-Ste9	Protist-ectosymbionts[a]	[21]
		NkW01-44; Nk01-024	Whole hindgut	[19]
N. castaneus	Berlin, Germany	SPN1	Whole hindgut	[6]
Rhinotermitidae (termites)				
Heterotermes tenuis	Ecuador	LQ-clones	Whole hindgut	This work
Reticulitermes grassei	Córdoba, Spain	LJ-clones	Whole hindgut	This work
R. flavipes	Dansville, MI, USA	RFS94; RFS12; RFS59	Whole hindgut	[17]
R. speratus	Japan	RS-A43; RS-H09; RS-B68	Whole hindgut	[11]
Coptotermes formosanus		BCf1-01	Whole hindgut	[27]
		CFS6; CFS124; CFS149p	Whole hindgut	[17]
Termopsidae (termites)				
Hodotermopsis sjoestedti	Yakushima, Japan	HsPySp20	Protist-ectosymbionts[b]	[14]
	Japan	HsW01-005	Whole hindgut	[19]
Zootermopsis angusticollis		*Treponema azotonutricium*; *T. primitia*	Whole hindgut	[9]
Mastotermitidae (termites)				
Mastotermes darwiniensis	Darwin, Australia	Sp5-18; Sp40-7;mpsp2; mpsp15	Whole hindgut	[1]
	Darwin, Australia	mp1; mp3; mp4	Protist-ectosymbionts[c]	[30]
Termitidae (termites)				
Nasutitermes lujae	Republic of Congo	NL1	Whole hindgut	[24]
N. takasagoensis	Irionote, Japan	Nt2-021; Nt2-070	Whole hindgut	[13]
Macrotermes michaelseni		MTG-91	Whole hindgut	(GenBank)
Ixodidae (ticks)				
Ixodes sp.		*Borrelia burgdorferi*	Midgut	(GenBank)
Non-insect spirochetes (reference sequences)		*Leptospira interrogans*	Aquatic environment	(GenBank)
		Treponema pallidum	Human genital tract	(GenBank)
		Escherichia coli		(GenBank)

[a]Spirochete ectosymbionts from the protists *Oxymonas* (NkS-Oxy phylotypes), *Stephanonympha* (NkS-Ste phylotypes) and *Devescovina* (NkS-Dev phylotype).
[b]Spirochete ectosymbionts from the protist *Pyrsonympha.*
[c]Spirochete ectosymbionts from the protist *Mixotricha paradoxa.*

LK047 (2), LK059 (5), LK006 (1), LR046 (2), LR045 (3), LJ026 (2) and LJ029 (1). Those phylotypes were compared with previous phylotypes identified by other authors. Table 1 summarizes the source of the 16S rDNA sequences compared in the phylotype analysis.

The 43 new spirochetal phylotypes are shown in Fig. 1. Thirty-seven phylotypes were grouped in termite Cluster I. Three phylotypes, two from *Reticulitermes grassei* (LJ029 and LJ012) and one from *Heterotermes tenuis* (LQ016), were assigned to Cluster II. Three phylotypes, LK057, LK050 and

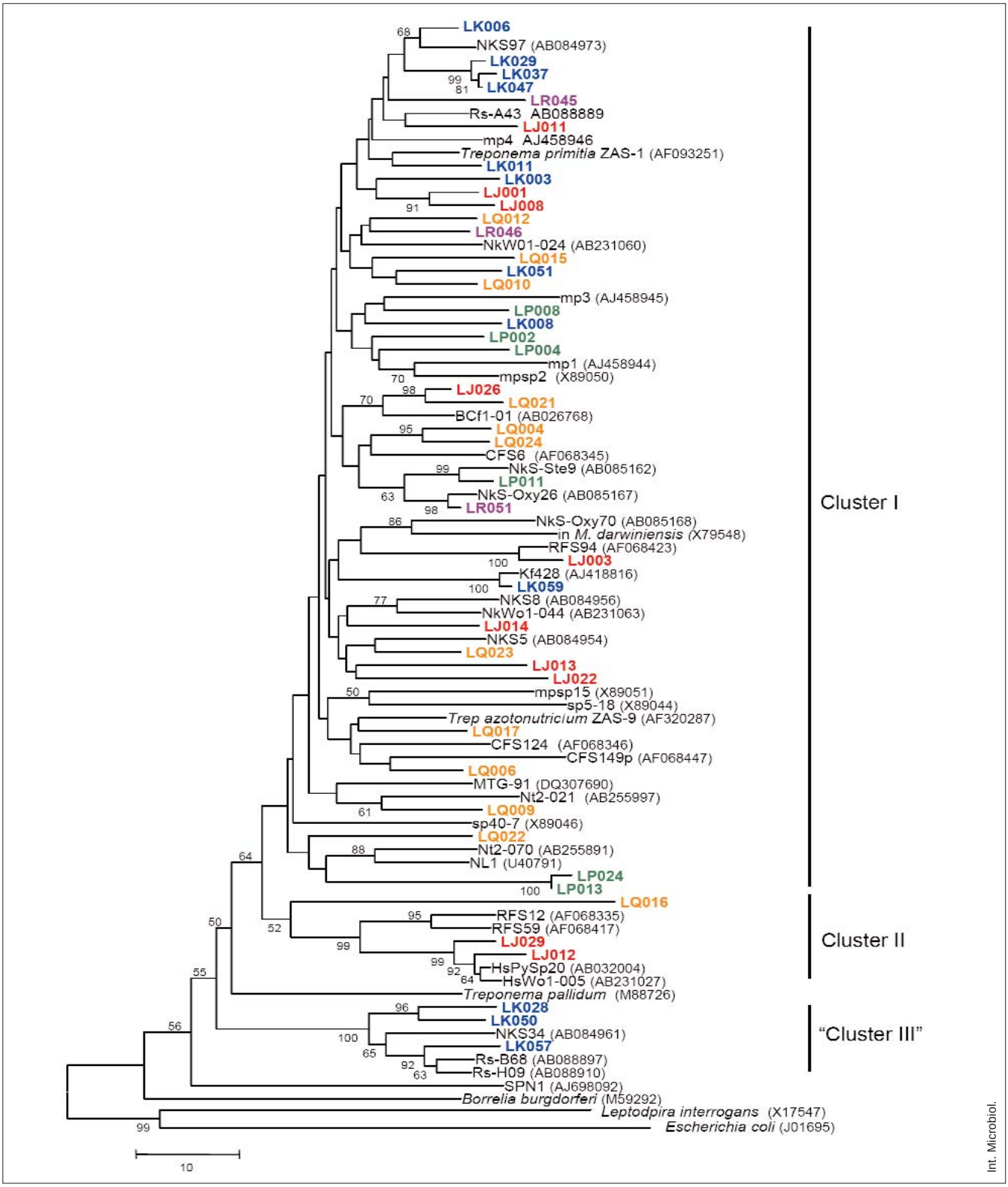
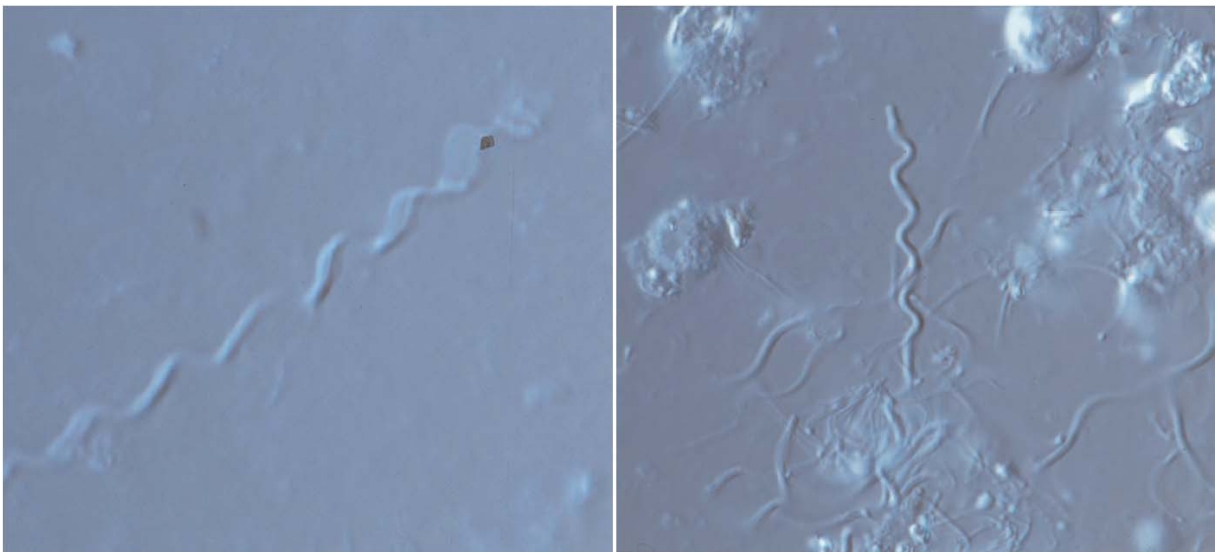


Fig. 1. Phylogenetic tree of the 16S rDNA partial sequences of spirochetes from five lower termites. *Treponema* Clusters I, II and III are indicated at the right side of the tree. Phylotypes obtained in this work: LJ (*Reticulitermes grassei*) [red], LK (*Kalotermes flavicollis*) [blue], LP (*Cryptotermes cavifrons*) [green], LQ (*Heterotermes tenuis*) [orange], LR (*Neotermes mona*) [purple]. Reference sequences were: *Escherichia coli*, *Leptospira interrogans*, *Borrelia burgdorferi*, *Treponema pallidum* and other phylotype spirochetes from *Reticulitermes flavipes*, *Hodotermopsis sjoestedti*, *Nasutitermes lujae* and *Mastotermes darwiniensis*. The database accession number is shown after the name of the clones from the reference sequences.



Spirochetes from the whole gut of *Kalotermes flavicollis* from the island of Crete, Greece.

LK028, were affiliated with a third and new Cluster III.

Spirochete phylotypes from a particular species of termites tended to be more closely related to each other than the phylotypes of other termites. Moreover, there was an affiliation relationship between spirochetes-phylotypes from genera belonging to the same family (e.g., spirochetes from the family Kalotermitidae, phylotypes LK, LP and LR, were also related to each other). A similar result was observed in Rhinotermitidae (phylotypes LJ and LQ). However, there were also a few exceptions: the phylotypes LK051 and LR046, from two species of the same family (*K. flavicollis* and *N. mona*, family Kalotermitidae), were present in clusters from another family (Rhinotermitidae) of termites.

Treponema Cluster I comprises both ectosymbionts attached to protists and free-swimming gut spirochetes [14,21]. Based on the affiliation with reported sequences, phylotypes LP013, LP024, LQ006, LQ009, LQ0017, LQ022 and LK011 could be considered free-swimming spirochetes because: (i) they grouped with NL1 (from *Nasutitermes lujae*), Nt2-021 and Nt2-070 (from *Nasutitermes takagoensis*), and MTG-91 (from *Macrotermes michaelseni*), which are higher termites (family Termitidae), and typically lack protists [13,24]; (ii) they grouped with mpsp15 of *Mastotermes darwiniensis*, which is a sequence derived from a free-swimming large spirochete in termite gut fluid [1]; and (iii) they also grouped with the culturable spirochetes *Treponema primitia* ZAS-1 and *T. azotonutricium* ZAS-9 (from *Zootermopsis angusticollis*) [9]. Paradoxically, neither spirochete phylotype obtained in this work nor phylotypes previously reported (used in this study) clustered with the recently cultivable spirochete SPN1, isolated from *Neotermes castaneus* [6].

Phylotypes LJ029, LJ012 (*Reticulitermes grassei*) and LQ016 (*Heterotermes tenuis*) grouped with several sequences previously reported as belonging to *Treponema* Cluster II (Fig. 1). Members of Cluster II are ectosymbiotic spirochetes of oxymonad protists. However, not all ectosymbiotic spirochetes are in Cluster II [14,17,19].

The phylotypes LK028, LK050 and LK057 were affiliated outside the *Treponema*-termite clusters and grouped with phylotypes RsB68 and RsH09 from *R. speratus*. The latter are related to the genus *Spirochaeta* [11]. The 16S rRNA gene sequence of strain SPN1 also belonged to the genus *Spirochaeta* but it was not closely related to sequences of Cluster III (Fig. 1). In contrast to all other known described spirochete species, strain SPN1 has a coccoid morphology and is immotile [6].

Discussion

The large number of spirochetal 16S rDNA clones obtained from each termite confirms the high resolution achieved by the use of group-specific primers prior to cloning [17]. It was previously reported that a single termite species may harbor >20 phylotypes of spirochetes [17], and indeed we detected 12 new phylotypes in 20 clones attributable to 16S rRNA of spirochetes in *Kalotermes flavicollis*.

Closely related spirochete phylotypes in geographically and taxonomically distinguishable kalotermitids may be due

to the presence of the protists *Stephanonympha* sp. and *Devescovina* sp. in different genera, e.g., *Cryptotermes* and *Neotermes*, or other spirochete-bearing protists common to the termite gut [5,8,21]. While termites support a characteristic community of gut protists, many protist species are not necessarily restricted to one termite species [5]. Furthermore, many protist species are simultaneously associated with different bacterial ectosymbionts [21,30], and common spirochete phylotypes are often shared among the different protist species [21]. Iida et al. [14] pointed out that ectosymbiotic spirochetes associated with a single protist include at least three phylotypes (species) of spirochetes. Thus, it seems that the dominant spirochete morphotypes are ectosymbionts rather than free-swimming spirochetes [2,28,33].

Spirochetes are specific symbionts that have coevolved with termites, are stably harbored, and are closely related particularly within single species but also within the same termite family. Termite gut bacteria are thought to be transmitted vertically from generation to generation basically via proctodeal trophallaxis as known for the gut symbiotic protists. Nonetheless, the coevolutionary process cannot be explained by a purely vertical transmission. Although a single colony of termites was used in the comparisons of the present study, our observations can most likely be generalized to most termite colonies because congeneric termites harbor similar spirochetes irrespective of colony or sampling location [12,19]. Variations in spirochete phylotypes within congeneric termites may be due to ambient temperature, food quality, humidity and other environmental factors that influence termite life.

In many microbial systems, the functionally active unit is not a single species or population (clonal descendence of the same bacterium), but a consortium of two or more types of cells living in close symbiotic association [10,26]. Ectosymbiotic and free-swimming spirochetes appear to specialize in metabolic interactions with the host or with other co-occurring microorganisms. The main compounds produced by spirochetes are acetate, H_2, and CO_2, which are normally consumed by sulfate-reducing bacteria and methanogens (with both groups represented in termites) [6,7,9,15]. Acetate produced by gut microbiota supports up to 100% of the respiratory requirement of termites [3,4]. Lilburn et al. [16] demonstrated that spirochetes from termite hindguts and freshwater sediments possess homologs of a nitrogenase gene (*nifH*) and exhibit nitrogenase activity. This observation essentially implicates spirochetes in the nitrogen nutrition of termites, whose food is typically low in nitrogen. Spirochetes populations can stably maintain the gut habitat by supplying carbon sources and electron donors to other resident microbial populations and to the host.

Termites preserved in amber provide direct paleontological evidence for the stable relationship between termites and their intestinal symbionts (protists and spirochetes) throughout at least 20 million years [32]. The coevolution of unique and diverse spirochetes with xylophagous social insects, as shown here, supports the hypothesis that these cosmopolitan microbial symbionts are obligatory for wood digestion and their presence is ancient.

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