

ORIGINAL ARTICLE

Intranuclear verrucomicrobial symbionts and evidence of lateral gene transfer to the host protist in the termite gut

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In 1944, Harold Kirby described microorganisms living within nuclei of the protists *Trichonympha* in guts of termites; however, their taxonomic assignment remains to be accomplished. Here, we identified intranuclear symbionts of *Trichonympha agilis* in the gut of the termite *Reticulitermes speratus*. We isolated single nuclei of *T. agilis*, performed whole-genome amplification, and obtained bacterial 16S rRNA genes by PCR. Unexpectedly, however, all of the analyzed clones were from pseudogenes of 16S rRNA with large deletions and numerous sequence variations even within a single-nucleus sample. Authentic 16S rRNA gene sequences were finally recovered by digesting the nuclear DNA; these pseudogenes were present on the host *Trichonympha* genome. The authentic sequences represented two distinct bacterial species belonging to the phylum *Verrucomicrobia*, and the pseudogenes have originated from each of the two species. Fluorescence *in situ* hybridization confirmed that both species are specifically localized, and occasionally co-localized, within nuclei of *T. agilis*. Transmission electron microscopy revealed that they are distorted cocci with characteristic electron-dense and lucent regions, which resemble the intranuclear symbionts illustrated by Kirby. For these symbionts, we propose a novel genus and species, '*Candidatus Nucleococcus trichonymphae*' and '*Candidatus Nucleococcus kirbyi*'. These formed a termite-specific cluster with database sequences, other members of which were also detected within nuclei of various gut protists, including both parabasalids and oxymonads. We suggest that this group is widely distributed as intranuclear symbionts of diverse protists in termite guts and that they might have affected the evolution of the host genome through lateral gene transfer.

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Introduction

Many protists harbor symbiotic microorganisms within their cytoplasm and occasionally within their nucleoplasm. Microorganisms living within the nuclei of protists were discovered in 1856 (Müller, 1856), and since then, numerous studies have reported both eukaryotic and prokaryotic intranuclear symbionts in diverse protist lineages (Görtz, 1986; Fokin, 2004). In the ciliates *Paramecium*, the macronucleus and micronucleus can be infected by specific bacterial species belonging to the genera *Holospora*, *Caedibacter* and '*Candidatus*

Gortzia' in the order *Rickettsiales* of *Alphaproteobacteria* (Boscaro *et al.*, 2013). These symbionts have two distinct life stages; the long, infectious form found in the cytoplasm and the short, reproductive form that divides in the host nucleus (Fujishima and Kodama, 2012). For other intranuclear symbionts, mainly because of the difficulty of their cultivation, information on their ecology, physiology and even the taxonomy is scarce.

Termites harbor abundant and diverse microorganisms in their hindgut, which comprise protists, bacteria and archaea. These gut symbionts are unique to termites and their closest relatives, cockroaches (Hongoh, 2010; Schauer *et al.*, 2012), and are essential to the nutrition of the host insects (Brune and Ohkuma, 2011; Hongoh, 2011). One of the prominent features of this symbiotic system is the cellular association between the protists and the prokaryotes. For example, the cellulolytic protist *Trichonympha agilis* in the gut of the termite

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Reticulitermes speratus is permanently associated with two species of cytoplasmic symbionts, ‘*Candidatus Endomicrobium trichonymphae*’ (phylotype Rs-D17) (Stingl *et al.*, 2005; Ohkuma *et al.*, 2007) and ‘*Candidatus Desulfovibrio trichonymphae*’ (phylotype Rs-N31) (Sato *et al.*, 2009). Furthermore, spirochetes and *Bacteroidales* bacteria are attached to its cell surface (Hongoh *et al.*, 2007b). The complete genome sequence analysis of phylotype Rs-D17 suggested that the endosymbionts contribute to the host protists and termites by the synthesis of amino acids and cofactors, which are deficient in the ingested wood materials (Hongoh *et al.*, 2008a). Likewise, termite gut protists generally harbor diverse intracellular and/or extracellular symbiotic prokaryotes comprising members of the orders *Spirochaetales*, *Bacteroidales*, ‘*Endomicrobiales*’, *Desulfovibrionales*, *Actinomycetales* and *Methanobacteriales* (Hongoh and Ohkuma, 2011; Desai and Brune, 2012; Strassert *et al.*, 2012).

Under light microscope observation, Kirby (1944) described several types of uncultured microorganisms living within the nuclei of *Trichonympha* in the gut of various termite species. He illustrated those symbionts in detail and tentatively assigned them to the bacterial genus *Caryococcus* and putatively chytrid fungi *Nucleophaga* and *Caryoletira* (Kirby, 1944). Bacteria-like cells within the nuclei of various protist species in termite guts were later observed by transmission electron microscopy (TEM) in numerous studies (Brugerolle and Radek, 2006). However, the taxonomic positions of these intranuclear symbionts of termite gut protists remain unknown.

In this study, we attempted to identify intranuclear bacterial symbionts of *Trichonympha agilis* based on small subunit rRNA sequences. Our results answer the long-standing question, ‘what are the symbionts living within the nuclei of the protists in termite guts?’ In addition, we present the evidence for the lateral transfer of genome fragments from the intranuclear symbionts to the host protist. This provides new insights into the evolution of the termite gut protists.

Materials and methods

Sample preparation

The wood-feeding termite *Reticulitermes speratus* was collected at Ogose in Saitama prefecture, Japan. The gut of a worker termite was removed using sterile forceps, and the gut contents were suspended in Trager’s solution U (Trager, 1934). A single cell of the gut protist *T. agilis* (phylum *Parabasalia*) was collected with a glass capillary attached to a TransferMan NK2 micromanipulator (Eppendorf, Hamburg, Germany). The isolated *T. agilis* cell was ruptured in the presence of 1% Nonidet P-40 or Tween 20 (Nacalai Tesque, Kyoto, Japan), and the exposed nucleus was collected and washed in the

same solution. The nucleus was transferred to a sterile 0.2-ml tube containing 2 µl of lysis buffer (0.3 N KOH, 10 mM EDTA, 100 mM dithiothreitol) and subjected to isothermal whole-genome amplification (WGA) using GenomiPhi HY or GenomiPhi V2 (GE Healthcare, Piscataway, NJ, USA) as described previously (Hongoh *et al.*, 2008a, b). The WGA samples were purified by ethanol precipitation and diluted samples were used as templates for PCR.

To digest the genomic DNA of *T. agilis*, the nucleus was dissected with a Microfeather Blade K-730 (Feather, Tokyo, Japan) attached to the micromanipulator, in a solution containing RQ1 RNase-free DNase (Promega, Madison, WI, USA). DNase was inactivated in the above alkaline lysis buffer.

PCR amplification, cloning and sequencing

PCR targeting the small subunit rRNA genes was performed using a proofreading DNA polymerase, Phusion (Finnzymes, Espoo, Finland), in a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) under previously described conditions (Hongoh *et al.*, 2007a). When PCR was performed directly using a single nucleus without WGA, the number of PCR cycles was raised from 15 to 35, and a second PCR with 20 cycles was performed. The primers and annealing temperature used in this study are listed in Table 1. Primers Verr-61F, Verr-361F, and Verr-1351R were designed in this study to specifically detect most members of subdivisions 1–4 and 6 in the phylum *Verrucomicrobia* (Hugenholtz *et al.*, 1998). Although primer Verr-61F also targets several other bacterial taxa, the combination with primer 1351R secured the specificity to the *Verrucomicrobia*. The products were separated by 1% agarose gel electrophoresis, purified using the Monofas DNA purification kit (GL Sciences, Tokyo, Japan), and cloned using the ZeroBlunt TOPO PCR Cloning Kit for Sequencing (Life Technologies, Carlsbad, CA, USA). The sequencing was performed using the BigDye Terminator Cycle Sequencing Kit V3.1 (Life Technologies) with an ABI 3730 genetic analyzer, basically as described previously (Hongoh *et al.*, 2003). The sequences reported in this study have been deposited in DDBJ/EMBL/GenBank under accession numbers AB826690–AB826745.

Phylogenetic analysis

16S rRNA sequences were subjected to BLASTn searches against the NCBI nr database for a preliminary taxonomic assignment (Altschul *et al.*, 1997). The sequences were sorted into phylotypes using the program package mothur 1.30.1 (Schloss *et al.*, 2009). Representative clones of the phylotypes were incorporated into an ARB database (Ludwig *et al.*, 2004). The alignment was manually corrected (Sato *et al.*, 2009), and 1389 unambiguously aligned

nucleotide sites, corresponding to positions 28–1491 in *Escherichia coli* (J01695), were used for phylogenetic analysis. Maximum likelihood (ML) trees were constructed using RAxML 7.0.3 (Stamatakis, 2006) implemented in ARB version 5.2 (Ludwig *et al.*, 2004). A general time-reversible model with a gamma distribution was used, and 500 bootstrap resamplings were performed to evaluate the robustness of the topology. Minimum evolution (ME) trees were also constructed in MEGA5 (Tamura *et al.*, 2011), using the maximum composite likelihood method with a gamma distribution to estimate a distance matrix.

Fluorescence in situ hybridization (FISH) analysis

FISH was performed as described previously (Noda *et al.*, 2006). The oligonucleotide probes used in this study are listed in Table 1. Probes specifically targeting 16S rRNA of verrucomicrobial phylotypes or clusters were designed using the probe-designing function in ARB. The sequence specificity of the probes was checked in the Probe Match program in the Ribosomal Database Project (<http://rdp.cme.msu.edu/probematch/search.jsp>) and by probeCheck (Loy *et al.*, 2008). The optimal conditions for specific hybridization were estimated *in silico* using DINAMelt (Markham and Zuker, 2005) and were determined by shifting the annealing temperature and/or formamide concentration in an actual sample. Probe V-RsD37-130 was designed to detect the majority of the verrucomicrobial termite-specific cluster, as designated in this study. This probe has one base mismatch to several phylotypes such as IswTcA-49 (JQ993599); therefore, we adjusted the conditions for specific hybridization to allow one base mismatch (Table 1). Phylotypes Cc3-048 (AB299564), Cc3-051 (AB299567), and RsaP110 (AY571501) are not detectable with this probe. For phylotype-specific probes Verr-K1-181 and Verr-K2-181,

we also designed helper probes (Table 1; Fuchs *et al.*, 2000). Probe EUB338-III was used to specifically detect the majority of the phylum *Verrucomicrobia* (Daims *et al.*, 1999). These probes were labeled at their 5' ends with either of Texas red or 6-carboxyfluorescein (6FAM). The specimens were observed under a BX-51 epifluorescence microscope (Olympus, Tokyo, Japan) or a TCS SP8 confocal laser scanning microscope (Leica, Wetzlar, Germany).

Transmission electron microscopy

TEM was conducted as described previously (Noda *et al.*, 2006). Briefly, large protist species, including *T. agilis*, were collected by low-speed centrifugation, prefixed with 2.5% (v/v) glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4 °C overnight, and then post-fixed in buffer containing 1% osmium tetroxide for 1.5 h. Thin sections were post-stained with uranyl acetate and lead citrate, and viewed under a 1230 transmission electron microscope (JEOL, Tokyo, Japan) or an H-7500 transmission electron microscope (Hitachi, Tokyo, Japan).

Results

Discovery of 16S rRNA pseudogenes related to *verrucomicrobia*

16S rRNA genes were amplified by PCR using bacteria-specific primers 27F-mix and 1492R (Table 1) from six WGA samples, each prepared from a single *T. agilis* nucleus. The PCR products were visualized in gel electrophoresis as multiple bands, the largest of which corresponded to the predicted size of the targeted region (lanes 1–6, Figure 1a). Both long and shorter products were separately excised from the gel and sequenced. Although ~200 clones were analyzed for the longest products, they represented only phylotypes Rs-D17 and Rs-N31, known cytoplasmic symbionts of

Table 1 PCR primers and FISH probes for small subunit rRNA used in this study

Target taxon	Primer/probe	Sequence (5' → 3')	Annealing/formamide	Reference
<i>For PCR</i>				
Bacteria	27F-mix	AGRGTTTGATYMTGGCTCAG	50 °C	Hongoh <i>et al.</i> (2007a)
	1390R	ACGGGCGGTGTGTACAA		
	1492R	GGHTACCTTGTTACGACTT		
Eukarya	E23F3	ACYTGGTTGATYCTGCC	50 °C	This study
	E1511R4	CWDCBGCAGGTTCCWCCWAC	50 °C	This study
Verrucomicrobia subdivisions 1–4 and 6	Verr-61F	GCGTGGYTAAGACATGCAAG	72 °C	This study
	Verr-361F	ACGGGTGGCAGCAGTTTC		
	Verr-1351R	GYCRTAGCTGATRCGCCA		
<i>For FISH</i>				
Bacteria except Verrucomicrobia	EUB338	GCTGCCTCCCGTAGGAGT	60 °C	Amann <i>et al.</i> (1990)
Mainly Verrucomicrobia	EUB338-III	GCTGCCACCCGTAGGTGT	60 °C	Daims <i>et al.</i> (1999)
Verrucomicrobia 'termite cluster'	V-RsD37-130	CGAGCTATCCCCAACTAA	50 °C	This study
RsTaN-K1	Verr-K1-181	CATGCAGAACGAAACAC	50 °C + 20%	This study
	helper-K1	ATTCGGTATTAATTCCGCTT		
RsTaN-K2	Verr-K2-181	CATGCGAAAAGAAATCAC	48 °C	This study
	helper-K2	ATTCGGTATTAATTCACC		

T. agilis (Stingl *et al.*, 2005; Ohkuma *et al.*, 2007; Sato *et al.*, 2009), from four of the WGA samples (1–3 and 6, corresponding to the lane numbers in Figure 1a).

On the other hand, the 126 clones of the shorter products from the above four WGA samples comprised exclusively pseudogenes of 16S rRNA, related to the bacterial phylum *Verrucomicrobia*. All the pseudogenes contained multiple large deletions (Figure 1b), and numerous base substitutions and indels in the highly conserved regions of 16S rRNA (data not shown). To discount the possibility that these were artifacts formed during WGA, we amplified 16S rRNA genes by PCR directly from two single *T. agilis* nuclei, bypassing WGA. Only pseudogenes with large deletions similar to those mentioned above were obtained. These pseudogenes, derived from the samples prepared with or without WGA, have numerous sequence variations even within a single-nucleus sample. Of 156 analyzed pseudogene sequences, 47 phylotypes, defined with 99.0% sequence identity, were recognized (Supplementary Table S1). In the case of the WGA sample 6 (Figure 1a), 12 phylotypes were identified from 14 analyzed clones (Supplementary Table S1). We used a proofreading DNA polymerase and found almost no variations in the Rs-D17 sequences obtained above; it is unlikely that these variants were artificially generated during PCR.

Acquisition of authentic 16S rRNA gene of verrucomicrobial phylotype RsTaN-K1

We assumed that these pseudogenes originated from intranuclear symbionts. Indeed, we detected coccoid cells within the nuclei of *T. agilis* by FISH using

the *Verrucomicrobia*-specific probe EUB338-III (Supplementary Figures 1a–f). To acquire an authentic 16S rRNA gene for the pseudogenes, we used verrucomicrobia-specific primers (Table 1), one of which, Verr-1351R was designed based on a deleted region in most of the pseudogenes (Figure 1b). This was an attempt not to amplify the sequences of the contaminating cytoplasmic symbionts and the verrucomicrobial pseudogenes. However, we could only amplify different variations of the verrucomicrobial pseudogenes: RsTaNv1-09, RsTaNv1-18, RsTaNv2-05 and RsTaN5v1-07 (AB826742–AB826745) (Figure 1b).

We hypothesized that these numerous pseudogenes are present on a chromosome or chromosomes of *T. agilis* as a result of lateral gene transfer (LGT). We therefore performed microsurgery of a *T. agilis* nucleus in the presence of DNase I, to digest the host genomic DNA. The DNase-treated nucleus sample was subjected to WGA, and PCR was performed using bacteria-specific primers 27F-mix and 1492R. As expected, the shorter products disappeared (lane 7, Figure 1a). Among 89 clones, five belonged to Rs-N31 and the rest consisted of a single phylotype, named RsTaN-K1, with identical, apparently authentic sequences, which were phylogenetically affiliated with the order *Puniceicoccales* in subdivision 4 of the phylum *Verrucomicrobia* (Figure 2). We also performed PCR using the verrucomicrobia-specific primers (Table 1), and obtained exclusively the same sequences.

Localization of RsTaN-K1 and the discovery of another phylotype, RsTaN-K2

FISH was performed to locate the cells of phylotype RsTaN-K1, using the phylotype-specific probe Verr-K1-181 in combination with the *Verrucomicrobia*-specific probe EUB338-III or probe EUB338 specific to bacteria except *Verrucomicrobia* and several other taxa (Daims *et al.*, 1999) (Table 1). We successfully detected the RsTaN-K1 cells exclusively within the nucleus of *T. agilis* (data not shown). Unexpectedly, however, we noticed that RsTaN-K1 frequently coexisted with another intranuclear bacterium that was detected with probe EUB338-III (data not shown). Thus, there must be another verrucomicrobial species present.

During our examination, we realized that the infection rate of RsTaN-K1 varied greatly, being dependent on the termite colony. In certain colonies, <1% of the *T. agilis* cells were infected with intranuclear verrucomicrobial symbionts, whereas in other colonies, for example, 10%, 38%, 42%, 53% and 87% of the *T. agilis* cells were infected with both RsTaN-K1 and the unidentified verrucomicrobia or with either of them alone. We used such a termite colony, where the nuclei of *T. agilis* were infected almost only with the unidentified verrucomicrobia. We repeated the same procedure as above (lane 8, Figure 1a) and obtained another

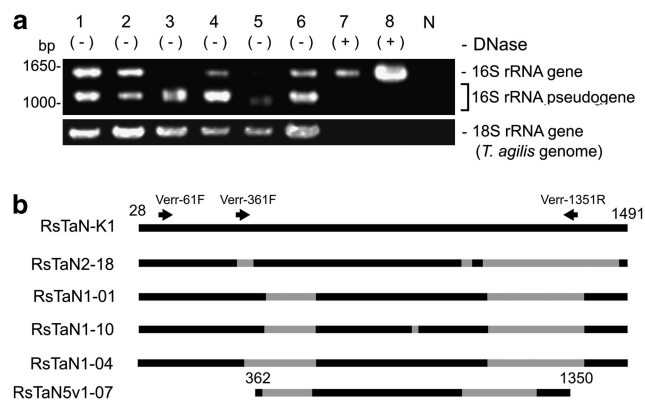


Figure 1 16S rRNA pseudogenes present within the nuclei of *T. agilis*. **(a)** Agarose gel electrophoresis of 16S rRNA genes amplified by PCR with a bacteria-specific primer set from single nuclei samples of *T. agilis*. Lanes 1–6, PCR products from each of six nuclei. Lane 7 and 8, PCR products from single DNase-treated nuclei. N, negative control for PCR. **(b)** Structures of representative pseudogenes. Gray color denotes deleted regions. The positions of PCR primers specific to verrucomicrobia (Table 1) are indicated. RsTaN-K1 is an authentic 16S rRNA phylotype. RsTaN2-18 originated from RsTaN-K2 and the others shown here originated from RsTaN-K1. The positions correspond to those in *Escherichia coli* (J01695).

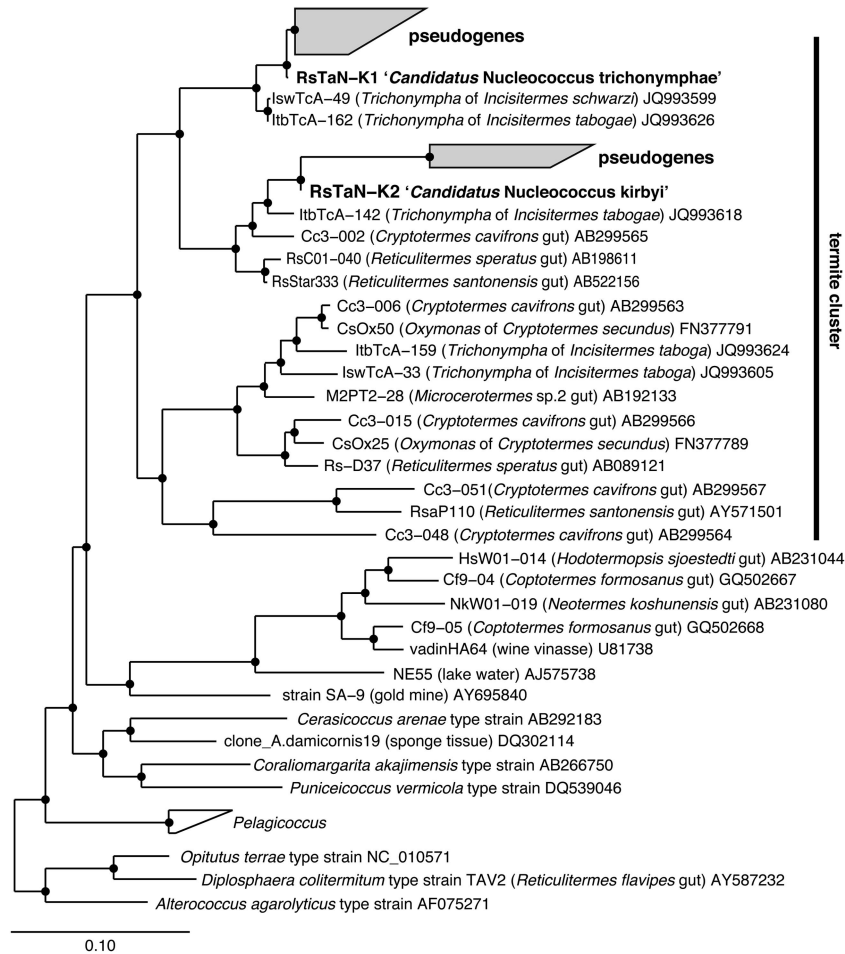


Figure 2 Phylogenetic positions of intranuclear symbionts based on 16S rRNA gene sequences. A maximum likelihood tree was constructed. Pseudogene phylotypes, RsTaNv1-09, RsTaNv1-18, RsNv2-05 and RsTaN5v1-07 were not included in this analysis because of their shorter length. The topologies, including the positions of pseudogenes and the formation of the termite-specific cluster, were basically consistent in a ME tree (data not shown). Open and closed circles at the nodes indicate the bootstrap confidence values of 70–94% and 95–100%, respectively. *O. terrae*, *D. colitermitum* and *A. agarolyticus* were used as outgroups.

verrucomicrobial phylotype, RsTaN-K2, which shared 90% sequence identity with RsTaN-K1 (Figure 2). By FISH analysis with probe Verr-K2-181 specific to RsTaN-K2 (Table 1), we confirmed that RsTaN-K2 inhabited only the nuclei of *T. agilis* and frequently coexisted with RsTaN-K1 within the same nuclei (Figure 3).

Morphology and enumeration of RsTaN-K1 and RsTaN-K2 cells

The cells of phylotypes RsTaN-K1 and RsTaN-K2, detected by FISH, were coccoid with a certain degree of distortion (Figure 3d and Supplementary Figures S1a–f). Under TEM observation of *T. agilis* nuclei, we found coccoid, distorted bacterial cells (Figure 4), although we could not discriminate RsTaN-K1 and RsTaN-K2. On FISH images, the dimensions of the RsTaN-K1 cells were $0.9 \pm 0.2 \mu\text{m}$ by $0.7 \pm 0.2 \mu\text{m}$ (mean \pm s.d., $n = 104$), and those of RsTaN-K2 cells were $1.1 \pm 0.3 \mu\text{m}$ by

$0.7 \pm 0.1 \mu\text{m}$ ($n = 98$). In a certain preparation, the numbers of RsTaN-K1 and RsTaN-K2 cells that were contained within the same nuclei were 31 ± 8 and 62 ± 26 ($n = 33$, t -test, $P < 0.0001$), respectively. In another preparation from a different termite colony, the numbers were 39 ± 10 and 55 ± 17 ($n = 5$, t -test, $P = 0.10$). Thus, RsTaN-K2 tended to outnumber RsTaN-K1 within a nucleus, when co-localized. When RsTaN-K2 alone inhabited a nucleus, the number of RsTaN-K2 cells was 57 ± 25 ($n = 7$) and 51 ± 17 ($n = 8$), in the former and latter preparations, respectively. This implied that the presence of co-inhabiting RsTaN-K1 does not affect the population of RsTaN-K2. The dimensions of the nuclei infected by both RsTaN-K1 and RsTaN-K2, in a preparation for FISH, were $129 \pm 23 \mu\text{m}^2$ ($n = 27$), which was not significantly different from those of non-infected nuclei, $119 \pm 27 \mu\text{m}^2$ ($n = 15$; t -test, $P = 0.09$). The morphological details are further explored in the description sections.

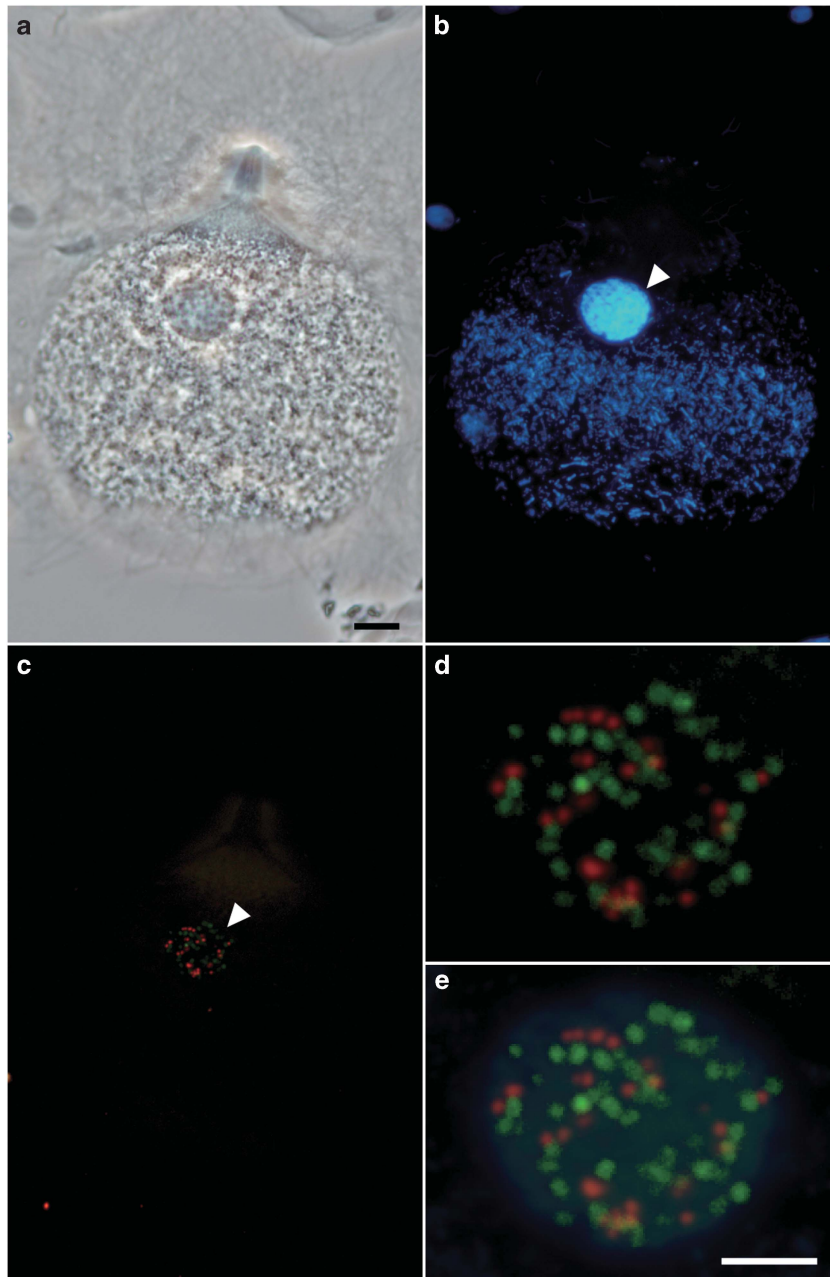


Figure 3 Specific detection of intranuclear symbionts of *Trichonympha agilis* by FISH. (a) Phase contrast image of a *T. agilis* cell. (b) DAPI-stained image. (c) Merged image of FISH analyses that detected RsTaN-K1 with the Texas red-labeled probe Verr-K1-181 (red) or RsTaN-K2 with the 6FAM-labeled probe Verr-K2-181 (green). (d) Magnified image of the nucleus (arrowhead) in panel c. (e) Panel d was overlaid with the DAPI image. Bars represent 10 μm (a); 5 μm (d).

Relationship between the intranuclear symbionts and the 16S rRNA pseudogenes

Phylogenetic analysis was conducted that included the pseudogenes (Figure 2). The pseudogenes were separated to two distinct clusters, which clearly originated from phylotypes RsTaN-K1 and RsTaN-K2, respectively. The obtained pseudogenes were more diverse and abundant in the RsTaN-K1-related cluster than in the RsTaN-K2-related one (Supplementary Figure S2a and Supplementary Table S1). There were almost no identical

pseudogene sequences among the six single-nucleus samples. The three phylotypes of pseudogenes that originated from RsTaN-K2 shared large deletions (Figure 1b and Supplementary Figure S2a); thus, the lateral transfer of the 16S rRNA gene of RsTaN-K2 to the *Trichonympha* genome was likely to have been a single event. Similarly, all but two phylotypes of the pseudogenes that originated from RsTaN-K1 shared large deletions (Figure 1b and Supplementary Figure S2a). The two phylotypes, RsTaNv1-09 and RsTaNv2-05 (Supplementary Figure S2a), had no

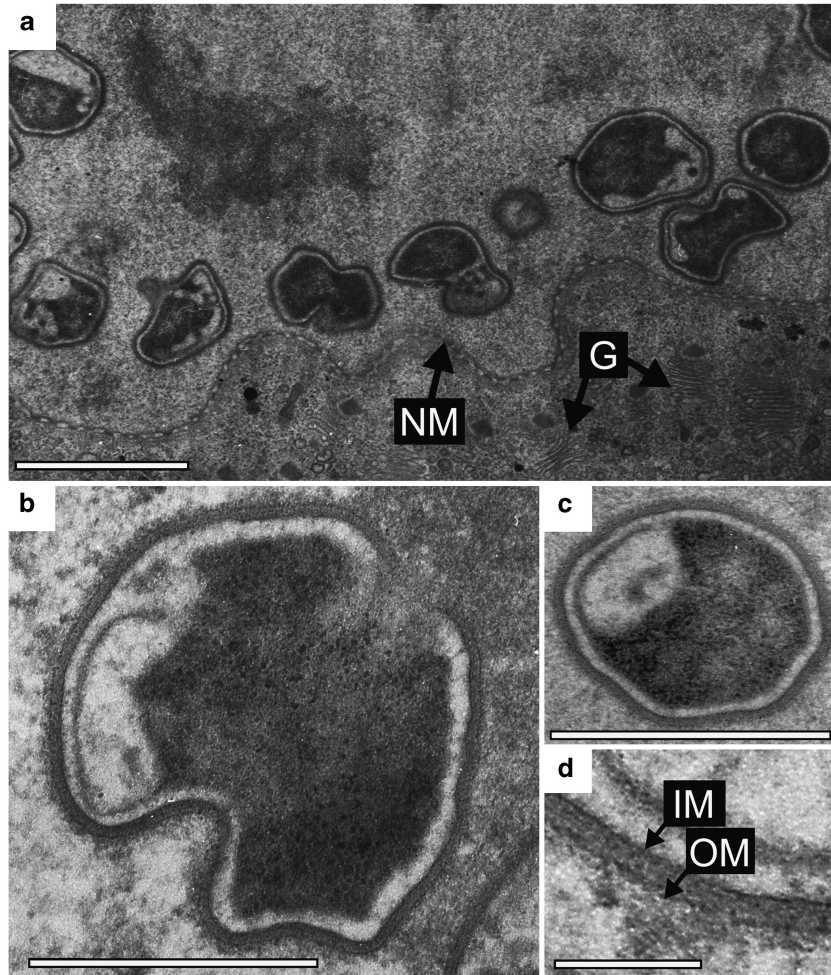


Figure 4 Transmission electron micrographs of intranuclear symbionts of *T. agilis*. (a) Irregular, distorted cocci within a *T. agilis* nucleus. NM, nuclear membrane; G, Golgi apparatus. (b) A coccus during division. (c) A coccus. (d) Magnified image of the cell wall of the intranuclear symbiont. OM, outer membrane; IM, inner membrane. The boundary between the electron-dense and lucent regions appears not to be a membranous structure, although it is unclear. Bars represent 1 μm (a); 0.5 μm (b, c); 0.1 μm (d).

large deletions, whereas they contained numerous base substitutions within conserved regions. Hence, the majority of the numerous pseudogenes in the RsTaN-K1-related cluster likely also derived from a single LGT event. This was supported by the data that the frequency of the pairwise genetic distances between the pseudogene phylogenies related to RsTaN-K1 formed nearly a single peak (Supplementary Figure S2b).

Phylogenetic positions of RsTaN-K1 and RsTaN-K2

Phylotype RsTaN-K1 shared 97% sequence identity with uncultured clones IswTcA-49 and ItbTcA-162 in the database (Figure 2). These were recovered from *Trichonympha* cells in the gut of the kalotermitid termites *Incisitermes schwarzi* and *I. tabogae*, respectively (Strassert *et al.*, 2012). Phylotype RsTaN-K2 shared 96% sequence identity with another uncultured clone, ItbTcA-142, from a *Trichonympha* cell in the gut of *I. tabogae* (Strassert *et al.*, 2012) and formed a monophyletic cluster

together with the other three clones from termite guts (Figure 2). These two clusters further formed a monophyletic cluster that exclusively consisted of uncultured clones from lower termites in the genera *Cryptotermes* and *Reticulitermes* and one clone from a wood-feeding higher termite, *Microcerotermes* sp. (Figure 2). This termite-specific cluster showed only 85% or lower sequence similarity to cultured strains, such as *Cerasicoccus arenae* and *Puniceicoccus vermicola*.

Detection of intranuclear symbionts in other protist species

We performed FISH using probe V-RsD37-130 in combination with EUB338-III or EUB338 to detect the cells of the majority of the termite-specific cluster in Figure 2. Cocci resembling the cells of RsTaN-K1 and RsTaN-K2 were detected within the nuclei of the parabasalid protist *Teranympha mirabilis*, in addition to *T. agilis*, and in nuclei of the oxymonad protists *Pyronympha grandis*, *Dinenympha*

exilis and *Dinenympha parva* (Figure 5). Occasionally, bacteria that were detected with EUB338 but not with EUB338-III or V-RsD37-130 were observed in the nuclei of *P. grandis* (Figures 5a–c). In several *P. grandis* cells, they were co-localized with the verrucomicrobial symbionts within the same nucleus (Supplementary Figures S1g–j). Thus, intranuclear bacterial symbionts other than verrucomicrobia were associated with *P. grandis*.

Based on the present molecular, morphological, and ecological data, we propose a novel genus and species, ‘*Candidatus Nucleococcus trichonymphae*’ and ‘*Candidatus Nucleococcus kirbyi*’ for phylogenies RsTaN-K1 and RsTaN-K2, respectively.

Description of ‘Candidatus Nucleococcus’ gen. nov.

Nucleococcus (nu.cle.o.coc’cus. L. masc. n. *nucleus*, a nucleus; N.L. masc. n. *coccus* from Gr. masc. n.

kokkos a coccus; N.L. masc. n. *nucleococcus* a coccus from a nucleus). The bacteria specifically colonize the nucleoplasm of protists in termite guts. They are distorted cocci of 0.3–1.9 μm and occasionally observed in a division form. They have a Gram-negative type cell wall and are directly in contact with the nucleoplasm. Using uranyl acetate–lead citrate TEM stain, the cytoplasm is sharply separated between electron-dense and electron-lucent regions. The peripheral region of the cytoplasm is consistently translucent. The ‘intracytoplasmic membrane’ that compartmentalizes the cytoplasm of certain verrucomicrobial species (Lee *et al.*, 2009) is not recognized, although further observation is needed to clarify its presence or absence. To date, the members of this genus have not been cultured. The assignment is based on 16S rRNA gene sequence. Currently, this genus comprises two species described below and other

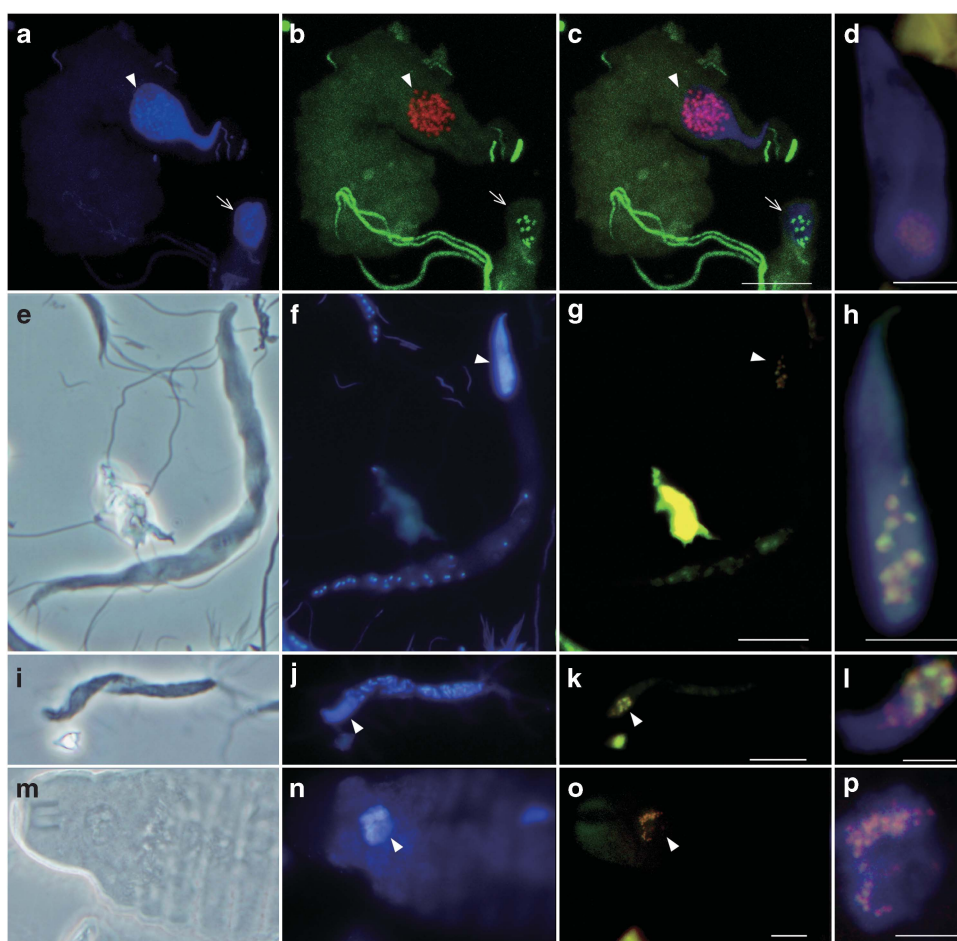


Figure 5 Detection of verrucomicrobial symbionts in nuclei of (a–d) *Pyrrsonympha grandis*, (e–h) *Dinenympha exilis*, (i–l) *Dinenympha parva* and (m–p) *Teranympna mirabilis*. (a, f, j, n) DAPI-stained images. (e, i, m) Phase contrast images. (b) Merged image of FISH analyses using the Texas red-labeled *Verrucomicrobia*-specific probe EUB338-III (red) and the 6FAM-labeled probe EUB338 specific to bacteria except *Verrucomicrobia* and several other taxa (green). (c) Merged image of a and b. (g, k, o) Merged images of FISH analyses using probe EUB338-III (red) and the 6FAM-labeled probe V-RsD37-130 specific to the majority of the termite-specific cluster in Figure 2 (green). (d, h, l, p) Magnified images of the DAPI-stained nuclei merged with FISH signals detected using probes EUB338-III (red) and V-RsD37-130 (green). Panel d was taken from a *P. grandis* cell that was different from the two cells in panels a–c. Arrowheads indicate the nuclei. Arrows in a–c indicate the *Pyrrsonympha* nucleus that contained non-verrucomicrobial intranuclear symbionts. Panels a–c were observed under a laser scanning microscope. Bars represent 10 μm (c, g, k, o); 5 μm (d, h, l, p).

uncharacterized 16S rRNA phylotypes IswTcA-49, ItbTcA-162, ItbTcA-142, Cc3-002, RsC01-040 and RsStar333. These phylotypes share more than 90% sequence identity.

Description of 'Candidatus Nucleococcus trichonymphae' sp. nov.

Nucleococcus trichonymphae (tri.cho.nym'phae. N.L. n. *Trichonympha*, a genus of flagellated protists; N.L. gen. *trichonymphae*, of *Trichonympha*, referring to the host genus). The basic characteristics of this species are the same as those for the genus. They specifically colonize the nucleoplasm of the protist *T. agilis* in the gut of the termite *Reticulitermes speratus*. The cell dimensions are 0.5–1.5 µm by 0.3–1.1 µm. The assignment is based on 16S rRNA gene sequence (AB826704) and hybridization with the 16S rRNA-targeted oligonucleotide probe (5'-CATGCAGAACGAAACCAC-3'). This species corresponds to phylotype RsTaN-K1.

Description of 'Candidatus Nucleococcus kirbyi' sp. nov.

Nucleococcus kirbyi (kir'byi. N.L. gen. n. *Kirbyi*, of Kirby; named after Harold Kirby, a microbiologist who discovered the bacteria of this genus). The basic characteristics of this species are the same as those for the genus. The cell dimension is 0.6–1.9 µm by 0.4–1.0 µm. They specifically colonize the nucleoplasm of the protist *T. agilis* in the gut of the termite *Reticulitermes speratus*. The assignment is based on 16S rRNA gene sequence (AB826705) and hybridization with the 16S rRNA-targeted oligonucleotide probe (5'-CATGCGAAAAGAAATCAC-3'). This species corresponds to phylotype RsTaN-K2.

Discussion

This is the first report of intranuclear symbionts belonging to the phylum *Verrucomicrobia*. Thus far, only members of the phylum *Proteobacteria* have been identified as intranuclear bacterial symbionts of protists and other eukaryotes (Alverca *et al.*, 2002; Fokin, 2004; Arneodo *et al.*, 2008; Zielinski *et al.*, 2009). Based on their morphology, '*Candidatus Nucleococcus*' (abbreviated hereafter as *Nucleococcus*) closely resembled *Caryococcus nucleophagus*, described by Kirby (1944) for the intranuclear symbionts of *Trichonympha corbula*, as 'Spherules with a diameter of 1/2 µm, sometimes arranged in pairs, sometimes with a thicker, crescentic, stainable area at the periphery on one side...' The genus *Caryococcus* was originally described for an intranuclear symbiont of the euglenid protist *Euglena deses* by Dangeard (1902). The assignment of the intranuclear symbionts of *Trichonympha* to the genus *Caryococcus* was not based on evidence and 'allocation to the genus *Caryococcus* is a matter of convenience' (Kirby, 1944). We therefore proposed a

novel genus for the intranuclear bacterial symbionts of *Trichonympha* in this study.

Phylum *Verrucomicrobia* is one of the dominant bacterial groups in soil and members are also detected, less abundantly, from various other environments including animal intestinal tracts (Wagner and Horn, 2006). *Verrucomicrobia* that inhabit a eukaryotic cell are rare; only two examples have been reported. Unique verrucomicrobial defensive symbionts are localized as 'epixenosomes' on the cell surface of the marine ciliates *Euplotidium* (Petroni *et al.*, 2000). '*Candidatus Xiphinematobacter*' are maternally inherited cytoplasmic symbionts of the ectoparasitic nematodes *Xiphinema* found on plants (Vandekerckhove *et al.*, 2002). These are phylogenetically distant from *Nucleococcus*.

In termite guts, verrucomicrobia are minor, but autochthonous residents. Several phylotypes have been found in subdivision 6 and in the orders *Opitutales* and *Puniceicoccales* in subdivision 4 (Hongoh *et al.*, 2003, 2005; Nakajima *et al.*, 2005; Yang *et al.*, 2005; Hongoh *et al.*, 2007a; Ikeda-Ohtsubo *et al.*, 2010; Strassert *et al.*, 2012). These phylotypes comprise one or more termite-specific clusters in each clade, and most members are as yet unculturable. Among them, TAV1–4 in the *Opitutales* are cultured strains isolated from the gut of *Reticulitermes flavipes* (Stevenson *et al.*, 2004), and genomic, transcriptomic, proteomic and physiological analyses of strain TAV2, described as *Diplosphaera colotermitum*, have been reported (Wertz *et al.*, 2012; Isanapong *et al.*, 2013). *D. colotermitum* is a microaerophilic coccus, and the studies suggested their symbiotic roles in fixation and recycling of nitrogen, amino-acid production and, possibly, degradation of cellulose and xylan. Similar physiological properties are found in other verrucomicrobial species (Martinez-Garcia *et al.*, 2012; Wertz *et al.*, 2012). However, '*Candidatus Nucleococcus trichonymphae*' (RsTaN-K1) and '*Candidatus Nucleococcus kirbyi*' (RsTaN-K2) (abbreviated hereafter as *N. trichonymphae* and *N. kirbyi*, respectively), shared only 85% or lower 16S rRNA sequence identity with known isolates, including *D. colotermitum*; thus, prediction of their functions is impossible at present.

The closest phylotypes to the two *Nucleococcus* species are those obtained from *Trichonympha* cells in the gut of *Incisitermes* termites (Strassert *et al.*, 2012) (Figure 2). The sequence similarity is as high as 96–97%; these are possibly also intranuclear symbionts of the *Trichonympha* species. These sequences appear to be authentic and it is not known whether similar LGT events have occurred between these phylotypes and their host *Trichonympha*. Other members of the termite-specific cluster in Figure 2 included uncultured phylotypes recovered from the cells of the protist *Oxymonas* sp. in the gut of *Cryptotermes secundus* (Desai *et al.*, 2010). In the present study, we identified members of this cluster as intranuclear symbionts of both

oxymonad and parabasalid protists (Figure 5). These intranuclear symbionts were detected from some, but not all of the population of each protist species; therefore, this symbiosis appears to be facultative. Thus, *Nucleococcus* might have a life stage outside the host nucleus, as do the alphaproteobacterial intranuclear symbionts of paramecia (Fujishima and Kodama, 2012). Indeed, Dolan *et al.* (2004) observed that cocci, morphologically resembling *Nucleococcus*, were released from a hypertrophied nucleus of *Staurojoenina assimilis*, although we did not observe such hypertrophy or rupture of infected nuclei of *T. agilis* and also did not find *N. trichonymphae* or *N. kirbyi* outside *T. agilis* cells.

The verrucomicrobial intranuclear symbionts appear to be widely distributed in diverse protists in termite guts. *Nucleococcus*-like intranuclear cocci with the characteristic electron-dense and lucent regions (Figure 4) could be observed in many published TEM images in previous studies. The host protists that harbored *Nucleococcus*-like intranuclear symbionts were parabasalids, including *Joenia annecteus*, *Pachyjoenia howa*, *Placojoenia sinaica*, *Spironympha kofoidi*, *Spirotrichonympha minor*, *Stephanonympha neulumbium* and the above-mentioned *Staurojoenina assimilis* (Radek *et al.*, 1992; Radek and Hausmann, 1994; Rösel *et al.*, 1996; Radek, 1997; Dolan *et al.*, 2004; Brugerolle and Bordereau, 2005, 2006). Intranuclear bacterial symbionts with cell morphologies distinct from *Nucleococcus* were also observed on TEM images of parabasalids, such as *Caduceia versatilis*, *Joenia annecteus*, *Metadevescovina* sp. *Microjoenia fallax*, *Spironympha vertisis*, and an oxymonad, *Monocercomonoides termitis* (Radek *et al.*, 1992; Radek, 1994; d'Ambrosio *et al.*, 1999; Brugerolle and Bordereau, 2006; Maaß and Radek, 2006). In *Joenia annecteus*, both *Nucleococcus*-like and a distinct morphotype coexisted in the same nucleus (Radek *et al.*, 1992), as in the oxymonad *Pyrsonympha grandis* in our observation (Supplementary Figure S1g–j).

Our discovery of the 16S rRNA pseudogenes present on the host *Trichonympha* genome strongly suggests that at least a portion of both the *N. trichonymphae* and *N. kirbyi* genomes have, independently, been laterally transferred, although this should be further confirmed by detailed genome analyses of the host *T. agilis*. A lateral transfer of genome fragments has been reported for the intracellular alphaproteobacterial symbionts *Wolbachia* and their invertebrate hosts (Hotopp *et al.*, 2007; Nikoh *et al.*, 2007). Interestingly, these pseudogenes on the host genome had numerous variants that likely derived from a single ancestral pseudogene. This implies that the laterally transferred *Nucleococcus* genome fragments have been multiplied within the host genome and that each copy has undergone independent molecular evolution. The differences in branch length and diversity between the pseudogenes of the two *Nucleococcus* species

possibly indicate the difference in the age of the LGT events (Supplementary Figure S2). Although it is not known whether there are verrucomicrobia-derived genes still functional in the host genome, it is conceivable that these intranuclear symbionts can be sources of laterally transferred genes to the host genome more easily than cytoplasmic symbionts or ingested microbes. In the pea aphid *Acyrtosiphon pisum*, it has been hypothesized that genes laterally transferred from *Wolbachia* to the host genome might be involved in the symbiosis with the primary intracellular symbiont *Buchnera* (Nikoh and Nakabachi, 2009). Considering the widespread infection of the verrucomicrobial intranuclear symbionts, they might have a non-negligible impact on the evolution of the termite gut protists and also on the intracellular symbiosis with other bacteria, through the lateral transfer of genome fragments and their multiplication within the host genome.

Conflict of Interest

The authors declare no conflict of interest.

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