Host-Symbiont Cospeciation of Termite-Gut Cellulolytic Protists of the Genera *Teranympha* and *Eucomonympha* and their *Treponema* Endosymbionts

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Cellulolytic flagellated protists inhabit the hindgut of termites. They are unique and essential to termites and related wood-feeding cockroaches, enabling host feeding on cellulose matter. Protists of two genera in the family Teranymphidae (phylum Parabasalia), *Eucomonympha* and *Teranympha*, are phylogenetically closely related and harbor intracellular endosymbiotic bacteria from the genus *Treponema*. In order to obtain a clearer understanding of the evolutionary history of this triplex symbiotic relationship, the molecular phylogenies of the three symbiotic partners, the Teranymphidae protists, their *Treponema* endosymbionts, and their host termites, were inferred and compared. Strong congruence was observed in the tree topologies of all interacting partners, implying their cospeciating relationships. In contrast, the coevolutionary relationships between the *Eucomonympha* protists and their endosymbionts was more complex, and evidence of incongruence against cospeciating relationships suggested frequent host switches of the endosymbionts, possibly because multiple *Eucomonympha* species are present in the same gut community. Similarities in the 16S rRNA and *gyrB* gene sequences of the endosymbionts were higher among *Teranympha* spp. (>99.25% and >97.2%, respectively), whereas those between *Teranympha* and *Eucomonympha* were lower (<97.1% and <91.9%, respectively). In addition, the endosymbionts of *Teranympha* spp. formed a phylogenetic clade distinct from those of *Eucomonympha* spp. Therefore, the endosymbiont species of *Teranympha* spp., designated here as “*Candidatus* Treponema teratomyphae”, needs to be classified as a species distinct from the endosymbiont species of *Eucomonympha* spp.

Key words: cospeciation, Teranymphidae protist, endosymbiotic bacteria, *Treponema*, termite

Numerous insects have established intimate mutualistic relationships with microorganisms, which play key roles in host adaptation to various environments (16, 40). This symbiotic relationship with microorganisms has a profound impact on the adaptation of termites to their xylophagous, wood-feeding lifestyle, and also contributes to the expansion of the termite niche in terrestrial ecosystems. Cellulolytic flagellated protists in the termite hindgut are unique to termites and related wood-feeding cockroaches, and are essential for host feeding on cellulose matter (2, 31).

The family Rhinotermitidae includes the global pest genera of termites, such as *Coptotermes*, *Reticulitermes*, and *Heterotermes* (20). In this termite family, cellulolytic protists from the genus *Pseudotrichonympha* (phylum Parabasalia) are widely distributed as gut symbionts (17). The cells of *Pseudotrichonympha* protists harbor endosymbiotic bacteria of a unique phylogenetic lineage within the order Bacteroidales (22). The members of this triplex symbiotic system appear to have cospeciated during their evolution (25), possibly because of the crucial roles played by symbionts in termite nutrition (13, 33). Various examples of species-specific symbiotic relationships between protists and bacteria have been identified in the termite gut, and include ectosymbioses on the protist cell surface (29); many ectosymbionts are Bacteroidales and Spirochaetes members (14, 21, 23). As expected for endosymbioses, most, if not all, have cospeciated (15, 25, 39, 41), with some exceptions (10); ectosymbiotic relationships appear to be less strictly cospeciated than endosymbiosis (26).

The composition of the gut protist fauna is specific to the host species, and is suggested to reflect the host’s phylogeny (18). However, in the family Rhinotermitidae, termites from the genus *Reticulitermes* typically lack *Pseudotrichonympha* protists. The gut protist composition in this genus is unique within the Rhinotermitidae, and is similar to that of the phylogenetically distant genus *Hodotermpsis* from the family Archotermopsidae, an early branching group of termites (1). These two termite genera are often distributed sympatrically, and the Japanese species *Reticulitermes speratus* or *R. amamianus*, and *Hodotermpsis sjostedti* frequently coexist in the same fallen log (18). A previous study reported that the protist fauna of a *Hodotermpsis* termite may have been laterally transferred to *Reticulitermes* termites (18), *Hodotermpsis* and *Reticulitermes* termites are both characterized by the rich species diversity of gut protists, and commonly harbor members of the family Teranymphidae (phylum Parabasalia) as large-size cellulolytic protists. Two genera of Teranymphidae, *Eucomonympha* and *Teranympha* (5), are phylogenetically closely related and inhabit *H. sjostedti* and *Reticulitermes* termites, respectively (27).

*Eucomonympha* and *Teranympha* protists both harbor bacterial endosymbionts (32). In *H. sjostedti*, a rod-shaped endosymbiiont of *Eucomonympha* sp. was shown to be a spirochete species from the genus *Treponema* and was
designated as “*Candidatus Treponema intracellularis*” (32). Recent biochemical and single-cell genome analyses revealed that this endosymbiotic species plays crucial roles in termite nutrition, e.g., reductive acetogenesis from H₂ plus CO₂ and nitrogen fixation (32). The former provides acetate as a major energy source for termites, while the latter provides nitrogen sources, which are poor in the termite diet (3). These dual functions of the endosymbiont as well as the cellulosytic ability of the protist may enhance the adaptability of the host termite to xylophagy, and are also responsible for the stability of the protist in the gut. In the gut of *R. speratus*, the endosymbiotic species of *Teranympha mirabilis* was shown to be closely related to the endosymbiont of *Eucomomyphna* sp. (32). Nevertheless, the distribution and phylogenetic relationships of the endosymbiotic *Treponema* among *Teranymphidae* protists have not yet been investigated.

In the present study, we identified the endosymbionts of *Teranymphidae* protists in *Reticulitermes* and *H. sjostedti* termites. The molecular phylogenies of the three symbiotic partners, *Teranymphidae* protists, their host termites, and their *Treponema* endosymbionts, were inferred and compared in order to obtain a clearer understanding of the evolutionary history of this triplex symbiotic relationship.

**Materials and Methods**

**Data collection**

Protist samples investigated in the present study and their host termites are listed in Table 1. All termite samples were collected in Japan (Fig. S1) and maintained in plastic boxes before use. Living termites were used as the material in the phylogenetic analyses of insects, protists, and endosymbiotic bacteria.

Termite DNA was extracted from the head and legs, and used as a template for PCR, as described previously (25). Mitochondrial COI, COII, and 16S rRNA genes were amplified, and used directly for DNA sequencing, as described previously (25). *Teranymphidae* protists were consistently found in the hindgut flora of the respective termites and were easily recognizable based on their morphological characteristics (6). The protist cells in the hindgut suspension of each termite were isolated manually and washed extensively under a microscope equipped with a micromanipulator (Cell Tram; Eppendorf, Hamburg, Germany), as described elsewhere (25). Each cell showing a typical morphology was isolated and was used as a template for isothermal whole-genome amplification (WGA), as previously described (27); amplified genomic DNA was used as a template for PCR.

Regarding the expressed small subunit ribosomal RNA (SSU rRNA), 20 cells were directly used as a template for first-strand cDNA synthesis with the primer PZR1 (25, 27) and the resulting cDNA was used for PCR amplification.

The protist gene encoding SSU rRNA, and bacterial 16S rRNA and gyrB genes were amplified using the WGA sample prepared from the same isolated cell as a template. The three genes were amplified by PCR, using the high fidelity DNA polymerase KOD Plus Neo (Toyobo, Osaka, Japan), with the previously described primers (25). The amplified products were separated by agarose gel electrophoresis, purified, and then cloned using the Zero Blunt pCR4-TOPO PCR cloning kit for sequencing (Invitrogen, Carlsbad, CA, USA) and Competent Quick DH5α (Toyobo). Clones containing inserts of the expected size were picked and partially sequenced, and the complete DNA sequence of each representative clone was obtained. In each sample, 3–9 clones were analyzed for the *Teranympha* SSU rRNA gene, while 1–4 clones were analyzed for the *Eucomomyphna* SSU rRNA gene. Most of the clone sequences of *Eucomomyphna* spp. were almost identical to either one of the three phyotype sequences identified in the whole gut community of *H. sjostedti* (unpublished data).

Eight clones were typically analyzed for the bacterial 16S rRNA and gyrB genes in each WGA sample; the partial DNA sequences of clones were sorted into phylotypes at >98.5% (16S rRNA gene) and >99.4% (gyrB gene) sequence similarities. The 16S rRNA gene sequences that were distantly related to those of endosymbiotic *Treponema* were excluded from further analyses. In the case of gyrB, the phylotype sequences sharing high sequence similarities with the *Treponema* sequences were phylogenetically analyzed (Fig. S2), and only the sequences identified as the endosymbiotic *Treponema* species were concatenated for the phylogenetic analysis. The sequences obtained in this study have been deposited in the DNA Databank of Japan and the accession numbers are shown in Tables S1 and S2.

**Phylogenetic analyses**

The DNA sequences obtained in the present study and publicly available sequences were aligned using ClustalW2 (19) and alignments were refined manually. Ambiguously aligned positions were omitted from the subsequent phylogenetic inference analysis. An appropriate model of sequence evolution was selected using the program JModelTest 2.4.8 (26). In the tree from each dataset, a maximum likelihood (ML) analysis was conducted in RAxML 8.2.38 using the GTR+GAMMA1 model. Bootstrap values were obtained from 10,000 replicates for the SSU rRNA gene. A Bayesian analysis was performed in MrBayes 3.2.1 (34) using the GTR+I+Γ model. The starting tree was random, and four simultaneous Markov chains in duplicate were run for 10,000,000 generations. Log likelihoods stabilized well before 2,500,000 generations, and the remaining generations were used to measure Bayesian posterior probabilities.

The alignments of bacterial 16S rRNA and gyrB gene sequences were concatenated manually, and the ML tree was estimated in RAxML using the GTR+GAMMA1 model. Parameters and branch lengths were individually optimized for each partition, and bootstrap values were obtained from 10,000 replicates. The GTR+I+Γ model was employed in the Bayesian analysis, and parameters and branch lengths were individually optimized for each partition. The starting tree was random, and four simultaneous Markov chains in duplicate were run for 10,000,000 generations. As described above, log likelihoods stabilized well before 2,500,000 generations, and the remaining generations were used to measure Bayesian posterior probabilities.

Three mitochondrial genes (mt16S rRNA, COI, and COII) were used in host termite phylogeny analyses, as described previously (25). The ML analysis was performed in RAxML using the GTR+GAMMA1 model. Parameters and branch lengths were individually optimized for each partition, and bootstrap values were obtained from 10,000 replicates. The GTR+I+Γ model was employed in the Bayesian analysis, and parameters and branch lengths were individually optimized for each partition. The starting tree was random, and four simultaneous Markov chains in duplicate were run for 10,000,000 generations. As described above, log likelihoods stabilized well before 2,500,000 generations, and the remaining generations were used to measure Bayesian posterior probabilities.

Cophylogenetic analyses

Cophylogenetic analyses were conducted using five host *Reticulitermes* spp. and 12 samples of the protists and their endosymbionts. Tree topologies reconstructed using the ML method for each phylogenetic analysis were used in the analysis of the congruence of host and symbiont phylogenies. The Jane 4 program with 100 generations and a population size of 100 was used (7). The analysis also tested the significance of topological congruence between the hosts and symbionts. Cophylogeny mapping in Jane 4 employed heuristics to find solutions that minimized the overall cost of a historical reconstruction. The default event costs were as follows: 0 for a codivergence event, 1 for duplication and host switch events, and 2 for loss events. TreeMap (Charleston, M. TREEMAP 3.0 beta. Available at http://sites.google.com/site/cophylogeny) was used to construct the tanglegram for hosts and symbionts.

**Fluorescence in situ hybridization (FISH)**

FISH was performed to detect endosymbionts, as described previously (21, 24, 35). Briefly, termite gut contents were fixed in 4%
paraformaldehyde; fixed cells were then spotted onto a silane-coated glass slide (Matsumani Glass, Osaka, Japan). After dehydration in ethanol, the slides were incubated with the hybridization solution (0.9 M NaCl and 0.1 M Tris-HCl) containing fluorescently-labeled probes at 48°C for 2 h. Specimens were washed for 20 min in a washing buffer (0.2 M NaCl and 0.1 M Tris-HCl) at 48°C. They were then mounted using a Fluoro-Keeper antifade reagent (Nacalai Tesque, Kyoto, Japan) and observed under an Olympus epifluorescence microscope; BX-63 (Olympus, Tokyo, Japan). Probes for the intracellular Treponema species and general eubacterial probes (EUB338) were described previously (32). The 5’-termini of the probes were labeled with 6-carboxyfluorescein (6-FAM) or Texas red.

Results

Host termites

Five Reticulitermes termites that harbored Teranympha protists in their guts were analyzed (Table 1). The distribution of these termites and sampling points are shown in Fig. S1. Reticulitermes termites have distinct geographical distributions in Japan, and H. sjostedti distributes sympatrically with R. speratus and R. amamianus. The phylogenetic relationship between these host termites was inferred from the concatenated data of the three mitochondrial genes of 16S rRNA, and cytochrome oxidase subunits I and II. In the ML tree (Fig. 1A), each sequence of the five termite species was closely related to that of the corresponding species in the database, and most of the sequences obtained from two independent single protist cells from each termite host typically shared high sequence similarity (>98.0%). However, two sequences sharing 88.0% sequence similarity in R. amamianus (Ra2Tera1bSSU3 and Ra2Tera1bSSU6) were obtained from one single-cell sample of Teranympha; two sequences (Ra2Tera1cSSU2 and Ra2Tera1cSSU1) sharing 90.0% similarity were obtained from the other sample. Among these four, Ra2Tera1bSSU3 and Ra2Tera1bSSU2 shared more than 98% sequence similarity. We examined the transcribed rRNA sequences by cloning the RT-PCR products from a pool of Teranympha cells in this termite; the sequences of fifteen clones were almost identical to one another, and to Teranympha and Eucomonympha protists

The molecular phylogeny of the gut symbiotic protists of the genera Teranympha and Eucomonympha was inferred based on their SSU rRNA gene sequences (Fig. 1B). Each Reticulitermes termite harbored a morphologically unique Teranympha species, and most of the sequences obtained from two independent single proist cells from each termite host typically shared high sequence similarity (>98.0%). However, two sequences sharing 88.0% sequence similarity in R. amamianus (Ra2Tera1bSSU3 and Ra2Tera1bSSU6) were obtained from one single-cell sample of Teranympha; two sequences (Ra2Tera1cSSU2 and Ra2Tera1cSSU1) sharing 90.0% similarity were obtained from the other sample. Among these four, Ra2Tera1bSSU3 and Ra2Tera1bSSU2 shared more than 98% sequence similarity. We examined the transcribed rRNA sequences by cloning the RT-PCR products from a pool of Teranympha cells in this termite; the sequences of fifteen clones were almost identical to one another, and to

Ra2Tera1bSSU3 and Ra2Tera1cSSU2 (>98%). Teranympha sp. in R. amamianus appear to have harbored at least two copies of the SSU rRNA gene in the genome, only one of which was functional. A highly similar sequence was obtained from the two single-cell samples of protists in R. kannonensis and R. yaeyamanus (two samples per termite); however, the distantly related sequences, RkTera1xSSU3 and RyTera1bSSU4, respectively, were also obtained and appeared to be the same as that in R. amamianus. Consequently, we analyzed the phylogenetic relationships of the protists with sequences of the presumed functional genes only, and the tree topology obtained was used for subsequent comparisons (Fig. S4 and Table S2). Based on these data, Teranympha species in R. okinawanus and R. amamianus were grouped together, and this group was a sister to the group of Teranympha species in R. kannonensis and R. yaeyamanus. T. mirabilis from R. speratus formed a clade together with the other Teranympha species, but branched out most basally.

In the case of the Eucomonympha protists, the sequences from seven single-cell samples were clustered into three sequence groups (A, B, and C in Fig. 1B), although group C was weakly supported. The clones in each group showed high sequence similarities (more than 99%), except for those in group C (94.3%). These sequences were detected in our analyses of hundreds of clones of the SSU rRNA gene amplified by RT-PCR from the whole gut community of H. sjostedti as abundant clones (unpublished data), suggesting that the three sequence groups identified herein are functional and represent major populations of Eucomonympha spp. in the gut of H. sjostedti. These sequences of Eucomonympha protists formed a sister group to Teranympha species from the five Reticulitermes termites. The currently described Eucomonympha lineages are paraphyletic (30). As reported previously (4, 30), Eucomonympha imla in the wood-feeding cockroach Cryptocercus punctulatus is distantly related to Eucomonympha spp. in H. sjostedti, and the genus of the latter species needs to be reclassified.

Endosymbiotic bacteria

The 16S rRNA gene sequences of the endosymbiotic bacteria were obtained from each single-cell sample of the Teranympha and Eucomonympha species. Only a single phylotype affiliated with the genus Treponema was detected in each sample. Most of the sequences shared more than 99.5% identity, except for one sample from which a sequence sharing 98.5% identity with others (RkTera1B) was obtained. The representative sequences derived from the same termite species shared high sequence similarities (99.6–100%), while the sequences from other termite species shared lower sequence identities than those from conspecies. As previously reported.

Table 1. Host termite species and single-cell samples of protist species used in gene identification and phylogenetic analyses.

| Termite          | Sampling location | Protist              | Sample name         |
|------------------|-------------------|----------------------|---------------------|
| R. speratus      | Kofu, Yamanashi   | Teranympha mirabilis | RsTera1a, RsTera1b  |
| R. amamianus     | Tokunoshima, Kagoshima | Teranympha sp. | Ra2Tera1b, Ra2Tera1c |
| R. kannonensis   | Shimonoseki, Yamaguchi | Teranympha sp. | RkTera1B, RkTera1x  |
| R. yaeyamanus    | Iriomote, Okinawa | Teranympha sp. | RyTera1A, RyTera1B  |
| R. okinawanus    | Kunigami, Okinawa | Eucomonympha sp. A | Hs1EA-a, Hs2EA-a, Hs2EA-b |
| H. sjostedti     | Yakuishima, Kagoshima | Eucomonympha sp. B | Hs1EB-c, Hs3EB-a   |
|                  |                   | Eucomonympha sp. C | Hs2EC-c, Hs3EC-c   |
Cospeciation of Termites and Symbionts

formed by the endosymbionts of a monophyletic clade, which was clearly separated from the clade II (14, 32). The endosymbionts of lineages of termite-gut protists in the termite protists; for (B), two R. flavipes termites (47). The outgroup taxa in the analyses were: for (A), three Eucomonympha spp. and for (C), three Teranympha species (47). The scale bars correspond to 0.01, 0.05, and 0.10 substitutions per site for (A), (B), and (C), respectively. Numbers at nodes indicate maximum likelihood bootstrap support as percentages and Bayesian posterior probability values, respectively. Values less than 50% or 0.5 are indicated with hyphens.

We also obtained sequences of the gyrB gene of the endosymbionts. Since the 16S rRNA gene sequences derived from two single-cell samples of Teranympha spp. shared high similarity, only one single-cell sample for each host Teranympha termite species was analyzed. Although many of the sequences obtained were closely related to the genomic sequence of the Treponema endosymbionts of Eucomonympha spp., several distantly related sequences were also obtained; some of these were affiliated with the genus Treponema and others were related to those encoded by bacteria from the family Opitutaceae (phylum Verrucomicrobia) or the genus Ureaplasma (phylum Tenericutes), both of which are the intracellular bacteria of Eukaryotes. However, except for the Treponema-like sequences, frame-shift insertions/deletions or stop codons were detected in these sequences. Since 16S rRNA pseudogenes have been reported in the intranuclear Verrucomicrobia symbionts of their host protist’s genome, and the related intranuclear bacteria are widely distributed in termite-gut protists (36), these gyrB pseudogenes appear to be derived from intranuclear symbionts. As in the case of phylogenetic analyses based on the 16S rRNA gene, the Treponema endosymbionts formed a monophyletic cluster, and two clades comprising Teranympha and Eucomonympha endosymbionts were detected (Fig. S2).

In both phylogenetic trees, the branching orders within each clade of the Teranympha and Eucomonympha endosymbionts were poorly resolved because of their high sequence similarities (Table 2). In the case of the endosymbionts of Eucomonympha spp., their phylogenetic relationships did not appear to correlate with the host protist’s phylogeny; the endosymbiont sequences from the same Eucomonympha species were not always grouped together. In order to overcome the poor phylogenetic resolution of the single marker gene analysis, the concatenated sequences of 16S rRNA and gyrB genes were used in the phylogenetic analysis (Fig. 1C). Although the supporting values at each node were not significant, the Teranympha endosymbionts formed a monophyletic clade that was a sister of, but clearly separated from the Eucomonympha endosymbionts.

Table 2. Nucleotide sequence similarities of 16S rDNA and gyrB genes of the endosymbiotic Treponema of the intra-genus or inter-genus of Teranymphidae protists

| Protist          | 16S rDNA (%) | gyrB (%) |
|------------------|--------------|----------|
| Teranympha       | 99.25–99.73  | 97.23–99.35 |
| Eucomonympha     | 98.64–99.86  | 92.02–99.92 |
| Teranympha spp.  | 96.46–97.07  | 90.23–91.86 |

The host and symbiont phylogenies reconstructed by the ML method were used to assess their phylogenetic congruence (Fig. 2). The tanglegram and relationships among the host Reticulitermes termites and Teranympha protists revealed a significant coevolutionary congruence, although the tree topologies did not perfectly match (Fig. 2A). The evolutionary event that was incongruent in the two trees occurred at a weakly supported node. We then used Jane 4 (7) to generate

![Fig. 1. Phylogenetic trees of Reticulitermes termites harboring Teranympha protists in the hindgut (A), Teranympha and Eucomonympha protists (B), and endosymbiotic Treponema bacteria of Teranympha and Eucomonympha protists (C).](image-url)

(A) The host and symbiont phylogenies reconstructed by the ML method were used to assess their phylogenetic congruence (Fig. 2). The tanglegram and relationships among the host Reticulitermes termites and Teranympha protists revealed a significant coevolutionary congruence, although the tree topologies did not perfectly match (Fig. 2A). The evolutionary event that was incongruent in the two trees occurred at a weakly supported node. We then used Jane 4 (7) to generate
coevolutionary events with the lowest cost sets (Fig. 2B). A significant global cost ($P=0.02$) was observed, which was 3 for cospeciation; 0 for duplication; 1 for host-switch; 1 for loss; and 0 for failure to diverge between the host termites and Teranympha protists (Table 3).

Reconciling the phylogenies of host protists and their endosymbionts indicated a significant topological congruence ($P=0.04$), with seven codivergence events out of a possible 11, four host switches, and three losses (Table 3). Similar to the relationship between the host termites and their gut protists, the tree topologies of the Teranympha protists and their endosymbionts were largely congruent with three codivergence events out of a possible four (Fig. 2C and D), and incongruence was observed at a weakly supported node.

In contrast, the coevolutionary relationship between the Eucomonympha protists and their endosymbionts was complex, with three host switch events (Fig. 2C and D). These incongruence events occurred at strongly supported nodes of the endosymbiont tree. The endosymbiont of the host protist group C (Hs2EC-c) was closely related to the endosymbionts of the two host protists in group A (Hs1EA-a and Hs2EA-b), and the endosymbionts of the host protists in groups B and C (Hs3EB-a and Hs3EC-c, respectively) were also closely related (see also Fig. 1C).

**Table 3.** Number of event types required for the reconciliation of host and symbiont trees

| Host and symbiont       | Total cost | Cospeciation | Duplication | Host switch | Losses/sorting | Failure to diverge | $P$-value |
|-------------------------|------------|--------------|-------------|-------------|----------------|--------------------|-----------|
| Termite and Teranympha  | 3          | 3            | 0           | 1           | 1              | 0                  | 0.02*     |
| Protist and endosymbion | 11         | 7            | 0           | 4           | 3              | 0                  | 0.04*     |

$P$-values were computed from 999 random reconstructions. Asterisks indicate a 5% level of significance. The event costs used for the analyses were as follows: 0 for cospeciation; 1 for duplication; 2 for host switching; 1 for sorting; and 1 for failure to diverge.

In situ identification of Teranympha endosymbionts and their morphology

A previously used oligonucleotide probe (IIC-637) targeting the 16S rRNA of the Treponema endosymbionts of Eucomonympha spp. was used to detect the endosymbionts of Teranympha spp. by FISH because, as shown above, the targeted sequence was conserved (32). This probe annealed to the rod-shaped endosymbiotic bacteria of Teranympha protists in the gut of all Reticulitermes termites examined (Fig. 3A, D, G, J, M, and Q). The same rod-shaped endosymbionts were detected during simultaneous hybridization of a universal bacterium probe (Fig. 3B, E, H, K, N, and R). Although the universal probe also detected ectosymbiotic bacteria, e.g., spirochetes attached at the posterior end of Teranympha cells (Fig. 3R), both probes simultaneously detected all bacteria in the protist cytoplasm. Most of the Teranympha cells harbored endosymbiotic bacteria that were homogeneously and densely packed in the cytoplasm, except for the anterior end of the cell, the rostrum (Fig. 3A, D, G, J, M, and Q). The endosymbionts of *T. mirabilis* in the gut of *R. speratus* (Fig. 3M, N, O, P, Q, R, S, T, and U) appeared to be more densely packed than Teranympha spp. in other termites. The Teranympha protists in *R. yaeyamanus* appeared to be colonized to a lesser
Discussion

Cophylogenetic analyses of gut protists from the family Teranymphidae, and their endosymbionts in five Reticulitermes termites and H. sjostedti revealed their possible cospeciating evolutionary relationships, particularly between the triplex symbiotic partners of Teranympha protists, their endosymbionts, and their host termites. Incongruence in evidence against a completely cospeciating relationship was observed, but occurred at nodes showing poor resolution, i.e., for the relationship between R. yaeyamanus and R. kamonensis, or between their symbionts. Therefore, we consider cospeciation to be the most plausible evolutionary scenario, albeit with the possibility of deviations.

A similar cospeciating relationship between the triplex symbiotic partners was reported for Pseudotrichonympha protists, their Bacteroidales endosymbionts, and their host termites from the family Rhinotermitidae (except for the genus Reticulitermes) (25). As previously discussed, the strict vertical transmission of gut symbionts is likely warranted by the proctodeal feeding behavior that characterizes the sociality of termites (31).

The termite gut fauna is discarded at every molting, but is acquired from nestmates; it is also carried over to a newly founded colony by the primary termite reproductives (31). Since endosymbionts are abundant in the protist cell, the cell is stably transmitted during protist cell division. The crucial roles played by the protists and their endosymbionts in their host termites, i.e., cellulose decomposition by the former and nutrient supply by the latter, appear to have had a significant impact on the maintenance of these symbiotic partners and their mutual adaptation.

In contrast to Pseudotrichonympha, which are widely distributed among termites from the family Rhinotermitidae, the Teranympha protists have only been found in Reticulitermes termites and are exclusively limited to the Asian species in this genus; however, the composition of other protist species in all Reticulitermes termites appears to be similar (17). In the present study, we confirmed this close phylogenetic relationship, which suggests a possible common ancestor of Teranympha spp. and Eucomonympha spp. in the H. sjostedti termite. This observation also supports the previously proposed horizontal transfer of the gut fauna between Reticulitermes and Hodotermitopsis termites (18). If this is the case, the absence of Teranympha likely represents a secondary loss after fauna transfer, with other members of the gut fauna possibly complementing the symbiotic roles of Teranympha and its endosymbionts. Furthermore, the Treponema endosymbionts of Teranympha spp. and Eucomonympha spp. form a closely related sister group. The results of our analyses imply that an ancestor of both protist groups acquired the endosymbiont before fauna transfer and that, after this transfer, the protists and their endosymbionts were vertically inherited together over a long evolutionary time period and cospeciated according to the species differentiation of the host termites.

In Teranympha and Pseudotrichonympha cases, only a single protist species appeared to be present in the gut microbial community of a single host termite species. This may affect the cospeciating evolutionary process because the spatial separation of host protist lineages creates the potential for accepting endosymbionts; hence, the opportunity for a host switch does not exist. In contrast, Eucomonympha spp. are sympatric and present simultaneously within the gut community of a single host termite. Although cospeciation appears to be a general rule for the Teranympha and Eucomonympha...
protists and their endosymbionts, as shown by the overall significance in the cophylogeny analysis (Table 3), the phylogenies of Eucomonympha spp. and their endosymbionts are not always congruent. For example, the endosymbiont phylotype Hs2EC-c from group-C protists was more closely related to those from group-A protists than to Hs3EC-c. Furthermore, the endosymbiont phylotypes Hs3EB-a and Hs3EC-c from protist groups-B and -C, respectively, were very closely related, and the endosymbiont phylotype Hs2EA-a from group-A protists was distantly related to the other endosymbionts from group-A protists. These examples of incongruence in evidence against cospeciation suggest more frequent host switches of endosymbionts in Eucomonympha spp. than in Teranympha spp., possibly because multiple protist species are present in the same gut community. A similar host switch-like relationship was reported for Trichonympha sp. and its endosymbiont “Ca. Endomicrobium trichonymphae” in the gut of H. sjostedti, with multiple Trichonympha species being present in the gut of this termite (15). However, this host switch-like relationship is the only exception among the strict cospeciating relationships of Trichonympha protists and Endomicrobium endosymbionts. The genomes of the endosymbionts of Trichonympha and Pseudotrichonympha are small in size, likely as a consequence of reductive evolution (12, 13). These endosymbiont species with reduced genomes may be more dependent on the host protists than the endosymbionts of Eucomonympha, and, thus, their opportunity for a host switch may be reduced. In contrast, the genome size of the endosymbiont of Eucomonympha was not as severely reduced, although its gene content, as evaluated by a cluster of orthologous group analysis, was similar to the genomes of the Trichonympha and Pseudotrichonympha endosymbionts; for example, the relative abundance of cell motility genes is universally lower, and that of the translation and coenzyme metabolism genes is universally higher in the genomes of the endosymbionts of termite-gut protists than in cultured treponemes (32). The acquisition of the endosymbiont by Eucomonympha protists appears to be a recent evolutionary event, and the endosymbiont species is at an initial developmental stage of adaptive evolution. The endosymbiont of Eucomonympha may be more independent of the host protists than the other two, and may be able to switch the host lineages more frequently.

In the present study, we confirmed that Treponema endosymbionts are widely and commonly distributed among the Teranympha and Eucomonympha protists examined to date. As discussed above and previously (32), Treponema endosymbionts are monophyletic and appear to have originated from an eucaryotic lineage of termite-gut protists. Nevertheless, the phylogenetic analyses revealed that the endosymbionts of Teranympha spp. clearly form a distinct clade from those of Eucomonympha spp. The sequence similarity of the 16S rRNA gene of the endosymbionts of Teranympha species (intra-genus) was greater than 99.25%, whereas the sequence similarity of the endosymbionts between Eucomonympha and Teranympha species (inter-genera) was less than 97.1% (Table 2). It is generally accepted that bacteria sharing less than 98.7% 16S rRNA gene sequence similarity need to be classified into distinct species because this sequence similarity corresponds to a DNA reassociation value less than 70%, i.e., a threshold of bacterial species delineation (37). The shared sequence similarity of the gyeB gene of the endosymbionts was also high among Eucomonympha spp., but low between Teranympha and Eucomonympha protists (Table 2). Based on these findings, we propose classifying the endosymbiont species of Teranympha into a species distinct from ‘Ca. T. intracellularis’, the endosymbiont of Eucomonympha spp. (32). Therefore, we propose a novel species “Candidatus Treponema teranymphae” for Teranympha endosymbionts. Although we used the popular original name of the genus “Teranympha” in the present study, its erroneous orthography was pointed out earlier (Teratonympha is the proper name) (11), and we adopted the proper form for the nomenclature of the Teronema endosymbiont species.

Description of “Candidatus Treponema teranymphae”

Ter.a.to.nym’phae. N.L. gen. n. teranymapha, of Teranympha, referring to the generic name of the host protist. Cells are rods, 1.64–2.39 μm×0.32–0.51 μm in size (average 2.09±0.29 μm×0.41±0.07 μm). They lack flagella and are surrounded by two membranes; the outer membrane is presumably the host-derived membrane. The bacterium is specifically found in the cytoplasm of the parabasalian protist Teranympha (or its amended Teratonympha spp.), in the hindgut of Reticulitermes spp. termites. Thus far uncultured, but forms a monophyletic group based on sequence analyses of the 16S rRNA and gyrB genes (DDBJ accession numbers: LC276704–LC276733).

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References

1. Bourguignon, T., N. Lo, S.L. Cameron, J. Šobotník, Y. Hayashi, S. Shigenobu, D. Watanabe, Y. Roisin, T. Miura, and T.A. Evans. 2015. The evolutionary history of termites as inferred from 66 mitochondrial genomes. Mol. Biol. Evol. 32:406–421.
2. Brune, A., and M. Ohkuma. 2011. Role of the termite gut microbiota in symbiotic digestion, p 439–475. In D.E. Bignell, Y. Roisin, N. Lo (ed.), Biology of Termites: A Modern Synthesis. Springer, Dordrecht.
3. Brune, A. 2014. Symbiotic digestion of lignocellulose in termite guts. Nat. Rev. Microbiol. 12:168–180.
4. Carpenter, K.J., and P.J. Keeling. 2007. Morphology and phylogenetic position of Eucomonympha imla (Parabasalia: Hypermastigida). J. Eukaryotic Microbiol. 54:325–332.
5. Cavalieri-Smith, T. 2013. Early evolution of eukaryote feeding modes, cell structural diversity, and classification of the protozoan phyla Loudzoza, Sulcozoa, and Choanozoa. Eur. J. Protistol. 49:115–178.
6. Cleveland, L.R. 1938. Morphology and mitosis of Teranympha. Arch. Protistenk. 91:441–451.
7. Conow, C., D. Fielder, Y. Ovadia, and R. Libeskind-Hadas. 2010. Jane: a new tool for the cophylogeny reconstruction problem. Algorithms Mol. Biol. 5:16.
8. Darriba, D., G.L. Taboada, R. Doallo, and D. Posada. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9:772–772.
9. Dedene, F., S. Dupont, S. Guyot, K. Matsuura, C.Wang, B. Habibpour, A-G. Bagñères, B. Mantovani, and A. Lucchetti. 2016. Historical biogeography of Reticulitermes termites (Isoptera: Rhinotermitidae) inferred from analyses of mitochondrial and nuclear loci. Mol. Phylogenet. Evol. 94:778–790.

10. Desai, M.S., J.F.H. Strassert, K. Meuser, H. Hertel, W. Ikeda-Ohtsubo, R. Radek, and A. Brune. 2010. Strict cospeciation of devescovinid flagellates and Bacteroidales ectosymbionts in the gut of dry-wood termites (Kalotermitidae). Environ. Microbiol. 12:2120–2132.

11. Dobell, O. 1939. On “Teranympha” and other monstrous Latin parasites. Parasitology 31:255–262.

12. Hongoh, Y., Y.K. Sharma, T. Prakash, S. Noda, T.D. Taylor, T. Kudo, Y. Sakaki, A. Toyoda, M. Hattori, and M. Ohkuma. 2008. Complete genome of the uncultured Termite Group 1 bacteria in a single host protist cell. Proc. Natl. Acad. Sci. U.S.A. 105:5555–5560.

13. Hongoh, Y., V.K. Sharma, T. Prakash, et al. 2008. Genome of an endosymbiotic coupling N2 fixation to cellulolysis within protist cells in termite gut. Science 322:1108–1109.

14. Iida, T., M. Ohkuma, K. Ohtoko, and T. Kudo. 2000. Symbiotic srichoetes in the termite hindgut: phylogenetic identification of ectosymbiotic srichoetes of oxymonad protists. FEMS Microbiol. Ecol. 34:17–26.

15. Ikeda-Ohtsubo, W., and A. Brune. 2009. Cospeciation of termite gut flagellates and their bacterial endosymbionts: Trichonympha species and Candidatus Endomicrobiurn trichonymphi. Mol. Ecol. 18:332–342.

16. Kinjo, Y., S. Saitoh, and G. Tokuda. 2015. An efficient strategy developed for next-generation sequencing of endosymbiont genomes performed using crude DNA isolated from host tissues: a case study of Blattabacterium cuenoti inhabiting the fat bodies of cockroaches. Microbes Environ. 30:208–220.

17. Kitade, O., and T. Matsumoto. 1998. Characteristics of the symbiotic flagellate composition within the termite family Rhinotermiteidae (Isoptera). Symbiosis 25:271–278.

18. Kitade, O. 2004. Comparison of symbiotic flagellate faunae between termites and a wood-feeding cockroach of the genus Cryptocer us. Environ. Microbiol. 16:345–352.

20. Lo, N., O. Kitade, T. Miura, R. Constantino, and T. Matsumoto. 2004. Molecular phylogeny of the Rhinotermitidae. Insectes Soc. 51:365–371.

21. Noda, S., M. Hattori, M. Ohkuma, A.C. Darby, and Y. Hongoh. 2017. Discovery and complete genome sequence of a bacteriophage from an obligate intracellular symbiont of a cellulolytic protist in the termite gut. Microbes Environ. 32:112–117.

22. Noda, S., T. Iida, O. Kitade, H. Nakajima, T. Kudo, and M. Ohkuma. 2005. Endosymbiotic Bacteroidales bacteria of the flagellated protist Pseudotrichonympha gravis in the gut of the termite Coptotermes formosanus. Appl. Environ. Microbiol. 71:8811–8817.

23. Noda, S., T. Inoue, Y. Hongoh, M. Kawai, C.A. Nalepa, C. Vongkhaluang, T. Kudo, and M. Ohkuma. 2006. Identification and characterization of endosymbiotic flagellates of distinct lineages in Bacteroidales attached to flagellated protists in the gut of termites and a wood-feeding cockroach. Environ. Microbiol. 8:11–20.

24. 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, Oxy monas sp. Microbes Environ. 21:16–22.

25. Noda, S., O. Kitade, T. Inoue, et al. 2007. Cospeciation in the triple symbiosis of termite gut protists (Pseudotrichonympha sp.), their hosts, and their bacterial endosymbionts. Mol. Ecol. 16:1257–1266.

26. Noda, S., Y. Hongoh, T. Sato, and M. Ohkuma. 2009. Complex coevolutionary history of symbiotic Bacteroidales bacteria of various protists in the gut of termites. BMC Evol. Biol. 9:158.

27. Noda, S., C. Mantini, D. Meloni, J. Inoue, O. Kitade, E. Viscogliosi, and M. Ohkuma. 2012. Molecular phylogeny and evolution of parasalasia with improved taxon sampling and new protein markers of actin and elongation factor-1a. PLoS One 7:e29938.

28. Ohkuma, M., H. Yuzawa, W. Amornsak, et al. 2004. Molecular phylogeny of Asian termites (Isoptera) of the families Termitidae and Rhinotermitidae based on mitochondrial COII sequences. Mol. Phylogenet. Evol. 31:701–710.

29. Ohkuma, M. 2008. Symbioses of flagellates and prokaryotes in the gut of lower termites. Trends Microbiol. 16:345–352.

30. Ohkuma, M., S. Noda, Y. Hongoh, C.A. Nalepa, and T. Inoue. 2009. Inheritance and diversification of symbiotic trichonymphid flagellates from a common ancestor of termites and the cockroach Cryptocer us. Proc. R. Soc. B 276:239–245.

31. Ohkuma, M., and A. Brune. 2011. Diversity, structure, and evolution of the termite gut microbial community, p. 413–438. In D.E. Bignell, Y. Rosin, N. Lo (ed.), Biology of Termites: A Modern Synthesis. Springer, Dordrecht.

32. Pramono, A.K., M. Ohkuma, A.C. Darby, and Y. Hongoh. 2015. Population structure of endomicrobia in single host cells of termite gut. Microbiol. Environ. 30:321–329.

33. Pramono, A.K., H. Kuwahara, T. Itoh, A. Toyoda, A. Yamada, and Y. Hongoh. 2017. Discovery and complete genome sequence of a bacteriophage from an obligate intracellular symbiont of a cellulolytic protist in the termite gut. Microbes Environ. 32:112–117.

34. Ronquist, F., M. Teslenko, P. van der Mark, D.L. Ayres, A. Darling, S. Holma, B. Larget, L. Liu, M.A. Suchard, and J.P. Huelsenbeck. 2012. MrBayes 3.2: Efficient bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61:539–542.

35. Sato, T., Y. Hongoh, S. Noda, S. Hattori, S. Ui, and M. Ohkuma. 2009. Candidatus Desulfovibrio trichonymphi, a novel intracellular symbiont of the flagellate Trichonympha agilis in termite gut environment. Microbiol. 11:1007–1015.

36. Sato, T., H. Kuwahara, K. Fujita, S. Noda, K. Kihara, A. Yamada, M. Ohkuma, and Y. Hongoh. 2014. Intranuclear verrucomicrobial symbionts and evidence of lateral gene transfer to the host protist in the termite gut. ISME J. 8:1008–1019.

37. Stackebrandt, E., and J. Ebers. 2006. Taxonomic parameters revisited: tanshined gold standards. Microbiol. Today 33:152–155.

38. Shimada, S., G. Zellers, S. Matsuura, and T. Hori. 2014. RAXML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:3122–3132.

39. Strassert, J.F.H., T. Köhler, T.H.G. Wienemann, W. Ikeda-Ohtsubo, N. Faivre, S. Franckenberg, R. Pfarrer, R. Radek, and A. Brune. 2012. ‘Candidatus Ancillula trichonymphi’, a novel lineage of endosymbiotic Actinobacteria in termite gut flagellates of the genus Trichonympha. Microbiol. Environ. 14:3259–3270.

40. Takeshita, K., Y. Matsuura, H. Itoh, R. Novaro, T. Hori, T. Sone, Y. Kamagata, P. Mergaert, and Y. Kikuchi. 2015. Burkholderia of plant-beneficial group are symbiotically associated with bordered plant bugs (Heteroptera: Pyrrhocoridae: Largidae). Microbes Environ. 30:321–329.

41. Zheng, H., C. Dietrich, C.L. Thompson, K. Meuser, and A. Brune. 2015. Population structure of endomicrobia in single host cells of termite gut flagellates (Trichonympha spp.). Microbes Environ. 30:92–98.