Endomicrobia in termite guts: symbionts within a symbiont

Phylogeny, cospeciation with host flagellates, and preliminary genome analysis

Dissertation

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Marburg, Oktober 2007

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1. Introduction

Termites: Ecology and taxonomy

Termites are ecologically-important terrestrial arthropods, which decompose lignocellulosic plant materials such as wood, grass, or plant litter at various stages of humification. They frequently occur in many tropical habitats and are also widely distributed in temperate zones with less ecologically importance. On the other hand, several species of wood-feeding termites are targets of the commercial pest control, since they have become major pests of households and wood industries in many countries. The costs for the recovery from termite damages and the continuing termite control may reach more than 1 billion dollar per year in the United States (Su and Scheffrahn, 1998).

On the other hand, such powerful and efficient cellulose degradation by termites have been an intriguing research target with various aspects, such as synergetic degradation activities with gut microorganisms and substrate specificities of a variety of cellulolytic enzymes (Tokuda and Watanabe, 2007). The catalytic ability of termites has a potential to be used for decomposition and fermentation of fibrous organic wastes in industries. In recent years, termites are even becoming prospective biofuel-producers that are able to decompose crops into usable ethanol (Schubert, 2006).

Termites constitute a distinct clade of insects classified into the Isoptera, one of major groups in the superorder Dictyoptera. The phylogeny of termites has been investigated using phylogenetic markers including the mitochondrial cytochrome oxidase II (COII) gene and rRNA genes (Eggleton, 2001). Accordingly, termites are classified into six families of lower termites and one higher termite family (Fig. 1). It should be noted that Inward *et al*. recently claimed that termites shall be affiliated with a subclass of Blattodea (cockroaches) (Inward et al., 2007). According to their phylogenetic analyses using multiple gene markers (mitochondrial *12S* and *cox II* genes and nuclear *28S*, *18S*, and *histone 3* genes), Isoptera (termites) should be reclassified as a family within the Blattodea clade and the extant termite families should be downgraded to subfamilies. Nevertheless, this new classification has not yet been widely acknowledged and still controversial; hence the traditional classification, which regards Isoptera as an order, is employed throughout this thesis.

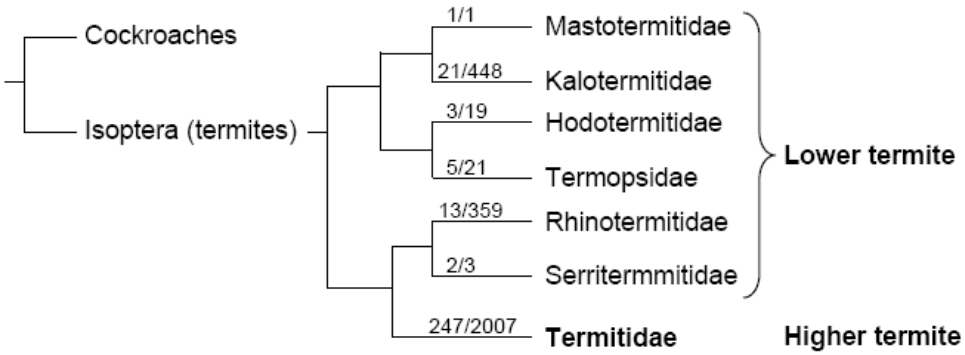

Figure 1. Schematic outline of phylogenetic tree of different termite families and closely related cockroaches (modified from Higashi and Abe, 1996). The numbers on the branches represent the number of genera/species of the respective termite families. The most current statistics were obtained from the Online Termite Database (http://www.unb.br/ib/zoo/docente/constant/catal/catnew.html).

Termite gut microhabitats

The intestinal tract of lower termites is compartmentalized into three sections: the foregut, the midgut, and the hindgut. The hindgut paunch is a major site of microbial metabolic activities and nutrient absorption. Physicochemical measurement using microsensensors has shown the existence of the steep gradients of oxygen partial pressures across the hindgut of *Reticulitermes flavipes* (Brune et al., 1995). Whereas the gut wall and epithelium remains microoxic by a continuous influx of oxygen from outside, anaerobic area is still found at the central lumen, where the low redox potential (–230 to –270 mV, Veivers et al., 1980) may be maintained by bacterial symbionts that consume oxygen (Veivers et al., 1982). Since the oxygen gradient can significantly influence the metabolic processes in the hindgut of *R. flavipes* (Tholen and Brune, 2000), it can be assumed it has a major impact on the composition of microbial community structure. H_2 is another major environmental parameter in the hindgut. Microelectrodes measurement revealed the presence of dynamics of H_2 partial pressure across the gut of *R. flavipes* (Ebert and Brune, 1997). The steep gradient of H_2 is attributed to the large number of H_2-producing (e.g., parabasalid flagellates) and H_2-consuming (e.g., homoacetogens) populations in the hindgut. Such various physicochemical conditions and the spatial organization of gut microorganisms adapting to the habitat are crucial factors to elucidate the comprehensive metabolic network in the hindgut of lower termites.

Gut microorganisms and their functions

Lignocellulose-degrading activities of lower termites largely depend on the metabolic capacity of their gut microorganisms. Various unique types of flagellate protozoa, which densely pack in the enlarged hindgut, are thought to be responsible for the degradation cascade starting from wood polysaccharides to the end products: CO_2, H_2, and acetate, the latter of which is resorbed by the host termites as major nutrients (Hungate, 1943; Yamin, 1980; see next chapter for detail). Prokaryotes also occur in a high density and diversity in the termite gut, free-swimming in the gut fluid or colonizing the gut wall, or more frequently, associating the gut flagellates (Brune, 2006). They are not believed to play a major role in cellulose degradation, but rather to participate in the fermentation of soluble metabolites such as short-chain fatty acids released in the gut fluids from other metabolic activities.

One of the most important metabolic activities conducted by prokaryotes in the hindgut of lower termites is reductive acetogenesis. H_2/CO_2-reductive acetogenesis dominates the H_2 sink in the termites and accounts for one of every three acetate molecules present in the hindgut paunch, contributing significantly to nutrition of the host termite (Odelson and Breznak, 1983). Analyses using the functional marker gene coding for formyl tetrahydrofolate synthase (FTHFS) in three termite species has revealed that homoacetogenic spirochetes predominantly contribute to the H_2-dependent reductive acetogenesis (Pester and Brune, 2006). The presence of methanogenesis, another important anaerobic metabolic activity in the termite gut, is also clear from the evidence of microbial activities (Odelson and Breznak, 1983) and retrievability of isolates from methanogenic species of *Methanobrevibacter* (Leadbetter and Breznak, 1996; Leadbetter et al, 1998). Interestingly enough, homoacetogenesis generally exceeds methanogenesis as the terminal H_2-oxidizing reaction, in most wood-feeding termites (Brauman et al., 1992).

With wood diets low in nitrogen, termites seem to rely on the capacity of nitrogen fixation by their prokaryotic symbionts for their nitrogen nutrition requirement. Whereas several bacterial strains with nitrogen-fixing ability have been isolated from termite guts, which are mainly represented by enterobacteria (e.g., French et al., 1976) and spirochetes (Leadbetter et al., 1999; Graber et al., 2004), no conclusive evidence has revealed which members of gut microbiota are primarily involved in the nitrogen fixation. Culture independent studies revealed a number of various *nifH* genes, which encode nitrogenase reductase, are present in the gut of *R. speratus* (Ohkuma et al., 1996; 1999). Diagnostic RT-PCR has detected alternative nitrogenase (*anf*) genes and anaerobe group or pseudo-*nifH* genes are primarily expressed in the lower termites *Neotermes koshunensis* and

Coptotermes formosanus, respectively (Noda et al., 1999; 2002). Importantly, a subsequent study by Lilburn *et al*. documented the presence of nitrogen fixation activity and *nifH* genes in some termite gut spirochetes, indicating they are potentially active nitrogen fixers in the termite gut (Lilburn et al., 2001).

These previous studies have shown that culture-independent molecular techniques are powerful tools to address possible nature and roles of microorganisms in termite gut community, most of which appear to defy axenic cultivation using standard cultivation methods.

Affiliation of termite gut flagellates

Termite gut flagellates are very unique and diverse. A great series of microscopic works on their distinctive morphology and other characteristic properties were conducted in the early 20th century by some prominent biologists, represented by L. R. Cleveland and H. Kirby. More than 430 species and subspecies have been described, which are possessed by approximately 200 termite species (Yamin, 1979), and an additional number of species have been added by molecular-based studies (e.g., Ohkuma et al., 2000).

Collective evidence indicates that the flagellates are involved in the cellulose fermentation in the gut microbiota. Some important attempts to isolate termite gut flagellates were made by Yamin, who established a culture medium that supports the continuous growth of *Trichomitopsis termopsidis* from *Zootermopsis* sp. and demonstrated the production of acetate, CO_2 and H_2 using radiolabeled cellulose as a substrate (1979; 1980). These works were followed up by Odelson and Breznak, who improved the growth of the strains and confirmed the cellulolytic activity in the culture and cell extracts (Odelson and Breznak, 1985a; 1985b) Molecular studies corroborated the results of cultivation studies regarding cellulolytic activities of termite gut flagellates. Diagnostic RT-PCR using cellulase-specific primers have obtained cDNA fragments from the flagellates encoding cellulases from glycoside hydrolase family 7 (GHF7) (Nakashima et al., 2002; Watanabe et al., 2002). Additionally, a large number of cellulases including GHF5 and GHF45 have been identified in gut flagellates of *R. speratus* and *Mastotermes darwiniensis*, indicating that gut flagellates are the major cellulose degraders in the termite gut (Ohtoko et al., 2000; Li et al., 2003; Todaka et al., 2007).

Classification of termite gut flagellates have been extensively systematized by a large number of molecular ecological studies in recent years using various phylogenetic markers, including genes encoding SSU rRNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), α-tubulin, β-tubulin, enolase, and elongation factor 1α (EF-1α) (e.g., Moriya et al., 2001; Gerbod et al., 2004). Currently,

termite gut flagellate has been classified into two distinct taxa: the Parabasalia and Oxymonadea (or commonly parabasalid and oxymonad). These two taxa both are thought to represent the Excavata, one of the six eukaryotic supergroups that comprise a wide array of flagellates, which was recently established by Adl *et al*. (2005). However, phylogenetic affiliation of Excavata is particularly controversial, since the verification of monophyly of this taxon has been confounded by the low number of available phylogenetic samples and by incongruity between phylogenetic analyses using different phylogenetic markers (Simpson et al., 2006).

Parabasalids are a group of anaerobic flagellates that are characterized by the presence of hydrogenosomes instead of mitochondria, a parabasal body. They are comprised of two historically separated two classes: the Trichomonadea and the Hypermastigea (commonly trichomonad and hypermastigid) according to their morphological characteristics, i.e. the number of flagella and parabasal bodies as well as the complexity of cytoskeleton structure (Brugerolle and Lee, 2000). However, recent studies by Ohkuma *et al*. have shown that the hypermastigid order is highly diverse and polyphyletic across the phylum (Ohkuma et al., 2005; 2007). Therefore, the group hypermastigid is no longer be regarded as a valid taxon in phylogenetic classification. In a currently established classification, the phylum Parabasalia contains four lower ranked groups: Trichomonadida, Cristamonadida, Spirotrichonymphida, and Trichonymphida (Adl et al., 2005).

Table 1. Current classification of flagellates present in the gut of lower termites (Affiliation is based on Adl et al., 2005).

First rank*	Second rank	Examples (genus)
Parabasalids	Cristamonadida	*Calonympha*, *Devescovina*, *Foaina*, *Joenia*, *Metacoronympha*
	Spirotrichonymphida	*Holomastigotes*, *Microjoenia*, *Spirotrichonympha*
	Trichomonadida	*Monocercomonas*, *Trichomitopsis*, *Trichomonas*
	Trichonymphida	*Eucomonympha*, *Hoplonympha*, *Staurojoenina*, *Trichonympha*
Preaxostyla	Oxymonadida	*Dinenympha*, *Oxymonas*, *Pyrsonympha*, *Streblomastix*

* These ranks are placed under the eukaryotic super-group Excavata.

Oxymonads are a small group of amitochondriate flagellates often distinguished by the presence of a large axostyle. A few studies documented phylogenetic positions of oxymonad species. Two contemporaneous studies clarified distinct phylogenetic positions of *Pyrsonympha*, *Dinenympha*, and

Oxymonas, using whole-cell hybridization using individually designed phylotype-specific probes (Moriya et al., 2003; Stingl et al., 2003). These termite gut-specific oxymonads formed a deep-branching cluster in the Oxymonadida tree, with an exception of *Streblomastix*, which forms the most basal branching group together with the genus *Monocercomonoides* (Hampl et al., 2005). In any cases, comprehensive investigation is still required for constructing more persistent and reliable phylogenetic trees of termite gut flagellates.

Flagellate-prokaryote symbioses in the gut microbiota

A large body of microscopic evidence has shown that most termite gut flagellates are associated with prokaryotic cells, which colonize on the cell surface, in the cytoplasm, or sometimes in the nucleus of the host flagellates. In the gut of *M. darwiniensis*, it has been reported that as many as 85% of prokaryotes is associated with flagellates (Berchtold et al., 1999). This suggests the gut microbiota is composed of a number of complex symbioses between flagellates and prokaryotes. Methanogens represented by *Methanobrevibacter* species are one of the most frequent prokaryotic symbionts attached to flagellates (Leadbetter and Breznak, 1996). Detection of the methanogenic symbionts has been conducted by visualization of auto-fluorescence from their indigenous coenzyme F_{420}, which have shown that methanogens occur as ecto- and endosymbionts of smaller gut flagellates (Lee et al., 1987; Tokura et al., 2000). Many spirochetes affiliated with *Treponema* also form a loose or tight attachment to the surface of many gut flagellates (Noda et al., 2003; Wenzel et al., 2003). A prominent example of this association is the locomotory spirochetes on the surface of *Mixotricha paradoxa* in the gut of *M. darwiniensis*, where the symbionts propel the host cell by coordinated undulation (Cleveland and Grimstone, 1964). Special attachment structures on the cell surface have been observed in many flagellates, confirming their intimate association with their ectosymbionts (e.g., Tamm, 1980; Radek et al., 1992). Several phylogenetic studies using manually isolated flagellate cells demonstrated that specific types of *Bacteroidales* ecto- and endosymbionts are associated with different flagellate species (Stingl et al., 2004; Noda et al., 2005; 2006a; 2006b). Despite the frequent occurrence of such association between termite gut flagellates and prokaryotic symbionts, the functional significance of these symbioses is still largely unknown.

Termite Group 1 (TG-1) and "Endomicrobia"

Termite Group 1 (TG-1) was first described as a new-branching lineage of bacteria found in the gut community of *R. speratus* (Ohkuma and Kudo, 1996). This lineage was subsequently defined as a candidate bacterial phylum that contains several sequences from environmental clones (Hugenholtz, 1998; Fig. 2). In following studies, TG-1 bacteria, along with *Spirochaetes*, *Bacteroidetes* and *Firmicutes*, represented a numerically dominant group in the 16S rRNA gene libraries from the gut of *Reticulitermes* termites (Hongoh et al., 2003; Yang et al., 2005). In the latter study, a larger number of TG-1 clones were obtained particularly in the flagellate (protozoa) fraction in *R. santonensis*, which led to a hypothesis that TG-1 bacteria occur as symbionts of the gut flagellates (Yang et al., 2005).

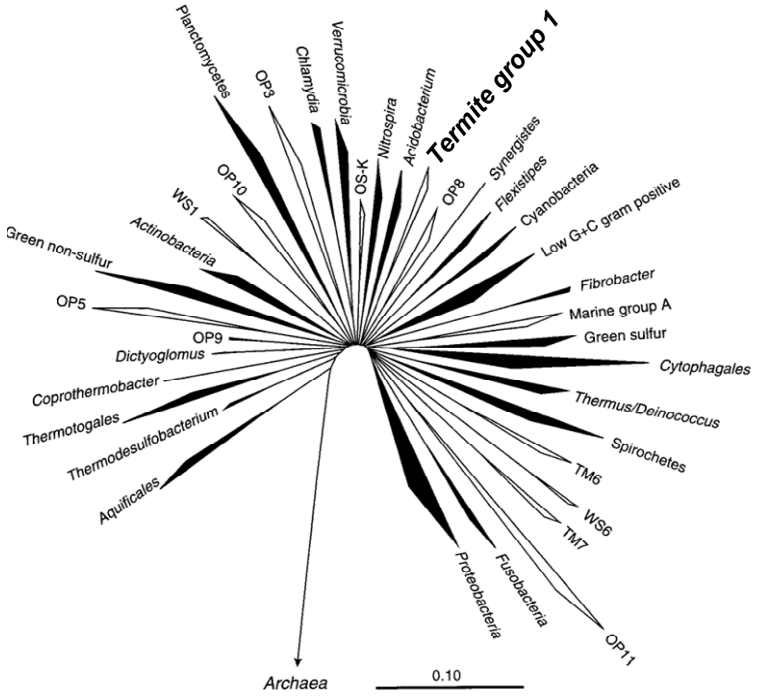

Figure 2. Termite group 1 (TG-1) as a candidate phylum of hitherto uncultivated bacteria (shown in outline) in the evolutionary distance tree of the bacterial domain (adapted from Hugenholtz et al., 1998)

Using the full-cycle rRNA approach, Stingl *et al*. recently documented that TG-1 clones retrieved from two distinct species of gut flagellates in *R. santonensis*, *Trichonympha agilis* and *Pyrsonympha vertens*, represent intracellular symbionts of respective gut flagellates (Stingl et al., 2005). In the same study, they demonstrated the presence of polyphyletic TG-1 species among different termite families and in the wood-feeding cockroach *Cryptocercus* by diagnostic PCR. Phylogenetic

analyses revealed that TG-1 bacteria are phylogenetically diverse (more than 1.5% sequence divergence) but collectively placed into a monophyletic cluster. The name "Endomicrobia" has been suggested for this lineage representing TG-1 bacteria occurring as intracellular symbionts of gut flagellates in lower termites and the *Cryptocercus*. The systematic position of this lineage is probably the class, but not yet determined.

In the meantime, separate studies have obtained an increasing number of TG-1 clones from various environments, including the cow rumen, rice field soil, contaminated groundwater, and other different natural habitats. The diversity of environment TG-1 clones was recently investigated using specifically designed primers based on the 16S rRNA gene (Herlemann et al., in press). Sequences obtained in this study formed several deep-branching lineages within the TG-1 phylum, clustering together with environmental clones previously obtained in other studies. This evidence suggests that the TG-1 phylum represents a group of bacteria with various characteristics, which are widely distributed in terrestrial environments worldwide. In current phylogenetic analyses, "Endomicrobia" still forms a distinct cluster within the TG-1 phylum (Fig. 3).

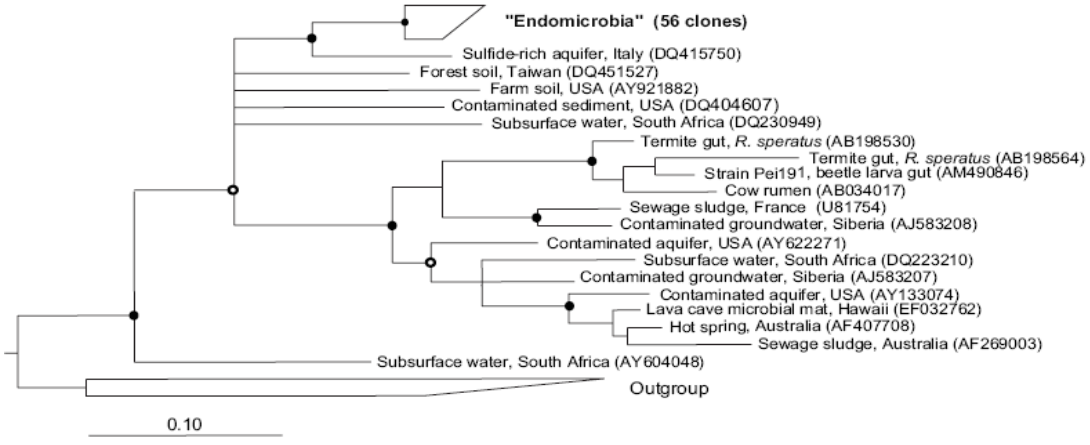

Figure 3. Phylogenetic tree of the phylum TG-1 consisting of "Endomicrobia" and several representative clones from different environmental samples (adapted from Herlemann et al., 2007).

The aims of the study

The aim of the study is to investigate the symbiotic properties of "Endomicrobia". With currently available information, we are only able to describe this group as a group of TG-1 bacteria consisting of clones obtained from the gut of termites and a wood-feeding cockroach. Although a previous study has documented that their representatives are present as specific intracellular symbionts of two flagellates in *R. santonensis* (Stingl et al. 2005), the available dataset is still too small to illustrate this whole group in terms of symbiosis with the termite gut flagellates. In this study, the following three aspects were investigated.

1. Diversity, distribution, and occurrence as intracellular symbionts among termite gut flagellates

"Endomicrobia" are highly diverse and exhibit multiple phylotypes even in the gut of a single termite species (Stingl et al., 2005). Where does this diversity come from? It may be possible that their phylogenetic diversity reflects their habitat variation, i.e., their host variation, when "Endomicrobia" are specific endosymbionts of the termite gut flagellates. Importantly, the phylogeny of "Endomicrobia" does not seem to be correlated to the phylogenetic affiliation of the termite hosts (Stingl et al., 2005). Therefore, this study investigated whether there is a correlation between the phylogeny of "Endomicrobia" and gut flagellates. It has also not been known what range of termite gut flagellates harbor "Endomicrobia" as endosymbionts, and whether they invariably occur in a host-specific manner. The present work is also aimed to document these features.

2. Cospeciation with the host flagellates

Evolutionary history of the symbiosis between "Endomicrobia" and termite gut flagellates has not been investigated before. If a symbiotic pair has established intimate association in its long evolutionary history, a concordant diversification (cospeciation) is expected to have occured between the two lineages (Hafner and Page, 1995). Such phenomenon has been documented in an enormous variety of symbiotic pairs of distinct organisms, such as animal and parasites, fungi and grasses, and insects and their obligate bacterial symbionts (Wade, 2007). In this study, the level of concordance across the phylogeny of "Endomicrobia" symbionts and flagellate hosts was investigated to validate the cospeciation between this symbiotic pair.

3. Physiological properties involved in the symbiosis

A symbiotic relationship can be established for different reasons, such as spatial limitation or defense-purpose, but it is most frequently attributed to functional dependencies of the symbiotic pair. Many obligate endosymbionts, particularly

those of insects, have been found to significantly contribute to the host nutrition requirements. For example, *Buchnera*, the intracellular symbionts of aphids, are able to produce essential amino acids and supply them to their own hosts (Douglas, 1998). At the same time, the symbionts are largely dependent on their host for their carbon and energy source, which details are not yet clarified. If "Endomicrobia" are obligate endosymbionts of termite gut flagellates, what kind of profitable element can they provide to the host? And importantly, do they also benefit from the host? So far, no functional evidence has been obtained from any endosymbionts originating from termite gut flagellates. Elucidation of such interspecies interaction poses highly challenging task, since no stable cultures of "Endomicrobia" or flagellates are currently available. In this study, the enrichment of "Endomicrobia" from termite gut contents was established to obtain high-molecular-weight DNA for a genome sequencing project. As other uncultivated obligate symbionts studied previously, the genome sequencing has been able to provide a large amount of important information for understanding their physiological properties (Wernegreen, 2002). In this study, a preliminary analysis of a genome fragment originating from the enrichment of "Endomicrobia" is documented.

The term “symbiosis” is used here in its broader definition, associations between or interactions between different organisms (“Zusammenlebens ungleichnamiger Organismen”), as it was coined and introduced by Anton de Bary (1878).

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2. Phylogenetic diversity of "Endomicrobia" and their specific affiliation with termite gut flagellates

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Abstract

"Endomicrobia", a distinct and diverse group of uncultivated bacteria in the candidate phylum Termite Group I (TG-1), have been found exclusively in the gut of lower termites and wood-feeding cockroaches. In a previous study, we had demonstrated that the "Endomicrobia" clones retrieved from Reticulitermes santonensis represent intracellular symbionts of the two major gut flagellates of this termite. Here, we document that "Endomicrobia" are present also in many other gut flagellates of lower termites. Phylogeny and host specificity of "Endomicrobia" were investigated by cloning and sequencing of the small subunit ribosomal RNA genes of the host flagellate and the symbionts, which originated from suspensions of individual flagellates isolated by micropipette. Each flagellate harbored a distinct phylogenetic lineage of "Endomicrobia". The results of fluorescent in situ hybridization with "Endomicrobia"-specific oligonucleotide probes corroborated that "Endomicrobia" are intracellular symbionts specifically affiliated with their flagellate host. Interestingly, the "Endomicrobia" sequences obtained from flagellates belonging to the genus *Trichonympha* formed a monophyletic group, suggesting cospeciation between symbiont and host.

Introduction

"Endomicrobia" are a distinct and diverse group of uncultivated bacteria in the candidate phylum Termite Group I (TG-1) (Hugenholtz et al., 1998; Stingl et al., 2005). Originally discovered as members of the hindgut community of Reticulitermes speratus (Ohkuma and Kudo, 1996; Hongoh et al., 2003), their occurrence seems to be restricted to the guts of phylogenetically lower termites and wood-feeding cockroaches (*Cryptocercus punctulatus*) (Hongoh et al., 2005; Stingl et al., 2005; Yang et al., 2005). Recently, we demonstrated that the "Endomicrobia" clones retrieved from *R. santonensis* represent intracellular symbionts of flagellate protists (as previously proposed by M. Ohkuma, N. S. Noda, T. Iida, and T. Kudo;

Abstract P.15.006, 9th International Symposium on Microbial Ecology, Amsterdam, 2001), and documented that the two major gut flagellates of this termite, *Trichonympha* agilis and *Pyrsonympha* vertens, each harbor a phylogenetically distinct lineage of "Endomicrobia" (Stingl et al., 2005).

Termite gut flagellates are a unique group of protists consisting of more than 430 species, which have been described mostly on a morphological basis (Brugerolle and Lee, 2000; Yamin, 1979). Phylogenetic studies using small-subunit (SSU) rRNA and other molecular markers confirmed the presence of two distinct phylogenetic lineages, i.e., Oxymonadida and Parabasalidea (Dacks et al., 2001; Gerbod et al., 2002; Stingl and Brune, 2003; Ohkuma et al., 2005). Although only little is known about the metabolic functions of termite gut flagellates (Brune and Stingl, 2005) — the majority of which are uncultivated — they are generally considered to play a major role in the cellulose metabolism of the hindgut (Yamin, 1980; Odelson and Breznak, 1985).

Most termite gut flagellates are associated with prokaryotic symbionts, which colonize the cell surface, the cytoplasm, or sometimes the nucleus of their hosts (Kirby, 1941; Berchtold et al., 1999; Brune and Stingl, 2005; Brune, 2006). The high frequency of such associations and an apparent specificity of the symbionts for their host flagellate (Noda et al., 2005, 2006; Stingl et al., 2004) are indicative of a functional significance of such symbioses for the termite gut ecosystem.

The symbiosis between "Endomicrobia" and termite gut flagellates might also represent such an intimate relationship, which has been supported by evidence that some "Endomicrobia" form host-specific associations with their host flagellate (Stingl et al., 2005). Furthermore, the wide distribution and phylogenetic heterogeneity of "Endomicrobia" among lower termites harboring various gut flagellates (Stingl et al., 2005) collectively suggests a strong connection between the phylogenetic diversity of the symbiont and their flagellate hosts. We hypothesize here that the phylogenetic diversity of "Endomicrobia" in the gut of lower termites reflects the diversity of their flagellate hosts. To prove this hypothesis, we phylogenetically analyzed SSU rRNA genes of the major flagellates and their symbionts in the termite *Hodotermopsis sjoestedti* and in selected flagellates of five other termite species.

Results

Host affiliation of "Endomicrobia" in *H. sjoestedti*

SSU rRNA genes were amplified from the whole gut DNA extract of the termite *H. sjoestedti* using "Endomicrobia"-specific primers. More than 10 distinct monophyletic lineages of "Endomicrobia" were identified by phylogenetic analysis

of the sequences in the clone library (Fig. 2). To test whether individual phylotypes of "Endomicrobia" can be assigned to their host flagellates, suspensions were prepared by carefully picking individual flagellate cells according to their characteristic morphotypes. The major populations among the flagellate community were formed by species of the genus *Dinenympha* (Oxymonadida; Fig. 1a), *Trichonympha*, and *Eucomonympha* (both Parabasalidea; Figs. 1b and 1c). DNA extracted from the respective suspensions yielded PCR products of the expected length with eukaryotic (1500–1800 bp), bacterial (~1500 bp), and "Endomicrobia"-specific (~1100 bp) SSU rRNA primers.

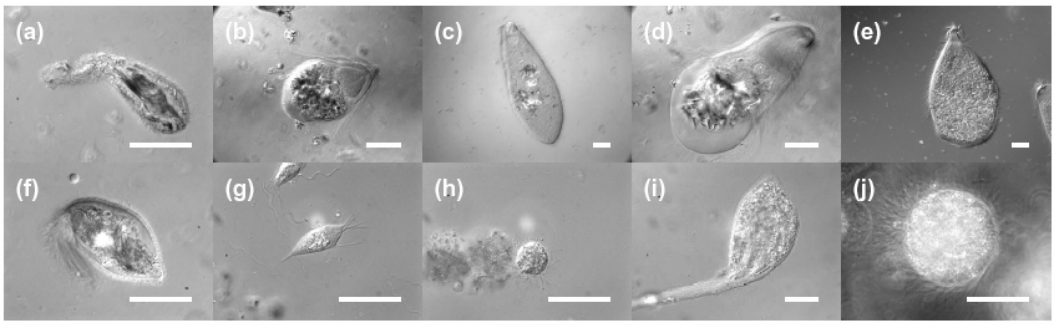

Fig. 1. Light micrographs of ten flagellate species used in this study: *Dinenympha* sp. (a), *Trichonympha* sp. (b), and *Eucomonympha* sp. (c) from *H. sjoestedti*; *Trichonympha* sp. (d) from *Z. nevadensis*; *Deltotrichonympha* sp. (e) from *M. darwiniensis*; *Joenia* sp. (f) *from K. flavicollis*; *Devescovina* sp. (g), *Calonympha* sp. (h), and *Oxymonas* sp. (i) from *N. castaneus*; and an unclassified parabasalid (j) from *C. secundus*. Bars represent 50 μm.

The eukaryotic SSU rRNA gene libraries prepared from the *Eucomonympha* and *Dinenympha* suspensions each contained a single phylotype (HsEcA and HsDnA, respectively; Table 1). In the library from the *Eucomonympha* suspension, the sequence obtained was virtually identical to that of clone HsL3 previously recovered in a clone library of mixed flagellate population of *H. sjoestedti* (Ohkuma et al., 2000), corroborating the tentative assignment of this clone to the genus *Eucomonympha* with morphological evidence. In the case of the *Dinenympha* suspension, the sequence showed 94% identity to that of a *Dinenympha* species from *Reticulitermes hesperus* (Moriya et al., 2003) and probably represents a new, hitherto unrecognized species of *Dinenympha*. The *Trichonympha* suspension yielded three different phylotypes (HsTcA, HsTcB, and HsTcC) of eukaryotic SSU rRNA genes, whose sequences were virtually identical to those of the three *Trichonympha* phylotypes previously obtained from the same termite by Ohkuma et al. (2000).

Table 1. Phylotypes of flagellates recovered in the flagellate suspensions prepared from hindgut contents of different termite species and closest relatives in public databases.

Termite species (family)	Flagellate suspension (Order*)	Flagellate phylotype (Accession number)	Closest relatives (Accession number)	Sequence similarity†
Hodotermopsis sjoestedti (Termopsidae)				
	Trichonympha (Trichonymphida)	HsTcA (AB326107)	*Trichonympha* sp. HsL5 from *H. sjoestedti* (AB032233)	99.9 %
		HsTcB (AB326371)	*Trichonympha* sp. Hs8 from *H. sjoestedti* (AB032229)	99.5 %
		HsTcC (AB326373)	*Trichonympha* sp. HsS9 from *H. sjoestedti* (AB032239)	99.6 %
	Eucomonympha (Trichonymphida)	HsEcA (AB326375)	*Eucomonympha* sp. HsL3 from *H. sjoestedti* (AB032231)	99.0 %
	Dinenympha (Oxymonadida)	HsDnA (AB326376)	*Dinenympha* sp. OS1 from *R. hesperus* (AB092933)	94.0 %
Zootermopsis nevadensis (Termopsidae)				
	Trichonympha (Trichonymphida)	ZnTcA (AB326378)	*Trichonympha* cf. *collaris* from *Z. angusticollis* (AF023622)	95.2 %
Mastotermes darwiniensis (Mastotermitidae)				
	Deltotrichonympha (Lophomonadida)	MdDtA (AB326380)	*Deltotrichonympha operculata* from *M. darwiniensis* (AJ583379)	99.5 %
Kalotermes flavicollis (Kalotermitidae)				
	Joenia (Cristamonadida)	KfJeA (AB326381)	Gut symbiont Kf5 from *K. flavicollis* (AF215857)	98.7 %
Neotermes castaneus (Kalotermitidae)				
	Devescovina (Cristamonadida)	NcDvA (AM747389)	*Devescovina* sp. D16 from *N. jouteli* (X97974)	98.9 %
	Calonympha (Cristamonadida)	NcClA (AM747388)	*Calonympha* sp. B14 from *N. jouteli* (X97976)	98.5 %
	Oxymonas (Oxymonadida)	NcOxA (AB326383)	*Oxymonas* sp. Nk_U08 from *N. koshunensis* (AB092931)	94.5 %
Cryptotermes secundus (Kalotermitidae)				
	Unclassified parabasalid (Cristamonadida)	CsSnA	Unclassified parabasalid from *C. brevis* (AF052699)	96.1 %

* Affiliation is based on Ohkuma *et al.*, 2005.

†Calculated based on the aligned dataset using ARB.

SSU rRNA gene libraries were constructed from the same flagellate suspensions using Bacteria-specific or "Endomicrobia"-specific primers. With either primer set, only a single phylotype of "Endomicrobia" was recovered from the *Dinenympha* ("Endomicrobia" phylotype: HsDn-1) and *Eucomonympha* (HsEc-1) suspensions, whereas two distinct phylotypes (HsTc-1 and HsTc-2) were identified in the *Trichonympha* suspension. Each of the phylotypes formed a distinct, host-specific cluster (Fig. 2). Additional clusters consisting only of clones from whole gut preparations were present (WG1–WG9, Fig. 2), suggesting that "Endomicrobia" might be present also in other flagellate species present in *H. sjoestedti* other than those investigated. However, we do not completely preclude the possibility that these unidentified clones are derived from free-living "Endomicrobia" species.

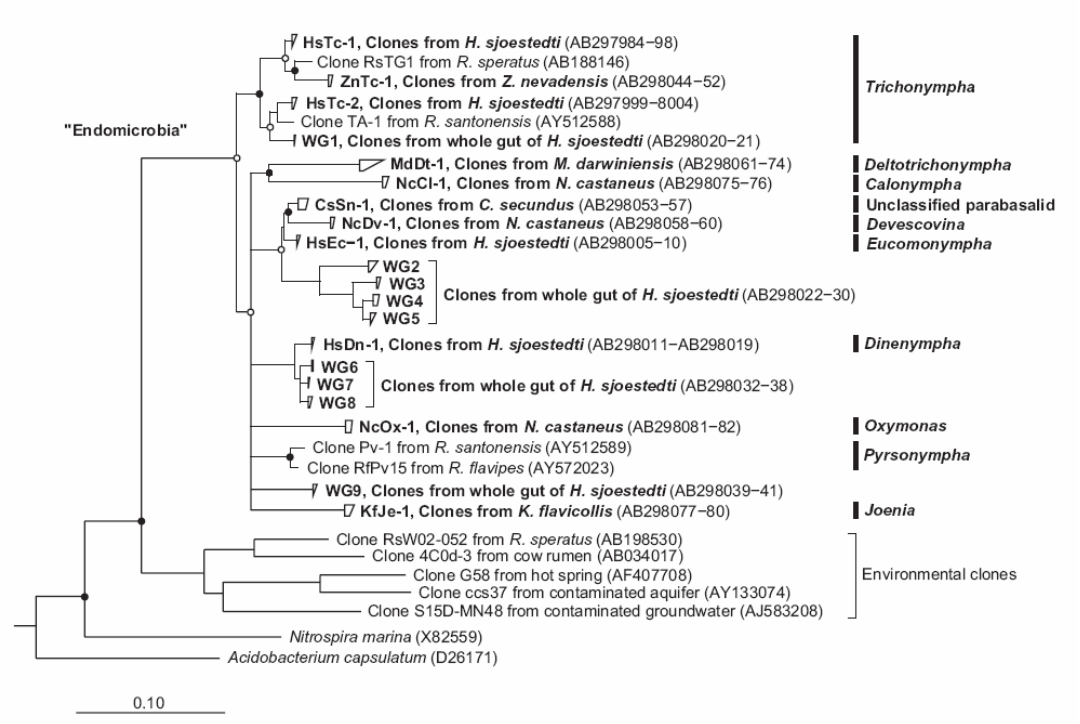

Fig. 2. Phylogenetic tree of "Endomicrobia" and selected environmental clones in the TG 1 phylum, based on SSU rRNA gene sequences. The core tree (maximum-likelihood) was constructed from almost-full-length sequences (>1300 bp). Tree topology was tested by neighbor-joining and parsimony analysis with bootstrapping (DNAPARS, 1000 replicates). Marked nodes have bootstrap values of > 95% (●) and > 50% (○), nodes not supported by all analyses are shown as multifurcation. WG1–WG9: clusters formed by shorter (~1070 bp) "Endomicrobia" sequences originating from whole gut contents added using the ARB parsimony tool. Sequences obtained in this study are marked in bold.

Using the same strategy, we phylogenetically analyzed the host flagellates and "Endomicrobia" in the flagellate suspensions prepared from five other termites, which represent seven additional flagellate genera of parabasalids and oxymonads (Table 1). Again, we were able to assign the eukaryotic SSU rRNA gene sequences obtained from each flagellate suspension to the identical or similar respective genera, whose SSU rRNA gene sequences have been previously published. A notable exception was the SSU rRNA gene recovered from the suspension of Joenia sp. of K. flavicollis, which showed the highest identity (98%) to clone Kf5 (AF215857) previously obtained from a clone library of the same termite and assigned to the flagellate genus Foaina by other authors (Gerbod et al., 2000).

"Endomicrobia" in representative flagellates from other termites

Each of the flagellate suspensions yielded a single and unique host-specific phylotype of "Endomicrobia" in the corresponding SSU rRNA libraries. The phylogenetic tree of all near-full-length (>1400 bp) SSU rRNA gene sequences obtained in this and previous studies clearly showed that the "Endomicrobia" sequences from each flagellate host always represent distinct phylotypes (Fig. 2). The "Endomicrobia" of flagellates originating from the same termite did not cluster among each other. Instead, the "Endomicrobia" from the *Trichonympha* species of *H. sjoestedti* and *Z. nevadensis* clustered together with those previously obtained from the *Trichonympha* species of *R. santonensis* and *R. speratus*, and collectively constitute a monophyletic cluster that forms a sister group of the "Endomicrobia" clones recovered from all other flagellates.

Localization of "Endomicrobia" by FISH

For selected termites, we conducted FISH to confirm the intracellular location of the "Endomicrobia" phylotypes obtained from the respective flagellate suspensions by the specific PCR amplification. It was not possible to design a specific probe for all "Endomicrobia". Moreover, the limited number of variable regions among different "Endomicrobia" did not allow designing specific probes for each phylotype. Therefore, we designed a set of oligonucleotide probes that covered the phylotypes in question (Table 2).

Simultaneous fluorescence in situ hybridization (FISH) was conducted with a fluorescein-labeled bacterial probe and a Cy3-labeled "Endomicrobia" probe. Fig. 3 shows representative examples in which the "Endomicrobia"-specific signal is exclusively localized within the corresponding host cells, whereas the Bacteria-specific probe also stained bacteria associated with the surface or content of these and other flagellate species (Fig. 3). In no case did we see evidence for a location of "Endomicrobia" on the cell surface or within the nucleus of the host.

Since the morphotypes of certain flagellates (*Dinenympha* spp. in the gut of *H. sjoestedti* and *Joenia* spp. in *K. flavicollis*) were difficult to distinguish in the fixed samples, the presence of "Endomicrobia" in the host cells was also confirmed by double hybridization with the respective combination of host and symbiont probes (Table 2; details not shown).

Table 2. Oligonucleotide probes newly designed for whole-cell hybridization of "Endomicrobia" and their host flagellates.

Probe name	Target*	Sequence (5' to 3')†	Formamide (%)
TG1End1023T1	Endomicrobia phylotypes ZnTc-1, HsTc-1, RsTG1	GCT GAC TCC CTT GCG GGT CA	20–50
TG1End1027	Most Endomicrobia lineages (including HsDn-1)	CTC TGC TAA CTC CCT TGC GG	40
TG1End1023	Some Endomicrobia lineages (including HsEc-1)	ACT AAC TCC CTT GCG GGT CA	20#
TG1-TriG1-Hsj	Symbiont HsTc-1 of *Trichonympha* sp. HsTcA	TTG GTC CAG AAG ACT GCT T	20
TG1-Joe-Kf	Symbiont KfJe-1 of *Joenia* sp. KfJeA	GCT AAC TCT CTT GCG AGT CA	20
TG1-Dev-Nca	Symbiont NcDv-1 of *Devescovina* sp. NcDvA	GCA TAG GAC CAC AGT TTG GC	20
Flg-Dine-Hsj	*Dinenympha* sp. HsDnA of *H. sjoestedti*	GCT TTT TGA GGC GGC TAT	35
Flg-Tricho1-Hsj	*Trichonympha* sp. HsTcA of *H. sjoestedti*	GCT AGA TTT CAA GAT AGT CT	10
Flg-Euc-Hsj	*Eucomonympha* sp. HsEcA of *H. sjoestedti*	AAA CCT CCA GAC CAC GCT	10#
Flg-Joe-Kfl	*Joenia* sp. KfJeA of *K. flavicollis*	GCT AGG TTG CAC ACT AGT GG	35

*All Endomicrobia probes had at least two mismatches against any other bacterial phylotype in public databases previously detected in termite guts.

†The oligonucleotide probe sequences have been submitted to probeBase (Loy et al., 2003; http://www.microbial-ecology.net/probebase/).

#Not optimized.

In the case of *Eucomonympha* cells, it was not possible to visualize single cells of "Endomicrobia" because of a high affinity of both the Bacteria-specific and nonsense probe to the dense cell content of the host flagellate. Fluorescence signals outside of the flagellate cells observed in dry-wood termites (see Fig. 3f) were present also in non-stained preparations and were caused by autofluorescence of wood-particles in the gut content.

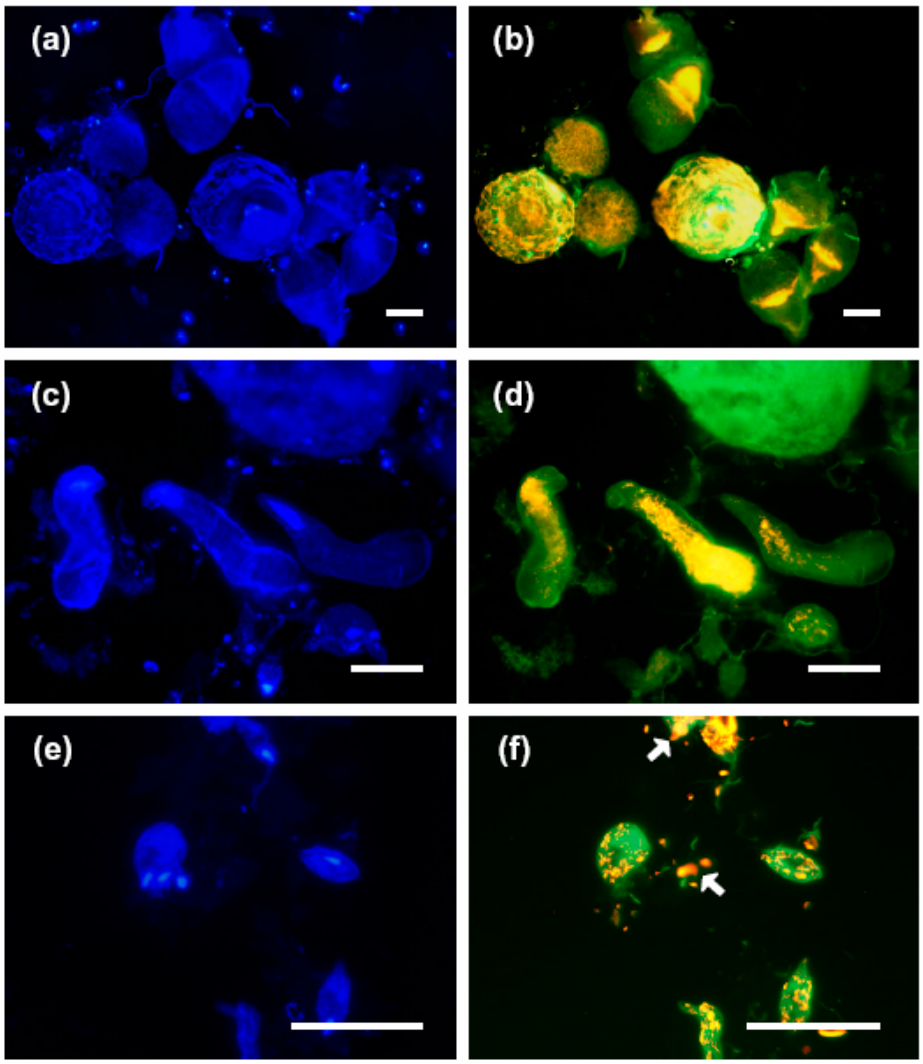

Fig. 3. Epifluorescence micrographs of hindgut preparations of *Z. nevadensis* (a, b); *H. sjoestedti* (c, d); and *Devescovina* from *N. castaneus* (e, f). The preparations were stained with DAPI (a, c, e) and simultaneously hybridized with the fluorescein-labeled (green) probe EUB338 and the Cy3-labeled (orange) probes TG1End1023T1 (b), TG1End1027 (d), or TG1-Dev-Nca (f). Bacteria hybridizing with both probes appear in yellow. Arrows (in f) indicate autofluorescence of wood particles. Bars represent 50 µm.

Discussion

The results of this study corroborate that "Endomicrobia" are host-specific intracellular symbionts of termite gut flagellates. Each of the flagellates investigated harbored a unique phylotype of "Endomicrobia", which proves our hypothesis that the diversity of "Endomicrobia" in each termite gut reflects the diversity of their flagellate hosts. Potential co-speciation between endosymbiont and host is suggested by the "Endomicrobia" phylotypes associated with flagellates of the genus *Trichonympha* constituting a monophyletic group.

Each of the termite gut flagellates analyzed in this study invariably harbored "Endomicrobia". Together with the phylotypes that remain to be assigned to a particular host, "Endomicrobia" represent the symbionts of up to 24 parabasalid and oxymonad species and probably more, in view of the presence of additional "Endomicrobia" phylotypes retrieved from whole gut homogenates of *H. sjoestedti* in addition to those retrieved from the flagellate suspensions. The wide host range and their consistent occurrence within the host indicate a broad host spectrum of "Endomicrobia" as symbionts of termite gut flagellates.

The "Endomicrobia" of each flagellate species form a unique phylogenetic lineage. The case of *H. sjoestedti*, in which the *Trichonympha* suspension contained three phylotypes of *Trichonympha* but from which only two distinct phylotypes of "Endomicrobia" were recovered, does not necessarily contradict the proposed host specificity. It is possible that the third phylotype of "Endomicrobia" was missed in this study because it had been underrepresented in the sample, or that one of the three phylotypes of *Trichonympha* in *H. sjoestedti* lacks "Endomicrobia". The first explanation is supported by the presence of another "Endomicrobia" lineage (WG1) recovered from total gut DNA that clusters with the two other lineages from the *Trichonympha* suspension (Fig. 2).

All "Endomicrobia" phylotypes associated with *Trichonympha* species collectively constitute a monophyletic group that is phylogenetically distinct from the phylotypes recovered from all other flagellates. The evidence that host-specificity is present also at the species level is indicative of cospeciation between the partners (Page and Charleston, 1998). This would imply that each of the extant *Trichonympha* flagellates harbors a specific lineage of "Endomicrobia" inherited by vertical transmission from their common ancestor – an issue that cannot be resolved based on the current dataset. However, it is conversely possible that at one point in time "Endomicrobia" have been horizontally transferred from one flagellate species to another within the same termite gut. This would explain why oxymonads (*Dinenympha*, *Oxymonas*) harbor "Endomicrobia" that are relatively closely-related to the symbionts of parabasalids, i.e., flagellates of a different phylum.

This study corroborates that "Endomicrobia" form a separate line of descent in the bacterial tree (Stingl et al., 2005). They are part of the TG-1 phylum, which consists of numerous diverse and deep-branching lineages (Herlemann et al., in press). While "Endomicrobia" seem to be restricted to termites and wood-feeding cockroaches, other representatives of the TG-1 phylum occur in a wide range of chemically and geographically distinct habitats, including soils, sediment, and intestinal tracts.

Although nothing is known about the metabolic function of "Endomicrobia", their constant occurrence as intracellular symbionts with a broad host range suggests that their nutritional requirements may be met by substances commonly available in the cytoplasm of gut flagellates. Likewise, also the host flagellates may benefit from their endosymbionts, e.g., by the provision of nutrients otherwise lacking in the diet of the termites (see also Stingl et al., 2005). Although certain termite gut flagellates have been shown to ferment cellulose to hydrogen and acetate (Hungate, 1955; Yamin, 1980; Yamin, 1981; Odelson and Breznak, 1985), the physiology of most termite gut flagellates is still completely unknown. This makes elucidation of the biology of "Endomicrobia" and their function in the symbiosis a most intriguing but very challenging subject.

Materials and methods

Termites

Hodotermopsis sjoestedti was collected on Yakushima Island, Japan. *Zootermopsis nevadensis* was collected on Mt. Pinos, Los Padres National Forest, California, USA. Cryptotermes secundus stemmed from a mangrove forest near Darwin, Australia. *Mastotermes darwiniensis*, *Kalotermes flavicollis*, and *Neotermes castaneus* were from cultures maintained at the Bundesanstalt für Materialforschung und -prüfung (BAM), Germany. In the laboratory, colonies were maintained in polyethylene containers at 25 °C on a diet of pinewood. Only termite workers/pseudergates were used in the experiments.

DNA extraction from whole hindguts

Ten hindguts were dissected using sterile forceps and pooled in 750 µl filter-sterilized sodium phosphate buffer (pH 8.0) in a polyethylene tube. The entire content of the tube was transferred into a polyethylene screw-cap tube containing 250 µl TNS solution (500 mM Tris-HCl, 100 mM NaCl, 10% SDS, pH 8.0) and 0.7 g Zirconium beads, and then homogenized in a FastPrep FP120 (Bio101 Savant Instruments, Inc., Holbrook, N. Y.) for 45 s at 6.5 m s–1. The homogenates were

centrifuged, and DNA in the supernatant was purified by phenol-chloroform extraction and ethanol precipitation.

DNA extraction from flagellates

The contents of 3–7 hindguts were suspended in Solution U (Trager, 1934) and diluted to a density of approximately 10 flagellate cells per µl. Aliquots (20 µl) of the diluted suspension were placed in the wells of a 10-well Teflon-coated glass slide (Erie Scientific Company, N. H.). Flagellate cells were sorted by morphology (Radek et al., 1992; Tamm, 1999; Brugerolle and Bordereau, 2004), and 150–200 flagellate cells of each morphotype were collected by micropipette using an inverted microscope. The cells were collected into a well containing sterile 15 µl phosphate-buffered saline (PBS) and washed by at least three transfers into fresh PBS-containing wells. Approximately 100 cells were finally suspended in 200 µl sterile PBS. Cells were disrupted by three cycles of freeze-thawing, and DNA was extracted from each sample using the NucleoSpin kit (Macherey-Nagel, Germany) following the manufacturer's instruction. The extracted DNA was finally eluted with 30 µl of distilled water and used as a template for PCR reactions.

PCR amplification

Flagellate SSU rRNA genes were amplified as previously described, using universal eukaryotic primers (Keeling et al., 1998). Bacterial SSU rRNA genes were amplified using 27F (Edwards et al., 1989) and 1492R (Weisburg et al., 1991) as described by Henckel et al. (1999). "Endomicrobia" SSU rRNA genes were amplified as previously described, using the forward primer TG1-209F (Stingl et al., 2005) and a slightly modified reverse primer TG1-1325R' (5'-GATTCCTACTTCATGTG - 3').

Cloning and sequencing. PCR products were ligated into plasmid pCR2.1-TOPO and introduced into *E. coli* TOP10F' by transformation using the TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.), following the manufacturer's instructions. Clones with flagellate SSU rRNA gene insert and clones with "Endomicrobia" SSU rRNA gene insert (~1070 bp) were screened by direct PCR using M13 primers. To obtain the almost-full length "Endomicrobia" SSU rRNA genes, bacterial SSU rRNA gene libraries (~1500 bp) were screened with "Endomicrobia"-specific primers (see above). PCR products of the expected size were digested separately with the restriction enzymes MspI and AluI, and subjected to electrophoresis on a 3% agarose gel. The clones were sorted according to their restriction patterns, and 2–10 representatives of each ribotype were sequenced using M13 primer sets. For each phylotype (sequence clusters with more than 1% sequence divergence) obtained in this study, several representative SSU rRNA gene sequences have been

submitted to GenBank under accession numbers AB297984–AB298082, AB326107, AB326370–AB326383, and AM747388–AM747389.

Phylogenetic analysis

The SSU rRNA gene sequences were imported into the database implemented in the ARB software package (Ludwig et al., 2004). The sequences were automatically aligned with the other closely related SSU rRNA sequences using the ARB Fast_Aligner, followed by manual refinement. Phylogenetic trees were constructed using almost-full-length SSU rRNA sequences (>1300 bases) by maximum-likelihood methods (AxML and fastDNAml), and the stability of the tree topology was tested by the neighbor-joining and maximum-parsimony methods implemented in ARB. Shorter sequences were added using the ARB parsimony tool. Chimeric sequences were identified using the Bellerophon server (Huber et al., 2004; http://foo.maths.uq.edu.au/~huber/bellerophon.pl) and by carefully checking for signature sequences in the alignment, and were subsequently removed from the dataset.

Whole-cell in situ hybridization

Fixed gut contents were prepared and in situ hybridization was performed as previously described (Stingl and Brune, 2003). Probe EUB338 (Amann et al., 1990) and the nonsense probe NON338 (Wallner et al., 1993) were used to identify bacterial cells and to distinguish unspecific probe binding in the same suspension. For each probe, hybridization stringency was optimized by testing formamide concentrations over a range of 0–50%.

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3. Cospeciation between intracellular symbionts "Endomicrobia" and their host *Trichonympha* flagellates in the termite gut

In preparation for submission

Abstract

Flagellate protozoa play a major role in cellulose degradation in the gut of lower termites. Recently, we have reported that bacterial endosymbionts "Endomicrobia" occur in a wide variety of termite gut flagellates and constitute host-specific clusters within the phylogenetic tree. Here, we analyzed the phylogenetic correlation of and divergence between "Endomicrobia" and their specific host flagellates *Trichonympha* to provide the evidence of cospeciation. Molecular phylogeny of "Endomicrobia" and *Trichonympha* flagellates was characterized based on their small-subunit rRNA (SSU rRNA) genes originating from manually isolated *Trichonympha* flagellates of 10 termite species. Congruence between the phylogenetic trees of the symbiont and the host was well supported over all three phylogenetic estimation analyses (maximum parsimony, maximum likelihood, and Bayesian inference). In the termite *Hodotermopsis sjoestedti* and *Reticulitermes lucifugus*, where multiple phylotypes of "Endomicrobia" and *Trichonympha* were identified, the phylogenetic congruence between the pairs was able to be confirmed by fluorescent in situ hybridization using newly-designed oligonucleotide probes specific for each phylotype. The evidence of cospeciation between "Endomicrobia" and *Trichonympha* in different termites suggests their intimate association during the evolutionary history for more than a hundred million years. A single case of incongruence was observed in one of three symbiotic pairs in *H. sjoestedti*, indicating that a single event of horizontal transmission of the symbiont from one specific host to another has once occurred in this termite. "Endomicrobia" were not detected in *Trichonympha* of *Incisitermes* termites, which suggests that there was at least one “sorting” or “acquisition” event of the symbiont during the divergence of this flagellate.

Introduction

Flagellate protozoa, which densely fill up the hindgut paunch of lower termites, play a major role in the cellulose degradation derived from wood polysaccharides. In addition to their significant contribution to the gut metabolism, the flagellates can provide a nutrient-rich stable habitat for many prokaryotes thriving in the termite gut. A large body of microscopic evidence has shown that prokaryotic cells frequently colonize on the surface or in the cytoplasm, and in rare cases, inside the nucleus of the flagellate host (Ball, 1969; Bloodgood and Fitzharris, 1976). Ultrastructural studies have revealed the evidence of co-adaptation between the prokaryotic symbiont and the flagellate host, e.g., the special attachment structures on the surface of flagellates and the organized arrangement of symbiont attaching to them (Radek et al., 1992; Leander and Keeling, 2004). The functional basis for such tight association is still largely unknown, except a few cases where the symbionts were shown to generate a locomotory force of the host (Cleveland and Grimstone, 1964; Tamm, 1982). The difficulties of studying the mechanism of symbioses between termite gut microorganisms are mainly attributed to the fact that the majority of them cannot be isolated using standard cultivation methods. Recently, culture-independent techniques based on small-subunit rRNA (SSU rRNA) genes or other molecular markers have become a powerful tool for investigation of the specific association between bacterial symbionts and flagellate hosts in the termite gut (Stingl et al., 2004; Noda et al., 2005; 2006). The standard procedure employed in these studies is extracting DNA from manually isolated gut flagellates and amplifying phylogenetic marker genes using different sets of primers. This method allows simultaneous identification of SSU rRNA genes of specific bacterial symbionts and their host flagellate, followed by confirmation of the specific association by fluorescence in situ hybridization (FISH) using oligonucleotide probes designed for each symbiont and host.

Host-specific association is often indicative of cospeciation, i.e., simultaneous speciation of symbionts and hosts, which may be inferred directly from the congruence between the phylogenies of the symbiotic pair (Johnson and Clayton, 2004). Several cases of cospeciation between various insects and their obligate primary endosymbionts (e.g., *Buchnera* and *Wigglesworthia*) have been reported, indicating that the endosymbionts have been vertically inherited from their common ancestral host (Chen et al., 1999; Clark et al., 2000; Downie and Gullan, 2004; Thao et al., 2000). On the other hand, cospeciation has not been well supported in the case of ciliates and their methanogenic archaeal symbionts, suggesting multiple acquisitions and replacements of these symbionts by the host (van Hoek et al., 2000). Only a single study has investigated cospeciation between

termite gut flagellates and their prokaryotic symbionts. The first evidence of cospeciation was provided by a phylogenetic comparison between intracellular *Bacteroidales* symbionts and their host *Pseudotrichonympha* (Noda et al., 2007). In this study, phylogenies of the bacterial symbiont and the flagellate host showed a significant congruence to a large extent, although some non-codivergent events were also indicated by the incongruence at the unresolved lineages obtained from the termite *Coptotermes* (Noda et al., 2007).

"Endomicrobia" form a distinct monophyletic lineage in the phylum “Termite group 1 (TG-1)”, which comprises a large number of clones derived from the gut of termites and cockroaches (Ohkuma and Kudo, 1996). Analyses of "Endomicrobia" SSU rRNA genes obtained from manually isolated termite gut flagellates have shown that this symbiont widely occurs in different termite families, establishing a host-specific association in the cytoplasm of flagellates of different genera (Stingl el al., 2005; Ikeda-Ohtsubo et al., 2007; Ohkuma et al., 2007). The basis of this association is completely unclear, but the frequent and consistent appearance of "Endomicrobia" as specific endosymbionts indicates their co-adaptative evolution. Cospeciation between "Endomicrobia" and their host flagellates is a particular interest, since such evidence can provide a better understanding of evolutionary history and symbiotic nature of these unique organisms. The specific association of "Endomicrobia" has been documented in a wide range of flagellates, which belong to either of two highly distinct taxa Oxymonadida (an order in the phylum Preaxostyla; Adl et al., 2005) and Parabasalia (currently designated as a phylum). When the phylogenetic correlation is compared, the flagellate hosts used in the analysis should be restricted to a group at lower phylogenetic level, considering that "Endomicrobia" are generally affiliated with the same genus-level monophyletic lineage (Stingl et al., 2005). In this study, therefore, the host flagellates affiliated with the genus *Trichonympha* were exclusively used for the phylogenetic comparison with their "Endomicrobia" symbionts. Importantly, "Endomicrobia" SSU rRNA genes that have been previously obtained from *Trichonympha* in different lower termites collectively fall into a monophyletic lineage (Ikeda-Ohtsubo et al., 2007); indicating the speciation of "Endomicrobia" to the *Trichonympha* hosts. In the present study, we performed a detailed evaluation of phylogenetic correlation between "Endomicrobia" and *Trichonympha*.

Results

Identification of SSU rRNA sequences of host *Trichonympha*

Three families, ten species of wood-feeding lower termites, which have been known to harbor *Trichonympha* flagellates in their hindgut, were used in this study (Table 1). A cell suspension of *Trichonympha* flagellates was prepared from gut contents of each termite, from which DNA was extracted for analyzing SSU rRNA genes. Whereas only one morphotype of *Trichonympha* was observed in most termites under the microscope, three morphotypes of *Trichonympha* flagellates differing in size and shape were present in the gut contents of *H. sjoestedti*. The three morphotypes (HsA, HsB, and HsC) were separately picked and a *Trichonympha* suspension was prepared from each morphotype. In the case of *Z. nevadensis* and *Z. angusticollis* that are known to harbor three species of *Trichonympha* (Chapter 7), morphotypes of different species were not distinguishable under the experiment condition with an inverted microscope (×50 magnification); therefore a cell suspension containing all three species was prepared from each termite. PCR using eukaryote-specific primers successfully amplified SSU rRNA gene sequences with length ranging from 1504 to 1524 bp from all *Trichonympha* suspensions, and the following phylogenetic analysis identified 16 phylotypes (sequence clusters with more than 1% sequence divergence) (Table 1). In *H. sjoestedti*, a single distinct phylotype (HsjTcA, HsjTcB, and HsjTcC) was obtained from each *Trichonympha* suspension (morphotype HsA, HsB, and HsC, see above). Unexpectedly, identical sets of four phylotypes of *Trichonympha* (ZooTcA, ZooTcB, ZooTcC, and ZooTcD) were retrieved from the suspensions from *Z. nevadensis* and *Z. angusticollis*. A single phylotype of *Trichonympha* (RsaTcA, RflTcA, RxxTcA, and RheTcA) was obtained from each of four *Reticulitermes* termites. Although the pairwise sequence divergences between these sequences were lower than 1% (0.1% to 0.7%, Table 2A), they were distinguished and used as individual phylotypes for the phylogenetic comparison with sequences from "Endomicrobia" symbionts. Two distinct phylotypes (RluTcA and RluTcB) were obtained in the case of *R. lucifugus*. In *Incisitermes* termites, two phylotypes (ImrTcA and ImrTcB) from *I. marginipennis* and a single phylotype of *Trichonympha* (ItbTcA) from *I. tabogae* were respectively identified. Pairwise sequence divergences among the individual phylotypes of *Trichonympha* ranged from 2.2% to 11.3% (5.7% in average; Table 2A), excluding the values between the four similar phylotypes in *Reticulitermes* (see above). The sequences of *Trichonympha* from *Incisitermes* termites much more differed from other *Trichonympha* (9.0% to 11.8%). When these sequences from *Incisitermes* are excluded, the average sequence divergence between

individual *Trichonympha* phylotypes fell to 3.6%, which still indicates a significant level of evolutionary distance. SSU rRNA sequences from other genera of parabasalid flagellates (*Deltotrichonympha operculata* and *Trichomonas vaginalis*) differed by more than 50%.

Table 1. Termite information and phylotypes of *Trichonympha* and "Endomicrobia"

Termite species (abbreviation, Family)	Source	Phylotype of the host *Trichonympha*	Phylotype of the symbiont "Endomicrobia"
Hodotermopsis sjoestedti (Hsj, Termopsidae)	Yaku island, Japan	HsjTcA*	HsjEm1*
		HsjTcB*	HsjEm2*
		HsjTcC*	HsjEm3
Zootermopsis nevadensis (Znv, Termopsidae)	California, USA	ZooTcA*, ZooTcB, ZooTcC, ZooTcD	ZnvEm1*
Zootermopsis angusticollis (Zan, Termopsidae)	Oregon, USA	ZooTcA*, ZooTcB, ZooTcC, ZooTcD	ZagEm1
Reticulitermes santonensis (Rsa, Rhinotermitidae)	Forêt de la Coubre, France	RsaTcA	RsaEm1
Reticulitermes flavipes (Rfl, Rhinotermitidae)	California, USA	RflTcA	RflEm1
Reticulitermes lucifugus (Rlu, Rhinotermitidae)	Elba island, Italy	RluTcA, RluTcB	RluEm1, RluEm2
Reticulitermes sp. (Rxx, Rhinotermitidae)	Oregon, USA	RxxTcA	RxxEm1
Reticulitermes hesperus (Rhe Rhinotermitidae)	California, USA	RheTcA	RheEm1
Incisitermes marginipennis (Ima, Kalotermitidae)	BAM (Mexico)	ImrTcA, ImrTcB	—†
Incisitermes tabogae (Ita, Kalotermitidae)	BAM (Mexico)	ItbTcA	—†

* SSU rRNA gene sequences from these phylotypes were identical to the sequences that have been published by Ikeda-Ohtsubo et al. (2007).

† No PCR product was detected when "Endomicrobia"-specific primers were used.

Table 2. Maximum-likelihood pairwise distances (GTR+I+Γ model) among SSU rRNA sequences. (A) Comparison between *Trichonympha* (shown in yellow and light blue) and other parabasalid flagellates (gray; MdDtA: *Deltotrichonympha operculata* from *Mastotermes darwiniensis*; Tvag: *Trichomonas vaginalis*). (B) Comparison between "Endomicrobia" symbionts from *Trichonympha* hosts (yellow) and from other flagellates (light blue; HsEc-1: symbiont from *Eucomonympha* sp. of *H. sjoestedti*; MdDt-1: symbiont from *D. operculata* of *M. darwiniensis*; HsDn-1: symbiont from *Dinenympha* sp. of *H. sjoestedti*; KfJe-1: symbiont from *Joenia* sp. from *Kalotermes flavicollis*). Distances from the cultivated TG-1 strain Pei191 are shown in gray.

A: Trichonympha

Phylotype	Pairwise sequence divergence																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1 HsjTcA																		
2 HsjTcB	0.0363																	
3 HsjTcC	0.0445	0.0329																
4 ZooTcA	0.0452	0.0369	0.0418															
5 ZooTcB	0.0475	0.0385	0.0385	0.0222														
6 ZooTcC	0.0349	0.0331	0.0373	0.0291	0.0283													
7 ZooTcD	0.0430	0.0332	0.0338	0.0272	0.0300	0.0240												
8 RsaTcA	0.0447	0.0316	0.0434	0.0375	0.0376	0.0398	0.0393											
9 RflTcA	0.0483	0.0359	0.0461	0.0404	0.0423	0.0424	0.0420	0.0041										
10 RluTcA	0.0332	0.0354	0.0434	0.0403	0.0412	0.0340	0.0342	0.0424	0.0442									
11 RluTcB	0.0382	0.0089	0.0346	0.0366	0.0393	0.0340	0.0349	0.0314	0.0356	0.0378								
12 RxxTcA	0.0473	0.0341	0.0442	0.0382	0.0412	0.0412	0.0408	0.0090	0.0076	0.0440	0.0338							
13 RheTcA	0.0455	0.0324	0.0424	0.0364	0.0394	0.0394	0.0390	0.0076	0.0062	0.0422	0.0321	0.0014						
14 *T. agilis*	0.0365	0.0426	0.0507	0.0439	0.0440	0.0383	0.0403	0.0440	0.0476	0.0276	0.0475	0.0513	0.0494					
15 ImrTcA	0.1028	0.0898	0.0942	0.0974	0.1045	0.1042	0.0997	0.0919	0.0925	0.1037	0.0940	0.0919	0.0895	0.1089				
16 ImrTcB	0.1127	0.0927	0.0961	0.1044	0.1086	0.1100	0.1057	0.1005	0.1004	0.1102	0.0984	0.0991	0.0967	0.1119	0.0104			
17 ItbTcA	0.1090	0.0950	0.0941	0.0997	0.0981	0.1057	0.1015	0.0985	0.0990	0.1121	0.0954	0.0998	0.0974	0.1184	0.0450	0.0432		
18 MdDtA	0.5720	0.5688	0.5459	0.6067	0.6227	0.5718	0.5885	0.5542	0.5822	0.6001	0.5544	0.5643	0.5592	0.6033	0.6441	0.6549	0.6345	
19 Tvag	0.5701	0.5867	0.5917	0.6141	0.6210	0.6077	0.6100	0.5741	0.5562	0.6103	0.5878	0.5865	0.5813	0.6056	0.6477	0.6291	0.6351	0.2236

B: Endomicrobia

Phylotype	Pairwise sequence divergence															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 HsjEm1																
2 HsjEm2	0.0356															
3 HsjEm3	0.0115	0.0308														
4 ZagEm1	0.0337	0.0396	0.0243													
5 ZnvEm1	0.0289	0.0356	0.0212	0.0212												
6 RsaEm1	0.0363	0.0167	0.0347	0.0419	0.0379											
7 RflEm1	0.0347	0.0167	0.0347	0.0453	0.0396	0.0042										
8 RluEm1	0.0235	0.0398	0.0167	0.0290	0.0242	0.0421	0.0422									
9 RluEm2	0.0347	0.0057	0.0299	0.0387	0.0363	0.0159	0.0159	0.0388								
10 RxxEm1	0.0355	0.0152	0.0339	0.0444	0.0387	0.0049	0.0035	0.0413	0.0137							
11 RheEm1	0.0363	0.0159	0.0347	0.0453	0.0395	0.0056	0.0042	0.0421	0.0144	0.0007						
12 RspTG1	0.0229	0.0368	0.0180	0.0322	0.0261	0.0375	0.0375	0.0147	0.0357	0.0366	0.0375					
13 HsEc-1	0.0515	0.0476	0.0481	0.0595	0.0559	0.0516	0.0499	0.0577	0.0465	0.0490	0.0482	0.0579				
14 MdDt-1	0.0744	0.0772	0.0734	0.0915	0.0838	0.0760	0.0761	0.0829	0.0741	0.0751	0.0742	0.0787	0.0705			
15 HsDn-1	0.0558	0.0474	0.0532	0.0646	0.0602	0.0514	0.0515	0.0639	0.0463	0.0523	0.0531	0.0587	0.0388	0.0763		
16 KfJe-1	0.0730	0.0753	0.0749	0.0883	0.0844	0.0777	0.0720	0.0865	0.0740	0.0749	0.0739	0.0825	0.0627	0.0985	0.0738	
17 Pei191	0.4113	0.4169	0.4000	0.4298	0.4251	0.4069	0.4049	0.4203	0.4086	0.4086	0.4067	0.4144	0.3830	0.4218	0.4137	0.3945

Identification of SSU rRNA sequences of "Endomicrobia"

SSU rRNA gene sequences of "Endomicrobia" were simultaneously obtained from the same *Trichonympha* suspensions, from which the eukaryotic SSU rRNA sequences were retrieved as described above. Sequences of "Endomicrobia" had an identical length (1442 bp), and 11 phylotypes were identified from different suspensions (Table 1). In *H. sjoestedti*, a single phylotype (HsjEm1, HsjEm2, and HsjEm3) was obtained from each suspension from *Trichonympha* with three different morphotypes (HsA, HsB, and HsC). The sequences of HsjEm1 and HsjEm3 were very similar and shared almost 99% sequence identity (Table 2B). Only one single phylotype of "Endomicrobia" (ZnvEm1 and ZanEm1) was

identified from each *Zootermopsis* termite. The "Endomicrobia" sequences obtained from each of four *Reticulitermes* termites (RsaEm1, RflEm1, RxxEm1, and RheEm1) were also similar each other (0.0–0.6% divergence; Table 2B) as observed in those of *Trichonympha* (see above). Two phylotypes (RluEm1 and RluEm2) were obtained from *R. lucifugus*, which was also in agreement with the number of phylotypes of *Trichonympha* identified in this termite. No SSU rRNA genes of "Endomicrobia" were amplified from the *Trichonympha* suspensions of *Incisitermes* termites, when "Endomicrobia"-specific primers were used (Table 1). We also investigated bacterial SSU rRNA gene libraries from the same suspensions, but no "Endomicrobia" sequences were obtained (details described in Chapter 4). Pairwise sequence divergences among the individual phylotypes of "Endomicrobia" ranged from 0.4% to 4.5% (2.8% in average; Table 2B). Divergence between "Endomicrobia" from *Trichonympha* and those from other flagellates (sequences from Chapter 2) ranged from 4.6% to 8.8% (6.6% in average). "Endomicrobia" symbionts differed from the recently isolated TG-1 strain Pei191 by more than 40%.

Phylogenetic reconstruction of symbionts and hosts

The phylogenetic inference of "Endomicrobia" and *Trichonympha* was performed using three individual methods: maximum-likelihood (ML), maximum-parsimony (MP), and Bayesian analysis. Sequences of an additional symbiotic pair, *T. agilis* and its "Endomicrobia" symbiont RsTG1, which were previously obtained from the gut of *R. speratus* in separate studies (Ohkuma et al., 1999; 2007), were also included in the analyses. Fig. 1 shows the reconstructed SSU rRNA phylogenetic trees of *Trichonympha* and "Endomicrobia" based on the ML analysis. An obvious overall similarity was observed between two trees, and most nodes were mostly well-supported (>75%) by all three different phylogenetic estimation methods. The phylogenetic tree of "Endomicrobia" consisted of two strongly-supported subclusters. The first subcluster is represented by symbionts of *Zootermopsis* (ZagEm1 and ZnvEm1) and the symbiont RsTG1 from *R. speratus*; the second cluster consists of the similar phylotypes of symbionts from four *Reticulitermes* termites (RsaEm1, RflEm1, RxxEm1, and RheEm1). Each subcluster also contained either of two distinct phylotypes of symbionts originating from the same termite; e.g., RluEm1 and RluEm2 or HsjEm1 and RsjEm2 (Fig. 1). These patterns were also conserved in the tree of *Trichonympha*, although the nodes of the subclusters were not well supported as those of "Endomicrobia". Three phylotypes of *Trichonympha* from *Incisitermes* termites (ImrTcA, ImrTcB, and ItbTcA), from which no "Endomicrobia" was

obtained, formed a strongly-supported cluster, which branches out from the rest of the tree. There was a single case of obvious incongruence: the symbiont HsjEm3 and the host HsjTcC. Whereas the HsjEm3 was placed into the proximity of HsjEm1 from the same termite, the *Trichonympha* HsjTcC fell between the out-branched *Trichonympha* from *Incisitermes* and the cluster containing all other *Trichonympha* (Fig. 1).

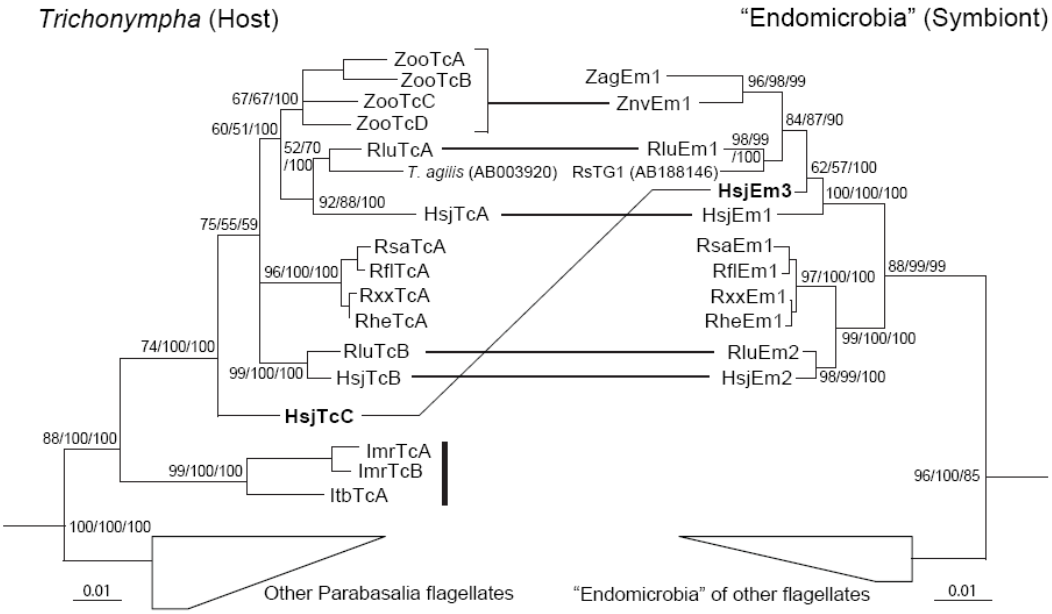


Fig. 1. The phylogenetic placement of *Trichonympha* host (left) and their symbiont "Endomicrobia" (right) shown in the maximum-likelihood trees based on SSU rRNA gene sequences. The numbers at the nodes indicate support values from ML (100 replications), Bayesian inference, and MP (1000 replications), separated by forward slashes. For the signature of the sequences, see the phylotype names in Table 1. *Trichonympha* tree was rooted with outgroups consisting of non-parabasalid flagellates, whereas "Endomicrobia" tree was rooted with outgroups consisting of non-"Endomicrobia" TG1-bacteria as well as bacteria from other phyla. The vertical bar at the *Trichonympha* tree indicates the group of phylotypes lacking respective "Endomicrobia" symbionts. Horizontal bars between hosts and symbionts indicate the symbiotic pair, which specific association was also confirmed by FISH analysis.

Confirmation of congruence between multiple phylotypes of "Endomicrobia" and *Trichonympha* in the same termite by fluorescent in situ hybridization (FISH)

Although the phylogenetic trees exhibited an overall topological congruence between "Endomicrobia" and *Trichonympha*, there were still unresolved cases remained to be further investigated. Most importantly, the specific association between "Endomicrobia": RluEm1 and RluEm2 and *Trichonympha*: RluTcA and RluTcB in *R. lucifugus* could not be demonstrated only by the phylogenetic analyses, since all these phylotypes were recovered from the same *Trichonympha* suspension (Table 1). Furthermore, the specific association of symbionts: HsjEm1, 2, and 3 to the respective hosts: HsjTc1, 2, and 3 in *H. sjoestedti* should also be confirmed, in order to confirm the incongruence found in the phylogenetic analyses (see above). It is also need to be examined whether a single phylotype of "Endomicrobia" is present in four phylotypes of *Trichonympha* in the gut of *Zootermopsis*; consistently or sporadically. To address all these issues, oligonucleotide probes specific for each phylotype were designed and used for simultaneous FISH detection of the symbiont and the host (Table 3).

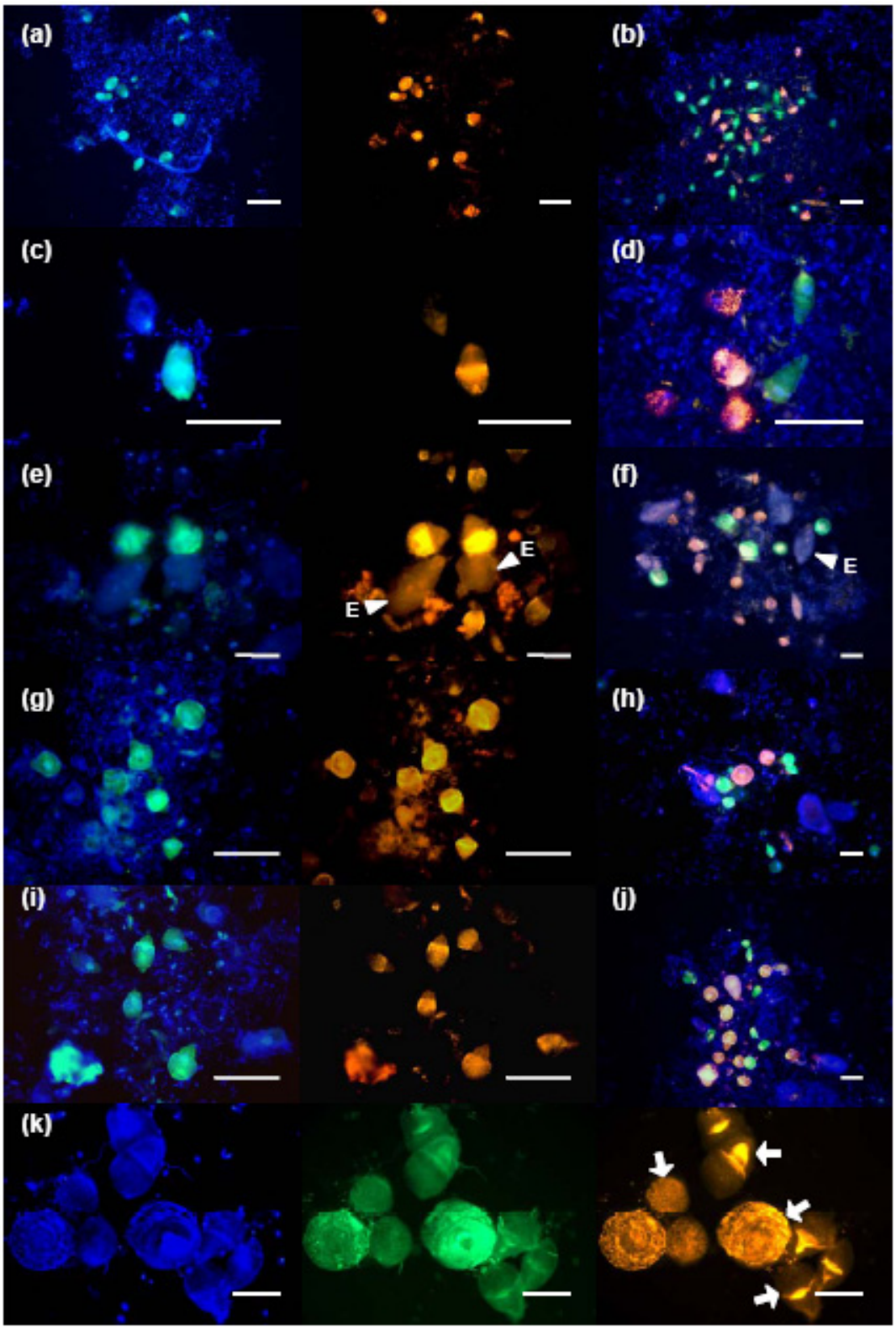
Table 3. Oligonucleotide probes used for in situ hybridization

	Target organism			
Probe name	*Trichonympha* (Host)	Endomicrobia (Symbiont)	Sequence (5'-3')	Image in the Fig. 2‡
Euk-TriGA-Rlu*	RluTcA		CTG CCC ATA AGC ATG GAC	(a), (b)
Euk-TriGB-Rlu*	RluTcB		GAT CTT GCC CAA TGA GCA	(c), (d)
TG1-TriG1-Rlu†		RluEm1	ACC CTT TAA GGA CGG ATA	(a), (d)
TG1-TriG2-Rlu†		RluEm2	CTT TGA CCT CTG CTT GAT G	(b), (c)
Flg-TriGA-Hsj*	HsjTcA		GCT AGA TTT CAA GAT AGT CT	(e), (f)
Flg-TriGB-Hsj*	HsjTcB		GCT TCA ACT AGA GGC TAG C	(g), (h)
Flg-TriGC-Hsj*	HsjTcC		GCT AGA TTT CAC GAT ATT CT	(i), (j)
TG1-TriG1-Hsj†		HsjEm1/HsjEm3	TTG GTC CAG AAG ACT GCTT	(e), (h), (i)
TG1-TriG2-Hsj†		HsjEm2	TTG GTC CAG GAG ATT GCT T	(f), (g), (j)
TG1End1023T1		ZnvEm1	GCT GAC TCC CTT GCG GGT CA	(k)

* Labeled with fluorescein.

† Labeled with Cy3.

‡ The signature of FISH images in Fig. 2 where the respective probe was used.


(a)
(b)
(c)
(d)
(e)
E
E
(f)
E
(g)
(h)
(i)
(j)
(k)

(Previous page) Fig. 2. Epifluorescence micrographs of hindgut preparations of *R. lucifugus* (a–d), *H. sjoestedti* (e–j), and *Z. nevadensis* (k). The preparations were stained with DAPI (blue; a–k) and simultaneously hybridized with the fluorescein-labeled probes specific for different phylotypes of the host *Trichonympha* (green) and Cy3-labeled probes for different phylotypes of the symbiont "Endomicrobia" (orange, or pink when the DAPI image is overlaid). The probes used for each micrograph are described in Table 3. The left micrographs of a, c, e, g, and i are merged images of DAPI staining and the hybridization with a fluorescein-labeled probe. The micrographs b, d, f, h, and j are merged images of DAPI staining and the hybridization with both fluorescein- and Cy3 labeled probes. Arrowheads in the micrographs e and f indicate *Eucomonympha* flagellates that are seen bright because of their autofluorescence. Arrows in the lower right image (k) indicate four different *Trichonympha* species in *Z. nevadensis*. Bars represent 100 μm.

Host-specific associations of "Endomicrobia" RluEm1 and RluEm2 to their respective host *Trichonympha* RluTcA and RluTcB were determined by double hybridization in the fixed gut contents of *R. lucifugus*, using probes specific for each phylotype (Table 3). The cells hybridized with the probe Euk-TriGA-Rlu specific for the host RluTcA (left, Fig. 2a) were also hybridized with TG1-TriG1-Rlu targeting the symbiont RluEm1 (right, Fig. 2a), confirming the specific association. On the contrary, the combination of the probe Euk-TriGA-Rlu and TG1-TriG2-Rlu specific for the other symbiont RluEm2 did not co-hybridize with the same cell (Fig. 2b). Similarly, the specific association between the symbiont RluEm2 and the host RluTcB was investigated using a combination of the probes TG1-TriG2-Rlu and Euk-TriGB-Rlu (Fig. 2c). When observed under a higher magnification, *Trichonympha* cells of the phylotype RluTcB (hybridized with Euk-TriGB-Rlu) were more spindle-shaped, while RluTcA (the host of RluEm1) looked more spherical (Fig. 2d). These results collectively supported evidence of congruence between the phylogeny of two pairs of "Endomicrobia" and *Trichonympha*, each of which was placed into two distinct subclusters in the SSU rRNA trees (Fig. 1).

It was not possible to design phylotype-specific probes that can distinguish the symbiont HsjEm1 and HsjEm3 in *H. sjoestedti*, because of the high sequence similarity between them (~99%, Table 2). Since the individual occurrence of these symbionts in their respective host HsjTcA and HsjTcC was already clear by the fractionation used for phylogenetic analysis, the simultaneous detection of each pair was only performed using the probe TG1-TriG1-Hsj, which detects both HsjEm1 and HsjEm3. The double hybridization using different combination of three probes for *Trichonympha* hosts and two probes for "Endomicrobia" was performed. The three phylotypes of *Trichonympha*: HsjTcA (Fig. 2e, f); HsjTcB (Fig. 2g, h); and HsjTcC (Fig. 2i, j) were clearly distinguished by each specific probe. The specific association of each phylotype of "Endomicrobia": HsjEm1 (Fig.

2e); HsjEm2 (Fig. 2g); and HsjEm3 (Fig. 2i) to the respective host was also confirmed. The absence of the symbiont HsjEm2 in *Trichonympha* HsjTcA and HsjTcC was also confirmed by the double hybridization (Fig. 2f, j) and so was the opposite case: the absence of symbionts HsjEm1 and HsjEm3 in the host HsjTcB (Fig. 2h). The in situ hybridization using all possible combination of probes supported the evidence from the phylogenetic analyses, i.e., the specific association of the "Endomicrobia" symbiont HsjEm1, HsjEm2, and HsjEm3 to their respective *Trichonympha* host HsjTcA, HsjTcB, and HsjTcC (Fig. 1).

The fixed gut sample of *Z. nevadensis* was examined to investigate whether all four phylotypes (or species) of *Trichonympha* harbor this symbiont. Fluorescent signals from the Cy3-labeled probe TG1End1023T1, designed for some "Endomicrobia" including ZnvEm1, were detected in all *Trichonympha* cells in the sample (Fig. 2k). The density and distribution patterns of the symbiont significantly differed between four phylotypes (see below).

Identification of four *Trichonympha* phylotypes in *Z. nevadensis*

Three species of *Trichonympha*: *T. collaris*, *T. campanula*, and *T. sphaerica*, have been morphologically described in *Zootermopsis* termites (Kirby, 1932). Among them, the phylogenetic position of *T. collaris* in *Z. angusticollis* has only been described (Dacks and Redfield, 1998). In this study, four phylotypes of *Trichonympha* (phylotype ZooTcA, B, C, and D) were identified in *Z. nevadensis*. In order to assign each phylotype to the previously described three species of *Trichonympha*, and to identify the fourth species found in this study, phylotype-specific probes were designed for each phylotype and used for whole cell hybridization (Table 1).

Table 4. Newly designed phylotype specific probes for four phylotypes of *Trichonympha* in *Zootermopsis* termites.

Probe	Target	Sequence (5'-3')
ZTcA-Euk-FLUO	Phylotype ZooTcA (*T. collaris*) in *Zootermopsis*	CCA CAT CGT GGA CAA TCC
ZTcB-Euk-FLUO	Phylotype ZooTcB (*T. campanula?*) in *Zootermopsis*	GCC CAT CTT GAT GGA CAA
ZTcC-Euk-FLUO	Phylotype ZooTcC (*T. campanula*?) in Zootermopsis	TTC TGC CCA TAA GGA TGG
ZTcD-Euk-FLUO	Phylotype ZooTcD (*T. sphaerica*) in *Zootermopsis*	GCT GCC CAT AAT TTG GAC

The probe ZTcA-Euk-FLUO specific for the phylotype ZooTcA hybridized with large *Trichonympha* cells, which are densely colonized by ectosymbionts (Fig. 1a) as well as by "Endomicrobia" (Fig. 1b). These ectosymbionts are probably δ-

Proteobacteria described in Chapter 4. These cells have a strong morphological resemblance to *T. collaris*, and SSU rRNA gene sequence obtained by Dacks and Redfield also fell into this phylotype in the tree (data not shown).

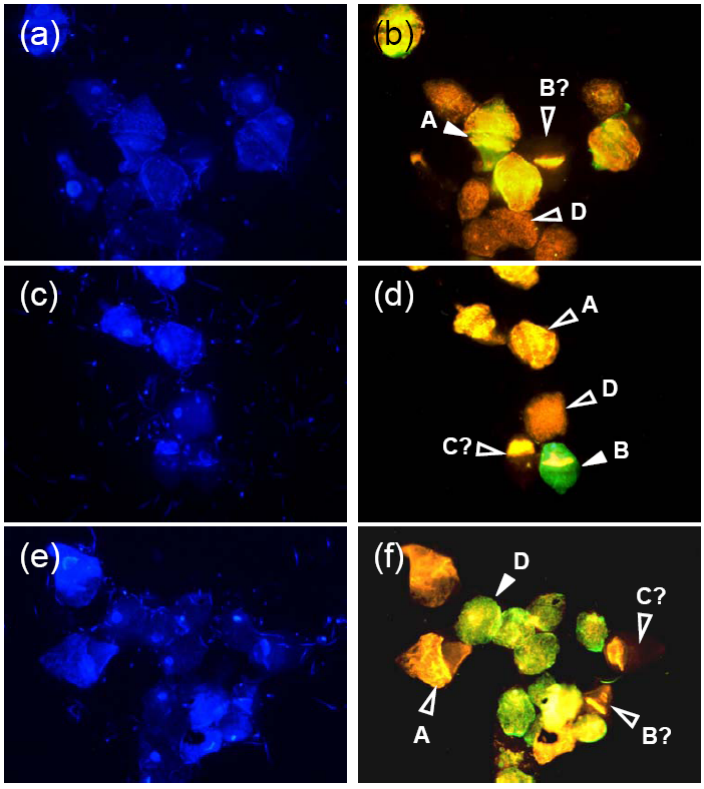


Fig. 3. Epifluorescence micrographs of hindgut preparation of *Z. nevadensis*. The preparations were stained with DAPI (a, c, e) and simultaneously hybridized with the Cy-3 labeled (orange) probe (TG1End1023T1, Ikeda-Ohtsubo et al., 2007) specific for "Endomicrobia" and fluorescein-labeled (green) *Trichonympha* phylotype-specific probes ZTcA-Euk-FLUO (b), ZTcB-Euk-FLUO (d), or ZTcC-Euk-FLUO (f). Filled arrowheads indicate representative cells hybridized with the *Trichonympha* phylotype-specific probe; open arrowheads indicate representative cells that was not hybridized. The phylotype of each *Trichonympha* is indicated: A, ZooTcA; B, ZooTcB; C, ZooTcC; and D, ZooTcD. Note that the appearance of the phylotype ZooTcC was not confirmed by this analysis.

The flagellates hybridized by the probe ZTcB-Euk-FLUO were slightly smaller than ZooTcA and apparently lacked ectosymbionts (Fig. 1c). "Endomicrobia" seemed to be restricted around the nucleus of the host, similarly to the intracellular distribution of "Endomicrobia" in *Trichonympha* of *R. santonensis* (Fig. 1d; Stingl et al., 2005). The sizes and appearance of the ZooTcB were similar to those of *T. campanula* described by Kirby. The probe ZTcC-Euk-FLUO yielded no strong signals from any cells in the sample that was tested in this study. The probe ZTcD-Euk-FLUO hybridized with spherical flagellates that were smaller than ZooTcA or

ZooTcB, indicating the phylotype ZooTcD represents the species *T. sphaerica*. Ectosymbionts were not observed on this flagellate (Fig. 1e), whereas "Endomicrobia" evenly distributed in the cytoplasm (Fig. 1f).

Although *Trichonympha* cells hybridized by the probe ZTcC-Euk-FLUO were not detected by this FISH analysis, the flagellate, which looks similar to the ZooTcB flagellate but was not hybridized by ZTcB-Euk-FLUO appearing in Fig. 1d, is probably the ZooTcC. This flagellate does not harbor ectosymbionts, and the occurrence of "Endomicrobia" seems to be restricted to the posterior end (Fig. 1d and 1f). We therefore concluded (i) *T. collaris* is represented by the phylotype ZooTcA; (ii) *T. sphaerica* is represented by the phylotype ZooTcD; and (iii) *T. campanula* is represented by either or both phylotypes ZooTcB and ZooTcC. In the future, the third evidence should be further investigated to re-describe these *Trichonympha* flagellates as two new species with both aspects of their morphology and phylogeny.

Discussion

The congruence of the phylogenetic tree between "Endomicrobia" and *Trichonympha* demonstrated in this study provides strong evidence of cospeciation. Although the nodal supports in the *Trichonympha* tree was not always as strong as those in the "Endomicrobia" tree, the overall tree topology was consistently supported by the inference of three phylogenetic estimation methods. Furthermore, the FISH analysis using different combinations of specific probes was proven here as a useful method for corroborating the result of phylogenetic analyses, which are sometimes not sufficient for evidencing phylogenetic correlation between multiple symbiotic pairs present in one termite. The co-occurrence and cospeciation of multiple phylotypes of "Endomicrobia" and *Trichonympha* in the gut of a single termite (*H. sjoestedti* and *R. lucifugus*) revealed here provides a new aspect of symbioses between gut-dwelling microorganisms. The highly diverged phylogeny of "Endomicrobia" and *Trichonympha* indicates the long-term history of this tripartite symbiosis. Although the evidence of cospeciation is the most prominent feature of this study, a few exceptional cases were also observed, which require further explanation. In this section, four possible cases of evolutionary events between "Endomicrobia" and *Trichonympha* are further discussed.

Case 1: Cospeciation

Among 11 phylotypes of "Endomicrobia" identified in this study, eight showed obvious phylogenetic congruence with their *Trichonympha* hosts (Fig. 1). This is the evidence of cospeciation, indicating that these symbionts originate from a

symbiont that was acquired by a shared ancestor of extant *Trichonympha*, and the divergence of the symbiont has been synchronized to the divergence of the host during the vertically transmission over the evolutionary history. The acquisition of the symbiont by the ancestor can be placed at the branching point of *Trichonympha* and non-*Trichonympha* flagellates, or *Trichonympha* possessing "Endomicrobia" and those without i.e., *Trichonympha* of *Incisitermes* termites (indicated by a vertical line in Fig. 1). When the widely-accepted value of 1 to 2% of the nucleotide substitution (1.5% in average) per 50 million years (Moran et al., 1993) is applied for estimating the rate of SSU rRNA evolution in "Endomicrobia", this first acquisition of the symbiont by *Trichonympha* (and also by another gut flagellates) can date back to 150–300 million years ago (calculated from the divergence among "Endomicrobia" from different parabasalid flagellates). The co-divergence of multiple phylotypes of "Endomicrobia" and *Trichonympha* in the termites *H. sjoestedti* and *R. lucifugus* seems to have occurred at early stages of divergence (150–100 million years ago), and symbionts in the four *Reticulitermes* termites (Rsa, Rfl, Rxx, and Rhe) are likely to have diverged more recently (within 0–30 million years ago). This divergence of "Endomicrobia" in the recent evolutionary history is in agreement with that the evolutionary rate of bacteria is highly accelerated once they become host-dependent symbionts (Moran, 1996). Cospeciation is also an indication of co-adaptation between the symbiont and the host, which can involve the physical association as well as functional interactions. Although the physiological properties of these organisms are still unclear, it can be assumed that "Endomicrobia" may benefit from the stable cytoplasmic environment with a high amount of carbon and energy source provided by the host. *Trichonympha* flagellates have been shown to digest cellulose and release H_2, CO_2, and acetate as end products (Yamin, 1981), but they are also likely to produce more diverse types of organic substrates. The question whether the presence of "Endomicrobia" is beneficial to the host entails future investigations on physiological properties of this symbiont.

Case 2: Acquisition and loss

The absence of "Endomicrobia" in three phylotypes of *Trichonympha* (ImrTcA, ImrTcB, and ItbTcA) in *Incisitermes* suggests three possible evolutionary hypotheses: 1) a shared ancestor of these flagellates, or each of them had lost the symbiont in the past (sorting event); 2) there was no acquisition event since these *Trichonympha* branched out from the other cluster containing all the rest of *Trichonympha* (Fig. 1); 3) these *Trichonympha* actually possess a distinct group of "Endomicrobia", which is not detected by universal bacterial primers or probes. The sorting event claimed by the first hypothesis seems to be rare, considering the

consistent occurrence of this symbiont among a wide range of termite gut flagellates (Ikeda-Ohtsubo et al., 2007). When the sorting event had really occurred, it may be possible to detect the remnant of the descendent of the “exiled” symbiont in the gut of these termites. Since *Trichonympha* in *Incisitermes* collectively form an early-branching cluster, it is also possible that they are affiliated with a sister group of the "Endomicrobia"-harboring *Trichonympha* (9–12% sequence divergence). The second hypothesis is well explained by the evidence that all *Trichonympha* on the other branch presently possess "Endomicrobia" and show cospeciation, indicating the acquisition of "Endomicrobia" occurred one-sidedly by a common ancestor of this clade. The third hypothesis assumes that these *Trichonympha* contain endosymbionts that cannot be recovered by presently available primers or probes. SSU rRNA gene sequences of some "Endomicrobia" (e.g., ZnEm1) contain one or two mismatches to universal eubacterial primers and probes (data not shown). Therefore, it is necessary in future studies to use newly-design new primers that cover all presently available "Endomicrobia" sequences.

Case 3: Horizontal transmission

In this study, a single case of incongruence between "Endomicrobia" and *Trichonympha* was observed in one of three symbiotic pairs in *H. sjoestedti*, HsjEm3 and HsjTcC. While SSU rRNA sequence of HsjEm3 was almost identical to that of another symbiont HsjEm1 and closely related to other symbionts, the host HsjTcC exhibited a distinct phylotype (divergence up to 5%) that branched out from the subcluster consisting of all other *Trichonympha* except those from *Incisitermes* (Fig. 1). This discrepancy implies that the symbiont might have been horizontally transmitted to the current host from another host in the past. Most probably, the host HsjTcC may have acquired a symbiont from the ancestor of HsjTcA, the specific host of the symbiont HsjEm1. This assumption is based on the high similarity between the SSU rRNA sequences of the symbiont HsjEm1 and HsjEm3 (~99%), indicating that their divergence occurred quite recently (within last 35 million years). The symbiont transmitted from the HsjTcA to the HsjTcC should be a common ancestor of HsjEm1 and HsjEm3, which had already speciated to HsjTcA. Although this is the first evidence that suggests the horizontal transmission of "Endomicrobia" between flagellates, it remains to be investigated whether it also occurs between phylogenetically distinct flagellates, e.g., oxymonads and parabasalids.

Case 4: Occurrence of a symbiont in multiple hosts

A single phylotype of "Endomicrobia" was identified in *Trichonympha* suspension of *Z. nevadensis* and *Z. angusticollis*, which yielded multiple phylotypes of *Trichonympha*. "Endomicrobia" from two *Zootermopsis* termites ZnvEm1 and ZagEm1 showed 2.1% distance to each other (~67 million years divergence time predicted). This result indicates the cospeciation of "Endomicrobia" with their two different termite hosts, rather than with their *Trichonympha* hosts. If "Endomicrobia" were already present in the gut of each termite since earlier than *Trichonympha*, four phylotypes of *Trichonympha* seem to have acquired a single phylotype of "Endomicrobia" from the gut fluid very recently, which have already been speciated to each termite. If flagellates had already possessed "Endomicrobia" when they appeared in the termite gut, it is more likely that other *Trichonympha* flagellates have acquired "Endomicrobia" via horizontal transfer from this flagellate.

To elucidate the evolutionary history of "Endomicrobia" in more details, the tripartite symbiosis considering the evolution of termites will need to be investigated in future studies.

Materials and methods

DNA preparation and PCR amplification

The termite species used in this study is presented in Table 1. Cell suspension of *Trichonympha* flagellates was prepared from gut contents of each termite as previously described (Ikeda-Ohtsubo et al., 2007). DNA extraction, amplification of SSU rRNA genes from *Trichonympha* and "Endomicrobia", cloning of SSU rRNA genes and sequencing were performed as described previously (Ikeda-Ohtsubo et al., 2007). Sequences were assembled using the SEQMAN program (DNAstar, Madison WI).

Phylogenetic analyses

The SSU rRNA gene sequences were aligned with nearest neighbor sequences by using the alignment tool of the ARB software package (Ludwig et al., 2004). SSU rRNA sequences that have more than 1400 bases were used for further phylogenetic estimation procedures. The multiple sequence alignment of each dataset was exported using an appropriate ARB filter and saved in a nexus format (Maddison et al., 1997). Phylogenetic trees of the alignment were constructed using maximum-parsimony (MP) and maximum-likelihood (ML) estimation implemented in PAUP version 4.0b10 (Swofford, 2003). In each case, gaps in the

alignment were treated as missing data. MP trees were inferred from heuristic searches using a starting tree generated by stepwise addition with 100 random replicates with tree bisection reconnection (TBR) branch swapping algorithm (dividing a tree into two and rejoining by every possible pair of branches). The support of the nodes of MP trees was evaluated by 1000 bootstrap replicates using the same heuristic search options. The appropriate model of nucleotide substitution for ML analysis was selected using the program MODELTEST version 3.7 (Posada and Crandall, 1998). The general time reversible model with corrections for invariant characters and gamma-distributed rate heterogeneity (GTR+I+Γ) was selected for the multiple sequence alignment of both "Endomicrobia" and *Trichonympha*, under the Akaike information criterion (AIC). AIC presents some advantages over the other criterion hierarchical likelihood ration tests (hLRT) for model selections, in that they are able to simultaneously compare all nested or non-nested models, assess model selection uncertainty, and allow for model-averaged parameters (Posada and Buckley, 2004). ML trees were inferred from heuristic searches using the selected model using a starting tree generated by stepwise addition with 10 random replicates TBR branch swapping. The nodal supports were assessed by bootstrap analysis consisting of 100 bootstrap replicates, using the same heuristic search options. Trees obtained in MP and ML analyses were displayed using the TREEVIEW program (Page, 1996). The maximum-likelihood pairwise distances between SSU rRNA gene sequences were calculated with PAUP using the GTR+I+Γ model.

Bayesian analyses were conducted using MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). The substitution model for each alignment was determined by MrModeltest version 2.2 (Nylander, 2004), using the GTR+I+Γ models for "Endomicrobia" and *Trichonympha* selected by AIC as described above. The 50% majority rule consensus Four Markov chains were simultaneously run for 1,000,000 generations and parameters and trees were sampled every 100 generations. The 50% majority rule consensus tree calculated from the 10001 trees sampled after the initial burn-in period provided estimation of posterior probabilities.

Probe design and whole cell hybridization

Hindgut contents of each termite were incubated for two hours at 4 °C in 3% (w/v) paraformaldehyde for the fixation. The cells were washed with ice-cold phosphate-buffered saline (PBS: 0.13 M NaCl, 7 mM Na_2HPO_4 and 3 mM NaH_2PO_4, pH 7.4) for three times and resuspended in PBS with the equal volume of ethanol and stored at –21 °C. Oligonucleotide probes targeting SSU rRNA of specific phylotypes of "Endomicrobia" and *Trichonympha* in different termites were designed using the

probe design functions of the ARB software (Ludwig et al., 2004). The number of mismatches among organisms in the databases was checked using the Probe Match function of Ribosomal Database Project II (Cole et al., 2007). Optimal hybridization stringency for probes was examined by changing formamide concentration in the hybridization buffer over a range of 0 to 40% in 5% intervals at a fixed temperature 46 °C. In this study, the optimal stringency was determined as 20% formamide for all Cy3-labelled probes except TG1End1023T1, which was used under 35% formamide concentration. 10% formamide was used for all fluorescein-labeled probes except Euk-TriGA-Rlu and Euk-TriGB-Rlu, for which 30% formamide was determined as the optimal stringency. The hybridization procedure was performed as described previously (Stingl and Brune, 2003), but the dehydration step in an ethanol series was omitted to minimize the distortion of flagellate cells. In cases when the optimal stringency (i.e., formamide concentration) of two simultaneously used probes is different, the hybridization was performed under the higher stringency at first, and the hybridization at the lower stringency was performed after the plate was once washed and air-dried. After the hybridization and washing, samples were quickly dried with compressed air and stained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI), washed with ice-cold 80% ethanol, air-dried, and cover-slipped with Citifluor (Citifluor Limited, London, UK). Samples were observed by epifluorescence microscopy using a Zeiss Axiophot microscope (Zeiss, Jena, Germany). Unspecific probe binding in the fixed samples was checked with the fluorescein-labeled EUB338 probe (Amann et al., 1990) and Cy3-labeled NON338 probe (Wallner et al., 1993).

New internal primers for sequencing SSU rRNA genes

A number of universal primers have been designed for amplification of SSU rRNA genes of a variety of microorganisms in various environments. Although published primers such as 27f or 1492r are able to amplify SSU rRNA genes of vast majority, they often fail to amplify sequences of certain groups of microorganisms that have mismatches, which may cause biased results in phylogenetic analyses. Termite Group 1 bacteria, which represent the major component of bacterial community in many lower termites and probably some other habitats, often have significant mismatches to such oligonucleotide probes and primers (Herlemann et al., in press). Additionally, SSU rRNA gene sequences of many termite gut flagellates also have unique nucleotide patterns, which often fail to be amplified with universal eukaryotic primers. Although some new set of primers that are able to amplify SSU rRNA genes from termite gut microorganisms have been described in several studies (e.g., Keeling et al., 1998) to address this problem, those primers are not sufficient for determining almost full-length SSU

rRNA genes (~1500 bp), when the sequencing reads are shorter than 800 bp. Therefore, four internal sequencing primers were newly designed here, which can be used for primer walking of longer SSU RNA gene sequences from most gut microorganisms in the termite gut. New primers were designed mainly based on published primers, 343f and 1100r (Lane, 1991) as well as 519f (Stubner et al.,, 2002). The primer match was tested against SSU rRNA sequences from termite gut microorganisms imported in ARB database. The 343f_BacTer and 1100r_BacTer can hybridize SSU rRNA of most of termite gut bacteria including "Endomicrobia". The 519f_EukTer and 1061r_EukTer hybridize SSU rRNA of most of termite gut including Trichonympha. Using these primers in addition to the universal or vector-specific primers, we are able to obtain almost full-length SSU rRNA genes from wider range of microorganisms originating from termite gut, even when an average sequence read was below 600 bp.

Table 5. Internal primers newly designed in this study for primer walking of SSU rRNA genes of termite gut microorganisms.

Primer name	Sequence (5'? 3')
343f_BacTer	CTA (C/T) GG GAG GCA GCA G
1100r_BacTer	GGG TT (A/G) CGC TCG TT
519f_EukTer	CAG CAG CTG CGG TAA TTC
1061r_EukTer	TCA ACC CAC GCA CCA CCA AC

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4. Phylogenetic affiliation of bacterial symbionts of *Trichonympha* species in the termite *Zootermopsis nevadensis* and *Incisitermes marginipennis*

Abstract

Many termite gut flagellates harbor dense populations of bacterial symbionts. The phylogenetic profiles of such bacterial symbionts are still largely unknown. In this study, the community structure of bacterial symbionts was investigated using *Trichonympha* flagellates in a damp wood-feeding lower termite *Zootermopsis nevadensis* and a dry wood-feeding lower termite *Incisitermes marginipennis*. 16S rRNA gene libraries constructed from manually isolated *Trichonympha* cells from each termite revealed a high diversity of bacteria occurring as symbionts of these flagellates. Several phylotypes of δ-proteobacteria bacteria were the most abundant member in both bacterial communities. Interestingly, the composition of major phylogenetic groups was otherwise significantly different between the two libraries: *Treponema*, Bacteroidetes, and several proteobacteria clones were predominant in the library from *I. marginipennis*, whereas *Endomicrobia* (Termite Group 1) and Mycoplasmatales represented a major fraction in *Z. nevadensis*. The result of T-RFLP analyses of the whole-gut DNA of each termite indicates that these symbionts are also well-represented in the entire community. Fluorescent in situ hybridization showed that *Trichonympha* flagellates are densely populated by δ-proteobacteria in *Z. nevadensis*. These bacteria hybridized with specific probes were shown to occur as ecto- and endosymbionts of *Trichonympha*. This study provides the first evidence of prospective sulfate-reducers occurring as major symbionts of termite gut flagellates.

Introduction

Symbiotic flagellate protozoa (flagellates) in the gut of wood-feeding lower termites are known to play an important role in the cellulose degradation (for reviews, see Brune, 2006). The flagellates swimming in the gut fluid ingest wood particles, by which 70–75% of the cellulose can be anaerobically degraded to acetate, H_2 and CO_2 (Hungate, 1939; 1943; Yamin, 1980). In addition to the prominent populations of flagellates, there are an enormous number of prokaryotes (estimated as 10^8–10^{10} cm^{-3} by Breznak, 1975) are present in the termite gut

ecosystem. Accumulative microscopic studies have shown that prokaryotes often appear as symbionts of termite gut flagellates in different forms; colonizing on the surface (ectosymbiosis), in the cytoplasm (endosymbiosis), or sometimes inside the nucleus (endonuclear symbiosis) (Ball, 1969; Brune and Stingl, 2005). Considering that the vast majority of the prokaryotes are associated with flagellates (e.g., ~90% in the gut of *Mastotermes darwiniensis*, Berchtold et al., 1999), the symbiosis between prokaryotes and flagellates seems to function as a highly diverse and significant micro-ecosystem in the gut of termites.

Bacterial community structure in the gut of lower termites has been recently studied by analyzing 16S rRNA genes retrieved from DNA extracted from the whole-gut or fractionated intestinal habitats (Hongoh et al 2003; 2005; Nakajima et al., 2005; Yang et al., 2005). These studies revealed that spirochetes affiliated with *Treponema* are the major bacterial groups in the total gut fraction, followed by Termite Group 1 (TG-1), Clostridiales, Bacteroidetes and Firmicutes, most of which constitute a "termite-specific" lineage within each phylum. There were also significant differences in bacterial community structures between habitats, e.g., the gut wall and the gut fluid, in which the spatial organization of the gut bacteria seems to be influenced by various physicochemical factors (Nakajima et al., 2005; Yang et al., 2005). It has been shown that spirochetes and TG-1 are also abundant in protozoa fractions, but only a few studies have determined the phylogenetic profiles of bacterial symbionts that are specifically attached to the flagellates.

Specific associations of bacterial symbionts to the termite gut flagellate have been recently investigated using 16S rRNA genes retrieved from flagellate cells, which were manually separated from termite gut contents. Bacteroidetes have been shown to occur as ecto- and endosymbionts (e.g., Stingl et al., 2004; Noda et al., 2005), and "Endomicrobia" are also widely-distributed endosymbionts in different termite gut flagellates (Stingl et al., 2005; Ikeda-Ohtsubo et al., 2007). These bacteria also represent a major phylogenetic group in the bacterial community in the gut of termites.

Trichonympha is a large flagellate (50 to 300 μm) occurring as common symbiotic flagellates in different families of lower termites. This flagellate has been long studied for its fine structure, phylogenetic affiliation, and physiological properties. The genus *Trichonympha* belongs to a phylum-level lineage Parabasalia, which is characterized by the presence of hydrogenosomes, well-developed flagellar system, and a parabasal apparatus (Brugerolle and Lee, 2000). *Trichonympha* constitutes a distinct cluster within the phylogenetic tree of Parabasalia, indicating the early divergence of this group from other clades (Ohkuma et al., 2005). A few cultivation studies of the axenic culture of *Trichonympha* have documented the digestion of cellulose and production of

acetate, CO_2, and H_2 from this flagellate (Gutierrez, 1956; Yamin, 1981). Although the morphological studies have documented that *Trichonympha* harbors a large number of bacterial symbionts with different morphotypes (Kirby, 1944; Yamin, 1979), the "Endomicrobia" are the only group that has been shown to occur as symbionts of *Trichonympha*. Since "Endomicrobia" seems to represent an obligate symbiont, it is of interest to investigate whether other groups of bacteria can cohabit in the same flagellate.

In the present study, we performed phylogenetic characterization of bacterial symbionts attached to *Trichonympha* flagellates in two termites, *I. marginipennis* and *Z. nevadensis*.

Results

Phylogenetic affiliation of bacteria in *Trichonympha* of *I. marginipennis*

Trichonympha of *I. marginipennis* is a large flagellate (~100 μm, Fig. 1A) and comprises a major fraction in the gut of this termite. 16S rRNA gene library was constructed from DNA extracted from the cell suspension of this flagellate. In the library containing 40 clones, three dominating ribotypes (I-A, I-B and I-C) were identified. All other ribotypes were represented by only one or two clones (singletons). Randomly selected clones from each ribotype were sequenced and the phylogenetic position of each sequence was determined. The ribotype I-A represented approximately 30% of the total clones. Most sequences from this ribotype assigned to the phylotype ImaTr-1, which was affiliated with a novel lineage of *Treponema* (only 91% similarity to known phylotypes, see Table 1) and placed into a so-called “Termite Cluster” in the tree of *Treponema* (Fig. 2). The second dominant ribotype I-B (~20% of the clones) consisted of clones that are affiliated with *Desulfovibrio* (phylotype ImaTr-5), probably representing an undescribed species (Fig. 3). Sequences from the third dominant ribotype I-C (~10% of the clones) were almost identical to those of *Pseudomonas fluorescens* (phylotype ImaTr-4). Sequences from singletons showed a large variety of phylogenetic groups, including three distinct phylotypes (ImaTr-9, ImaTr-10, ImaTr-11) in the phylum ‘*Bacteroidetes*’ (Fig. 4), two phylotypes of ε-proteobacteria (ImaTr-6, ImaTr-7) and a single phylotype from Actinobacteria (ImaTr-12), α-proteobacteria (ImaTr-3), and Mycoplasmatales (ImaTr-8, Fig. 5) (Table.1). In most cases, the closest relatives of each phylotype were sequences that were previously retrieved from comprehensive 16S libraries of the gut of other termites (Table 1).

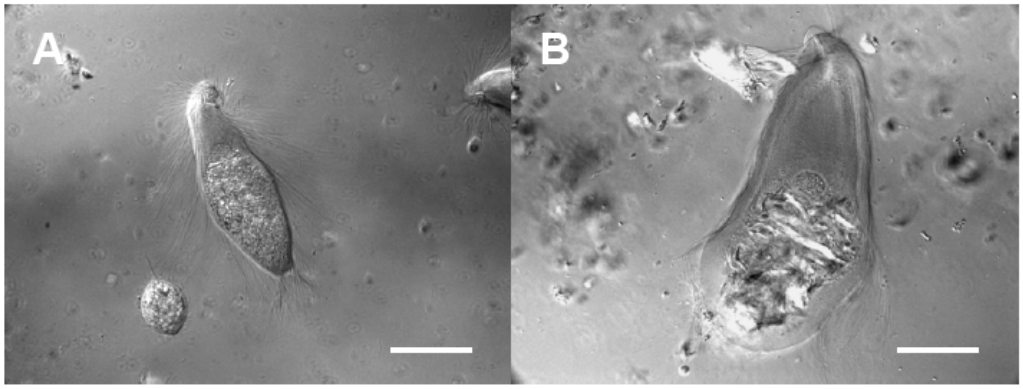

Fig. 1. Light micrographs of *Trichonympha* from *I. marginipennis* (A) and *Z. nevadensis* (B). Bars represent 50 µm.

Phylogenetic affiliation of bacterial symbionts from *Trichonympha* of *Z. nevadensis*

Z. nevadensis harbors the largest *Trichonympha* (~200 µm, Fig 1B) among lower termite species. Three ribotypes (Z-A, Z-B and Z-C) dominated in the 16S rRNA gene library with 40 clones derived from the cell suspension of *Trichonympha*. Ribotype Z-A, which constituted approximately 30% of clones in the library, was affiliated with an uncultured group of δ-proteobacteria (phylotype ZnvTr-3, Table 1). The second phylotype of δ-proteobacteria ZnvTr-2, which was less represented in the library, clustered together with *Desulfovibrio* species (Fig. 3). The ribotype Z-B accounting for about 30% of total clones was from the phylotype ZnvTr-4 (Table 1), which was almost identical to the phylotype ZnTc-1 affiliated with "Endomicrobia" obtained in our recent study (see Chapter 2, Ikeda-Ohtsubo et al., 2007). The third abundant phylotype Z-C was represented by 8% of clones, which comprised two distinct phylotypes of Mycoplasmatales (phylotype ZnvTr-5 and ZnvTr-6). These phylotypes were placed into a cluster consisting exclusively of clones, which were previously obtained from termite guts (Fig. 5). This cluster also comprises the phylotype ImaTr-8 from *I. marginipennis*, as well as a symbiont of *Koruga Bonita* isolated from other lower termite *Mastotermes darwiniensis* (AJ142569, Fig. 5). Singletons yielded a single phylotype of *Treponema* (ZnvTr-2, Fig. 2), Acidobacteria (ZnvTr-7), and Verrucomicrobia (ZnvTr-8). The closest relatives of these clones were among clones that were previously retrieved from the gut of different termite species (Table 1). Unexpectedly, 10% of clones contained sequences highly similar to Euryarchaeota (BLASTn, data not shown). These sequences were not further investigated, since they were very short (~350 bp) and of low quality.

Table 1. Phylogenetic affiliation of 16S rRNA genes obtained from *Trichonympha* of *I. marginipennis* and *Z. nevadensis*.

			I. marginipennis	*Z. nevadensis*
Phylogenetic Group (Specific lineage*)	Closest neighbor in public database (Accession No.)	Sequence similarity (%)	Phylotype (Accession No.)	Phylotype (Accession No.)
Spirochaetes (*Treponema*)	Clone RFS88 from *R. flavipes* (AF068344)	91	ImaTr-1 (I-A*)	
	Clone NkS-Oxy26 from *Oxymonas* of *N. koshunensis* (AB085167)	99	ImaTr-2	
	Clone ZAS-1 from *Z. angusticollis* (AF093251)	95		ZnvTr-1
α-Proteobacteria	RsTu-1-42 from *R. speratus* (AB192231)	95	ImaTr-3	
γ-Proteobacteria (*Pseudomonas*)	*Pseudomonas fluorescens* genome (DQ178234)	99	ImaTr-4 (I-C)	
δ-Proteobacteria (*Desulfovibrio*)	*Desulfovibrio desulfuricans* genome (DQ517287)	94	ImaTr-5 (I-B)	
	Zn-FG10 from *Z. nevadensis* (DQ420319)	99		ZnvTr-2
(uncultured)	Rs-K70 from *R. speratus* (AB089106)	95		ZnvTr-3 (Z-A)
ε-Proteobacteria	NkW01-011 from the gut wall of *N. koshunensis* (AB231075)	95	ImaTr-6	
	NkW01-035 from the gut wall of *N. koshunensis* (AB231076)	98	ImaTr-7	
TG-1 Phylum ("Endomicrobia")	ZnTc-1 from *Z. nevadensis* (AB298044)	99		ZnvTr-4 (Z-B)
Mollicutes (Mycoplasmatales)	Rs-E42 from *R. speratus* (AB089057)	92	ImaTr-8	
	Rs-K63 from *R. speratus* (AB089060)	94		ZnvTr-5 (Z-C)
	BCf4-19 from *Coptotermes formosanus* (AB062824)	88		ZnvTr-6
Bacteroidetes (*Bacteroides*)	HBG_A2V3-2 from honeybee intestines (DQ837638)	93	ImaTr-9	
(*Flavobacterium*)	Cc3-012 from *Cryptotermes cavifrons* (AB299530)	92	ImaTr-10	
	NkW01-012 from the gut wall of *N. koshunensis* (AB231050)	94	ImaTr-11	
Actinobacteria (*Actinomyces*)	Rs-Q7 from *R. speratus* (AB089082)	92	ImaTr-12	
Acidobacteria	RsTu1-92 from *R. speratus* (AB192240)	96		ZnvTr-7
Verrucomicrobia	Rs-P07 from *R. speratus* (AB089122)	98		ZnvTr-8

* Phylotypes that are not assigned to specific lineages are affiliated with uncultured lineages within each phylum.

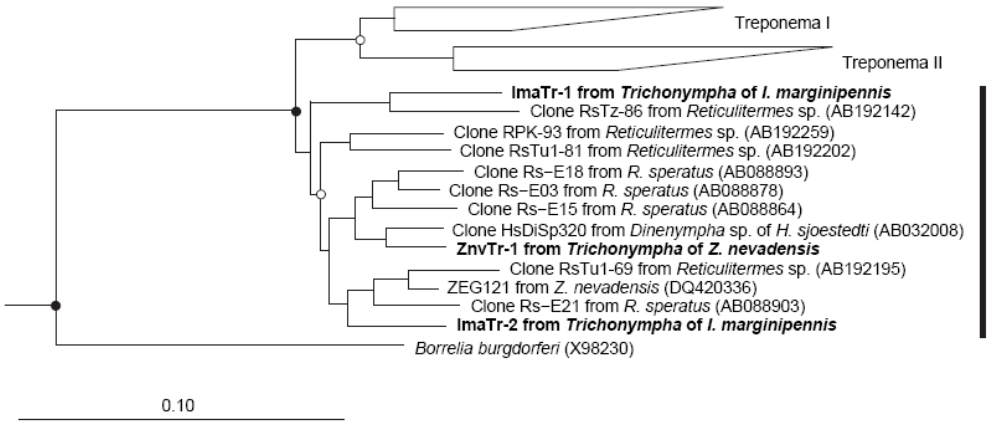

Fig. 2. Phylogenetic position of *Treponema* clones (ImaTr-1, ImaTr-2, ZnvTr-1) obtained in this study (shown in bold) in a 16S rRNA based maximum-likelihood tree. Outgroups used for rooting of the tree (*Escherichia coli* and *Bacillus cereus*) are not shown. The scale bar indicates 0.10 substitutions per nucleotide position. Nodes with bootstrap values (DNAPARS, 1000 replicates) with >90% (●) and >50% (○) are marked. The vertical bar indicates the "Termite Cluster" that consists of sequences originating from termite gut flagellates or termite gut homogenates.

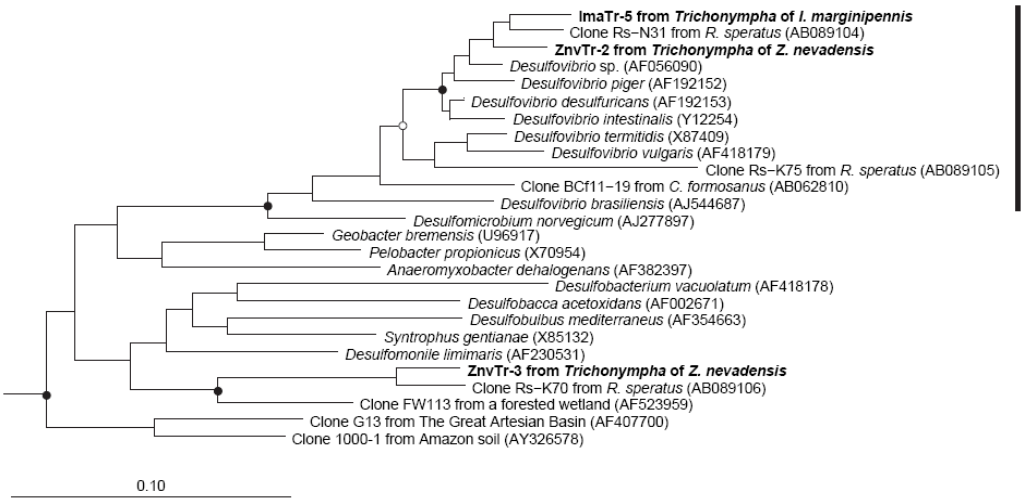

Fig. 3. Phylogenetic position of δ-proteobacteria clones (ImaTr-5, ZnvTr-2, ZnvTr-3) obtained in this study (shown in bold) in a 16S rRNA based maximum-likelihood tree. The vertical bar indicates the cluster consisting of *Desulfovibrio* species. Description of marked nodes, see Fig. 2 for details.

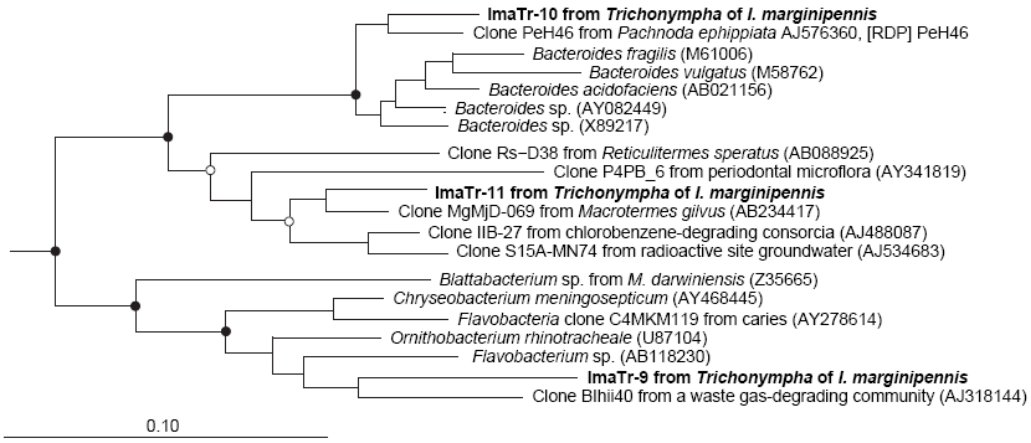

Fig. 4. Phylogenetic position of Bacteroidetes clones (ImaTr-9, ImaTr-10, ImaTr-11) obtained in this study (shown in bold) in a 16S rRNA based maximum-likelihood tree. Description of marked nodes, see Fig. 2 for details.

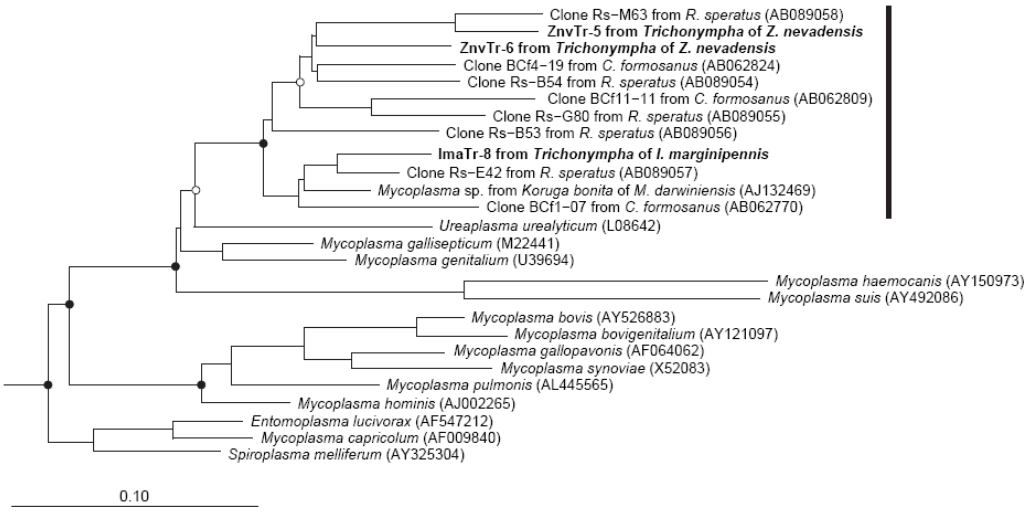

Fig. 5. Phylogenetic position of Mycoplasmatales clones (ImaTr-8, ZnTr-5, ZnTr-6) obtained in this study (shown in bold) in a 16S rRNA based maximum-likelihood tree. The vertical bar indicates a cluster consisting of clones originating from termite gut flagellates or termite gut homogenates. The short 16S rRNA sequence of *Mycoplasma* sp. manually isolated from *K. bonita* of *M. darwiniensis* (AJ132469) was subsequently added using the ARB parsimony tool. Description of marked nodes, see Fig. 2 for details.

T-RFLP fingerprinting of the whole-gut DNA from *I. marginipennis* and *Z. nevadensis*

The diversity of bacterial community in the gut of *I. marginipennis* and *Z. nevadensis* was analyzed by the T-RFLP fingerprinting obtained from the *Msp*I-digested whole-gut DNA (Fig. 2). As is shown in the terminal restriction fragment (T-RF) patterns, a significant difference was also observed in the gut community structure between the two termites. T-RFs that were assigned to Spirochaetes, ε-proteobacteria and Bacteroidetes were represented by prominent peaks in the profile of *I. marginipennis*, indicating that the bacterial symbionts of *Trichonympha* also constitute the major population in the entire gut microbial community. The T-RF assigned to "Endomicrobia" was well represented in the gut of *Z. nevadensis*, although the prominent peak at 281 bp should also contain a substantial number of T-RFs from spirochetes. On the other hand, δ-proteobacteria that dominated the symbionts of *Trichonympha* in both termites ware not well-represented in the whole gut bacterial community (predicted T-RFs of 490 bp in *I. marginipennis*; of 132 and 163 bp in *Z. nevadensis*), indicating their specific occurrence in the flagellate fraction. The peak representing the T-RF of Firmicutes was prominent (60 bp) in the whole gut of *I. marginipennis*, whereas it was below the detection limit in *Z. nevadensis* (538 bp). No peak that can be assigned to *Pseudomonas* was detected. T-RFLP fingerprinting of DNA extracted from *Trichonympha* suspension was not successful, which was probably caused by the low yield of PCR product when a labeled forward primer was used.

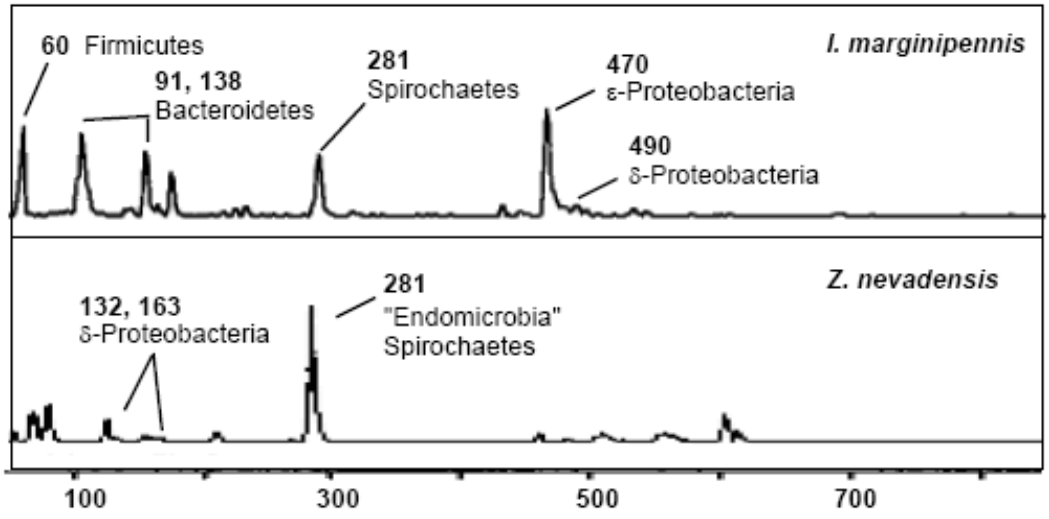

Fig. 5. T-RFLP profiles of 16S rRNA gene fragments. PCR products amplified from whole-gut DNA from *I. marginipennis* and *Z. nevadensis* were digested with MspI. T-RFs that matched the predicted size in base are marked together with the phylogenetic affiliation. The horizontal axis is the size of the T-RF (bp).

Localization of δ-proteobacteria in *Trichonympha* of *Z. nevadensis*

The δ-proteobacteria symbionts, which dominated the 16S rRNA gene library from *Trichonympha* of both termites, were investigated for their distribution and location by fluorescent in situ hybridization (FISH). Since the gut sample of *I. marginipennis* had a problem of autofluorescence (see materials and methods), only the sample from *Z. nevadensis* was used. Approximately two third of *Trichonympha* harbored either or both of these symbionts (data not shown). The rostrum of *Trichonympha* was densely colonized by both symbionts (Fig. 3a, b, g). The *Desulfovibrio* symbionts represented by the phylotype ZnvTr-2 was hybridized with the probe DSV698, which revealed their occurrence on the anterior part of the host *Trichonympha* (Fig. 3a). Another phylotype ZnvTr-3, which was hybridized with the probe Delta-ZnvTr3 was also found to colonize the anterior part of the host in a similar manner (Fig. 3b). Both symbionts were small (~1 µm) rod-shaped cells but ZnvTr-3 symbionts had a more spherical shape (Fig. 3c and 3d). Interestingly, more than half of both symbionts were found to vertically line up on the anterior surface of the host (Fig. 3e and 3f). Double-hybridization using the probes DSV698 and Delta-ZnvTr3 confirmed that no cells were hybridized by both probes and that these two symbionts are abundantly and homogeneously distributed in and on the *Trichonympha* cell (Fig. 3g).

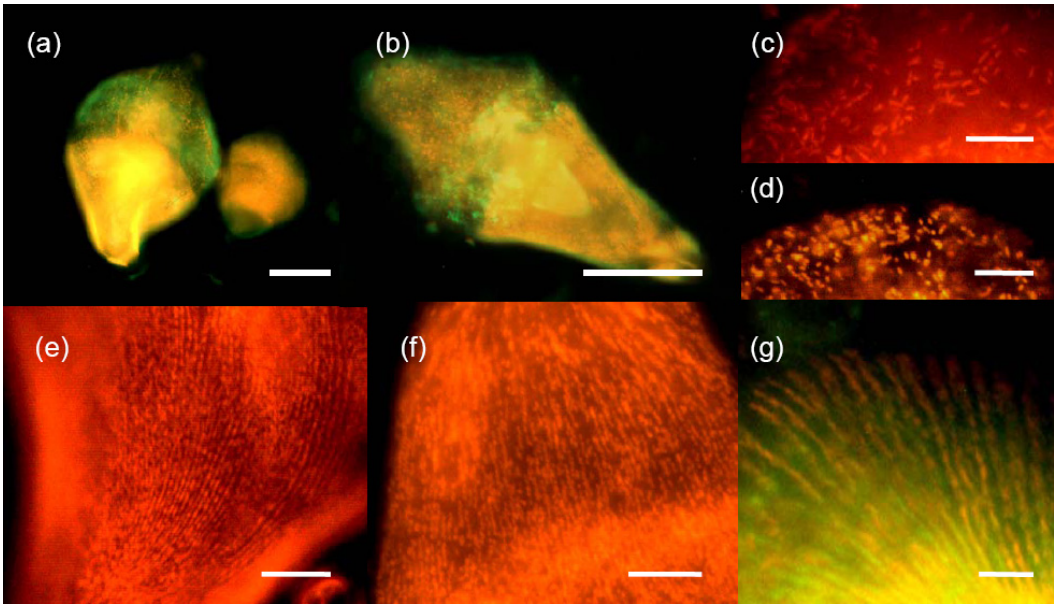

Fig. 6. Epifluorescent micrographs of *Desulfovibrio* (phylotype ZnvTr-2) and uncultured δ-proteobacteria (phylotype ZnvTr-3) in *Trichonympha* of *Z. nevadensis*. Symbiont ZnvTr-2 was hybridized with the probe DSV698 labeled with Cy3 (orange; a, c, e, g), and Symbiont ZnvTr-3 was hybridized with the probe Delta-ZnvTr3 labeled with Cy3 (orange; b, d, f) or fluorescein (green; g). EUB338 labeled with fluorescein was used for hybridization of bacteria (green; a and b). Bacteria hybridizing both Cy3 and fluorescein probes appear yellow. Bars indicate 50 µm in a and b; 10 µm in c, d, e, f, and g.

Discussion

Symbiosis between δ-proteobacteria and *Trichonympha*

A common feature of the bacterial symbionts identified in *Trichonympha* of two termites was the abundance of δ-proteobacteria. Although they did not seem to dominate the bacterial community in the whole gut, they emerged as prominent ecto- and endosymbionts of *Trichonympha* flagellates in *Z. nevadensis* (Fig. 3). The dense colonization and characteristic spatial organization of δ-proteobacteria symbionts on termite gut flagellates have not been reported before. δ-proteobacteria occurring as ectosymbionts of anaerobic ciliates has been reported previously (Fenchel and Ramsing, 1992).

Kirby described detailed morphologies of *Trichonympha* in *Zootermopsis* and identified "peripheral granules" that are located in the outer zone of the ectoplasm of *T. collaris* (Kirby, 1932). His documentation "the rostrum in *T. collaris* has the appearance of a collar striped with granular bands" bears a strong resemblance to the appearance of FISH-stained δ-proteobacteria on the *Trichonympha* cell (Fig. 6). He also made some illustrations showing these granules lined up in the grooves between the surface ridges on the anterior part of *Trichonympha* flagellates, which seems to depict the spatial organization of this symbiont as shown in Fig. 6g. Although he was not able to assign these granules to certain organisms at that time, his documentation of these symbiont-like granules is highly elaborate.

In previous studies, several strains of sulfate reducing bacteria affiliated with *Desulfovibrio* have been isolated from the gut of lower termite *R. santonensis* and *M. darwiniensis* (Trinkerl et al., 1990; Kuhnigk et al., 1996; Fröhlich et al., 1999). These isolates were able to oxidize H_2 and different low-molecular-weight organic compounds such as lactate, formate, and ethanol in the presence of sulfate, thiosulfate, and sulfite, as well as O_2. Since two of the phylotypes (ImaTr-5 and ZnvTr-2) retrieved from *Trichonympha* in this study are also members of *Desulfovibrio* (Fig. 4), the physiological evidence found in these isolates can be applied to predict their functions in terms of symbiosis. For example, the ability to utilize a large variety of organic substrates may allow these symbionts to establish a stable niche in the proximity of flagellates where dense populations of other bacteria are constantly present. The high rate of O_2 reduction in the presence of H_2 indicates that these symbionts can remove a substantial amount of O_2 from surroundings and contribute to the fitness of the flagellate host, which is strictly anaerobic. Furthermore, they may also provide the hosts with nitrogen nutrition by fixing nitrogen. Another phylotype ZnvTr-3, which was predominant among the symbionts of *Trichonympha* from *Z. nevadensis*, appeared to be affiliated with a novel cluster of sulfate reducers (Fig. 4). Although there is no evidence of

functional roles of this symbiont, they may also be involved in a H_2-consuming process in this habitat.

The low sulfate concentration and lack of major sulfate-reducing activities in the gut of wood-feeding termites have generally led to an assumption that sulfate reducers do not contribute to the gut metabolism as spirochetes and methanogens. However, δ-proteobacteria, which are closely related to the described sulfate reducers, are actually as abundant in certain habitats and likely to play a significant metabolic role. It has been shown that sulfate-reducers can dominate in the microbial community in freshwater sediment with a sulfate concentration as low as 60 to 105 μM (Lovley and Klug, 1983). Since a certain amount of sulfate is present in the termite gut (0.3-0.7 mM, Kuhnigk et al., 1996), sulfate reducers have a great chance to constitute a major H_2-consuming population, when the reduced sulfur compounds are able to be reoxidized. The *Desulfovibrio* strain obtained from the gut of *R. santonensis* has been shown to oxidize some reduced compounds such as sulfide with oxygen (Kuhnigk et al., 1996).

Sulfate reducers can also occur in large numbers in human intestine (Gibson et al., 1993), although they seem to be outcompeted by methanogens (Stroucchi et al., 1994). Their functional role in the human gut is not well understood.

Interestingly, methanogens are apparently absent on *Trichonympha* flagellates of *Zootermopsis*, whereas they are densely attached to other smaller flagellates in this termite: *Trichomitopsis termopsidis*, *Tricercomitus termopsidis*, and *Hexamastix termopsidis* (Lee et al,. 1987; Lee and Messer, 1989; Pester, 2006). It has not been clarified why methanogens are missing from *Trichonympha*, despite a large amount of H_2 produced by hydrogenosomes (Stingl, 2004). The result presented here indicates that the dense population of δ-proteobacteria symbionts occupying the restricted habitable area of *Trichonympha* leaves no space for the attachment of methanogens.

Do sulfate reducers play a significant role in the termite gut? If so, which organic substrates do they use and how the reducing equivalents are transferred? In order to answer these questions, future studies should focus on biochemical and metabolic properties in the gut of termites. For example, monitoring the change in H_2 and acetate metabolism during the inhibition of sulfate-reducing activities may give as a hint to understand how significantly sulfate-reducers are involved in the gut metabolism. It is also important to investigate the basis of symbiotic relationships between sulfate reducers and other symbionts and their host flagellates.

Spirochetes as symbionts of *Trichonympha* flagellates

Spirochetes were also found to be the most abundant bacterial symbiont of *Trichonympha*, especially in *I. marginipennis*. The phylogenetic position and abundance of *Treponema* as symbionts of *Trichonympha* are documented here for the first time. The result corroborates previous findings, which have shown the predominance of spirochetes that may account for 50% of all bacteria in the hindgut (Paster et al., 1996; Hongoh et al., 2003). The three phylotypes of *Treponema* (ImaTr-1, ImaTr-2, and ZnvTr-1) from this study are affiliated with the "termite cluster" consisting of *Treponema* clones and cultivated strains, all of which originate from termite guts (Lilburn et al., 1999; Ohkuma et al., 1999). It is not clear whether there is a subcluster formed by the symbiotic members of termite gut flagellates. Previous studies have documented that multiple phylotypes of *Treponema* can occur as symbionts of different species of flagellates, and a certain degree of host-specificity of the symbiont has been evidenced by FISH (Iida et al., 2000; Noda et al., 2003). The small number of clones representing *Treponema* in the library from *Z. nevadensis* in this study indicates that these spirochetes are loosely attached to the surface of *Trichonympha* and therefore not likely to establish a strict symbiosis with the host flagellate. Considering that spirochetes are the major H_2-consuming homoacetogens in the termite gut (Breznak, 1973), it is not surprising that many of them prefer the proximity of *Trichonympha*, which has shown to produce a high amount of H_2 (see above). Possible benefit of this association to the host *Trichonympha* is uncertain, but the presence of *nifH* homologs and nitrogenase activities in many termite gut spirochetes suggests their contribution to the nitrogen source of the host flagellate (Ohkuma et al., 1996; Lilburn et al., 2001).

Other phylogenetic groups

"Endomicrobia" occur in a wide range of flagellate species in different lower termites, including *Trichonympha* flagellates of *Z. nevadensis* (Stingl et al., 2005; Ikeda-Ohtsubo et al., 2007). The absence of "Endomicrobia" in the 16S rRNA gene library from *Trichonympha* flagellates of *I. marginipennis* is also in agreement with a previous result (Chapter 3), where PCR amplification using specific primers for "Endomicrobia" 16S rRNA genes yielded no products from DNA of *Trichonympha* of this termite. "Endomicrobia" seem to be a strict endosymbiont of termite gut flagellates, and all flagellates so far investigated except *Trichonympha* of *Incisitermes* have been found to possess this symbiont. Stingl *et al.* have reported that PCR products were also obtained from the whole gut of *Incisitermes* termites,

when the "Endomicrobia"-specific primers were used (Stingl et al., 2005). The origin of these "Endomicrobia" sequences needs to be clarified in the future.

Different phylotypes of bacteria belonging to the phylum Bacteroidetes (CFB/Chlorobi group) were recovered from *Trichonympha* of *I. marginipennis* (Fig. 4). Many bacteria affiliated with *Bacteroidales* have been often found in the gut bacterial community of *Reticulitermes* termites (Hongoh et al., 2003; 2005; Yang et al., 2005). Some lineages of these bacteria have been identified as host-specific ecto- or endosymbionts of different types of flagellates (Stingl et al., 2004; Noda et al., 2005; 2006). Although the occurrence of *Bacteroidales* symbionts in termite gut flagellates seems is sporadic, they seem to dominate the bacterial symbionts of their specific host flagellates (Noda et al., 2005). Since the *Bacteroidales* obtained in this study was only represented by a singleton, they are not likely a prominent symbiotic member of *Trichonympha*. Two other phylotypes of Bacteroidetes detected in this study independently represented an uncultivated cluster (Fig. 4). These clusters consist of clones from different environmental samples and do not exclusively represent the symbionts of termite gut flagellates. Although Bacteroidetes were not detected in *Trichonympha* of *Z. nevadensis*, they appear to form a minor population in this flagellate (Chapter 5).

Bacteria affiliated with the order Mycoplasmatales are known to constitute a major fraction in the bacterial community in the gut of *Reticulitermes* (Hongoh et al., 2003; Yang et al., 2005). It is not surprising that they occur in *Trichonympha* flagellates, considering that members of this group are often found in various eukaryotic cells as chronic parasites. It has been previously reported that a bacterial cell that was directly aspirated from the cytoplasm of the flagellate *Koruga Bonita* in *M. darwiniensis* was affiliated with *Mycoplasma* (Fröhlich and König, 1999). Interestingly, the phylogenetic analysis in this study revealed that sequences obtained from termite gut contents in separate studies collectively form a distinct cluster within the Mycoplasmatales clade (Fig. 6). Since all phylotypes retrieved in this study fell into this cluster, this clade probably consists exclusively of symbionts of termite gut flagellates. A strain *Mycoplasma hominis*, which is distantly related to the symbionts of termite gut flagellates, is known to occur as a parasite of the pathogenic parabasalid flagellates *Trichomonas vaginalis* (e.g., *Rappelli* et al., 2001). The tight association between these two organisms has been thought to be a key cause of the infectious disease of human being and therefore extensively studied. Understanding the nature of the symbiosis between mycoplasma and termite gut flagellates may provide insights into the origin of such effective association.

It was rather accidental that several clones containing archaeal 16S rRNA genes were recovered from the library. Since the universal reverse primer 1492r

can also hybridize 3'-end of archaeal 16S rRNA genes, archaeal sequences could be amplified when DNA is abundantly present. Although all archaeal 16S sequences retrieved here were of bad qualities, most of these sequences were affiliated with a group of Euryarchaeota. In *Z. nevadensis*, methanogens are not likely to occur on *Trichonympha* flagellates, because they cannot be detected by the visualization of the coenzyme F420 under the epifluorescence microscope (see above). Therefore, the Euryarchaeota sequences detected here are likely to originate from non-methanogenic archaea. However, evidence obtained here is not sufficient for evaluating its credibility.

The phylogenetic study of 16S rRNA library from the *Trichonympha* suspension revealed a significant diversity of symbionts attached to this flagellate. Future studies should focus on the functional significance of each symbiont, which may lead to a better understanding of metabolic properties of termite gut microorganisms.

Materials and methods

Termites

I. marginipennis was from cultures maintained at the Bundesanstalt für Materialforschung und -prüfung (BAM). *Z. nevadensis* was originally collected in the Mt. Pinos in California and maintained in a polyethylene container with pine woods and water. 3–5 individual worker caste termites were used for the experiments.

DNA preparation, PCR, and Cloning

Cell suspensions of *Trichonympha* flagellates from each termite were prepared as described previously (Ikeda-Ohtsubo et al., 2007). DNA extracted from 50–100 cells was eluted with 50 µl distilled water, and 2–5 µl of the DNA was subsequently used for PCR amplification in a 50 µl reaction volume. Amplification of 16S rRNA genes was performed using universal bacterial primers 27f (Edwards et al., 1989) and 1492f (Weisburg et al., 1991), with a reaction profile of 95 °C for 3 min, then 30 cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 45 s, followed by 72 °C for 7 min. 2 µl of the PCR product was directly added to a ligation mix with the components of 1.3 µl distilled water, 1µl salt solution, and 0.7 µl pCR2.1-TOPO vector (Invitrogen) and incubated overnight at 4 °C. TA cloning of the ligated vector was performed following the manufactures' instruction (TA TOPO cloning kit, Invitrogen).

Clone libraries and RFLP analysis

Clones containing 16S rRNA genes were screened by colony PCR using M13 primers. The PCR products from positive clones were digested with a restriction enzyme *Msp*I overnight at 37 °C. Digested DNA was separated by electrophoresis in 3.0% agarose gels in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA-Na_2). Clones were sorted by their restriction patterns (ribotypes) appearing on the visualized gels and one to seven representative clones from each pattern were subsequently sequenced from both ends using M13 primers. The partial fragments from each sequence read were assembled using the SEQMAN program (DNAstar).

Phylogenetic analysis

Blastx (NCBI, Bethesda, USA) was used to find the sequences with the highest similarities to the 16S rRNA sequences obtained in this study. New sequences were imported into a database in the ARB program (Ludwig et al., 2004). Sequences were aligned with the phylogenetic species that showed high similarities in the database using the Fast Aligner function. Phylotypes were defined as single clones that have more than 1% sequence divergence from a published sequence. A neighbor joining tree containing all sequences obtained in this study for rough estimation of the position of each phylotype and its closest relatives. The percentage similarity was calculated according to the aligned dataset in ARB using the similarity matrix. A maximum-likelihood tree was constructed by the ARB AxML program using almost full length (>1300 bp) sequences obtained in this study. The topology of the tree was tested by bootstrapping for maximum parsimony (DNAPARS) with 1000 replicates and for neighbor joining with 10000 replicates, both of which are implemented in ARB.

T-RFLP analysis

DNA from the whole gut of termite was extracted as described in Chapter2. Bacterial 16S rRNA genes from the extracted DNA were amplified using 27f primer labeled with the fluorescent dye IRD 800 (pentamethine carbocyanine, MWG), and unlabeled 907r primer (Muyzer et al., 1993). The PCR reaction was performed in a 50 µl reaction volume as described above. 5 µl of PCR products were separated in 0.8% agarose gel to check the quantity. 50 ng DNA from each product was digested with the restriction enzyme *Msp*I overnight at 37 °C.

2.5 µl of the digested DNA was mixed with 1.9 µl of formamide dye and 0.6 µl of internal dye standard (ROX-2500) and incubated for 2 min at 95 °C. 1 µl of each mixture was loaded onto 5.5% polyacrylamide gel containing 7 M urea, and electrophoresed using an automated DNA sequencer (LI-COR Biosciences). Length of T-RFs was measured using the Gel-Pro Analyzer (version 4.5, MediaCybernetics) by comparing with the molecular size marker (LI-COR).

Epifluorescent microscopy and in situ hybridization

Hindgut contents of each termite were fixated with 3% (w/v) paraformaldehyde as described in Chapter 3. The nonsense probe NON338 (Wallner et al., 1993) was used to distinguish unspecific probe binding. In *I. marginipennis*, most flagellates in the fixed sample were nonspecifically hybridized with this probe. This gave false signals and hampered detecting signals from specific probes. A significant amount of autofluorescence from wood particles also did not allow further FISH analysis using this sample in this study. Therefore, only the fixed sample from *Z. nevadensis* was used for the FISH analysis. A previously published probe DSV698 (Manz et al., 1998), which has a specificity to some *Desulfovibrio* species and has more than two mismatches to any 16S rRNA gene sequences obtained in this study, was used for detection of *Desulfovibrio* bacteria represented by the phylotype ZnvTr-2. Another probe ALF968 (Neef, 1997), which targets a wide range of Proteobacteria but has one significant mismatch to 16S rRNA of *Desulfovibrio*, was used for detection of another δ-proteobacteria represented by the phylotype ZnvTr-3. To exclude the possibility of cross-hybridization, a new specific probe Delta-ZnvTr3 (5'- CTT GAA CCG AAG TTC CTG -3') was used for specific detection of the phylotype ZnvTr-3. When published probes were used, the formamide concentration in the buffer for optimal hybridization conditions was used. 20% formamide concentration was used for hybridization with the Delta-ZnvTr3.

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5. Preparation of genomic DNA from the cell enrichment of "*Candidatus* Endomicrobium trichonymphae" from hindgut contents of *Z. nevadensis*

Abstract

"*Candidatus* Endomicrobium trichonymphae" (CET) was enriched from the gut of *Z. nevadensis* by physical separation. Sedimentation of the *Trichonympha* cells and separation of contaminating bacteria with filters were effective for obtaining the cell enrichment of CET from termite gut contents. The contaminating bacteria in the preparation were able to be reduced to less than 25%. 3.2 µg of high-molecular weight DNA from CET was recovered from the enrichment prepared from 150 termite guts. The extracted DNA was used for genome sequencing project of CET.

Introduction

Many termite gut flagellates contain host-specific endosymbionts "Endomicrobia", which represent a distinct cluster in a novel phylum Termite Group 1 (TG-1). The lifestyle and symbiotic status of this symbiont are completely unknown. Since "Endomicrobia" reside in the cytoplasm of uncultured flagellates, which are strictly anaerobic, it seems challenging to cultivate this symbiont using standard isolation techniques. Physical separation of symbionts from the host flagellates would be therefore much more straightforward to retrieve the cell fraction of "Endomicrobia", which can be further used for investigating their physiological properties. Physical separation of primary endosymbionts of insects such as *Buchnera* or *Wigglesworthia* has been commonly performed by isolating a specialized organ called bacteriome where the endosymbiont cells are densely packed. Filtration using filters with different pore sizes and centrifugation steps have shown to be effective for removing host cell debris and contaminating bacteria from the fraction (Charles and Ishikawa, 1999; Baumann et al., 2006). These bacteriocyte endosymbionts occur in the tissue of their host insect as a bacteriome containing only few other cohabiting bacteria and the resulting cell preparation is therefore almost free of contaminating bacteria. "Endomicrobia", on the other hand, colonize in the cytoplasm of flagellates that can be densely populated by other prokaryotic symbionts (see Chapter 4). Furthermore, one termite gut can harbor more than one phylotypes of gut flagellates, which indicates the occurrence of multiple distinct phylotypes of "Endomicrobia" (Stingl et al., 2005; Ikeda-Ohtsubo

et al., 2007). Therefore, removal of a substantial fraction of flagellates and prokaryotes will be necessary to isolate "Endomicrobia" from termite gut contents. Fortunately, "Endomicrobia" are small rod-shaped bacteria (average length, ~0.6 μm; diameter, ~0.3 μm), and the difference in size from between this symbiont and other larger contaminating bacteria would facilitate the fractionation using filters.

The aim of this study was to obtain the cell enrichment of "Endomicrobia", from which high molecular weight genomic DNA can be yielded. The amount of the genomic DNA should be sufficient for conducting a genome sequencing project of "Endomicrobia". Therefore, the procedure established here should concern not only the purity of "Endomicrobia" in the sample, but also the quantity.

We have selected the symbiont ZnTc-1 occurring specifically in the cytoplasm of large and predominant *Trichonympha* flagellates of *Zootermopsis nevadensis* for this study (Ikeda-Ohtsubo et al., 2007). The ZnTc-1, "*Candidatus* Endomicrobium trichonymphae" (CET) as an official designation, represents the only "Endomicrobia" phylotype in the hindgut of this termite (Stingl et al., 2005). *Trichonympha* flagellates in *Z. nevadensis* comprise four phylotypes, three of which are represented by the described species: *T. collaris*, *T. campanula*, and *T. sphaerica*. All of these *Trichonympha* were shown to harbor the same phylotype of "Endomicrobia", i.e., CET (Ikeda-Ohtsubo et al., 2007; see also Chapter 3 and 7). The hindgut of *Z. nevadensis* also contains other four smaller flagellates: *Trichomitopsis termopsidis*, *Tricercomitus termopsidis*, *Hexamastix termopsidis*, and *Streblomastix strix*.

A previous study suggested that CET may account for 20% of the clones in the cDNA library originating from bacterial 16S rRNA in the total gut of *Z. nevadensis* (Ottesen et al., 2006). 30% of bacteria in manually isolated *Trichonympha* of this termite have been represented by CET (Chapter 4). Other major phylogenetic groups attached to *Trichonympha* cells are two phylotypes of δ-Proteobacteria (>30% of total symbionts), Mycoplasmatales (~10%), and spirochetes (Chapter 4). This chapter explains the procedure of the cell enrichment of CET by fractionation of termite gut contents mainly using filters. The subsequent evaluations of the enrichment and extraction of high-molecular-weight DNA from the enrichment are also documented. A preliminary experiment using density gradient centrifugation with Percoll did not yield sufficient number of "Endomicrobia" cells in the fractions, and is not described further in this chapter.

Estimation of DNA content of CET per termite gut

Before the experiments, the number of termites necessary for this study was estimated by calculation and by cell counts. CET is present in the cytoplasm of all three *Trichonympha* flagellates: *T. collaris*, *T. campanula*, and *T. sphaerica*, which sizes range from 100–300 μm in length and 100–200 μm in width. The relative number of each species can significantly vary among termite individuals (Kirby, 1932; also observed in this study). Given that an average *Trichonympha* cell is a sphere with a diameter of 150 μm (radius=75 μm), the volume of one cell can be estimated as 1.8×10^{-3} mm^3. The volume of the gut fluid from one individual of *Z. nevadensis* is 10–15 μl; this gives an estimate that 5555–8333 *Trichonympha* cells are present in a gut (note that flagellates are highly deformable and packaged in the gut). This number was consistent with the estimation made by counting cells in dilutions of gut contents (~7000 cells/gut, in this study). The number of CET per flagellate varies between different *Trichonympha* species (Chapter 7, Fig. 1). Since "Endomicrobia" appear to have irregular shapes and often form cell aggregates, the resulting number of cell counting of CET in *Z. nevadensis* differed in the range of 10^3–10^5 (Chapter 7). It seems reasonable to estimate that CET accounts for at least 1% of the total volume of a *Trichonympha* host, judging from the FISH micrograph of CET in *Trichonympha* of *Z. nevadensis* (Chapter 7, Fig. 1). When endosymbionts with a size of 1 μm^3 account for 1% of the total volume of an average *Trichonympha* cell, they would be counted as 1.8×10^4 cells per flagellate (calculated from the estimated cell sized of *Trichonympha*, see above). The number of CET per flagellate would be higher than this, considering its smaller size (~0.6 μm diameter). Stingl *et al.* have reported that the number of "Endomicrobia" in a cell of *T. agilis* (~6.5×10^{-5} mm^3, 1/25 of the average size of *Trichonympha* in *Zootermopsis*) from *Reticulitermes santonensis* is about 800 (Stingl et al 2005). In view of this result, 1.8×10^4 cells per *Trichonympha* flagellate estimated here seems to be a reasonable estimation. Using the values described above, the number of CET per gut of *Z. nevadensis* was estimated as $1.0–1.5\times10^8$. Although the DNA content per cell largely depend on its genome size and G+C content, most bacteria seem to contain at least 1.1 femtogram (=10^{-15} g) of DNA per cell (Button and Robertson 2001). Even if the genome of CET is as small as 1 Mb, 0.11–0.16 μg of DNA from CET can theoretically be obtained from a gut of *Z. nevadensis*. The whole genome sequencing of a bacterium requires at least 10 μg of high-molecular-weight DNA, CET cells from at least 60–90 guts of *Z. nevadensis* seemed to be the minimum requirement for this purpose.

Preparation of cytoplasmic fraction from *Trichonympha* cells

Z. nevadensis was originally collected in the Mt. Pinos in California and maintained in a polyethylene container with pine woods and water. Species affiliation of the termite was confirmed by sequencing mitochondrial cytochrome oxidase II gene originating from DNA of termite heads as described previously (Pester and Brune, 2006). The termites were degutted and gut fluid was carefully suspended in solution U (Trager, 1934) in a centrifuge tube. Gut wall fraction and tissue debris from termite was removed thoroughly in order to eliminate contaminating prokaryotes attached to them. When the suspension was incubated on ice for 10 min, *Trichonympha* cells were sedimented at the bottom of the tube (Fig. 1A), whereas smaller flagellates including *Trichomitopsis*, *Tricercomitus*, and *Streblomastix* as well as substantial amount of wood particles stayed in the upper part of the solution (Fig. 1B). Repeating the washing steps with ice-cold solution U (5–6 times) allowed the enrichment of all three species of *Trichonympha* cells (Fig. 1A), in which no other smaller flagellates were observed. This procedure also removed the vast majority of spirochetes, which constitute a major fraction in the gut of this termite. Since *Trichonympha* cells are fragile, these steps should be gently performed at the constant temperature 4 °C. The *Trichonympha* cells were resuspended in isolation buffer (330 mM sorbitol 20 mM MOPS, 13 mM Tris-base, 3 mM $MgCl_2$, 0.1% [w/v] BSA, Prechtl et al., 2004) and readily disrupted by ultrasonication under the condition: 10 cycles of 0.5 seconds ultrasonication at 30 % amplitude (Ultrasonic Processor 50H, Rose). After the sonication, the cytoplasmic fraction was separated from large particles of *Trichonympha* cell debris by a quick centrifugation at 500×g and stained with DAPI and examined by fluorescent microscopy (Fig. 1C).

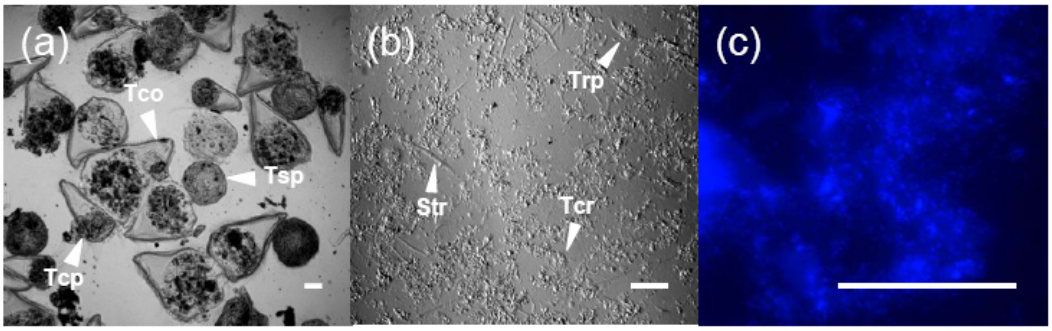

Fig. 1. Micrographs of fractionated samples. Light micrographs of *Trichonympha* cells (Tco, *T. collaris*; Tcp, *T. campanula*; Tsp, *T. sphaerica*) prepared by sedimentation (a) and smaller flagellates (Str, S. strix; Trp, *Trichomitopsis*; Tcr, *Tricercomitus*) remained in the supernatant (b); an epifluorescent micrograph of DAPI-stained cytoplasmic fraction (unfiltered) prepared from disrupted *Trichonympha* cells (c). Bars represent 50 µm.

Filtration series and host-DNA digestion

The cytoplasmic fraction was passed through 18-gauge 40 mm needles twice to dissolve the major cell clumping (Fig. 1C). The fraction was filtered through an 80-μm nylon mesh using a Swinex filter holder (Millipore). This procedure removed large wood particles and cell debris from *Trichonympha*. There was still a large amount of cell clumping consisting of ~100 bacterial cells present in the fraction, which caused low yield of filtered cells after the following separation steps using filters with smaller pore sizes. This problem was able to be avoided by treating the fraction by DNaseI (RQ1 DNase, Promega) for 15 min at 4 °C (Fig. 2A and B). This treatment seemed to be also effective for eliminating a large quantity of free DNA in the fraction, most of which originate from host *Trichonympha* or termite. The fraction was treated with 20 mM EDTA to inhibit the endonuclease activity, and filtered with 20-μm nylon mesh. The following filtration steps were performed using centrifugal filter tubes (Ultrafree-CL, amicon), in order to minimize the dead volume on the filtration system. 2 ml of the fraction was applied onto the filter cup with a 5-μm pore size membrane filter and centrifuged for at 3000 ×g for 20 min at 4 °C. The filtrate was further filtered through a membrane filter with a 0.65-μm pore size at the same condition. These filtration steps were able to remove a large amount of small wood particles as well as larger bacteria, including spirochetes and unidentified rod-shaped bacteria. The centrifugal cup was further centrifuged at 8000×g for 20 min to sediment the bacterial cells in the filtrate. The cell pellet was resuspended in PBS buffer (Chapter 3). The suspension was heated at 65°C for 10 min for the complete inactivation of DNase I. The following procedure was conducted to perform DAPI-staining and fluorescent in situ hybridization (FISH) of the cells in the fractions. Cells in the fraction were harvested by centrifugation and resuspended in PBS buffer. The fixation of cells using paraformaldehyde was performed as described in Chapter 3. Hybridization was conducted by suspending the fixed cells in the hybridization buffer (20% formamide) in a 1.5 ml centrifugation tube. Cells were washed by washing buffer for several times, and resuspended in PBS containing DAPI. The DAPI-stained bacterial cells were aspirated onto polycarbonate membrane filters (0.2 μm pore size, Millipore). The membrane was subsequently washed with 96% ethanol and transferred onto a glass slide. Cells were observed at ×1000 magnification by using an epifluorescent microscope. The probe TG1End1023T1 (Ikeda-Ohtsubo et al., 2007) was used for the detection of CET in the enrichment, while EUB338 (Amann et al., 1990) was used for all bacterial cells in the sample.

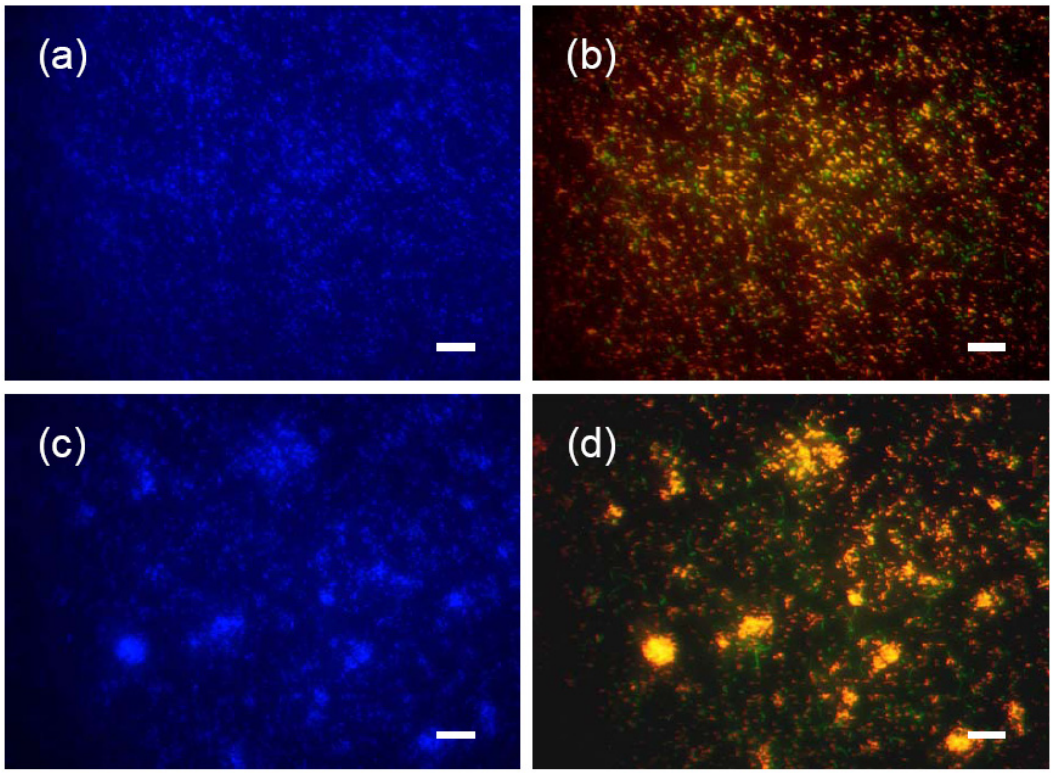

Fig. 2. Epifluorescent micrographs of DNaseI treated (a, b) and untreated (c, d) cells in the fraction after filtrated through a 20-µm pore size membrane filter. Cells were stained with DAPI (a, c) and simultaneously hybridized with fluorescein-labeled EUB338 (bacteria-specific, shown in green) and Cy-3-labeled TG1End1023T1 (CET-specific, shown in orange) probes (b, d). Bars represent 10 µm.

Evaluation of the enrichment of CET

The cells in the final preparation (cell enrichment of CET) were analyzed by FISH as described above. The total DAPI count of bacterial cells recovered from one gut of termite was ~3.0×10^7. The number of CET hybridized with a specific probe was counted in the same sample, which revealed that CET constituted >80% of bacterial cells in the enrichment (1.65×10^3 CET cells among 2.02×10^3 total bacterial cells). To summarize, the described procedure was able to enrich 2.45×10^7 cells of CET per termite gut, which accounts for 24.5% of the estimated value (see above). Most of the contaminating bacteria stained by the bacterial-specific probe EUB338 were small rods or spirochetes.

The CET enrichment was further evaluated using rRNA approach. DNA was extracted from the enrichment using the NucleoSpin kit (Macherey-Nagel, Germany) and eluted with 50 µl water. 1 µl of the extracted DNA was used for PCR amplification of 16S rRNA genes. T-RFLP analysis was conducted as described in Chapter 4. In this experiment, the T-RFs of 16S rRNA genes obtained from the final cell enrichment was compared with that from the cell

fraction before the filtration steps. As shown in Fig. 3a, a single prominent peak at 281 bp was detected in the final cell enrichment, which length was predicted as the fragment from 16S rRNA gene of CET. δ-Proteobacteria ZnTr-3 (Chapter 4) represented by the T-RF of 160 bp, constituted a major group of bacteria in the cytoplasmic fraction of *Trichonympha* before being applied to the filtration steps (Fig. 3b). Considering that the ZnTr-3 symbionts as well as *Desulfovibrio* symbionts (ZnTr-2) attached to the surface of *Trichonympha* flagellates, they have probably been substantially removed from the fraction during the sedimentation of the host cell debris. Many contaminating bacteria occurring in the cytoplasm of *Trichonympha* (Chapter 4) seem to have been eliminated by the filtratiotion steps.

16S rRNA gene clone library from the DNA from the CET enrichment was constructed as described in Chapter 4. Clones were sorted by their restriction patterns with *Msp*I as described in Chapter 4. Of 24 clones, 18 clones were assigned to CET, which accounted for 75% of the 16S rRNA gene library. This result corresponds to the result of FISH and T-RFLP analyses. 4 clones were assigned to ZnTr-3, confirming the major contamination by this group of bacteria.

CET cells were successfully enriched from the gut contents of *Z. nevadensis*. Although there was a certain amount of contamination by δ-Proteobacteria (ZnTr-3), the majority of small flagellates and non-CET bacteria were removed from the enrichment by fractionation steps. This result suggests that CET cells were stable in the buffer solutions used in this study and tolerant for several physical stresses during the filtration and resuspention procedures. The ultrastructure of "Endomicrobia" studied in *R. santonensis* showed that this symbiont is surrounded by two membranes (Stingl et al., 2005). The authors questioned about the condition of the outermost membrane, whether it is the typical outer membrane of gram-negative cell or formed by the host (Stingl et al., 2005). The latter case would not be the case, since the stability that CET cells retained outside of the host cell was observed in this study. However, there is still a possibility that CET owes its lipid bilayer to the host, as *Buchnera* symbionts of aphids or mitochondria, which apparently depend on the host cell for the biosynthesis of the phospholipids components (Shigenobu et al., 2000). Although the membrane structure of "Endomicrobia" has not been closely studied, a recent microscopic study of a strain Pei191, the first isolate from the phylum TG-1, suggests that they possess a unique membrane structure (Geissinger et al., unpublished results).

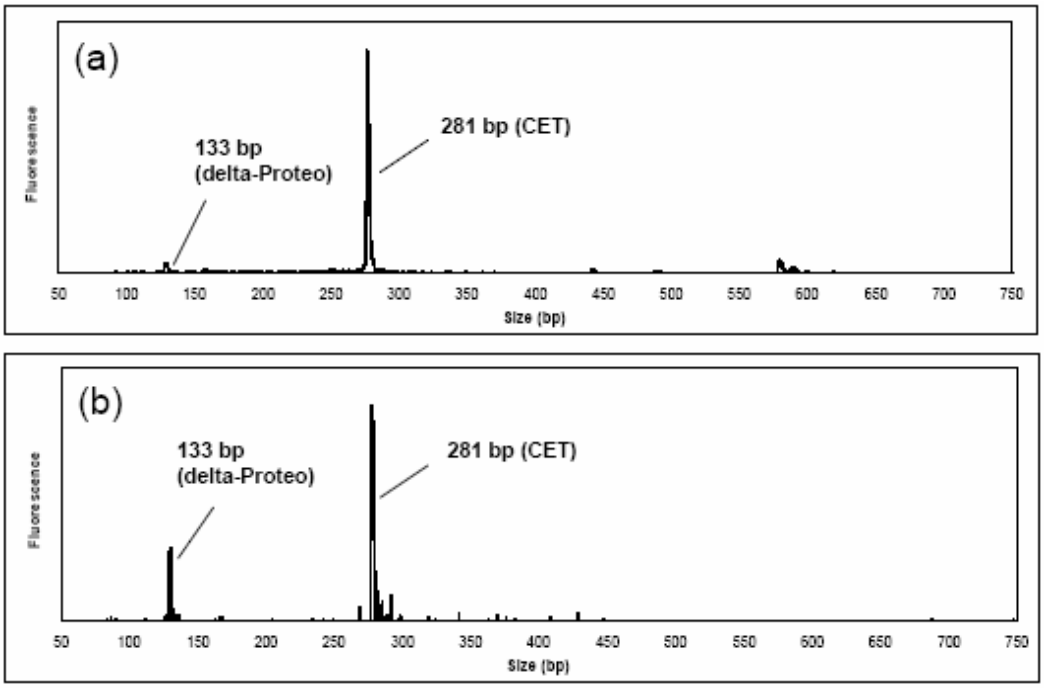

Fig. 3. T-RFLP profiles of 16S rRNA genes amplified from DNA extracted from the cell enrichment of CET (a), and from the unfiltered cytoplasmic fraction (b).

Genomic DNA extraction from the cell enrichment of CET

High-molecular-weight DNA was extracted from the cell enrichment of CET as following procedure. The enriched cells was pelleted by centrifugation and resuspended in TE buffer (10 mM Tris-HC1, 1 mM EDTA, pH 8.0). For cells in 567 µl TE buffer, 30 µl of 10%SDS and 3 µl of proteinase K (20 mg/ml) were added and well mixed. The mixture was incubated at 37°C for 1 h to until the cells are lysed. DNA from the lysate was extracted with the same volume of the phenol-chloroform-isoamyl alcohol (49:49:1) and the phases were separated by microcentrifugation at 10,000×g for 10 min at 20 °C using Phase Lock Gel tubes (Eppendorf AG, Hamburg, Germany). The extraction was repeated three times. The supernatant was transferred into a fresh tube and 0.6 volume of isopropanol was added. The DNA was pelleted by centrifugation at 10,000×g for 10 min at 4 °C. The pellet was washed in ice-cold 80% (vol/vol) ethanol and air-dried. The pellet was dissolved in 1 ml TE buffer and the quality and quantity was checked by electrophoresis on a 0.8% agarose gel at 120 mV for 1 h. The lambda DNA/*Hin*dIII marker was prepared by digesting 5 µg of lambda DNA with *Hin*dIII for 2 h at 37 °C. As shown in Fig. 4, a clear band of high-molecular-weight DNA (>23 kb) was observed on the gel, whereas the amount of eukaryotic host DNA, which often appear as a smear band in the range of 1–10 kb, was not significant. RNA in the sample, which appeared as multiple bands of ~1 kb (Fig. 4), was subsequently

digested with RNase (Promega) for 1h at 37 °C. Fig. 4 shows the high-molecular DNA (0.5, 1.0, 5.0 µl from 1 ml preparation) extracted from 150 termites. The concentration of DNA was 40–50 ng/µl, indicating 4 µg DNA is present in this preparation. Since CET constitutes ~80% of total bacteria in the sample (see above), 3.2 µg DNA/150 termites was obtained in this study. This accounts for ~16% of the first estimation calculated above (10 µg of CET DNA/60–90 termites). Additional 8 µg DNA was subsequently prepared using the same procedure from 400 termites. The DNA prepared in this study pelleted and shipped to Joint Genome Institute (JGI, Walnut Creek, Calif.) for the Community Sequencing Program (CSP, Project ID 4043170) funded by US Department of Energy.

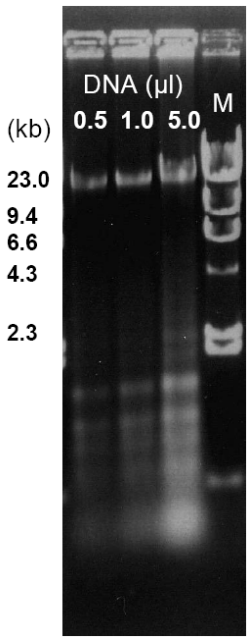

Fig. 4. High-molecular-weight DNA extracted from the cell enrichment of CET. Different volume (0.5, 1.0, 5.0 µl per lane) of the DNA preparation was applied on a 0.8% agarose gel and electrophoresed to illustrate the quality and quantity. The marker is prepared with lambda DNA digested with *Hin*d III (500 ng).

Appendix: DNA extraction from *Elusimicrobium minutum* Pei191

Elusimicrobium minutum Pei191 is the first cultivated representative from the TG-1 phylum, which was recently discovered from the hindgut of the humivorous beetle larva of *Pachnoda ephippiata* (a scarab beetle) using a filtration technique (Geissinger et al., in preparation). It is a tiny (<0.4 µm), slow-growing, glucose-fermenting motile rod, which morphology is similar to that of "Endomicrobia". It can utilize glucose and produces acetate, ethanol, and hydrogen. The genomic and physiological properties of this strain would provide an important opportunity to

estimate the metabolic potential of TG-1 bacteria including the uncultivated "Endomicrobia". Furthermore, comparing the conserved structure and properties of genomes between the free-living strain Pei191 and the endosymbiotic CET may provide valuable insights into the evolutionary events leading from the free-living to the symbiotic lifestyle. For these reasons, the genome sequencing project of the strain Pei191 has been performed simultaneously with the genome project of CET, which was described in Chapter 5 and 6 in this thesis. Genome sequencing of a cultured bacterium requires 75–100 µg genomic DNA from the strain, since the sequencing is usually performed using 40-kb clone library at the JGI. To obtain a sufficient amount of high-molecular-weight DNA from Pei191, 400 ml of a well-grown culture of this strain was used for DNA extraction. The extraction was performed as described in Chapter 5. The DNA pellet was collected using a glass rod and air-dried. DNA was finally dissolved in 4 ml Tris-EDTA buffer at 4 °C for 3 days. Fig. 3 shows the gel image of the DNA of Pei191. The concentration of the DNA preparation was approximately 1 µg/µl (4 mg genomic DNA in total). 1 mg of the DNA was pelleted and shipped to Joint Genome Institute (JGI, Walnut Creek, Calif.) for the Community Sequencing Program (CSP, Project ID 4043170) funded by US Department of Energy.

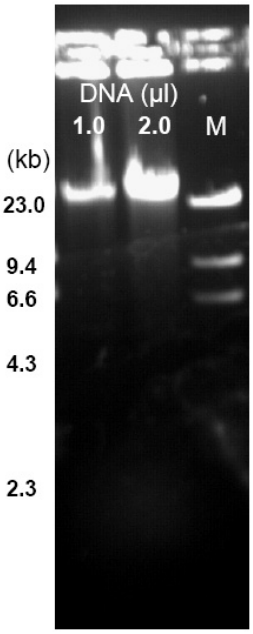

Fig. 4. High-molecular-weight DNA extracted from the culture of the strain Pei191. The genomic DNA (1 or 2 µl/lane) was analyzed on a 0.8% agarose gel to illustrate the quality and quantity. The markers are lambda DNA digested with Hind III (500 ng).

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6. Preliminary characterization of a partial genome sequence from the intracellular symbiont "*Candidatus* Endomicrobium trichonymphae"

A brief report of the ongoing genome project

Abstract

This chapter describes a preliminary characterization of an 80 kb contig of the genome of "*Candidatus* Endomicrobium trichonymphae" (CET), which was recently recovered from the metagenomic library constructed from DNA prepared from the enrichment of CET. 52 ORFs and a set of rRNA genes were identified. The average G+C content of the ORFs is 37%. ~30% of the ORFs showed the highest similarities to the corresponding genes in the genome of a strain *Elusimicrobium minutum* Pei191, the first isolate in the phylum "Termite Group 1" (TG-1), supporting that the contig represents the CET genome. A large number of ORFs in this fragment encode proteins involved in transcription and translation. Two ORFs show similarities to genes encoding enzymes for biosynthesis of coenzymes. Seven ORFs adjacent to the 16S rRNA gene are highly homologous to a set of genes that encode proteins responsible for uptake and catabolism of uronic acids. Three ORFs are similar to genes of CRISPR-associated proteins, which are also found in the genome of strain Pei191.

Introduction

"Endomicrobia" represent widely distributed intracellular symbionts of termite gut flagellates, which physiological properties are totally unknown. The metagenomic approach has been employed to understand their functional properties from their genomic information, since these symbionts cannot be cultivated by standard techniques. As described in Chapter 5, CET was enriched from the gut contents of the termite *Z. nevadensis* and the metagenomic library was constructed using DNA extracted from the enrichment. The sequencing of 60 Mb was conducted in Joint Genome Institute (JGI) in Walnut Creek in California. The sequences were assembled into a large number of contigs, which were subsequently sorted by the binning program Phylopythia to exclude sequences derived from contaminating bacteria in the sample (McHardy et al., 2007). This process was able to assign a

substantial amount of genomic sequences to CET. An 80 kb contig containing 16S rRNA of CET was recovered from the assembly of these sequences. Preliminary characterization of this contig is briefly described in this chapter.

Result and discussion

General overview

49 ORFs in the 80 kb contig have been identified by the automated annotating program in JGI, and additional 3 ORFs were found manually using IMG Genome Blast search (Fig. 1). The average G+C content of ORFs was 37%. This value is higher than that has been found in the genomes of endosymbiotic bacteria in insects (<30% G+C) and slightly lower than that of the strain Pei191 (40%), which is a free-living representative of Termite Group 1. The three rRNA genes retain a typical arrangement (16S-23S-5S) that is commonly found in prokaryotes. Most ORFs could be assigned to a COG (Cluster of Orthologous Groups, Table 1), which allowed sorting them into functional categories (Table. 2). The BLASTX search using a current IMG database containing the genome sequences of Pei191 showed that 15 ORFs have their most homologous ORFs in the genome of Pei191 (Table 1, shown in bold).

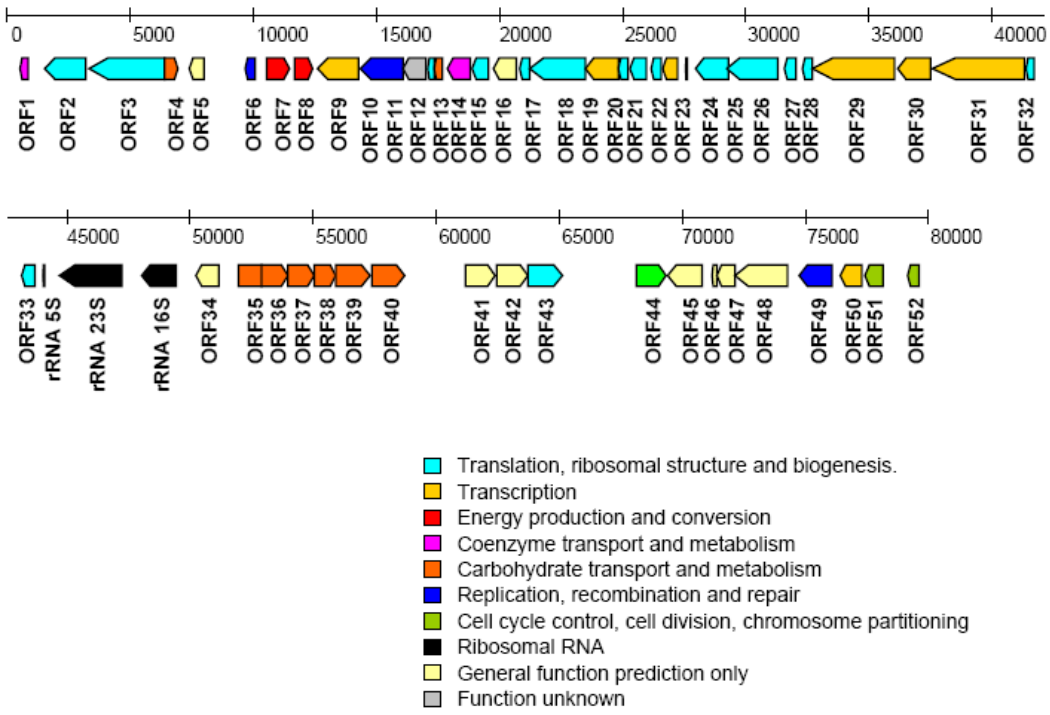

Fig. 1. Schematic representation of the 80 kb contig consisting of genomic sequences from CET. The color of the arrows represents the category of predicted functions of COG for each ORF. The scale represents the length of the sequence (bp).

Almost the half of ORFs encodes putative proteins involved in transcription or translation, which are commonly retained by most prokaryotes. Many obligate endosymbionts commonly possess seven conserved genes responsible for transcription, which encode four individual subunits of RNA polymerase and associated proteins NusA (termination), NusG (elongation) and GreA (elongation) (Klasson and Andersson, 2004). Among them, two beta subunits (ORF29, 30, 31) and one sigma subunit (ORF9) of RNA polymerases, NusA (ORF19), and NusG (ORF23) were found in this contig.

Energy metabolism

Two ORFs were highly homologous to the genes encoding alpha- and beta-subunit of the pyruvate:ferredoxin oxidoreductase (PFOR), which belongs to one of four groups of 2-oxoacid:ferredoxin oxidoreductase (Fukuda and Wakagi, 2002). This enzyme reversibly catalyzes the decarboxylation of pyruvate converting to acetyl-CoA, involving ferredoxin or flavodoxin as an electron acceptor. This enzyme is found in most anaerobic bacteria such as *Clostridium*, in which the electrons from the reduced ferredoxin are transferred to protons by H_2-evolving hydrogenase, resulting in the formation of H_2 (Kaji et al., 1999). Amitochondriate eukaryotes including parabasalid flagellates such as *Trichomonas vaginalis* also harbor pyruvate:ferredoxin oxidoreductase, which is involved in the energy metabolism of hydrogenosomes (Horner et al., 1999). It is not known whether CET produces H_2, but the strain Pei191 have been shown to produce H_2 (Geissinger et al. unpublished result).

Hexuronate catabolism

Six ORFs (ORF35–40) comprising a putative transcription unit were found to encode putative proteins involved in hexuronate catabolism. Hexuronates are widely distributed as a major component of plant cell wall and can be utilized by a wide range of bacterial groups as a single source of carbon and energy (Rivolta et al., 1998). These bacteria uptake hexuronates via specific transporters and hexuronates are converted to 2-keto-3-deoxygluconate (KDG), which is subsequently phosphorylated and catabolised via glycolysis or the pentose phosphate pathway. ORF40 encodes homologs of the gene products of KdgT, a glucuronate permease in *Escherichia coli*, and ORF37, 38 and 39 are homologous to *uxuA*, *uxuB*, and *uxaC* in *E. coli*, which encode proteins involved in the conversion of glucuronate to KDG via isomerization to fructuronate. ORF35 and 36 are homologous to genes encoding 2-keto-3-deoxygluconate kinase and 2-Keto-3-deoxy-6-phosphogluconate aldolase, respectively, which catalyzes the final steps converting KDG to pyruvate via phosphorylation (Entner-Doudoroff pathway).

UxuR, a regulator of the uxuAB operon in *E. coli* responsible for the catabolite repression was not identified within this contig. Glucuronates are often found to be associated with xylan (Kormelink et al., 1993), which is a major component of hemicelluloses. It can be assumed that CET could utilize the set of genes for degrading glucuronates released from hemicellulose-containing wood particles digested by *Trichonympha* host. Since wood contains low content of pectin (~4% Green and Clausen, 1999), galacuronates could also be taken up by CET and utilized. The set of homologs identified here have not been found in the genome of Pei191. This also indicates that these genes are specifically conserved in the endosymbionts, which occur in the cytoplasm of wood-digesting flagellates.

CRISPR

Three ORFs were identified as members of “CRISPR-associated proteins”. CRISPR (clustered regularly interspaced short palindromic repeats) consists of direct repeats from 21 to 47 bp, interspersed with similarly sized nonrepetitive sequences, which have been found in noncoding chromosomal regions in a wide range of prokaryotes (Godde and Bickerton, 2006). CRISPR-associated (Cas) protein families are found only in species containing CRISPR, and always located near a repeat cluster (Haft et al., 2005). Although there has been no conclusive evidence for functional roles of CRISPR or Cas proteins in prokaryotic cells, the structure of CRISPR elements implies the defense system against bacteriophage, whereas Cas proteins are thought to be involved in DNA and RNA metabolisms (Mojika et al., 2005). ORF 46, 47, and 48 are homologous to proteins Csn1, Cas1, and Cas2, respectively, and the same set of ORFs with the same arrangement is also present in the genome of Pei191. CRISPR elements were not found in the adjacent region when analyzed by CRISPRFinder (Grissa et al., 2007).

Others features

Two ORFs are homologs of genes responsible for coenzyme biosynthesis. ORF1 is highly homologous to thiamine biosynthesis enzyme ThiH and related uncharacterized enzymes. ORF14 is a homolog of FAD synthase, which are usually found as a bifunctional complex with another key enzyme, flavokinase. A substantial number of genes responsible for biosynthesis of cofactors have been found in *Wigglesworthia glossinidia*, an endosymbiont of tsetse flies (Akman et al., 2002). The abilities to synthesize amino acids or co-factors are thought to contribute to the fitness of the host organism.

Table 1. Description of predicted open reading frames and rRNA genes identified in the 80 kb fragment of CET.

ORF	Locus (bp)	Size (aa)	Putative function (COG accession number)	Species with the highest identity* (Phylogenetic group)	Identity (%)
1	1552–1881	109	Thiamine biosynthesis enzyme ThiH and related unc haracterized enzymes (COG1060)	*Thermotoga maritima* (Thermotogae)	63
2	2511–4247	578	Threonyl tRNA synthetase (COG0441)	Desulfotalea psychrophila (*Deltaproteobacteria*)	57
3	4379–7418	1013	Glycyl tRNA synthetase beta subunit (COG075)	*Protochlamydia amoebophila* (Chlamydiae)	39
4	7494–7986	163	Deoxycytidylate deaminase (COG2131)	*Dehalococcoides ethenogenes* (Chloroflexi)	61
5	8428–9070	213	Predicted phosphatases (COG0546)	*Solibacter usitatus* (Acidobacteria)	40
6	10748–11159	136	G:T/U mismatch specific DNA glycosilase (COG3663)	*Actinobacillus pleuropneumoniae* (*Gammaproteobacteria*)	35
7	11525–12560	345	Pyruvate:ferredoxin oxidoreductase and related 2 oxoacid:ferredoxin oxidoreductases, alpha subunit (COG0674)	*Halothermothrix orenii* (Firmicutes)	56
8	12743–13492	250	Pyruvate:ferredoxin oxidoreductase and related 2 oxoacid:ferredoxin oxidoreductases, beta subunit (COG1013)	*Thermoanaerobacter ethanolicus* (Firmicutes)	63
9	13593–15327	578	DNA-directed RNA polymerase, sigma subunit, sigma70/sigma32 (COG0568)	***Elusimicrobium minutum* Pei191 (Termite group 1)**	53
10	15339–17088	583	DNA primase bacterial type (COG0358)	*Thermoanaerobacter tengcongensis* (Firmicutes)	34
11	17091–18051	320	Uncharacterized conserved protein (COG0327)	*Desulfotomaculum reducens* (Firmicutes)	49
12	18166–18343	59	Ribosomal protein S21	***Elusimicrobium minutum* Pei191 (Termite group 1)**	55
13	18394–18742	116	Diadenosine tetraphosphate (Ap4A) hydrolase and other HIT family hydrolases (COG0537)	*Desulfuromonas acetoxidans* (*Deltaproteobacteria*)	59
14	18957–19842	295	FAD synthase (COG0196)	*Clostridium difficile* (Firmicutes)	35
15	19920–20613	231	Pseudouridine synthase (COG0130)	*Dehalococcoides* sp. (Chloroflexi)	47
16	20759–21746	328	Exopolyphosphatase-related proteins (COG0618)	*Clostridium acetobutylicum* (Firmicutes)	37
17	21892–22300	136	Ribosome-binding factor A (COG0858)	*Nostoc* sp. (Cyanobacteria)	39
18	22324–24565	746	Translation initiation factor 2 (IF-2; GTPase) (COG0532)	*Desulfotomaculum reducens*	55
19	24582–25839	418	Transcription elongation factor (COG0195) Transcription termination factor NusA	***Elusimicrobium minutum* Pei191 (Termite group 1)**	43
20	25853–26315	154	Uncharacterized protein conserved in bacteria (COG0779)	*Clostridium phytofermentans* (Firmicutes)	43
21	26391–27069	225	Ribosomal protein L1 (COG0081)	***Elusimicrobium minutum* Pei191 (Termite group 1)**	59
22	27223–27658	144	Ribosomal protein L11 (COG0080)	*Pelobacter propionicus* (*Deltaproteobacteria*)	69
23	27730–28261	176	Transcription antiterminator NusG (COG0250)	***Elusimicrobium minutum* Pei191 (Termite group 1)**	54
24	28603–28744	47	Ribosomal protein L33 pfam00471	***Elusimicrobium minutum* Pei191 (Termite group 1)**	63
25	29041–30341	399	Translation elongation factors Tu (COG0050)	*Anaeromyxobacter dehalogenans* (*Deltaproteobacteria*)	74
26	30277–32336	686	Translation elongation factors (GTPases) Factor G (COG0480)	*Moorella thermoacetica* (Firmicutes)	67
27	32646–33126	159	Ribosomal protein S7 (COG0049)	***Elusimicrobium minutum* Pei191 (Termite group 1)**	63
28	33400–33796	132	Ribosomal protein S12 (COG0048)	*Nostoc punctiforme* (Cyanobacteria)	82

Table 2. continued

ORF	Locus (bp)	Size (aa)	Putative function (COG accession number)	Species with the highest identity* (Phylogenetic group)	Identity (%)
30	37206–38565	452	DNA-directed RNA polymerase, beta' subunit/ 160 kD subunit (COG0086)	***Elusimicrobium minutum* Pei191 (Termite group 1)**	51
31	38623–42342	1240	DNA-directed RNA polymerase, beta subunit/ 140 kD subunit (COG0085)	***Elusimicrobium minutum* Pei191 (Termite group 1)**	57
32	42446–42826	126	Ribosomal protein L7/L12 (COG0222)	***Elusimicrobium minutum* Pei191 (Termite group 1)**	70
33	43214–43729	171	Ribosomal protein L10 (COG0244)	*Halothermothrix orenii* (Firmicutes)	47
RNA	44070–44118		5S ribosomal RNA		
RNA	44696–47258		23S ribosomal RNA		
RNA	48016–49457		16S ribosomal RNA		
34	50277–51182	302	Uncharacterized Fe-S protein PflX, homolog of pyruvate formate lyase activating proteins (COG1313)	*Geobacter sulfurreducens* (*Deltaproteobacteria*)	48
35	52006–53304	333	2-keto-3-deoxy-6-phosphogluconate aldolase (COG0800)	*Clostridium phytofermentans* (Firmicutes)	57
36	52989–54014	341	Sugar kinases, ribokinase family (COG0524)	*Treponema denticola* (Spirochaetes)	61
37	54022–55116	364	D-mannonate dehydratase (COG1312)	*Clostridium beijerincki* (Firmicutes)	65
38	55103–55937	277	Dehydrogenases with different specificities (related to short chain alcohol dehydrogenases) (COG1028)	*Bacillus* sp. (Firmicutes)	56
39	55959–57365	468	Glucuronate isomerase (COG1904)	*Clostridium phytofermentans* (Firmicutes)	67
40	57417–58821	468	Sugar phosphate permease (COG2271)	*Flavobacterium johnsoniae* (Bacteroidetes)	50
41	61204–62427	408	Predicted Zn-dependent peptidases (COG0612)	*Nostoc* sp. (Cyanobacteria)	
42	62514–63754	414			30
43	63775–65220	481	tRNA nucleotidyltransferase/poly(A) polymerase (COG0617)	***Elusimicrobium minutum* Pei191 (Termite group 1)**	35
44	68212–69399	396	Collagenase and related proteases (COG0826)	*Roseobacter* sp. (*Alphaproteobacteria*)	59
45	69481–70872	464	ATPase components of ABC transporters with duplicated ATPase domains (COG0488)	***Elusimicrobium minutum* Pei191 (Termite group 1)**	54
46	71261–71440	60	CRISPR-associated protein Cas2	*Helicobacter pylori* (*Epsilonproteobacteria*)	63
47	71479–72169	230	CRISPR-associated protein Cas1	***Elusimicrobium minutum* Pei191 (Termite group 1)**	38
48	72172–74382	737	CRISPR-associated protein, Csn1 family	***Elusimicrobium minutum* Pei191 (Termite group 1)**	46
49	74816–76090	424	ATP-dependent exoDNAse (exonuclease V), alpha subunit helicase superfamily I member (COG0507)	*Bacteroides fragilis* (Bacteroidetes)	41
50	76493–77353	286	Predicted transcriptional regulators (COG1475)	*Myxococcus xanthus* (*Deltaproteobacteria*)	44
51	77418–78182	254	ATPases involved in chromosome partitioning (COG1192)	*Listeria monocytogenes* (Firmicutes)	56
52	79191–79720	176	Predicted phosphatase/phosphohexomutase (COG0637)	*Methanosarcina barkeri* (Euryarchaeota)	37

* Organisms in the IMG database showing the highest score at BLASTX (IMG Genome BLAST)

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7. Phylogenetic placement of *Trichonympha* and other parabasalid flagellates in the wood-feeding cockroach *Cryptocercus punctulatus*

Abstract

Phylogenetic placement of parabasalid flagellates including *Barbulanympha* and *Trichonympha* in the gut of wood-feeding cockroach *Cryptocercus punctulatus* were determined based on small-subunit rRNA (SSU rRNA) genes. The SSU rRNA sequence obtained from manually isolated *Barbulanympha* clustered together with *Hoplonympha* sp. Phylotypes that are closely related to *Monocercomonas* in termite guts were also identified from the whole gut DNA. Interestingly, sequences obtained from *Trichonympha* and from the whole gut collectively formed a distinct cluster, branching out from the phylogenetic tree of Parabasalia. This indicates that flagellates that have been described as *Trichonympha* species in *C. punctulatus* actually belong to a novel lineage of Parabasalia, which is only distantly related to previously known parabasalid flagellates. The SSU rRNA gene sequence from "Endomicrobia", which frequently occur as endosymbionts of termite gut flagellates, was also obtained from the isolated "*Trichonympha*" of *C. punctulatus*. The sequence could not be placed into the *Trichonympha*-specific cluster consisting of symbionts from the termite gut *Trichonympha*, supporting that the *Trichonympha*-like flagellates from this cockroach are not affiliated with *Trichonympha*.

Introduction

A wood-feeding cockroach *Cryptocercus punctulatus* shares many properties with lower termites, which are particularly characterized by the presence flagellate protozoa in the gut. The flagellates found in *C. punctulatus* have been classified into twelve genera and twenty-five species according to their morphology (Cleveland et al., 1934). Although the flagellates in *C. punctulatus* have been thought to be affiliated with the same types of organisms as those of lower termites, these flagellates are poorly studied with respect to their phylogenetic placements. Phylogenetic positions of termite gut flagellates have been widely studied using SSU rRNA genes. The phylogenetic evidence has revealed the polyphyly of Hypermastigea which has been traditionally classified into a class consisting of

parabasalid flagellates with highly developed cellular structures (Ohkuma et al., 2005). Recently, a SSU rRNA sequence from a parabasalid flagellate *Eucomonympha imla* has been determined by separating the cells from the gut contents of this cockroach. The sequence was placed into a cluster of the order Trichonymphida, which comprises different termite gut symbionts including *Eucomonympha* from *H. sjoestedti* (Carpenter and Keeling, 2007; also see Fig. 2). While this study confirmed that *C. punctulatus* and termites can share flagellates in the same phylogenetic groups, the new sequence of *E. imla*, which was placed outside of two genera *Eucomonympha* and *Teranympha*, has raised a question about the present phylogenetic assignment of parabasalid flagellates.

Besides *Eucomonympha*, *Trichonympha* is another parabasalid flagellate that has been described both in termites and *C. punctulatus* (Cleveland et al., 1934). Cleveland documented that there are seven *Trichonympha* species in *C. punctulatus*, which differ in sizes and organelle structures. They share a lot of morphological features with *Trichonympha* in the termite gut, except that the presence of sexual cycles is prominent only in the *Trichonympha* of the cockroach (Cleveland, 1949; 1965). The comparative analysis of phylogenetic positions of *Trichonympha* from termites with those of *C. punctulatus* may provide insights into the divergence of this group of flagellates and also into the history of the acquisition of flagellates by these insects. In this study, the phylogenetic position *Trichonympha* flagellates of *C. punctulatus* of SSU rRNA gene sequences obtained from manually isolated cells. Additionally, the phylogeny of *Barbulanympha* and other gut flagellates in this cockroach was also investigated.

Results and discussion

The whole gut DNA of *C. punctulatus* was prepared as described previously (Ikeda-Ohtsubo et al., 2007). *Trichonympha* and *Barbulanympha* flagellates, besides *E. imla*, appear as conspicuous members in the gut of this cockroach, for their large sizes (100–200 μm length) and high density. DNA from each population was prepared from manually isolated cells and used for the amplification of SSU rRNA gene sequences. The experimental procedure of cloning of SSU rRNA gene sequences was performed as described previously (Ikeda-Ohtsubo et al., 2007). The six new SSU rRNA sequences obtained in this study were shown to be affiliated with Parabasalia by initial BLASTn searches in GenBank database. These sequences were therefore aligned with sequences of other parabasalid flagellates in the database and used for the maximum-likelihood (ML) inference (Fig. 1). The SSU rRNA sequence obtained from *Trichonympha* (clone CpEuk1) clustered neither with sequences of any known *Trichonympha* sequences nor those of

parabasalid flagellates in the database, and branched out from all other parabasalids (Fig. 1). This suggests that the sequence of CpEuk1 represents a novel lineage, which is apparently not affiliated with *Trichonympha* (*Trichonympha*-like flagellate). The sequence obtained from *Barbulanympha* (clone CpEuk7) clustered together with *Hoplonympha* sp. from the termite *H. sjoestedti*. This is in agreement with the previous morphological classification where the genus *Barbulanympha* was placed together with *Hoplonympha* into the family Hoplonymphidae (Light, 1926). The SSU rRNA sequences of the clones CpEuk2 and CpEuk4 obtained from the whole gut DNA of *C. punctulatus* were placed into two distinct undiscribed clusters, both of which contain *Monocercomonas*-type flagellates (Fig. 1). The sequences of the other three clones CpEuk3, CpEuk6 and CpEuk9 from the whole gut clustered with each other, and further clustered together with the "branched-out" sequence of CpEuk1 from the *Trichonympha*-like flagellate (Fig. 1).

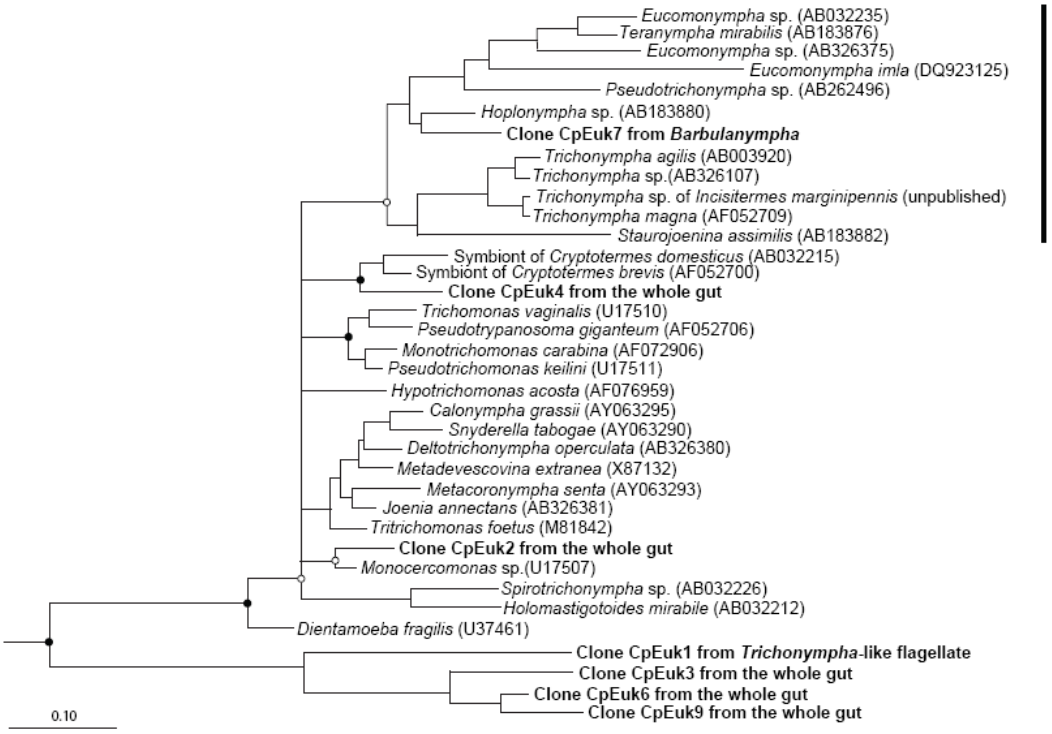

Fig. 1. Maximum likelihood tree of Parabasalia based on SSU rRNA. Sequences obtained in this study from the wood-feeding cockroach *C. punctulatus* are marked in bold. Nodes with bootstrap values (DNAPARS, 1000 replicates) with >90% (●) and >50% (○) are marked. The vertical bar indicates the order Trichonymphida.

Considering that the flagellates described as *Trichonympha* in *C. punctulatus* consist of seven species (Cleveland, 1934), the sequences of the clones CpEuk3, CpEuk6 and CpEuk9 may also originate from another species of *Trichonympha*-like flagellates, which morphologically differ from the flagellate isolated in this

study. The distinct phylogenetic position of this novel lineage indicates that these flagellates represent the most early-branching parabasalian clade that may have branched out before the divergence of Parabasalia. Nevertheless, despite their distinct phylogenetic position from that of other known parabasalid flagellates, the *Trichonympha*-like flagellates in *C. punctulatus* retain typical morphological features of parabasalids such as the unique flagellar/cytoskeletal apparatus and the presence of a parabasal body (Brugerolle and Lee, 2000). This discrepancy may have arisen from the insufficient resolution of SSU rRNA as a phylogenetic marker. The phylogenetic position of this new lineage determined by the SSU rRNA sequence seems to have been significantly affected by the unique nucleotide patterns in the aligned regions and the frequently-occurring poorly-aligned regions. On the other hand, ~30% of the sequence of this flagellate displayed high partial sequence identities (78–93%) to those of known parabasalid flagellates (BLASTn), indicating that these flagellates could essentially be affiliated with a typical parabasalid flagellate. Therefore, phylogenetic analyses using additional phylogenetic markers such as actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or alpha- and beta-tubulin (Ohkuma et al., 2007), in order should be performed in future studies to obtain more precise phylogenetic estimation of this group of flagellates.

In termite guts, a group of bacteria "Endomicrobia" occurs as a host-specific intracellular symbiont of gut flagellates (Stingl et al., 2005). It has recently been shown that "Endomicrobia" are specifically affiliated with their flagellate host (Ikeda-Ohtsubo et al., 2007). Although the occurrence of this symbiont in the gut of *C. punctulatus* has been shown by diagnostic PCR using "Endomicrobia"-specific primers (Stingl et al., 2005), it is still unclear which flagellates in this cockroach possess this symbiont. The SSU rRNA gene sequences of "Endomicrobia" were amplified from the DNA from the *Trichonympha*-like flagellates, and two phylotypes (sequences with more than 1% divergence) represented by the clones CpEm1 and CpEm4 were identified (Fig. 2). Whereas sequences previously obtained from *Trichonympha* of termites collectively form a *Trichonympha*-specific cluster (Fig. 2; Ikeda-Ohtsubo et al., 2007), the sequences from these *Trichonympha*-like flagellates were not placed into this cluster but clustered together with "Endomicrobia" from *Joenia* of the termite *K. flavicollis*. Short sequences (Cp-10, 12, 13, 16, 18, and 26) previously obtained from the whole gut DNA of *C. punctulatus* were also fell into this cluster (Stingl et al., 2005). This result supports that the *Trichonympha*-like flagellates in *C. punctulatus* are phylogenetically distinct from *Trichonympha* of termites.

In summary, this study determined the phylogenetic position of novel lineages parabasalid flagellates in the cockroach *C. punctulatus*. This includes the first

phylogenetic evidence of *Trichonympha*-like flagellates from this cockroach forming a novel lineage only distantly related to all other parabasalid flagellates.

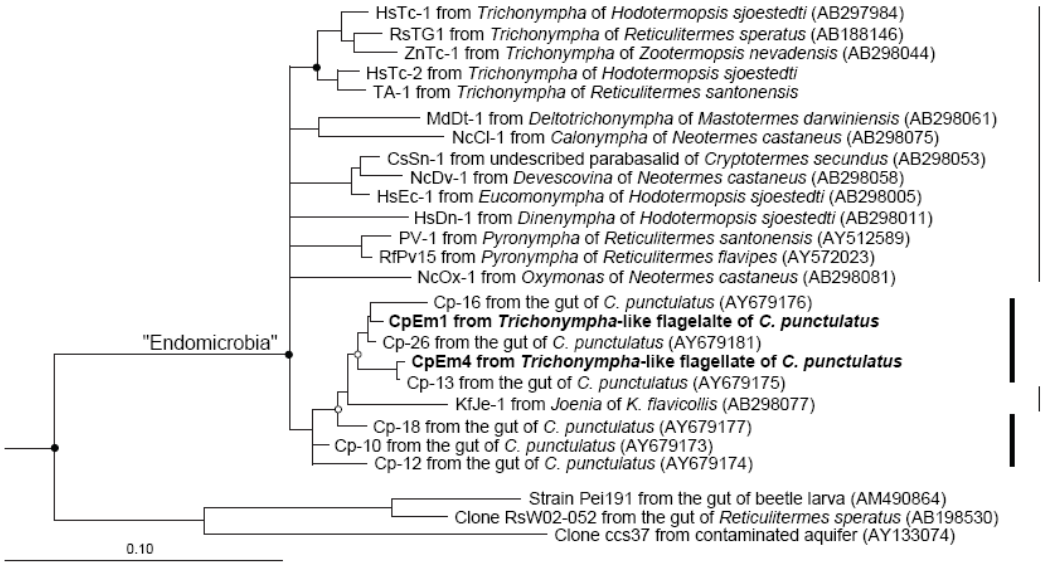

Fig. 2. Phylogenetic position of the CpEm1 and CpEm4 obtained from the *Trichonympha* (phylotype CpEuk1) of *C. punctulatus.* Sequences are designated by the clone name and their origin. Sequences obtained in this study are marked in bold. The thick vertical bars on the right indicate the clones originating from the cockroach *C. punctulatus*; thin bars indicate the clones originating from termites. The SSU rRNA tree was inferred by maximum-likelihood method using the ARB AxML. Shorter sequences were added to the tree using ARB parsimony tool. Nodes with bootstrap values (DNAPARS, 1000 replicates) with >90% (●) and >50% (○) are marked.

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8. General discussion

"Endomicrobia": mutualistic symbionts or parasites?

This study provided important perspectives on symbiotic properties of "Endomicrobia" in termite gut flagellates: wide distribution among a wide range of hosts in different termite families, consistent occurrence in the cytoplasmic space, host-specific association and simultaneous divergence with their host (Chapter 2 and 3). These properties are typically seen among obligate endosymbiotic organisms, which lifestyles are largely dependent on their specific hosts. Therefore, the current evidence collectively emphasizes that "Endomicrobia" are strictly host-dependent endosymbionts. One of the most important questions still remained is whether "Endomicrobia" are mutualists providing benefits to their host, or merely parasites that one-sidedly benefit from the host. Prospective information on genome of CET may lead to answering this question. When the genome has lost a substantial number of genes involved in biosyntheses of essential cell components, it is likely that the symbiont is a chronic parasite as many bacteria belonging to *Mycoplasma*, *Rickettsia*, or *Chlamydia* (Himmelreich et al., 1996; Andersson et al., 1998; Read et al., 2000). Although a mutualist can also largely dependent on its host to some extent, its genome tends to retain different sets of genes responsible for biosynthesis of metabolic substrates and cofactors, which are required for the fitness of the host. For example, many bacteriocyte-associated endosymbionts of insects have shown these genetic properties. *Wigglesworthia glossinidia* seems to provide its host tsetse fly with various cofactors and vitamins (Akman et al., 2002), while *Buchnera* symbionts and *Carsonella ruddii* are probably capable of provisioning of amino acids of their plant sap-feeding insect hosts, aphids and psyllids, respectively (Shigenobu et al., 2000; Nakabachi et al., 2006). It is a major interest whether "Endomicrobia" possess genes, which may contribute to the nitrogen nutrition requirement of the host flagellates, because wood particles are as nitrogen-poor as the plant sap. Provisioning of cofactors and vitamins is more plausible function of CET, considering that the genome fragment investigated in this study contained two ORFs homologous to the genes encoding a key enzyme in the biosyntheses of cofactors (Chapter 6). Nevertheless, it may be still challenging to elucidate the role of "Endomicrobia" solely by the genomic information, and the expression of each gene and functional interaction with the host should be further investigated.

Another important question is how the presence or absence of "Endomicrobia" affects the host. The most straightforward method that has been employed to investigate such a question is to remove the symbionts from the host by physically (e.g., heat treatment) or chemically using antibiotics. The removal of bacteriocyte-associated endosymbionts from their host has shown a significant negative effect on the fitness of the host insects (Ohtaka and Ishikawa, 1991, Yusuf and Turner, 2004). It seems, however, more difficult to apply this method to "Endomicrobia" in termite gut flagellates, since there is no conventional method for maintaining a stable culture of intact termite gut flagellates. Yamin, who was able to establish a culture for some gut flagellates in *Z. nevadensis*, documented that removal of symbiotic bacteria by antibiotics did not affect the fitness of flagellates as long as heat-killed bacterial cells are provided (Yamin, 1978; 1981). However, he did not explicitly state how he examined the absence of endosymbionts in the cytoplasm of these flagellates, and it is not clear whether endosymbionts were completely missing from these flagellates. Some indirect evidence obtained in this study, the absence of "Endomicrobia" in some early-branching *Trichonympha* and the possibility of horizontal transmission between the hosts (Chapter 3) indicates the removal of "Endomicrobia" from the host flagellate probably has no lethal effect. The symbiotic status of "Endomicrobia" and host flagellates would not be as absolute as that between bacteriocyte-associated endosymbionts and their insect hosts, at least on the host side. Nevertheless, the direct evidence of interaction between this symbiotic pair needs to be confirmed in prospective studies.

"Endomicrobia" and cohabiting prokaryotes: friends or foes?

This study revealed that a single cell of flagellate can be a complex micro-ecosystem consisting of various prokaryotic symbionts. Unlike endosymbionts in insect bacteriocytes or many known intracellular parasites, "Endomicrobia" have to share the intracellular habitat with many other phylogenetically distinct symbionts. Although "Endomicrobia" seem to be dominant in the cytoplasm of some flagellates such as *Trichonympha*, their relative abundance among the symbionts is apparently restricted in other flagellates such as *Eucomonympha* and *Oxymonas*, which are densely colonized by other concomitant endosymbionts. Nevertheless, considering their evolutionary history encompassing cospeciation (Chapter 3), "Endomicrobia" should be among the oldest members of endosymbionts in the termite gut flagellates. The cohabiting symbionts such as sulfate-reducing bacteria (δ-Proteobacteria) or spirochetes may also have been present for long evolutionary

time and seem to play central roles in H_2-consuming metabolism in this habitat. Spirochetes have been shown to be responsible for reductive acetogenesis, which is an important process to recycle the majority of reducing equivalent produced by gut flagellates (Pester and Brune, 2006). The involvement of "Endomicrobia" in H_2 metabolism is still not clear. In *Trichonympha* flagellates, "Endomicrobia", δ-Proteobacteria, and spirochetes do not seem to compete for the space but rather well compartmentalized (Chapter 4), colonizing the posterior part of cytoplasm; anterior part; and surfaces of membrane and flagellar of the host, respectively. Competition for carbon and energy sources is also not likely, since utilizable carbon compounds should be sufficiently present in the cytoplasm of cellulolytic flagellates. The genomic information suggests that "Endomicrobia" are probably able to utilize hexuronate as a carbon source, which is apparently not a major substrate for spirochetes or δ-Proteobacteria. On the other hand, nitrogen supply may be limited in this habitat, unless one or some members in this microecosystem are able to fix molecular nitrogen. Spirochetes isolated from the termite gut have been shown to fix dinitrogen, and some sulfate reducers are also known to possess this ability (Lilburn et al., 2001; Steppe and Paerl, 2002). Therefore, if "Endomicrobia" are not capable of fixing nitrogen, they may benefit from the presence of these cohabiters, who provide them with nitrogen compounds. The relationship between "Endomicrobia" and other bacterial symbionts in such complex microbial community seems to be difficult to elucidate, but genomic information of "Endomicrobia" will allow comparing their functional properties with those of cohabiting symbionts.

Vertical and horizontal transmission of "Endomicrobia"

Cospeciation between "Endomicrobia" and *Trichonympha* indicates the vertical inheritance of the symbiont among generations. Understanding the mechanism of vertical transmission entails detailed information of reproduction mechanism of *Trichonympha* flagellates. Bacteriocyte-associated endosymbionts in insects are transmitted to both developing embryos and eggs from maternal bacteriocytes (transovarial transmission, Baumann et al., 2006); therefore the symbionts are strictly transmitted within their host lineage. In termites, the gut flagellates are thought to enter reproduction cycle at each molting period. However, the flagellates substantially disappear from the gut contents when the molting starts (Messer and Lee, 1989) and only a few studies have been able to describe the reproductive procedure of *Trichonympha*. The sexual cycle is conspicuous in *Trichonympha* in the gut of the cockroach *C. punctulatus*, where one asexual cell converts to a gametocyte that fuse into one male and one female cell (Cleveland, 1949). In this

case, "Endomicrobia" in an asexual cell can be transmitted to progenies after the mitosis. Nevertheless, *Trichonympha* in this cockroach is apparently not related to the *Trichonympha* (Chapter 7), this process can differ from that in termites. Indeed, Cleveland could not find the evidence of gametogenesis or mitosis development in *Trichonympha* of termite species at the molting period. He documented that some specimen occasionally exhibited what he called “the ruminant of sexual reproduction” in living *Trichonympha* in *Porotermes adamsoni* after ecdysone treatment (Cleveland, 1960), but no conclusive evidence for the sexuality of *Trichonympha* in termites has been reported so far. Therefore, it is likely that *Trichonympha* reproduce simply by binary fission and "Endomicrobia" may accompany the migration of cytoplasmic components.

Horizontal transmission of "Endomicrobia" should not frequently occur between different genera of extant flagellates (Chapter 2), but seems to have occurred between *Trichonympha* flagellates in the same termite gut (Chapter 3). "Endomicrobia" can be easily released into the gut fluid when the host cells die and burst, but the symbionts are not likely to survive until they are endocytotically ingested by other flagellates and establish a new symbiosis. Therefore, the lateral transfer of the symbiont probably involves a direct physical contact between the flagellates.

Evolutionary scenario of divergence of "Endomicrobia": acquisition and transmission between gut flagellates, termites, and cockroaches

The evolutionary history of "Endomicrobia" contains multiple events: their acquisition by flagellates, termites, and cockroaches; vertical transmission and co-divergence; horizontal transmission; and complete loss from the hosts. Each event was indicated by several findings in this study, but the historical order of these events is still unclear. Particularly, the origin of symbiosis between "Endomicrobia" and flagellate hosts is one of the major interests for a better understanding the development of their symbiotic relationship. Recent studies have revealed that Termite Group 1 (TG-1) bacteria are not restricted to the gut of termites or wood-feeding cockroaches, but rather widely distributed in various environments, such as guts of other organisms, soil, and contaminate aquifers (Herlemann et al., in press). Furthermore, several SSU rRNA sequences obtained from guts of the “flagellate-free” higher termites and cockroaches apparently cluster together with "Endomicrobia" (Ohkuma et al., 2007), indicating the presence of free-living relatives of this symbiont in termite guts. This evidence leads to a hypothesis that the occurrence of "Endomicrobia" in the gut of termites precedes the establishment of the symbiosis with the flagellates. The sequence divergence between

"Endomicrobia" from different termites and cockroaches reaches up to ~9%, indicating that the first appearance of an ancestor of "Endomicrobia" in the gut of these insects can date back to as early as 300 million ago. This is approximately the same evolutionary time as a fossil of proto-dictyopterans originate from (Upper Carboniferous) (Grimaldi, 1997), which predates the split and the divergence of termites and cockroaches with wood-eating habits in the early Cretaceous (~140 million years ago) (Thorne et al., 2000). Therefore, the cellulolytic flagellates probably appeared during this period in the gut of termites or cockroaches with the herbivorous habit and acquired "Endomicrobia", which had already been in these insects for the long evolutionary history.

Even the species-level co-divergence of the flagellates such as *Trichonympha* seems to have occurred in the gut of termites, the phylum-level divergence of between parabasalids and oxymonads could date back to as early as 520 million years ago (Cavalier-Smith, 2003). This means, the flagellates of distinct order, family or genera could have been introduced into the gut of the herbivorous insects independently and acquired "Endomicrobia" by separate events. This explains the multifurcation of the SSU rRNA tree of "Endomicrobia" from distinct phylotypes of flagellates (Chapter 2).

The cospeciation between "Endomicrobia" and flagellate hosts can be accompanied by the simultaneous speciation of the termite host. This has been suggested by a previous study, which showed cospeciation between different termites and their gut flagellates of the genus *Pseudotrichonympha* (Noda et al., 2007). However, considering that the flagellate fauna can be rather easily transferred between termite species (or at even higher taxonomical level) by proctodeal trophallaxis or other physical contacts (Andrew, 1930), there is a great possibility that the symbionts have been often introduced to a new termite host, which is distantly related to their original host. Therefore, the cospeciation of the tripartite symbiosis between "Endomicrobia", flagellates, and termites should exhibit more complex phylogenetic features.

The findings in this study are supported by a number of morphological evidence obtained by Cleveland and Kirby in the early 20s century. Their description of morphological difference between *Trichonympha* flagellates in *Z. nevadensis* and *C. punctulatus* is almost consistent with the present phylogenetic evidence (Chapter 4 and 7). Their robust morphological studies will be useful for future investigation for discovering new lineages of symbionts attaching to the gut flagellates. Furthermore, the reproduction procedure of different termite gut flagellates described by Cleveland can be reevaluated using immuno-staining targeting specific proteins and microscopic monitoring, which may provide new insights into

the transmission of the symbionts between flagellate hosts. It is necessary to revalue important findings in the past cumulative morphological studies more thoroughly and reevaluate unanswered questions posed by these authors.

In addition to the genomic characterization of "Endomicrobia", future studies should also consider detailed functional properties of their host symbionts. Several attempts have been made recently to obtain information concerning functional properties of termite gut flagellates from cDNA libraries, which retrieved enormous amount of cellulases, confirming their significant roles in cellulose degradation in the gut of termites (Todaka et al., 2007). Nevertheless, there are still many undocumented questions concerning their energy metabolism as well as substrate biosynthesis, which support the lifestyles of their prokaryotic symbionts.

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Summary

"Endomicrobia" are intracellular symbionts of termite gut flagellates that represent a distinct lineage in the novel bacterial phylum Termite Group I (TG-1). The evolutionary history of "Endomicrobia" with respect to their symbiosis with host flagellates was investigated using phylogenetic analyses and in situ identification based on small-subunit ribosomal RNA (SSU rRNA) sequences.

By analyzing SSU rRNA sequences extracted from manually separated flagellate cells, "Endomicrobia" were shown to be widely distributed among termite gut flagellates. Symbionts originating from the same genus of flagellates invariably formed a host-specific monophyletic cluster in the phylogenetic tree. Their intracellular location in the cytoplasm was confirmed by fluorescent in situ hybridization (FISH) using oligonucleotide probes designed specifically for each symbiont and for the host. The phylogeny of "Endomicrobia" and host flagellates belonging to the parabasalid genus *Trichonympha* was investigated further in detail. SSU rRNA trees of the symbiont and the host exhibited overall congruence, which suggested cospeciation. Pairwise distance analysis and FISH corroborated the phylogenetic evidence, and these results allowed the construction of evolutionary scenarios for the divergence of "Endomicrobia" and their acquisition by flagellate hosts.

"Endomicrobia" share their intracellular habitat with other bacterial symbionts. Bacterial SSU rRNA sequences originating from *Trichonympha* flagellates of *Incisitermes marginipennis* and *Zootermopsis nevadensis* revealed the occurrence of several distinct phylogenetic groups, representing *Treponema* spp., "Endomicrobia", δ-Proteobacteria, Bacteroidetes, and Mycoplasmatales. The δ-Proteobacteria symbionts were shown to densely colonize the surface and the cytoplasm of the flagellates in high abundance.

Since no pure cultures of "Endomicrobia" or their host flagellates are available, a method for the physical enrichment of "Endomicrobia" was established to gain more insights into the nature of these symbionts. "*Candidatus* Endomicrobium trichonymphae" (CET), the symbiont of *Trichonympha* flagellates, was selected as representative and enriched from gut contents of *Z. nevadensis*. High-molecular-weight DNA extracted from the enrichment is currently used for genome sequencing at the DOE Joint Genome Institute. A recently assembled 80-kb contig of CET revealed first insights into its metabolism, including hexuronate metabolism and the possible formation of H_2.

"Endomicrobia" are also present in the gut of the wood-feeding cockroach *Cryptocercus punctulatus*, which is considered to share a common ancestor with

termites. Analysis of SSU rRNA sequences obtained from whole-gut DNA of this cockroach revealed the phylogenetic positions of six lineages (morphotypes) of parabasalid flagellates. Sequences obtained from manually isolated flagellates, which have long been assigned to the genus *Trichonympha*, turned out to be a previously undescribed lineage of Parabasalia. Since this new lineage may represent one of the earliest branches of parabasalid flagellates, the recovery of “Endomicrobia" sequences also from this flagellate underscores the presence of these endosymbionts already in the flagellates of the hypothetical dictyopteran ancestor of termites and cockroaches.

The results of this study collectively document that "Endomicrobia" are prevalent and persistent endosymbionts of termite gut flagellates. This study also provides a better understanding of the phylogenetic properties of their biotic environment, i.e., the host flagellates and the cohabiting bacteria, which may help to explain the functional roles of "Endomicrobia" and their symbiotic interactions.

Zusammenfassung

"Endomicrobia" sind intrazelluläre Symbionten, die eine eigene Linie im bakteriellen Phylum "Termite Group I" (TG-1) darstellen. Sie konnten bisher nur in Darmflagellaten von Termiten nachgewiesen werden. In dieser Arbeit wurde die Stammesgeschichte (Phylogenie) der "Endomicrobia" anhand von Analysen der Sequenzvariabilität und in situ Hybridisierung der rRNA der kleinen ribosomalen Untereinheit (SSU rRNA) untersucht.

Das für die SSU rRNA von "Endomicrobia" kodierende Gen konnte in allen manuell sortierten Flagellatensuspensionen nachgewiesen werden. Die phylogenetische Analyse der Gensequenzen ergab, dass aus der gleichen Flagellatengattung stammenden Symbionten jeweils eine monophyletische Gruppe im Stammbaum der TG-1 bildeten. Mittels "Fluoreszenz-in-situ-Hybridisierung" (FISH) der SSU rRNA mit spezifischen Oligonukleotid-Sonden konnten die Symbionten in den jeweiligen Wirtszellen lokalisiert werden.

Die Phylogenie der "Endomicrobia" und deren Wirtsflagellaten wurden im weiteren bei *Trichonympha*-Arten (Parabasalida) im Detail untersucht. Die phylogenetische Analyse zeigte eine klare Kongruenz der Stammbäume von Wirt und Symbiont, was auf Kospeziation der Partner hinweist. Paarweise-Distanz-Analyse und FISH bestätigten dieses Ergebnis und ermöglichten die Entwicklung eines evolutionären Szenarios, welches die Aufspaltung der "Endomicrobia" mit ihrer Aufnahme in Flagellaten erklärt.

"Endomicrobia" teilen sich das intrazelluläre Habitat mit anderen bakteriellen Symbionten. Die Untersuchung der bakteriellen SSU-rRNA-Gene aus den *Trichonympha*-Arten im Darm der Termitenarten *Incisitermes marginipennis* und *Zootermopsis nevadensis* ergab die Anwesenheit verschiedener weiterer Bakterien aus den Stämmen der Proteobacteria, Bacteroidetes und Mycoplasmatales. Vertreter des Unterstammes der δ-Proteobacteria bildeten dichte Rasen auf der Oberfläche der Flagellaten, waren aber auch in deren Zytoplasma vertreten.

Da keine Reinkulturen von "Endomicrobia" oder derer Wirtsflagellaten existieren, wurde für weitere Untersuchungen eine Methode zur physikalischen Anreicherung der Symbionten erarbeitet. Als Zielorganismus wurde „*Candidatus* Endomicrobium trichonymphae“ (CET), der Symbiont von *Trichonympha*-Spezies gewählt und aus *Z. nevadensis* angereichert. Die hochmolekulare DNA aus einer solchen Anreicherung wird momentan am JGI sequenziert, um die Genomsequenz des Symbionten zu erhalten. Die bisher assemblierten Sequenzen

(80 kb) ergaben bereits erste Hinweise auf den Stoffwechsel von CET (z.B. den Hexuronat-Metabolismus).

"Endomicrobia" wurden auch im Darm der holzfressenden Schabe *Cryptocercus punctulatus* gefunden, die einen gemeinsamen Vorfahren mit den Termiten hat. Die Analyse der 16S rRNA-Gensequenzen aus Darmextrakten der Schabe ergab sechs Linien von Parabasalia Flagellaten, die bisher stammesgeschichtlich nicht zugeordnet waren. Das Vorkommen von "Endomicrobia" in einer dieser Linien, die einen sehr frühen Zweig der parabasaliden Flagellaten darstellt, unterstützt zudem die Theorie, dass Termiten und Schaben einen gemeinsamen Vorfahren haben.

Die Ergebnisse dieser Arbeit zeigen, dass "Endomicrobia" häufige und stetige Endosymbionten von Termitendarmflagellaten sind. Sie vermitteln außerdem ein besseres Verständnis ihrer biotischen Umgebung, und bilden damit eine Grundlage für die weitere Erforschung der funktionellen Interaktionen von "Endomicrobia" mit dem Wirtsflagellaten und anderen symbiotischen Bakterien.

Contribution by other people to this work

Unless otherwise stated, all experiments were planned, conducted and evaluated by myself under the supervision of Prof. Andreas Brune.

Cloning and sequencing of cytochrome oxidase II gene for confirming termite taxa were conducted by Katja Meuser.

SSU rRNA gene sequences of "Endomicrobia" and flagellates from *N. castaneus* and *C. secundus* described in Chapter 2 were obtained by Mahesh Desai and Sibylle Frankenberg.

Initial experiments including PCR, cloning, RFLP, and T-RFLP analysis of *I. marginipennis* and *Z. nevadensis* described in Chapter 4 were conducted by an undergraduate student Sibylle Frankenberg, who I supervised during her practicum between January and March, 2006.

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