Bacterial symbionts of termite gut flagellates: cospeciation and nitrogen fixation in the gut of dry-wood termites

Dissertation

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Marburg, November 2008

List of publications

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Strassert, J., Desai, M. S., Radek, R., and Brune, A. Identification and localization of the multiple bacterial symbionts of the termite gut flagellate *Joenia annectens*.

For my beloved parents

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1 General introduction

Biology of lower termites

Termites are terrestrial, social insects that have gained attention both ecologically and commercially owing to their high abundance and pest nature (Collins, 1989; Lax and Osbrink, 2003). Termites (order: Isoptera) are phylogenetically closely related to cockroaches (Deitz *et al*., 2003; Inward *et al*., 2007) and are divided into seven families (Fig. 1) (Abe *et al*., 2000). Depending on the presence/absence of cellulolytic flagellate protozoa in the hindgut, termites are further distinguished into phylogenetically lower and higher termites, respectively. While higher termites can be fungus-cultivating, wood-feeding or soil-feeding (Abe *et al*., 2000), lower termites are strictly wood-feeding and depend on their flagellate symbionts for the degradation of lignocellulose (Cleveland, 1926).

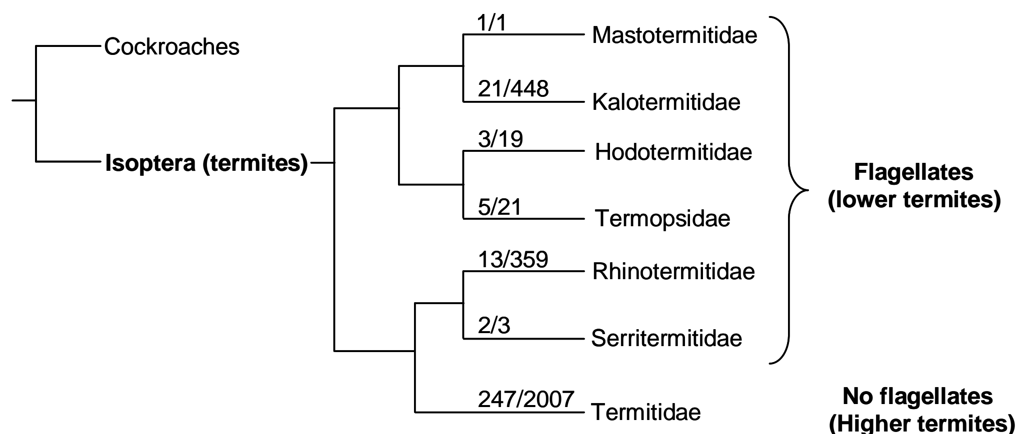

Figure 1. A simplified scheme of the phylogeny of different termite families and closely related cockroaches (modified from Abe *et al*., 2000). The lower termites harbor flagellates. The numbers on the lines represent the number of genera/species in the different families.

The relationship between the lower termites and their gut flagellates is a textbook example of symbiosis. Given the high diversity of lower termites, hardly any species of these termites are thoroughly studied. Notably, termite species of the family Kalotermitidae are very ill studied. Kalotermitidae—found mainly in the tropics—colloquially known as “dry-wood termites” and “primitive termites of

warm region"—live entirely within the single piece of dry wood, and obtain water adsorbed onto wood fibers and by metabolic processes (Noirot, 1970; Abe *et al.*, 2000). Despite being primitive and unique, only a few studies have been performed on the behavioral biology and gut microbial ecology of Kalotermitidae (Fuchs *et al.*, 2003; Korb and Lenz, 2004; Pester and Brune, 2006; Pester and Brune, 2007).

Termite hindgut: a structured environment

The gut of lower termite consists of foregut, midgut and hindgut (Escherich, 1909). The enlarged hindgut, also known as paunch, is considered as the "hotspot" of the microbial activity (Breznak, 2000; Brune, 2005, and references therein). In the hindgut, the symbiotic microbiota polymerizes cellulose and hemicellulose, which are further fermented to short-chain fatty acids; these short-chain fatty acids are then used as the main energy source by the host termite (Breznak and Brune, 1994). While other studies assumed the termite hindgut to be a completely anoxic fermenter, microelectrode measurements showed steep gradients of oxygen and hydrogen in the hindgut periphery (Fig. 2) (Brune *et al.*, 1995; Ebert and Brune, 1997).

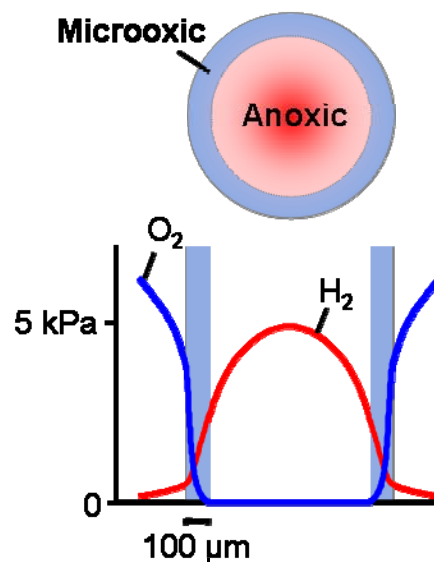

Figure 2. Oxygen and hydrogen profiles in the hindgut of the lower termite *Reticulitermes flavipes*. Employing the microsensor electrodes, both the gases were measured radially in an agarose-embedded hindgut. Oxygen (blue) penetrating into the gut periphery is rapidly consumed by the respiratory activity of the gut microbiota. Hydrogen (red) concentration is the highest at the centre of the gut. (Figure from Brune and Friedrich, 2000).

The rapid removal of oxygen by the respiratory activity of the bacteria creates a microoxic periphery (50-200 µm) around the anoxic centre (Fig. 2). Since termite guts have a high surface area per unit volume, it seems that more than 40% of the paunch is oxic; this clearly makes oxygen an important electron acceptor in the paunch (Brune, 1998). Hydrogen, produced by the anaerobic parabasalid flagellates, is another important metabolite in the paunch. Depending on the termite species, hydrogen concentration can reach up to 70 kPa (Pester and Brune, 2007). The concentration curve of hydrogen shows a steep gradient (Fig. 2). Altogether, the structured environment in the microliter-scaled paunch contains several microhabitats (Brune, 1998).

Flagellate symbionts: diversity and functions

The hindgut of lower termites is filled with numerous species of oxygen-sensitive flagellates (numbers up to 600,000 per gut), which make up to 33% of the total fresh weight of a termite (Hungate, 1955). 430 described species of flagellates unique to lower termites and wood-feeding cockroach *Cryptocercus* were listed by Yamin (1979). Electron microscope studies have improved the previously reported classifications of flagellates (Radek, 1992; Brugerolle, 2000 and references therein). Additionally, construction of molecular phylogenies of flagellates from several marker genes has helped to better understand their classification and evolution. (Ohkuma *et al*., 2000, 2005, 2007; Stingl and Brune, 2003; Gerbod *et al*., 2004). These flagellates belong to the phylum *Parabasalia* or the order *Oxymonadida* (phylum: *Preaxostyla*) and are believed to be specific to host termites (Kirby, 1937; Kirby, 1949; Honigberg, 1970; Kitade, 2004).

The reason behind the host-specificity of flagellates is their supposed vertical transmission, which occurs by the process of proctodeal trophallaxis (Kitade, 2004). Molecular phylogenetic congruence between rhinotermitid termites and their *Psuedotrichonympha* flagellates supported the notion of vertical transmission (Noda *et al*. 2007). Furthermore, a recent study showed that the common ancestor of termites and cockroaches acquired flagellate symbionts, and flagellates codiversified with their host termites and cockroaches (Ohkuma *et al*., 2008).

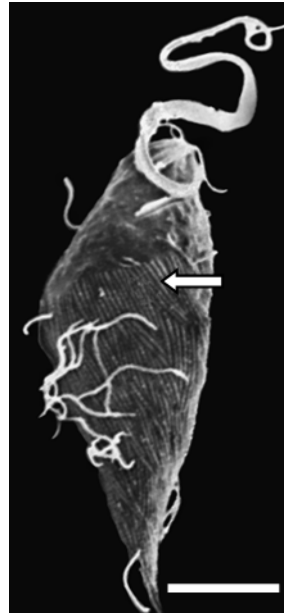

Figure 3. Scanning electron microscope image of a parabasalid flagellate (*Devescovina glabra*) from the gut of the dry-wood termite *Cryptotermes dudleyi*. Laterally attached filamentous bacteria (arrow) cover the posterior part of the flagellate cell. Scale bar 10 µm. (Figure from Radek *et al.*, 1996).

These primitive associations between the cellulolytic flagellates and termites underscore the importance of flagellates in the cellulose degradation by host insects. Defaunation of termites made it clear that termites depend on flagellates for the digestion of the lignocellulose (Yamin, 1926). Degradation of lignocellulose by termites demands a dual cellulase system, comprising cellulases of both termite and flagellate origin (Nakashima *et al.*, 2002; Tokuda *et al.*, 2007). The amorphous cellulose is degraded by the endogenous endoglucanase (termite origin). The product of this degradation (crystalline cellulose) is then depolymerized by flagellates, which possess several endo- and exo-type cellulases belonging to glycosyl hydrolase families (GHFs) 7, 45 and 5 as well as b-glucosidase and hemicellulases (Watanabe *et al.*, 2002; Watanabe *et al.*, 2006; Inoue *et al.*, 2007; Todaka *et al.*, 2007; Tokuda and Watanabe, 2007).

The other important feature of the flagellates is the production of molecular hydrogen. Hydrogen is known to be the principal metabolic intermediate in the gut of lower termites (Pester and Brune, 2007). Parabasalid flagellates (e.g., Fig. 3) lack mitochondria and instead possess anaerobic energy- and hydrogen-generating organelles called hydrogenosomes (Müller, 1993), where stoichiometric amounts of

acetate, CO_2 and H_2 are formed (Hungate, 1943; Yamin, 1980; Odelson and Breznak, 1985a, 1985b). Nothing, however, is known about the physiology of the oxymonad flagellates, which are believed not to possess hydrogenosome-like organelles (Brugerolle and Radek, 2006). Physiological studies on termite gut flagellates are hindered, as none of the flagellates are in permanent culture. Termite gut flagellates *Trichomitopsis termopsidis* and *Trichonympha sphaerica* were temporarily cultured (Yamin and Trager, 1979; Yamin, 1980; Yamin, 1981; Odelson and Breznak, 1985a, 1985b). The closest cultivated representative of the (termite gut) parabasalid flagellates is the human pathogen *Trichomonas vaginalis* (Steinbüchel and Müller, 1986).

Bacterial symbionts of flagellates: diversity and functions

Lower termites harbor a high number of morphologically distinct bacterial symbionts in their hindguts (Breznak, 1975). This morphological diversity is supported by the presence of more than three hundred 16S rRNA gene phylotypes in the termite *Reticulitermes speratus* (Hongoh *et al*., 2003a; Hongoh *et al*., 2003b; Hongoh *et al*., 2005). The long-held notion that the majority of these bacteria are associated with flagellates (Ball, 1969) was experimentally supported in the termite *Mastotermes darwiniensis*, as 90% of the bacteria present in the hindgut were shown to be symbionts of flagellates (Berchtold *et al*., 1999).

Despite their high diversity and abundance, none of the bacterial symbionts of the flagellates have been cultured (Breznak, 2000; Brune and Stingl, 2005). On the other hand, phylogenetic positions of bacterial symbionts can be determined using culture-independent techniques (Stingl *et al*., 2005; Ohkuma *et al*., 2007). The full-cycle-rRNA approach has proven to be a useful to localize bacterial symbionts (Fig. 4) (Noda *et al*., 2003; Stingl *et al*., 2004; Stingl *et al*., 2005). The majority of ectosymbionts of flagellates are affiliated with numerous lineages of Spirochaetes and *Bacteroidales* (Noda *et al*., 2003; Noda *et al*., 2006). “Endomicrobia” (phylum: Termite group 1) represent most of the endosymbionts of flagellates (Stingl *et al*., 2005).

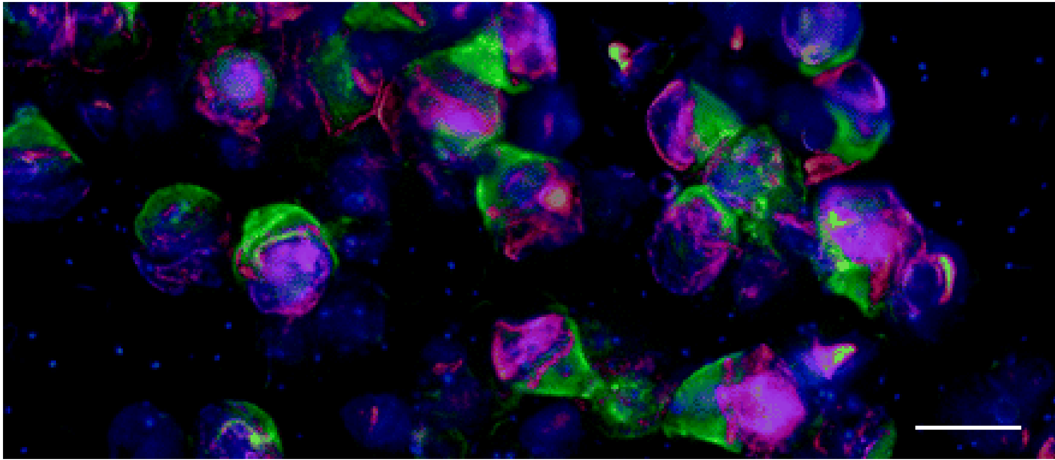

Figure. 4. Fluorescence microscopic image of flagellates *Trichonympha* species from the termite *Zootermopsis nevadensis*. The cell surface and cytoplasm are colonized by symbiotic bacteria. The epibionts (green) and the endobionts (pink) were hybridized with specific oligonucleotide probes. Endobionts are “Endomicrobia”. Scale bar 100 µm. (Franckenberg and Brune, cover page, Microbiology, 2007).

These ecto- and endosymbionts are believed to be specific to their host flagellates, which was confirmed for numerous of host-symbiont pairs (for review, see Ohkuma 2008). The cospeciation resulting from the host-specificity (Hafner and Page, 1995; Wade, 2007) was demonstrated for the *Bacteroidales* endosymbionts and *Psuedotrichonympha* flagellates (Noda *et al*., 2007), and “Endomicrobia” and *Trichonympha* flagellates (Ikeda-Ohtsubo and Brune, in press). In contrast, symbiotic spirochetes appear to be multiply acquired by their host flagellates (Noda *et al*., 2003), displaying the complexity of flagellate-bacteria symbioses in the termite gut.

The physiological basis of symbioses between flagellates and bacteria is not well known. The possible physiological roles of bacterial symbionts are mentioned in the following. Recently, Hongoh *et al*. (2008) sequenced the complete genome of the “Endomicrobia”. The reduced genome (1.1 Mbp) contains genes which encode 15 amino acids and several cofactors. Based on these data, “Endomicrobia” were suggested to provide nitrogenous compounds to their host flagellates. Since several members of the *Bacteroidales* are involved in the polysaccharide degradation, it is tempting to speculate that the symbiotic *Bacteroidales* play similar roles in the termite gut (Stingl *et al*., 2004). Moreover, endosymbiotic *Bacteroidales* were shown to rapidly consume the hydrogen produced by host flagellates (Inoue *et al*.,

2008). As suggested by Inoue *et al*. the rapid removal of hydrogen could enhance the fermentation by flagellates; the electron acceptor in this reaction is unknown. Spirochetes were shown to help host flagellates in motility (Cleveland and Grimstone, 1964; Wenzel *et al*., 2003). Furthermore, pure cultures of termite gut spirochetes perform reductive acetogenesis (Leadbetter *et al*., 1999; Graber *et al*., 2004) and nitrogen fixation (Lilburn *et al*., 2001). Expression profiles of the genes encoding formyltetrahydrofolate synthetase, a key enzyme for reductive acetogenesis, showed that spirochetes perform reductive acetogenesis also in the termite gut (Pester and Brune, 2006). However, both reductive acetogenesis and nitrogen fixation by spirochetal symbionts of flagellates remain to be elucidated.

Nitrogen fixation: a crucial process in the termite gut

Since termites can live on a diet of cellulose-filter paper, which has an extremely high C/N ratio, Cleveland (1925) suggested that termite gut bacteria fix dinitrogen. The first experimental proof for the nitrogen fixation by termites was provided by two independent studies using the sensitive acetylene reduction (AR) assay (Benemann, 1973; Breznak *et al*., 1973), which showed high rates of nitrogen fixation in several phylogenetically distinct termites. Live termites were incubated with acetylene for defined amounts of time. The production of ethylene was measured with the gas chromatograph as described by Postgate (1972) and Hardy (1973) e.g., the dry-wood termite *Cryptotermes brevis* formed 1.705 nmol C_2H_4 per hour per g termites. (Breznak *et al*., 1973). By treating termites with antibiotics, Brezank *et al*. (1973) demonstrated that the nitrogen fixation activity is associated with bacteria. In a later study (Bentley, 1984), incorporation of the fixed nitrogen in the termite tissue was demonstrated, confirming the symbiotic nature of nitrogen fixation. Moreover, stable isotope analysis showed that the wood-feeding termite *Neotermes koshunensis* (Kalotermitidae) obtains 30-50% of its nitrogen from the atmosphere (Tayasu *et al*., 1998).

Rates of nitrogen fixation vary among termite species and castes (Breznak 1982; Collins 1983; Hewitt *et al*., 1987; Curtis and Waller, 1998). Several factors influence nitrogen fixation by termite gut bacteria. High oxygen partial pressure and low pH of the diet decrease nitrogenase activity. Seasonal variation in the nitrogen

fixation activity has also been observed (Curtis and Waller, 1998). After isolation from the nest, some termites lose nitrogenase activity within hours (Prestwich *et al*., 1981; Lovelock *et al*., 1985). When termites are fed with nitrogen-rich diet, nitrogen fixation activity vanishes (Breznak *et al*., 1973; Noda *et al*., 1999). Rates of nitrogen fixation are higher for dry-wood termites (Breznak *et al*., 1973; Ohkuma *et al*., 1999). As pointed out by Breznak (2000), large differences in the rates of nitrogen fixation for different species of termites could be owing to the lack of standard acetylene reduction assay protocol, which would closely mimic conditions existing in the termite nest. Another reason for different rates of nitrogen fixation could be the presence of different types of nitrogen-fixing bacteria in the gut of different termite species.

Several nitrogen-fixing bacteria were isolated from termite guts. Two strains of *Enterobacter* (*Pantoea*) from the termite *Coptotermes formosanus* (Potrikus and Breznak, 1997), and *Citrobacter freundii* from termites *Coptotermes lacteus* and *Mastotermes darwiniensis* (French *et al*., 1976) were isolated. Moreover, Lilburn *et al*. (2002) isolated spirochetes (*Treponema* spp.) and showed that spirochetes fix nitrogen. All the isolates mentioned here fixed nitrogen *in vitro*. However, it is not clear whether these bacteria are responsible for the nitrogen fixation *in vivo*.

Aims of this study

1. **Evolutionary history of devescovinid flagellates and their bacterial symbionts**

   Devescovinid flagellates are the dominant flagellates in the gut of dry-wood termites (Kalotermitidae) (Kirby, 1941, 1942, 1945). Kirby described 12 genera of devescovinid flagellates; among these genera, the highest species diversity was documented for the genus *Devescovina* (total 20 species, in more than 60 termite species). All species of this genus possess filamentous ectosymbiotic bacteria (Kirby, 1941). Recently, Noda *et al*. (2006) showed that the filamentous ectosymbionts form a deep-branching novel lineage in the order *Bacteroidales*. These findings raised several interesting questions: (i) Do filamentous bacteria present on the surface of all *Devescovina* species belong to the *Bacteroidales*, and share a common ancestor? (ii) What are the other bacterial symbionts associated with *Devescovina* species? (iii) Are different species of *Devescovina* described by Kirby also different at the molecular level? (iv) Do all *Devescovina* species had a common ancestor? (v) What are the bacterial symbionts of other devescovinid flagellates (e.g., *Metadevescovina* species)? In order to understand the evolutionary histories of the devescovinid flagellates (*Devescovina* and *Metadevescovina* species) and their bacterial symbionts, I used the full-cycle-rRNA approach, and investigated the small-subunit ribosomal RNA (SSU rRNA) gene phylogenies of capillary-picked flagellates and their bacterial symbionts from several Kalotermitidae. Phylogenies of the *Bacteroidales* ectosymbionts of numerous *Oxymonas* species were also studied.

2. **Nitrogen-fixing bacteria in the gut of Kalotermitidae**

   Kalotermitidae are known to fix high amounts of nitrogen (Breznak *et al*., 1973; Noda *et al*., 1999), however, identities of the nitrogen-fixing bacteria in these termites are unknown. Therefore, the possibility of nitrogen fixation by the symbionts of flagellates in the gut of Kalotermitidae was examined. Nitrogen fixation genes (homologs of *nifH*), were cloned from the capillary-picked flagellate suspensions, and whole guts of four species of

Kalotermitidae. The expression of nitrogen fixation genes was studied with the DNA- and mRNA-based terminal restriction fragment length polymorphism (T-RFLP) analysis.

3. **Hydrogen microsensor measurements in the gut of Kalotermitidae**
   The lower termites accumulate high amounts of hydrogen (Brune *et al*., 1995; Ebert and Brune, 1997; Pester and Brune, 2007). Hydrogen is known to be a competitive inhibitor of nitrogen reduction by nitrogenase (Guth and Burris, 1983; Rasche and Arp, 1989). Therefore, to analyze the possible implications of hydrogen accumulation on the nitrogen fixation in the termite gut, I measured hydrogen in three species of Kalotermitidae using the hydrogen microsensor.

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2 The true diversity of devescovinid flagellates in the termite *Incisitermes marginipennis*

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Abstract

More than 40 years ago, ten species of devescovinid flagellates were described in the gut content of the termite *Incisitermes marginipennis*. Based on light microscopic examinations, the flagellates were then classified into the two genera *Devescovina* and *Metadevescovina*. Here, we combined molecular phylogenetic analysis of the small subunit rRNA genes of the gut flagellates with the first ultrastructural investigation of the genus *Metadevescovina*. Our results show that *I. marginipennis* contains only one species of devescovinid flagellates, *Metadevescovina modica*, which comprises three closely related phylotypes (sequence similarity >99.4%). Monophyly of the cluster and the dense colonization with spirochetal epibionts corroborate the validity of the genus *Metadevescovina* and allow its differentiation from other devescovinid flagellates.

***Authors' contribution:** The study was designed by J. F. H. S., M. S. D., A. B., and R. R. The molecular analysis was performed by M. S. D. The results of the molecular analysis were written by M. S. D. Electron microscopy was carried out by J. F. H. S. under the supervision of R. R. The draft was prepared by J. F. H. S., and J. F. H. S., M. S. D., A. B. and R. R. worked on the final manuscript.

Technical assistance: Katja Meuser carried out sequencing of the COII gene of the termite *Incisitermes marginipennis.*

Introduction

The guts of lower termites constitute a habitat for a diverse microbiota essential for the digestion of cellulosic food of their hosts (Brune and Stingl 2005; Inoue et al. 2000; Radek 1999). The microbiota comprises numerous species of flagellates, bacteria, archaea, and yeasts. Yamin (1979) listed more than 430 species of flagellates in 205 investigated termite species, and it is generally assumed that each termite species harbors a typical assemblage of flagellate species (Honigberg 1970). Termite gut flagellates, all of them amitochondriate anaerobic protists, can be classified into two groups: (i) the parabasalids, characterized by the possession of parabasal filaments connected with dictyosomes (parabasal bodies) and anaerobic ATP- and molecular-hydrogen-generating organelles (hydrogenosomes); and (ii) the oxymonads, which lack such organelles. The two groups are phylogenetically distinct (Parabasalia and Preaxostyla; Adl et al. 2005). Based on rRNA gene sequence analyses and other molecular markers, the Parabasalia are subdivided into four major taxa: Trichomonadida, Cristamonadida, Trichonymphida, and Spirotrichonymphida (Adl et al. 2005; Brugerolle and Radek 2006), although the exact phylogeny of parabasalids remains under discussion (e.g., Gerbod et al. 2004; Hampl et al. 2004, 2006, 2007; Keeling 2002; Noël et al. 2007; Ohkuma et al. 2005).

Since most termite gut flagellates were described more than 40 years ago solely by using light microscopy (Brugerolle and Radek 2006), it is not astonishing that individual species have been classified or identified erroneously. For example, different species or genera were classified as stadiums of a life cycle, as with the genera *Pyrsonympha* and *Dinenympha* (see Brugerolle and Lee 2000a). A particularly interesting example is the classification of devescovinid flagellates (Devescovinidae). After the original description of the genus *Devescovina* (Foà 1905), several other devescovinid genera such as *Foaina*, *Caduceia*, and *Metadevescovina* were established (see Brugerolle and Lee 2000b). However, especially the creation of the genus *Metadevescovina* by Light (1926) remained controversial. While the justification for the establishment of a separate genus is supported by De Mello (1941) and Kirby (1945), *Metadevescovina* is considered as a synonym for *Devescovina* by Grassé (1952).

Additional controversy concerns the number of species of devescovinid flagellates present within the gut of a given species of termites. There are several examples for a discrepancy between the number of species reported by individual researchers (see

Kirby 1941, 1942, 1945). The most prominent case is that of *Incisitermes marginipennis*. In his light-microscopic investigations, Kirby (1945) found only two species of *Metadevescovina* (*M. modica*, *M. magna*), whereas Pérez-Reyes and López-Ochoterena (1965) described as many as ten species in the two genera *Devescovina* and *Metadevescovina* (*D. foliacea*, *D. piriformis*, *D. vestitiformis*, *M. cicis*, *M. difficilis*, *M. grandis*, *M. modica*, *M. ovoidea*, *M. pavicula*, *M. sphaerica*). This again contradicts Kirby (1945), who postulated that *Devescovina* and *Metadevescovina* do not occur simultaneously in one termite (with the exception of *Glyptotermes angustus*).

A clarification of such questions based purely on morphological features is difficult. However, molecular-based studies allow the clarification of the number of phylotypes and their assignment to their respective morphotypes. In the case of *Pyrsonympha* and *Dinenympha* (see above), small subunit (SSU) rRNA gene sequence analysis combined with fluorescent in situ hybridization not only showed that both species are phylogenetically distinct, but also resolved different phylotypes within a single morphotype (Stingl and Brune 2003).

Therefore, we decided to combine molecular and morphological techniques to investigate the true diversity of devescovinid flagellates in *Incisitermes marginipennis*. Besides phylogenetic and light microscopy analyses, we present also the first ultrastructural study of the devescovinid symbionts of this termite.

Results

Phylogenetic diversity of devescovinids

A clone library of SSU rRNA genes (34 clones) from the hindgut homogenate of *I. marginipennis* contained six different phylotypes, representing four different genera of flagellates (Table 1). All sequences are most closely related to sequences of flagellates previously obtained from other dry-wood termites and are in agreement with previous reports on the presence of *Trichonympha*, *Metadevescovina*, *Tricercomitus*, and *Oxymonas* species in this termite (Kirby 1945; Pérez-Reyes and López-Ochoterena 1965). The results were corroborated by microscopic observations, which confirmed the presence of the above genera on the basis of their typical morphological characteristics. The presence of two phylotypes of *Trichonympha*

species (98.4% sequence similarity) is in agreement with the results of W. Ikeda-Ohtsubo and A. Brune (submitted for publication).

Table 1. Flagellate phylotypes from the hindgut of *Incisitermes marginipennis*, their proportion in the clone library, and their closest relatives in public databases.

Flagellate phylotypes (accession number)	Proportion in library (%)	Closest BLAST hit (accession number)	Sequence similarity (%)[a]
GhImp07 (AB434791)[b]	14.3	*Trichonympha magna* from *Porotermes adamsoni* (AF052712)	97.1
GhImp19 (AB434792)[b]	17.9	*Trichonympha magna* from *Porotermes adamsoni* (AF052712)	97.1
GhImp22 (FM160643)[c]	10.7	*Metadevescovina polyspira* from *Pterotermes occidentis* (U17506)	95.5
GhImp43 (FM160644)	3.6	*Metadevescovina polyspira* from *Pterotermes occidentis* (U17506)	95.6
GhImp44 (FM160646)	7.1	Uncultured parabasalid from *Incisitermes minor* (AB183887)[d]	97.7
GhImp29 (FM160647)[e]	46.4	*Oxymonas* sp. NcOxA from *Neotermes castaneus* (AB326383)	90.2

[a] Calculated based on the aligned and unfiltered dataset using ARB.

[b] Accession numbers are for the sequence-identical phylotypes ImrTcA and ImrTcB from picked flagellates (W. Ikeda-Ohtsubo and A. Brune, in press).

[c] Accession number is for the sequence-identical phylotype ImDev12 from picked flagellates (this study).

[d] Sequence tentatively assigned to *Tricercomitus* or *Macrotrichomonas* (Ohkuma et al. 2005).

[e] Partial sequence, 954 bp (sequenced with only one M13 primer).

However, the apparent absence of a *Devescovina* sequence and the presence of only two phylotypes (99.5% sequence similarity) of *Metadevescovina* are in clear contradiction to the report of Pérez-Reyes and López-Ochoterena (1965), who described the simultaneous presence of ten species of *Devescovina* and *Metadevescovina* in this termite. Therefore, we obtained a second clone library (36

clones) from a suspension of more than 200 capillary-picked flagellates with devescovinid morphology. All clones showed the same two RFLP patterns, indicating that only one ribotype was present. Five clones each were sequenced, resulting in a set of highly similar sequences (99.4% sequence similarity).

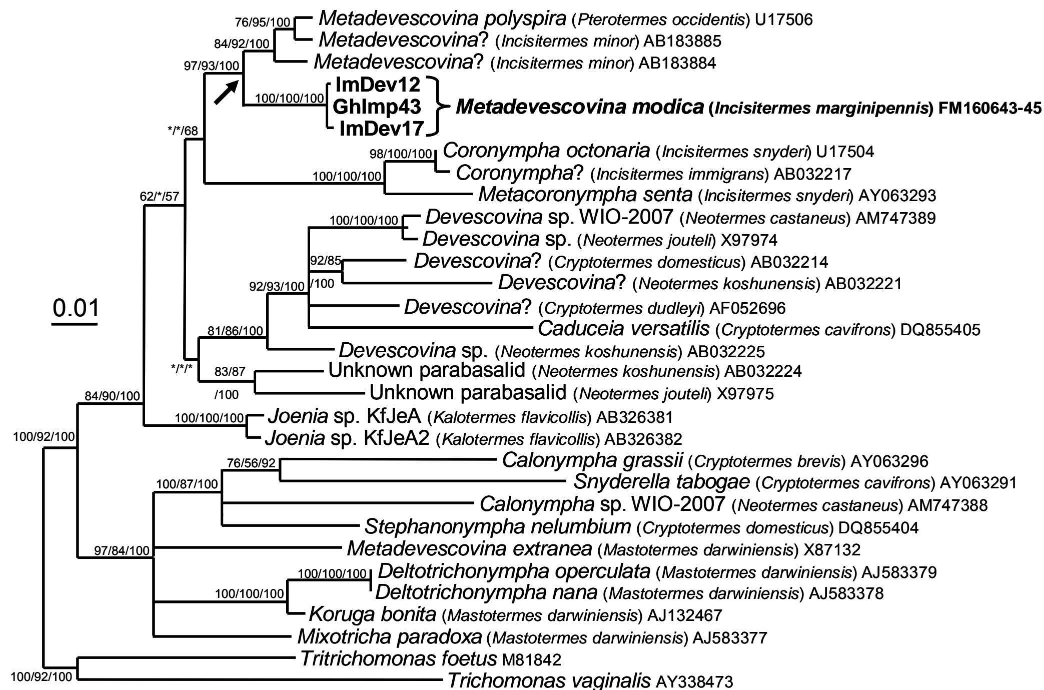

Figure 1. Phylogenetic position of the devescovinid phylotypes obtained from *Incisitermes marginipennis* (marked in bold), which were assigned to *Metadevescovina modica* (see Discussion). The SSU rRNA gene tree contains the sequences of all devescovinids and selected other parabasalid taxa. It is based on maximum-likelihood analysis of 1312 unambiguously aligned nucleotide positions. Tree topology was tested by neighbor-joining and parsimony analysis with bootstrapping (DNAPARS, 1000 replicates). Marked nodes have bootstrap values of >90% (●) and >80% (○). Multifurcations are introduced for the nodes not supported in all analyses. Names of host termites are given in parentheses. Tentative identifications of unidentified flagellate sequences are indicated with question marks.

Signature analysis of the variable sequence positions revealed the presence of two major phylotypes (Table 2), diverging only in individual positions from the consensus (<0.3% divergence). One of the phylotypes had been already recovered from the clone library obtained from hindgut homogenates. Combined with the gut homogenate clone library we found a total of three major phylotypes of devescovinid flagellates. Phylogenetic analysis (Fig. 1) showed that the sequences are most closely related to the SSU rRNA gene sequence of *Metadevescovina polyspira* (Gunderson et

al. 1995) and two other sequences from gut homogenates of *Incisitermes minor* (Ohkuma et al. 2005), a termite that reportedly contains *Metadevescovina* sp. (Kirby 1945).

Table 2. Signature analysis of the devescovinid small subunit rRNA genes obtained from *Incisitermes marginipennis*. Base positions that were identical in all sequences were omitted; bases that deviate from the consensus are given in bold. Signature positions of the three major phylotypes are highlighted; GhImp: clone obtained from the gut homogenate. ImDev: clone obtained from picked devescovinid flagellates.

Clone	Position (bp)[a]																			
	214	300	374	404	489	544	616	701	731	863	869	1071	1171	1330	1350	1468	1469	1478	1482	1484
GhImp 43	A	T	T	T	A	A	G	T	T	C	T	T	T	G	T	T	**C**	C	G	G
GhImp 12	A	**C**	T	T	A	A	A	T	T	T	T	T	T	G	T	T	T	T	G	A
GhImp 22	A	T	T	T	A	**G**	A	T	T	T	T	T	T	G	**C**	T	T	T	G	A
GhImp 23	A	T	T	T	A	**G**	A	T	T	T	T	T	T	G	**C**	T	T	T	G	A
ImDev 07	**G**	T	T	T	A	A	A	T	T	T	T	T	T	G	T	T	T	T	G	A
ImDev 12	A	T	T	T	A	A	A	T	T	T	T	T	T	G	T	T	T	T	G	A
ImDev 03	A	T	T	T	A	A	G	T	T	C	T	T	C	**A**	T	T	T	C	T	G
ImDev 04	A	T	T	T	A	A	G	T	T	C	T	T	C	G	T	T	T	C	T	G
ImDev 05	A	T	T	T	**T**	A	G	T	T	C	T	T	C	G	T	T	T	C	T	G
ImDev 06	A	T	T	T	A	A	G	T	T	C	T	T	C	G	T	T	T	C	T	G
ImDev 11	A	T	T	T	A	A	G	T	T	C	T	T	C	G	T	**C**	T	C	T	G
ImDev 14	A	T	T	T	A	A	G	T	T	C	T	T	C	G	T	T	T	C	T	G
ImDev 16	A	T	T	**C**	A	A	G	**C**	**C**	C	**C**	T	C	G	T	T	T	C	T	G
ImDev 17	A	T	**C**	T	A	A	G	T	T	C	T	**C**	C	G	T	T	T	C	T	G

[a] Position relative to 5' end of amplicon.

Morphological diversity of devescovinids

The devescovinid flagellates in the hindgut of *I. marginipennis* were not morphologically distinguishable. All devescovinids have a spindle-shaped body, which measures 28 to 52 µm (mean value 41 µm; n = 78) in length and 11 to 25 µm (mean value 17 µm; n = 78) in width (Figs 2A–C). They all show four flagella arising at an anterior papilla. Three flagella are directed anteriorly and measure about 1/2 to a full body length. The fourth, so-called recurrent flagellum is

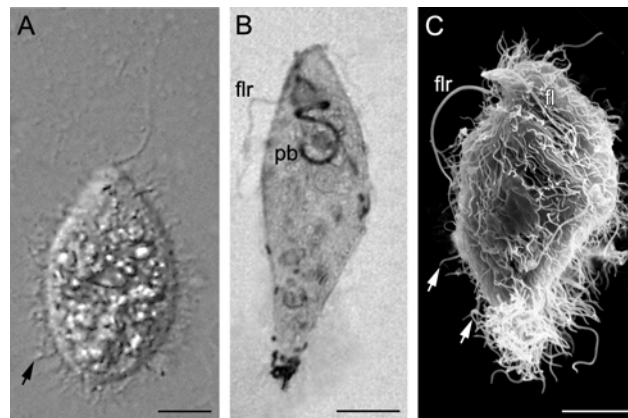

Figure 2. Morphology of *Metadevescovina modica*. **A:** View of a living cell. Differential interference contrast. **B:** Protargol-stained cell. pb – parabasal body. **C:** Scanning electron micrograph. The whole surface of the cell is covered by spirochetes (arrows; **A, C**). In addition to the three slender anterior flagella (fl; **C**) a thicker, recurrent flagellum could be observed (flr; **B, C**). Scale bars: 10 µm (**A–C**).

directed posteriorly without being attached to the cell body. It is nearly twice as thick as and longer than the anterior flagella (Figs 2B–C). Its length often measures somewhat more than the body length.

Structure of the karyomastigont system

In fixed and DAPI-stained smears, the nucleus of the devescovinids appears either elongated or rounded. The elongated nucleus is 5.7 ± 0.9 µm long and 3.9 ± 0.7 µm wide (n = 23) and lies perpendicularly or obliquely to the long axis of the cell. When the cells are viewed from the lateral side, the nucleus appears kidney-shaped, with a large furrow at its posterior end (Figs 3A, C). In rounded nuclei (4.3 ± 0.8 µm; n = 20) a furrow could not be seen (Fig. 3B). However, three-dimensional analyses with a digital fluorescence microscope (data not shown) revealed that the appearance of rounded or elongated nuclei was just a matter of orientation relative to the observer.

Seen from dorsal or ventral, the nuclei of the flagellates appears rounded without a furrow, but when the stack of images is turned 90°, the nuclei are elongated with a furrow. Therefore, a division of the devescovinids into two or more groups according to different nucleus shapes, as proposed by Pérez-Reyes and López-Ochoterena (1965), is not justified. Transmission electron microscopy showed that the chromatin mass extends up to the nuclear membrane; thus, there is no clear zone between the central chromatin mass and the membrane in all investigated devescovinids (Figs 3A, F–G).

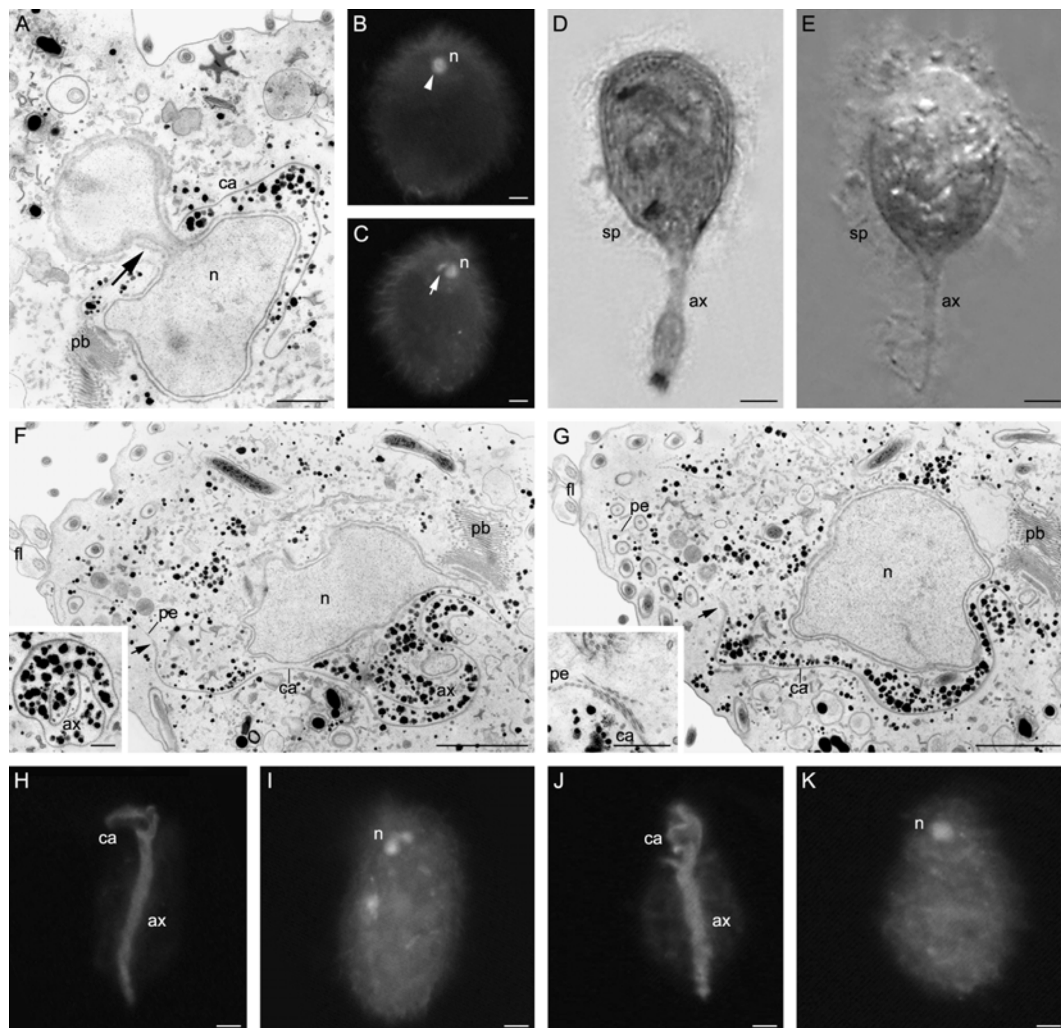

Figure 3. Structure of the nucleus and the pelta-axostyle complex. **A–C:** Transmission electron microscopy (**A**) and DAPI-staining (**B, C**) revealed a notched (arrow) or rounded (arrowhead) nucleus (n). ca – capitulum, pb – parabasal body. **D, E:** Protargol staining (**D**) and differential interference contrast microscopy (**E**) showed the axostyle (ax) that projects at the posterior cell pole. sp – spirochetes. **F, G:** Transmission electron microscopy. Below the nucleus (n) the transition of the axostyle (ax) into a capitulum (ca) is visible. More anteriorly, a pelta (pe) arises (arrows). fl – flagella, pb – parabasal body. **F; inset:** The axostyle (ax) is

composed of microtubular lamellae. **G; inset:** At the origin of the pelta (pe), its microtubules overlap with those of the capitulum (ca). **H–K:** Double-labeling with an anti-tubulin antibody (**H, J**) and DAPI (**I, K**) revealed that the axostyle (ax) widens into a conspicuously developed (**H**) or a narrow and short (**J**) capitulum (ca). The flagella were also labeled with the anti-tubulin antibody but are not in focus. **I:** The nucleus (n) appears notched. **K:** The nucleus (n) appears roundish. Scale bars: 1 µm (**A**), 5 µm (**B–E**), 2 µm (**F, G**), 0.5 µm (**F, G**; insets), 5 µm (**H–K**).

Another criterion that was used by Kirby (1945), and Pérez-Reyes and López-Ochoterena (1965) to differentiate the devescovinids of *I. marginipennis* is the construction of the axostyle. Generally, the axostyle does not protrude at the posterior cell pole. Only in a few cases was a projection visible, but it differed greatly in length. In these cases, the body appears more spherical (Figs 3D–E). It is conspicuous that protruding axostyles were rarely present in freshly prepared flagellates but increased in frequency already after a few minutes, and that they were present in protargol-stained smears but were rarely observed by scanning electron microscopy. Transmission electron microscopy revealed that the axostyles of all devescovinids resemble each other closely. Cross-sections showed that an axostyle consists of one to three lamellae of spirally rolled up microtubules (Fig. 3F inset).

Below the nucleus, the axostyle widens into a capitulum that encases the nucleus (Figs 3F–H, J). Ultra-thin sections showed that the capitulum consists of only one lamella of microtubules. A microtubular pelta arising from the inside of the capitulum supports the anterior cell pole (Fig. 3G inset). Also the capitulum–pelta architecture is a trait used by Kirby (1945) and Pérez-Reyes and López-Ochoterena (1965) to differentiate the devescovinids of *I. marginipennis*. Again, three-dimensional analyses (data not shown) of preparations double-labeled with anti-tubulin antibodies and DAPI revealed that the variants of the capitulum–pelta architecture were merely caused by different aspects of the flagellate cells. When the cells are viewed from the lateral side, where the nucleus appears elongated and furrowed (see above), the capitulum seems long and extends in an almost right angle towards the axostyle (Figs 3H–I). In a frontal view, when the nucleus appears small and roundish (see above), the capitulum seems short and inconspicuous (Figs 3J–K).

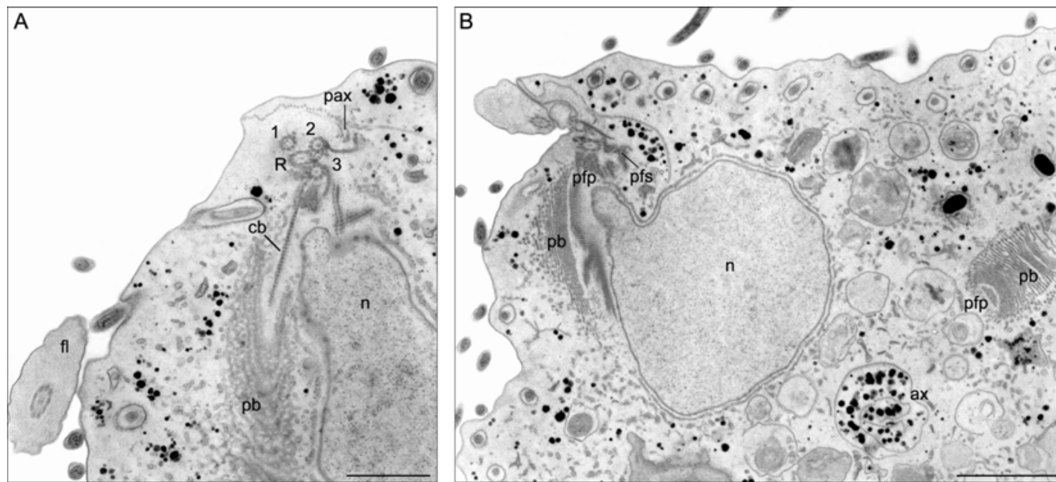

Figure 4. Transmission electron micrographs of the anterior cell pole. **A:** The basal bodies (1, 2, 3) of the anterior flagella and the recurrent flagellum (R) are visible. Sigmoid fibers (fsig) arise at basal body 2. A comb-like structure (cb) arises below basal body R. fl – flagellum, n – nucleus, pb – parabasal body. **B:** Micrograph showing the parabasal body (pb) attached alongside the primary parabasal fiber (pfp). Above the nucleus (n), a second parabasal fiber (pfs) arises between basal body 2 and basal body 3. ax – axostyle. Scale bars: 1 µm (**A**), 2 µm (**B**).

An important trait for the classification of parabasalids is the characteristics of the basal bodies and their associated structures (see Brugerolle 1975/76, Brugerolle and Lee 2000b). In all ultra-thin sections investigated, the basal bodies 1, 2, and 3 of the anterior flagella are parallel to each other, whereas basal body R of the recurrent flagellum is positioned at a slight angle to it (Fig. 4A). Sigmoid fibers arise at the basal body 2 and form a fan (Fig. 4A). Next to basal body 3, a primary parabasal fiber arises. Alongside this fiber, the parabasal body (dictyosome) is attached. It is composed of about 30 Golgi cisterns (Figs 4A–B). Between the basal bodies 2 and 3, a second parabasal fiber arises and runs ventrally along the nucleus (Fig. 4B). Under basal body R, there is a comb-like structure (Fig. 4A) linked to a thin fiber, resembling a structure in *Devescovina striata* reported by Joyon et al. (1969). Nevertheless, the possibility that this structure represents a longitudinal section of a parabasal fiber cannot be excluded.

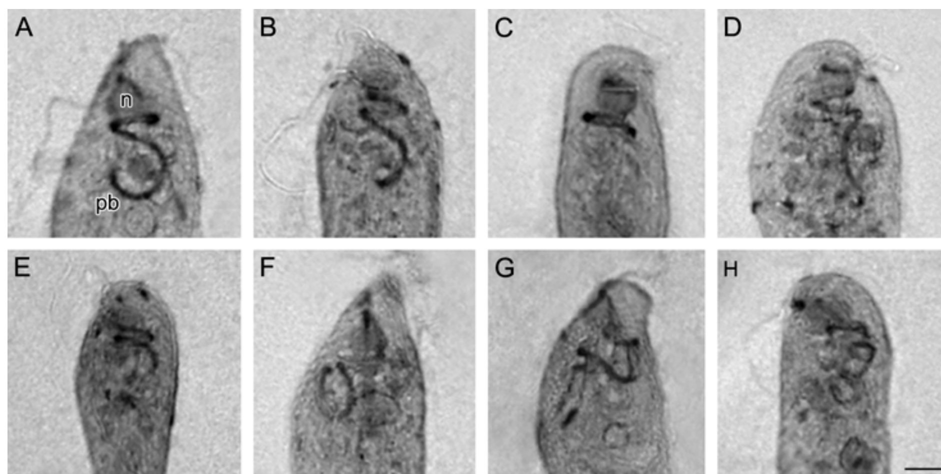

Figure 5. Protargol-stained cells showing the course of the parabasal body. **A–H:** The great variability of shape and length of the parabasal body (pb) is conspicuous. n – nucleus. Scale bar: 5 µm.

Together with the primary parabasal fiber, the parabasal body is typically wound around the nucleus and the axostyle in 1.5 to 2.5 circles on its course towards the posterior cell pole. However, protargol stained cells show a great variability in length, course, or shape of the parabasal body. In some cells, the turns are loosely wound around the axostyle (Figs 5A–B), whereas in other cells the windings of the parabasal body are closer (Figs 5C, E). There were also cells in which the parabasal bodies describe a circle not around but beside the axostyle. Here the end of the parabasal body points towards the anterior cell pole (Fig. 5F). Furthermore Z- or J-shaped parabasal bodies (Fig. 5G) were noticeable and even mazy forms appeared (Figs 5D, H). However, there were transitions between all described types of parabasal body.

Prokaryotic symbionts

The devescovinid flagellates in the hindgut of *I. marginipennis* were indistinguishable also based on the morphology of their prokaryotic symbionts. The entire surface of all devescovinid cells is densely covered with spiral-shaped bacteria (Figs 2A, C), identified as spirochetes by the presence of an axial filament in ultra-thin sections (not shown). Scanning electron microscopy showed that two morphotypes are present, which can be distinguished by their dimensions. The larger morphotype has a diameter of 0.4 µm and varies in length from 10 to 20 µm (n = 21) and is located specifically at the posterior cell pole (Fig. 6A).

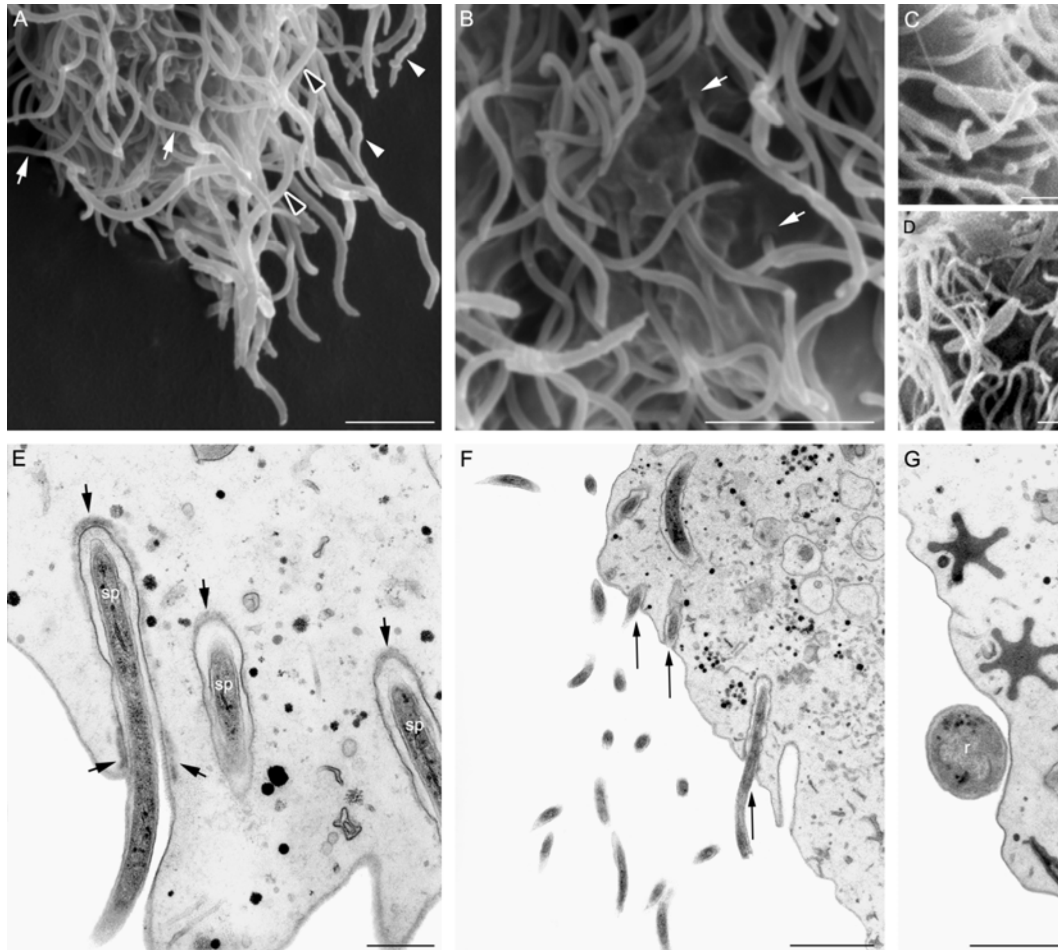

Figure 6. Bacterial symbionts of *Metadevescovina modica*. **A–D:** Scanning electron micrographs. **A:** Slender (arrows) and thicker (arrowheads) spirochetes at the posterior cell pole. White arrowheads: Spirochetes showing a rough surface. Black arrowheads: Spirochetes showing a smooth surface. **B:** Thin spirochetes encased by the flagellates' plasma membrane at their anterior cell poles (arrows). **C, D:** Straight (**C**) and slightly curved (**D**) rod-like prokaryotes at the cell surface. **E–G:** Transmission electron micrographs. **E:** Spirochetes (sp) encased by the flagellates' plasma membrane. An electron dense layer (arrows) supports the flagellate's plasma membrane. **F:** Encased parts of the spirochetes orientated in the same direction (arrows). **G:** Cross section of a rod-like prokaryote (r) located at the flagellates' surface. Scale bars: 2.5 µm (**A, B**), 1 µm (**C, D**), 0.4 µm (**E**), 1.5 µm (**F**), 1 µm (**G**).

Some of these large spirochetes have a rough surface, others a smooth surface (Fig. 6A). The shorter morphotype is considerably thinner, with a diameter of 0.2 µm and a length varying from 4 to 9 µm (n = 42). This morphotype is distributed over the whole surface of the flagellate and is attached to a special structure. One end of the spirochetal cell is inserted into a deep pouch formed by the plasma membrane of the flagellate, adding another 0.6 to 1.8 µm to the total length of the spirochete (Figs 6B,

E–F). At the base and the tip of this pouch, the flagellates' plasma membrane is underlain by an electron dense layer (Fig. 6E). All spirochetes are orientated in the same direction, i.e., the encased parts slant to the anterior cell pole of the flagellate (Fig. 6F).

Occasionally, two types of rod-shaped bacteria were present among the spirochetes, but only in very low numbers (1–2 per flagellate). One is slightly curved and measures about 0.8 µm × 4.0 µm; the other is straight and measures about 0.6 µm × 2.7 µm (Figs 6C–D). Occasional cross sections of such bacteria showed no special attachment structures; an assignment of these cells to one of the morphotypes was not possible (Fig. 6G).

Discussion

The results of this study suggest that the devescovinid flagellates in *I. marginipennis* belong exclusively to the genus *Metadevescovina*. There are three closely related SSU rRNA phylotypes (sequence similarity >99.4%) of *Metadevescovina*, but a thorough analysis of numerous ultrastructural traits indicates that only one morphotype is present. The presence of ten different devescovinid species, as proposed by Pérez-Reyes and López-Ochoterena (1965), can be excluded.

The validity of the three different devescovinid phylotypes (obtained in this study) is clearly documented by the signature analysis. Although Taq polymerase has an error rate of about 1.1×10^{-4} errors / base pair (Tindall and Kunkel 1988), it can not be assumed that random errors came up at the same position in all clones of the two clone libraries. Therefore, we exclude the possibility that the different bases at the signature positions are caused by PCR errors. Nevertheless, the sequence similarity (>99.4%) of the phylotypes combined with our morphological analyses indicates only one devescovinid species in *I. marginipennis*.

There are several, partially contradictory studies of the devescovinid flagellates of *I. marginipennis*. An early study by Light (1933) reported the presence of a single species of *Metadevescovina*, but the significance of this result is dubious, because he also reported the presence of a *Staurojoenina* species and did not find any *Trichonympha* species — two statements that are in contradiction to any other study of this termite species.

The first reliable report of devescovinids in *I. marginipennis* was published by Kirby (1945), who described two devescovinid species, *Metadevescovina modica* and

Metadevescovina magna. The morphotype reported in our study resembles Kirby's description of *M. modica*. We could not find any flagellates with a spirally rolled-up capitulum, which Kirby described as *M. magna*. It is possible that the description of *M. magna* is based on an artifact caused by the staining procedure. Also other features distinguishing *M. magna* from *M. modica* such as the length of the ectobiotic spirochetes or the shape of the nucleus were not found in our study, indicating that *M. modica* is the only species of *Metadevescovina* present, at least in the batch of *I. marginipennis* used in this study. Although generally the species composition of symbiotic flagellates is specific for a termite species (Honigberg 1970), differences in composition including loss of flagellates are documented. For example, different geographical positions as well as changes in experimental conditions (temperature, cellulose sources) can have an impact on the gut community (see Cook and Gold 2000; Kitade and Matsumoto 1993). Therefore, we cannot safely exclude the possibility that a second devescovinid species, *M. magna*, can be found in a different batch of *I. marginipennis*.

The subject of gut flagellates in *I. marginipennis* was picked up again by Pérez-Reyes and López-Ochoterena (1965). Based on light-microscopic observations, they described a total of ten different species of both *Devescovina* and *Metadevescovina*. However, the results of our study document that the morphological features used as criteria to create new species are either based on obvious artifacts or simply represent different aspects of the same structure. For instance, devescovinids resembling *Metadevescovina pavicula* were only found when the axostyles protruded at the posterior cell pole, a characteristic that was identified as an artifact. Other distinguishing traits, such as the presence of different ectobionts, different shapes of nuclei, different length of flagella, etc., were simply not supported by our observations and may just be morphological variations within a single species. Completely unjustified is the differentiation of the genera *Devescovina* and *Metadevescovina* based on smallest differences in the orientation of the anterior part of the parabasal body relative to the body axis of the flagellate, a criterion so far unprecedented in the literature.

The validity of *Metadevescovina* as a separate genus has been discussed controversially since the genus was established by Light (1926), who found a devescovinid flagellate in *Kalotermes hubbardi* that differed from *Devescovina* spp. by the presence of 12 additional flagella at the anterior cell pole. Grassé (1938) and

Kirby (1945) pointed out that these flagella are in fact spirochetes. Nevertheless, Kirby (1945) justified the validity of the genus *Metadevescovina* by a distinct posterior projection of the axostyle absent from *Devescovina*. However, a study of the illustrations of *Devescovina* and *Metadevescovina* species made by Kirby (1941, 1945) reveal that this classification was ignored several times by Kirby himself. Therefore, Grassé (1952) considered *Metadevescovina* as a synonym for *Devescovina*.

Clearly, the highly similar features of devescovinid flagellates do not allow the distinction of the genera *Devescovina* and *Metadevescovina* solely based on morphological data. Also our ultrastructural investigations could not reveal any differences between these two genera. However, the validity of the two genera is strongly supported by the results of the phylogenetic analysis of the SSU rRNA genes, where the two genera form distinct lineages in the radiation of devescovinid flagellates. The *Metadevescovina* species from *I. marginipennis* and *Pterotermes occidentis* form a monophyletic group together with the unidentified sequences from *Incisitermes minor*, which most likely originated from *Metadevescovina cuspidata* present in this termite (Kirby 1945). The only exception is *Metadevescovina extranea* from *Mastotermes darwiniensis*, which clusters among the Calonymphidae, as already pointed out by Noël et al. (2007). In this context, the phylogenetic basal position of that termite is remarkable. *M. darwiniensis* is the most primitive termite representing symbiotic flagellates of the genera *Deltotrichonympha*, *Koruga*, and *Mixotricha* that can be found in no other termite species (Brugerolle and Lee 2000b). In the same way, an early separation of *M. extranea* could be responsible for the unexpected phylogenetic position of this flagellate.

Another feature that corroborates the distinct position of the flagellates in the genus *Metadevescovina* is the colonization with bacterial epibionts. At this, most probably the epibionts represent the only distinguishing feature between *Devescovina* and *Metadevescovina* based on morphological characteristics. Whereas *Devescovina* species are generally colonized by rod-shaped bacteria (e.g., Kirby 1941; Noda et al. 2006; Radek et al. 1996; Tamm 1982), the cell surface of *Metadevescovina* species is covered by spirochetes (Kirby 1945; this study), suggesting that also the nature of the bacterial symbionts is of evolutionary relevance.

Experimental procedures

Termites

False workers (pseudergates) of *Incisitermes marginipennis* were obtained from the Federal Institute for Materials Research and Testing (BAM) in Berlin, where they are in culture. Termites were maintained in polyethylene containers on a diet of pinewood at 25°C.

DNA extraction, PCR amplification, cloning; and sequencing

Hindguts of four termites were carefully removed with fine forceps and suspended in solution U (Trager 1934). DNA from whole hindguts was extracted as previously described (Ikeda-Ohtsubo et al. 2007). DNA was extracted with the NucleoSpin kit (Macherey-Nagel; following the manufacture's instructions) from approx. 200 flagellates with devescovinid morphology collected by micropipetting using an inverted microscope (see Ikeda-Ohtsubo et al. 2007). They could be easily differentiated from the other genera present in *I. marginipennis* since *Trichonympha* is larger and multiflagellate, *Tricercomitus* is much smaller and has a long trailing flagellum, and *Oxymonas* possesses an anterior rostellum.

SSU rRNA genes of devescovinid flagellates were amplified using Taq DNA Polymerase (Invitrogen). The PCR condition was 5 min at 94°C (initial denaturation), 34 cycles 1 min at 94°C, 1 min at 50°C, 1.5 min at 72°C, and 7 min at 72°C (final extension). The SSU rRNA genes were amplified with universal eukaryotic primers (Ohkuma et al. 1998). PCR products were purified with the MinElute PCR Purification Kit (Manufacturer) and were eluted in 10 µl elution buffer. PCR products were ligated into plasmid pCR2.1-TOPO and introduced into *E. coli* TOP10F' by transformation using the TOPO TA cloning kit (Invitrogen). White colonies (transformants) were checked by direct PCR with M13 primers, and clones with correct-sized inserts were sorted into ribotypes by RFLP analysis using the restriction enzymes MSpI and HhaI. Due to these two enzymes, each single ribotype is represented by two RFLP patterns. For each ribotype, representative clones were sequenced with M13 primers. With exception of the two *Trichonympha* phylotypes (see Table 1), for each phylotype one representative SSU rRNA gene sequence was selected for submission. Sequences have been submitted to GenBank under accession numbers FM160643–FM160647.

Phylogenetic analyses

SSU rRNA gene sequences were imported into the Silva database (http://www.arb-silva.de/download) using the ARB software package (Ludwig et al. 2004). Sequences were automatically aligned with closely related SSU rRNA sequences using the ARB Fast Aligner tool, followed by manual refinement. Phylogenetic trees were calculated with almost-full-length sequences (>1300 bases) using the maximum-likelihood method (AxML) implemented in ARB. The tree topology was also tested in ARB using neighbor-joining and maximum-parsimony methods.

Light microscopy

To extract the flagellates, the hindgut of members of the functional worker cast was pulled out. For live observations, the hindgut was opened in 0.6% NaCl. Protargol staining was done according to Foissner (1991) after fixation with OsO_4 vapor. The nuclei of the flagellates and the prokaryotic symbionts were stained by incubation for 15 min in 2.5 µg/ml DAPI after a previous fixation for 15 min in 4% formaldehyde in 100 mM Soerensen phosphate buffer (SPB; pH 7.2). The preparations were rinsed three times for 5 min in 100 mM SPB before and after the incubation of the flagellates in DAPI. For tubulin immunofluorescence staining, the gut content was fixed 15 min in 4% formaldehyde in 20 mM SPB. After two 15-min washes in 20 mM SPB, the sample was incubated for 1 h in 400 mM glycine in double-distilled water. The flagellates were then washed twice for 15 min in 0.25% Triton X-100 in 20 mM SBP to permeabilize the flagellate membranes. The flagellates were then washed 15 min in 0.25% bovine serum albumin (BSA) in 20 mM SPB and incubated overnight with monoclonal mouse anti-bovine brain β-tubulin antibodies (Manfred Schliwa, Ludwig-Maximilians-Universität München). For this purpose, the antibodies were diluted 1:30 with 0.1% sodium azide, 0.25% BSA in 20 mM SPB. After three 30-min washes in 20 mM SPB and one 30-min wash in 0.25% BSA in 20 mM SPB, the flagellates were incubated with polyclonal fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibodies (Sigma) for 2 h. The polyclonal antibodies were diluted in the same way as the monoclonal antibodies. Thereafter the flagellates were washed three times for 10 min in 20 mM SPB. Prior to fluorescence microscopy, the flagellates were mixed with a drop of DABCO-glycerol solution (250 mg DABCO in 10 ml SPB plus 90 ml glycerol) to reduce fading. Some flagellates were double-labeled with DAPI by adding one to two drops of a DAPI solution (5 µg/ml).

In addition to standard light microscopic investigations with a Zeiss Axiophot, three-dimensional, digital fluorescence microscopic analyses of the nucleus and the axostyle with its capitulum were made with a Keyence BZ-8000. During a presentation of this microscope, we had the chance to investigate our double-labeled flagellates. Results were unequivocal, but since videos of electronically turned picture staples were produced, we cannot show single images in the present paper.

Scanning electron microscopy

Cells were fixed 30 min in 2.5% glutaraldehyde in 100 mM SPB, washed three times in the same buffer and fixed in 1% OsO_4 in 100 mM SPB on ice for 1 h. The specimens were washed again three times and transferred into small cups covered with planktonic gauze. After dehydration in a graded series of ethanol, the cells were dried with a Balzer CPD 030 and coated with gold in a Balzer SCD 040. The cells were examined using a FEI Quanta 200 ESEM.

Transmission electron microscopy

Flagellates were fixed 1 h in 2.5% glutaraldehyde in 50 mM SPB. The cells were rinsed three times in 50 mM cacodylate buffer (pH 7.2) and postfixed for 2 h in a 1:1 solution of 2% OsO_4 and 3% $K_4[Fe(CN)_6]$ according to Karnovsky (1971). After three further rinses in 50 mM cacodylate buffer, the cells were dehydrated in a graded series of ethanol and embedded in Spurr's resin. Sections were stained with saturated uranyl acetate and lead citrate (Reynolds 1963) and examined with a Philips EM 208.

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3 Strict cospeciation of devescovinid flagellates and *Bacteroidales* ectosymbionts in the gut of dry-wood termites (Kalotermitidae)

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Abstract

Although the majority of termite gut flagellates are associated with diverse ectosymbionts of the order *Bacteroidales*, little is known about their evolutionary relationship with their host flagellates. In this study, we investigated the molecular phylogenies of devescovinid flagellates (*Devescovina* spp.) and their symbionts in the gut of dry-wood termites (Kalotermitidae). Species-pure suspensions of flagellates were isolated with micropipettes from a wide range of termites. Employing the full-circle-rRNA approach, we obtained SSU rRNA gene sequences of numerous phylotypes of the host flagellates and their bacterial symbionts. Phylogenetic analysis confirmed that the *Devescovina* species present in many species of Kalotermitidae form a monophyletic group. They were consistently associated with a distinct lineage of ectosymbionts, which formed a monophyletic group among the *Bacteroidales*. The well-supported congruence of their phylogenies document that the ectosymbionts are co-speciating with their specific hosts. In contrast, the endosymbionts that were simultaneously present in

***Authors' contribution:** The study was designed by M. S. D. under the guidance of A. B. All the experiments (except electron microscopy and technical assistance by K. M.) were planned and performed by M. S. D. The draft was prepared by M. S. D., and M. S. D. and A. B. worked on the final manuscript. Electron microscopy was carried out by J. F. H. S. under the supervision of R. R. W. I.-O. introduced M. S. D. to capillary-picking and phylogenetic analysis. Most of the termites used in this study were provided by H. H. **Technical assistance:** K. M. prepared some of the SSU rRNA gene clone libraries and performed sequencing of the COII genes of termites.

all *Devescovina* flagellates investigated and belong to the so-called "Endomicrobia" (TG-1 phylum) are clearly polyphyletic and must have been acquired independently by different *Devescovina* species. The same was true for the *Bacteroidales* ectosymbionts of *Oxymonas* flagellates present in several Kalotermitidae, which formed several distantly related lineages in the phylogenetic tree, underscoring the notion that the evolutionary history of flagellate–bacteria symbioses in termite guts is quite complex.

Introduction

Termites are suitable examples to study the symbiosis (Brune, 2005). The cellulolytic flagellates in the hindgut of lower termites presumably play an important role in the digestion of the lignocellulose (Yamin, 1980; Odelson and Breznak, 1985). Although termite gut flagellates are difficult to cultivate, their phylogenetic identity can be determined by using the full-cycle-rRNA approach (Gerbod *et al.*, 2004; Moriya *et al.*, 2003; Stingl and Brune, 2003). Molecular phylogenies of flagellates have improved the knowledge about the classification and evolution of many flagellate genera (Ohkuma *et al*., 2000, 2005, 2007; Stingl and Brune, 2003; Gerbod *et al*., 2004).

Little is known about the molecular evolution of the devescovinid flagellates (*Devescovinidae*), which are present mainly in the gut of dry-wood termites (Kalotermitidae), and were thoroughly described on the basis of the morphological characteristics (Kirby, 1941, 1942, 1945). Among the devescovinid flagellates, the genus *Devescovina* comprises the highest number of species (Kirby, 1941). Since the small-subunit ribosomal RNA (SSU rRNA) gene sequence of the devescovinid flagellate *Caduceia versatilis* clustered within the members of the genus *Devescovina* (Noël *et al*., 2007), the monophyly of the genus *Devescovina* is under question.

All the described species of *Devescovina* harbor filamentous ectosymbiotic bacteria, which is the major criterion used by Kirby (1941) to describe this genus. Recently, Noda *et al*. (2006a) showed that the SSU rRNA gene sequences of the filamentous ectosymbionts of two *Devescovina* species from two different Kalotermitidae clustered together (~7% sequence divergence), forming a deep-

branching novel lineage in the order *Bacteroidales*. Most of the ectosymbionts of termite gut flagellates represent several novel lineages in the order *Bacteroidales* (Wenzel *et al.*, 2003; Stingl *et al.*, 2004; Noda *et al.*, 2006a). Interestingly, the SSU rRNA gene sequence of the filamentous ectosymbionts of *C. versatilis* formed a sister lineage of the ectosymbionts of the *Devescovina* species (Hongoh *et al.*, 2007), suggesting that the association between the devescovinid flagellates and the filamentous ectosymbionts is quite old.

Devescovina species harbor yet another bacterial symbiont affiliated with the "Endomicrobia" (phylum: Termite group 1) (Ikeda-Ohtsubo *et al.*, 2007; Ohkuma *et al.*, 2007). "Endomicrobia" are cytoplasmic symbionts (endosymbionts) of termite gut flagellates (Stingl *et al.*, 2005). Recently, Hongoh *et al.* (2008) sequenced the complete genome of the representatives of the "Endomicrobia". The reduced genome (1.1 Mbp) contained genes, which encode 15 amino acids and several cofactors. Based on these data, "Endomicrobia" were suggested to provide nitrogenous compounds to their host flagellates.

Unlike *Bacteroidales* ectosymbionts, "Endomicrobia" sequences of two *Devescovina* species (Ikeda-Ohtsubo *et al.*, 2007; Ohkuma *et al.*, 2007) do not cluster together in the SSU rRNA gene phylogenetic tree. Moreover, "Endomicrobia" from several phylogenetically distinct flagellates cluster together, with the exception of the "Endomicrobia" of *Trichonympha* species (see below). Altogether, these data provide weaker indications that the "Endomicrobia" may not represent an old association with different *Devescovina* species, and on the contrary, the filamentous *Bacteroidales* might have codiverged with the host devescovinid flagellates.

Codivergence, also known as cospeciation, is joint speciation of host and symbiont, which results from intimate and long-standing association. The test of cospeciation involves congruence analysis between the molecular phylogenies of hosts and symbionts. Cospeciation is believed to exist if the molecular phylogenies are significantly more similar than would be expected due to chance alone, e.g., cospeciation between insects and their bacterial symbionts, birds and their ectoparasites, etc., (Baumann *et al.*, 1997; Peek *et al.*, 1998; Hughes *et al.*, 2007; Hosokawa *et al.*, 2007). In contrast, hosts can randomly acquire symbionts,

leading to the incongruence in the host and symbiont phylogenies (van Hoek *et al.*, 2000).

Cospeciation in the triplex symbiosis involving termites (Isoptera, Rhinotermitidae), their symbiotic flagellate protists (*Pseudotrichonympha* spp.), and the *Bacteroidales* endosymbionts of flagellates was recently studied (Noda *et al.*, 2007). Another example of cospeciation was provided by Ikeda-Ohtsubo and Brune (2008), which showed significant congruence between the phylogenies of *Trichonympha* species and “Endomicrobia”. These two studies collectively suggest that the flagellates and their bacterial symbionts have evolved together in the termite gut.

In the present study, we investigated the molecular phylogenies of several *Devescovina* species and their bacterial symbionts. Species-pure suspensions of *Devescovina* species from a wide range of termite species in the family Kalotermitidae were isolated with micropipettes. SSU rRNA gene sequences of the host flagellates and their bacterial symbionts were obtained using the full-cycle-rRNA approach. Phylogenies of the host *Devescovina* flagellates and their bacterial symbionts were built and compared with each other. Molecular phylogenies of the *Bacteroidales* ectosymbionts of *Oxymonas* species (phylum: Preaxostyla) were also investigated from two species of Kalotermitidae.

Results

Phylogeny of *Devescovina* spp.

With the exception of *Neotermes castaneus*, all dry-wood termites, (Kalotermitidae) investigated in this study (Table 1), harbored only one morphotype of *Devescovina* flagellates. The measurement of *Devescovina* flagellates' sizes in *N. castaneus* showed a bimodal distribution, and thus the presence of two morphotypes (Fig. 1). Based on the previously described morphological features (Kirby, 1941), these morphotypes were assigned to *Devescovina lepida* and *Devescovina arta*. Separate flagellate suspensions prepared by capillary picking of each morphotype, including also the two morphotypes present in *N. castaneus*, yielded only a single major phylotype of SSU rRNA genes (> 99.8% sequence similarity). Two minor phylotypes were obtained from the flagellate suspension of *Devescovina lepida*. Similar minor phylotypes of *Devescovina lepida* were recovered in two independent clone libraries (details not shown). Phylogenetic analysis revealed that all phylotypes clustered together with the previously published sequences of flagellates assigned to the genus *Devescovina* (Fig. 2A). Similar tree topologies (with high bootstrap support) were observed in all the phylogenetic methods. The previously analyzed devescovinid flagellate *Caduceia versatilis* from the termite *C. cavifrons* (Noël *et al.*, 2007) formed a sister lineage of all *Devescovina* species.

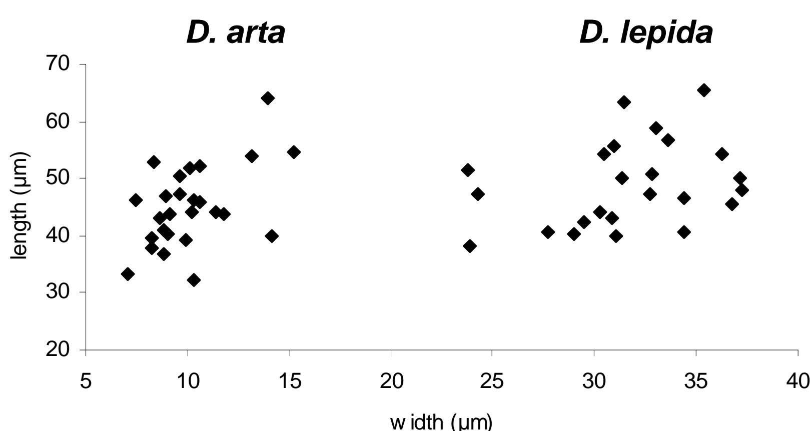

Figure 1. Bimodal distribution of sizes of *Devescovina* flagellates in the gut of termite *Neotermes castaneus*. These two morphotypes were assigned to *Devescovina lepida* and *Devescovina arta*; the latter is smaller in width.

Phylogeny of *Bacteroidales* ectosymbionts of *Devescovina* spp.

16S rRNA gene clone libraries for each picked flagellate suspension (flagellate phylotype) yielded one phylotype (> 99.8% sequence similarity) (Fig. 2B) of *Bacteroidales*. Two minor phylotypes of *Bacteroidales* were obtained from the flagellate suspension of *Devescovina lepida*. Phylogenetic analysis showed that the *Bacteroidales* ectosymbionts of all *Devescovina* spp. clustered together in the phylogenetic tree (Fig. 4). Previously studied *Bacteroidales* ectosymbionts of *Caduceia versatilis* (Hongoh *et al.*, 2007) formed a sister lineage of *Bacteroidales* ectosymbionts of *Devescovina* spp. (Fig. 2B).

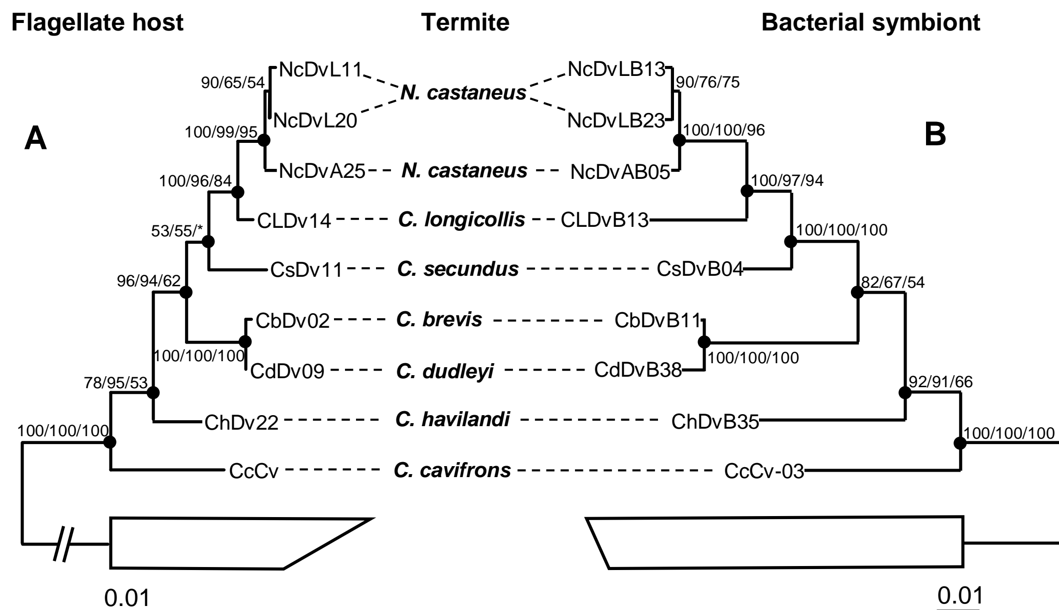

Figure 2. Tangled phylogenetic trees (maximum likelihood) of devescovinid flagellates (A) and their *Bacteroidales* ectosymbionts (B). The phylogeny of devescovinid flagellates is based on the 18S rRNA gene sequences (1408 unambiguously aligned nucleotide sites). The tree was rooted with *Trichomonas vaginalis* (AY338474) and *Tritrichomonas foetus* (AY055799). For simplicity, branch length of outgroups has been reduced to 50%. Phylogeny of *Bacteroidales* ectosymbionts of devescovinid flagellates is based on the 16S rRNA gene sequences (1363 unambiguously aligned nucleotide sites). The tree was rooted with *Bacteroides fragilis* (M11656) and *Tannerella forsythensis* (X73962). Solid circles are cospeciation events inferred from reconciliation analysis in Treemap (Page, 1994; Page, 1998). For reconciliation analysis only one of the phylotypes was considered in case of minor phylotypes of *Devescovina lepida*, and their *Bacteroidales* ectosymbionts. Sequence of the nodal support is Bayesian/Maximum parsimony/Maximum likelihood.

Table 1. Termites, flagellates and ecto- and endosymbiotic bacteria of flagellates

Termite (origin)	Flagellate suspension	Flagellate phylotype	Ectosymbiotic *Bacteroidales* phylotype	Endosymbiotic "Endomicrobia"
Neotermes castaneus (Cuba)	*Devescovina lepida*[a]	NcDvL11 NcDvL20	NcDvLB13 NcDvLB23	NcDv-1[c]
	Devescovina arta	NcDvA25[b]	NcDvA05	NcDvAE10
	Oxymonas sp.	NcOxA[c]	NcOxAB12 NcOxAB26	NcOx-1[c]
Cryptotermes longicollis (Mexico)	*Devescovina* sp.	CLDv14	CLDvB13	CLDvE07
Cryptotermes secundus (Australia)	*Devescovina* sp.	CsDv11	CsDvB04	CsDvE04
	Oxymonas sp.[d]	Not determined	CsOxB27 CsOxB40	Not obtained[e]
Cryptotermes brevis (Brazil)	*Devescovina* sp.	CbDv02	CbDvB11	CbDvE07
Cryptotermes dudleyi (Kenya)	*Devescovina* sp.[f]	CdDv09	CdDvB38	CdDvE17
Cryptotermes havilandi (Ghana)	*Devescovina* sp.	ChDv22	ChDvB35	ChDvE11
Cryptotermes cavifrons (USA)[g]	*Caduceia versatilis*	CcCv[h]	CcCv-03	Not obtained[i]

a. Kirby (1941) also described *D. lepida* in *C. longicollis*. However, *Devescovina* phylotype obtained from *C. longicollis* is different than in *N. castaneus*. Therefore, we have named *Devescovina* in *C. longicollis* as *Devescovina* sp.

b. Sequence is identical with the sequence in the Genbank (X97974) which was reportedly obtained from *N. jouteli* (J. Branke, unpublished, 1996). Nevertheless, it should be noted that *N. jouteli* (obtained from Florida, USA) does not contain *Devescovina* flagellates (details not shown). Therefore, it is highly likely that the flagellate sequence present in the public database (X97974) originated from *N. castaneus*. This observation is supported by the fact that Kirby (1941) did not describe any *Devescovina* species in *Neotermes jouteli*.

c. Sequences were submitted as a part of another manuscript (Ikeda-Ohtsubo *et al*., 2007).

d. Although individuals of *C. secundus* were collected at the same time and place (Darwin, Australia), *Oxymonas* flagellates were not observed in all the batches stored in separate containers.

e. The clone library did not contain any "Endomicrobia" sequences.

f. *Cryptotermes dudleyi* from Australia presumably has a different phylotype of *Devescovina* sp. (AF052696) than obtained in this study (Keeling *et al*., 1998).

g. All the data for this termite was used from previous studies (Noël *et al*., 2007; Hongoh *et al*., 2007).

h. Since this sequence in the Genbank has no phylotype name, we named it for its use in this manuscript.

i. Hongoh *et al.* (2007) did not find any "Endomicrobia" associated with *Caduceia versatilis*.

Cospeciation analysis of devescovinids and their *Bacteroidales* ectosymbionts

Reconciliation analysis (Treemap) of the phylogenetic trees of devescovinid flagellates and their *Bacteroidales* ectosymbionts showed that the host and symbiont trees perfectly mirror each other (Fig. 2). Cospeciation test (Page, 1994; Page, 1998) produced seven major cospeciation events, which are indicated by bold circles (Fig. 2). P value was 0.0008.

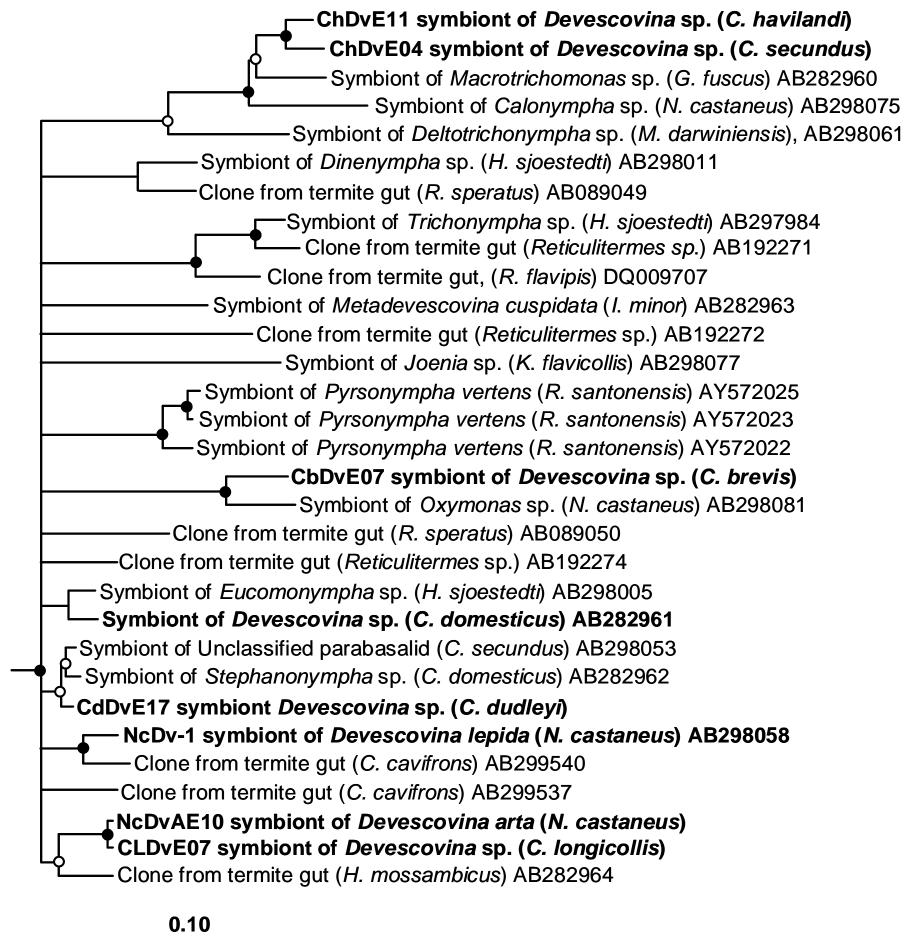

Figure 3. Phylogenetic tree of "Endomicrobia". The tree is based on the SSU rRNA gene sequences. "Endomicrobia" of *Devescovina* flagellates are shown in the bold. The tree topology was tested with neighbor joining and parsimony methods. Nodes that were not supported in all analyses are shown as multifurcations. Open and closed circles indicate bootstrap values of >70% and >90%, respectively.

Phylogeny of "Endomicrobia"

In addition to the *Bacteroidales* ectosymbionts, all investigated *Devescovina* species from several Kalotermitidae also contained the so-called "Endomicrobia" (Termite group 1) endosymbionts. Each *Devescovina* suspension (phylotype) possessed a unique 16S rRNA gene phylotype (> 99.8% sequence similarity) belonging to the "Endomicrobia". Phylogenetic analysis of these sequences showed that the "Endomicrobia" from majority of the *Devescovina* species are distantly related with each other (Fig. 3). Each "Endomicrobia" phylotype from several *Devescovina* species clustered together with "Endomicrobia" of numerous phylogenetically distinct flagellates (Fig. 3).

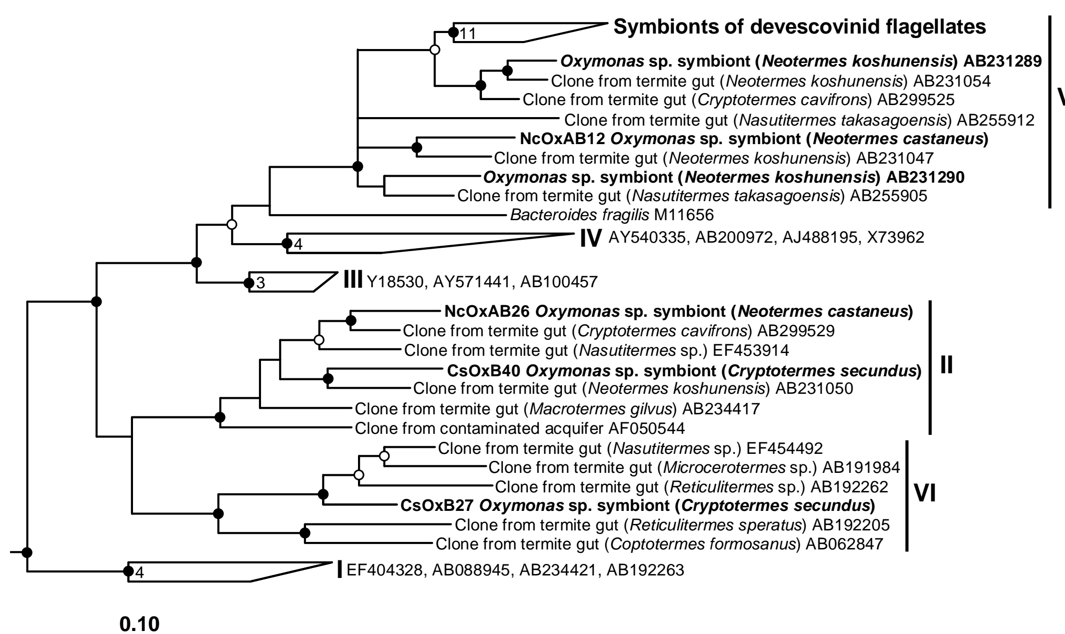

Figure 4. Phylogenetic relationships of the ectosymbiotic *Bacteroidales* of *Oxymonas* species. The tree is based on the 16S rRNA gene sequences (maximum-likelihood tree based on 1273 unambiguously aligned nucleotide sites). Names of termite species are given in parentheses. Previously named clusters (Ohkuma *et al.*, 2002) are shown. A new cluster (VI) was named in this study. *Bacteroidales* ectosymbionts of devescovinid flagellates formed a monophyletic group with a high bootstrap support. Previously published sequences of *Bacteroidales* ectosymbionts of two *Devescovina* flagellates are also included in the group (accession numbers: AB194939 and AB194938). *Bacteroidales* ectosymbionts of *Oxymonas* sp. from *N. koshunensis* are also shown in

bold. Filled and closed circles indicate bootstrap values of >90% and >70%, respectively. Tree was rooted with outgroups of the suitable taxa.

Phylogeny of *Bacteroidales* ectosymbionts of *Oxymonas* spp.

16S rRNA gene clone libraries were made from picked suspensions of *Oxymonas* spp., which originated from two different species of termites (*N. castaneus* and *Cryptotermes secundus*). 26 and 27 clones were analyzed for *Oxymonas* from *N. castaneus* and *C. secundus*, respectively. The clone libraries yielded several bacterial phylotypes (details not shown). In both the clone libraries, two major phylotypes belonging to the *Bacteroidales* were obtained. *Bacteroidales* phylotypes NcOxAB12, NcOxAB26, CsOxB27 and CsOxB40 (Table 1) were 30.80%, 23.10%, 37.0 and 14.8% abundant, respectively. Since electron microscopy (Fig. 6) showed the presence of two morphotypes of rod shaped bacteria on the surface of *Oxymonas* sp., and Noda *et al.*, (2006b) also found two types of *Bacteroidales* ectosymbionts associated with the *Oxymonas* sp. in *N. koshunensis*, phylotypes NcOxAB12, NcOxAB26, CsOxB27 and CsOxB40 were considered as ectosymbionts. Phylogenetic analysis showed that the *Bacteroidales* ectosymbionts of *Oxymonas* flagellates clustered together with the previously published sequences from different species of termites (Fig. 4). All the four *Bacteroidales* sequences originating from two *Oxymonas* suspensions formed novel lineages in the earlier described termite gut specific clusters (Ohkuma *et al.*, 2002). Collectively, these data show that the *Bacteroidales* ectosymbionts of *Oxymonas* species from several Kalotermitidae are polyphyletic.

Localization of *Bacteroidales* ectosymbionts by FISH

In order to confirm that the *Bacteroidales* present in the flagellates suspensions are specific symbionts of *Devescovina* spp., *in situ* hybridization was carried out with phylotype- and group-specific probes. A probe (DVB178) was designed for the *Bacteroidales* ectosymbionts of *Devescovina* spp. in *N. castaneus*. DVB178 had two strong mismatches with all the database sequences. DVB178 hybridized with the filamentous ectosymbionts of *Devescovina lepida* and *Devescovina arta* in the termite *N. castaneus* (Fig. 5). It did not hybridize with symbionts of the other flagellates and also with the free-living bacteria in the termite gut. 20%

formamide was found to be the optimum for the probe DVB178. Additionally, FISH with a general *Bacteroidales* probe BAC319a showed that all *Devescovina* species from several Kalotermitidae harbor filamentous ectosymbionts (*Bacteroidales*). Moreover, FISH with the probe BAC319a confirmed that all *Devescovina* cells present in the termite gut possess filamentous ectosymbionts (*Bacteroidales*). Taken together, results of FISH convincingly show that the *Bacteroidales* obtained in the *Devescovina* flagellate suspensions come from the filamentous ectosymbionts of *Devescovina* spp. In our previous study (Ikeda-Ohtsubo *et al.*, 2007), "Endomicrobia" of one of the *Devescovina* sp. (*D. lepida*) were *in situ* localized with a phylotype-specific probe.

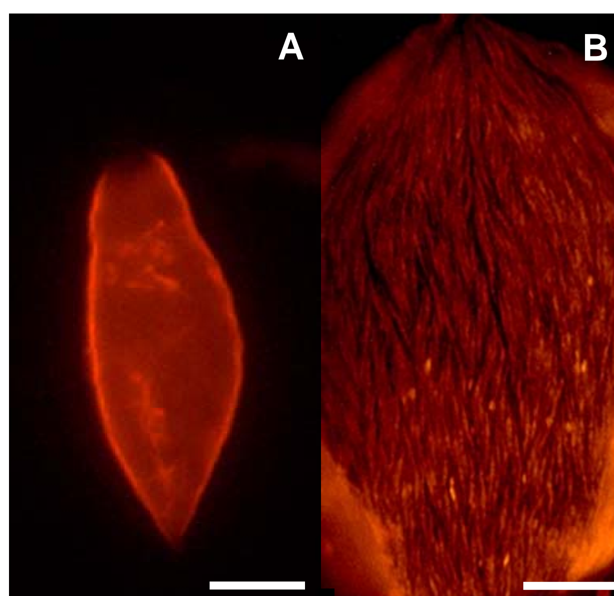

Figure 5. *In situ* identification of ectosymbiotic *Bacteroidales* of the flagellate *Devescovina lepida* in the termite *N. castaneus*. A and B: different magnifications. A, specific Cy3-labeled probe (DVB178) for the ectosymbiotic *Bacteroidales* of *Devescovina* spp. hybridized with the ectosymbionts. The filamentous ectosymbionts (B) cover the entire surface of the flagellate cell. Probe also hybridized with the symbionts of *Devescovina arta* (not shown). Scale bars, 10 µm.

Electron microscopy

Scanning electron microscopy (SEM) of the *Devescovina* sp. (present in *Neotermes* sp.) revealed the spindle-shaped body, three anterior flagella, and a

thicker, ribbon-shaped recurrent flagellum typical for all members of the genus *Devescovina* (Fig. 6A). The whole body of each cell of *Devescovina* sp. is densely

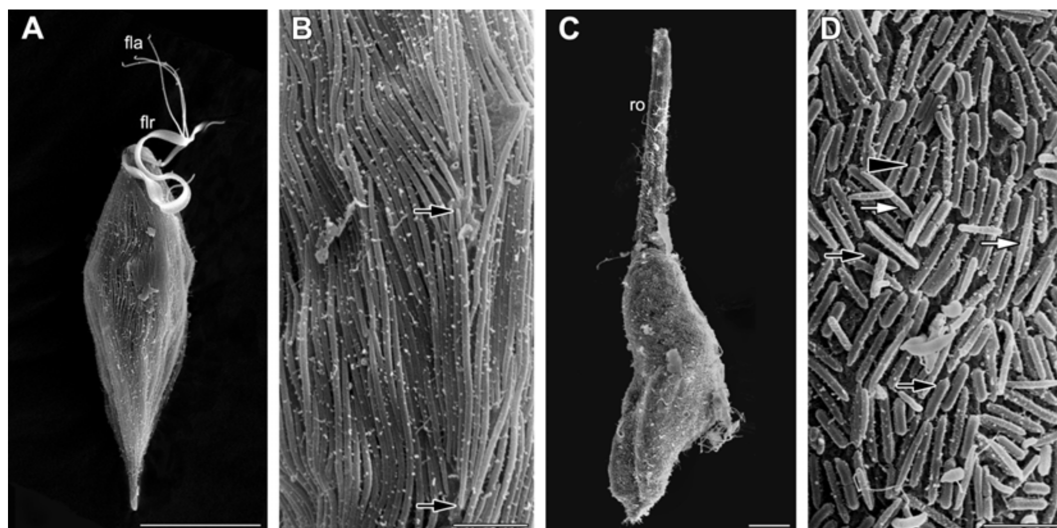

Figure 6. Scanning electron micrographs of *Devescovina* sp. (A) and *Oxymonas* sp. (C) and their ectosymbiotic bacteria (B, D) in *Neotermes* sp., exemplifying the two major groups of flagellates investigated in this study. *Devescovina* species possess three anterior flagella (fla) and one recurrent flagellum (flr). In *N. castaneus*, the entire flagellate is covered by a layer of uniform, filamentous bacteria; the arrows indicate beginning and end of a single cell. The *Oxymonas* species possess a rostellum (ro); in *Neotermes* sp., the flagellate is colonized by two types of ectosymbiotic bacteria: long, slender rods with tapered ends (white arrows) and short, thick rods with rounded ends (black arrows), sometimes in division (arrowhead). Scale bars: 20 µm (A, C), 3 µm (B, D).

Table 2. Measurements of flagellates (*Devescovina* sp. and *Oxymonas* sp. from *Neotermes* sp.) and their ectosymbiotic bacteria.

Flagellate	Length (µm), width (µm)	Morphology of the attached bacteria	Mean size of the attached bacteria (number of bacterial cells investigated) (µm)	Mean number of the attached bacteria/225 µm^2 (number of measurements)
Devescovina sp.	40—60 13—27	Thin long rods	10.9 x 0.3 (20)	65 (8)
Oxymonas sp.	46—150 20—60	Rods with rounded ends	1.4 x 0.4 (30)	28—67 (4)
		Rods with tapered ends	2.9 x 0.3 (30)	34—83 (4)

covered by laterally attached long, thin rods (Fig. 6A–B). They are arranged in the same orientation, i.e., their longitudinal axes lie parallel to the longitudinal axis of the host flagellate. Spirochetes were never found attached to the flagellates' membrane. The dimensions and morphology of *Devescovina* flagellates and their ectosymbiotic bacteria are summarized in Table 2.

The *Oxymonas* sp. (present in *Neotermes* sp.) had the typical club-shaped body with the so-called rostellum at the anterior cell pole, a holdfast serving as anchor to the termite gut wall (Fig. 6C). The surface of the flagellates was covered with two morphotypes of irregularly attached rods. Since the rods seem to be orientated randomly and some of them are lying on each other, their numbers were estimated. Many specimens were also covered with spirochetes, albeit at different numbers. When spirochetes were abundant, the density of rod-shaped ectosymbionts was markedly reduced (not shown). The dimensions and morphology of *Oxymonas* flagellate and their ectosymbiotic bacteria are summarized in Table 2.

Discussion

This is the first report of strict cospeciation of an ectosymbiotic bacterium (present on the body surface) with its host. The SSU rRNA gene phylogenies of devescovinid flagellates (*Devescovina* species and *Caduceia versatilis*), and their *Bacteroidales* ectosymbionts exactly mirror each other. The most parsimonious explanation for the congruent phylogenetic trees is the common ancestor of devescovinids acquired *Bacteroidales* ectosymbionts, and the *Bacteroidales* then cospeciated with the host flagellates into different species. In contrast, the endosymbiotic bacteria ("Endomicrobia") of several *Devescovina* species were found to be polyphyletic. The polyphyletic nature of "Endomicrobia" suggests their multiple acquisitions by *Devescovina* species during the course of evolution. The same was true for the *Bacteroidales* ectosymbionts of *Oxymonas* species, which formed several novel lineages in the phylogenetic tree, rejecting the possibility of cospeciation with the host flagellates.

The family *Devescovinidae* comprises 12 genera of flagellates (Kirby, 1941, 1942, 1944), found mainly in the gut of dry-wood termites (Kalotermitidae), and

defined mostly on the basis of the morphological characteristics. The highest species diversity was documented for the genus *Devescovina* (Kirby, 1941). Kirby described 20 species of *Devescovina* in several Kalotermtidae (Kirby, 1941). In a previous study, we showed the phylogenetic position of the devescovinid flagellate *Metadevescovina* sp., and also demonstrated that *Metadevescovina* species and *Devescovina* species form two distinct lineages (Strassert *et al.*, in revision), which supports differentiation of these two genera based on the morphological descriptions of Kirby. Another devescovinid flagellate *C. versatilis* from the termite *Cryptotermes cavifrons*, however, clustered within the group of *Devescovina* species (Noël *et al.*, 2007). In contrast, in this study, the phylogenetic analysis with a larger dataset of *Devescovina* species showed that *Caduceia versatilis* forms a sister group of *Devescovina* species. It is possible that the *Caduceia versatilis* belongs to the genus *Devescovina*, since the morphological features which allow a separation of *Caduceia* from *Devescovina* are weak (Kirby 1942). Even though Grassé supported the existence of the genus *Caduceia* in 1937, a synonymy of these two genera, as suggested by Duboscq and Grassé (1927), can not be declined.

The synonymy of these two genera is well supported by the presence of filamentous bacteria on the surface of *C. versatilis* and *Devescovina* species (Kirby, 1941, 1942, 1944; Tamm, 1982). Since the filamentous bacteria present on the surface of all the investigated *Devescovina* species (in this study) were *Bacteroidales*, we propose that the filamentous ectosymbionts of all other *Devescovina* species described by Kirby also belong to the presently studied lineage of *Bacteroidales*. Moreover, since filamentous *Bacteroidales* are not present as symbionts of devescovinid flagellates other than *Devescovina* and *C. versatilis*, it is clear that the common ancestor of all *Devescovina* species, and *Caduceia versatilis* acquired filamentous *Bacteroidales* at one point in time. Since their acquisition, *Bacteroidales* have been strictly cospeciating with their host flagellates.

The strict cospeciation between devescovinids and *Bacteroidales* indicate host–symbiont coadaptation (Woolhouse *et al.*, 2002). The filamentous ectosymbionts of devescovinid flagellates represent a special case because

Bacteroidales ectosymbionts of other flagellates (e.g., *Oxymonas* species) do not show cospeciation with their host flagellates. Therefore, it is tempting to speculate that during the evolution a physiological interaction might have developed between *Devescovina* flagellates and their *Bacteroidales* ectosymbionts. As suggested by several other authors (Stingl *et al*., 2004; Noda *et al*., 2006a), the *Bacteroidales* could be involved in several physiological roles such as reduction of oxygen or the provision of nutrients to the host flagellates. Previous studies showed that the *Devescovina* sp. in *C. dudleyi*, *Devescovina* sp. in *N. koshunensis* and *Staurojoenina* sp. in *N. cubanus* phagocytose their *Bacteroidales* ectosymbionts (Radek *et al*., 1996; Stingl *et al*., 2004; Noda *et al*., 2006). As discussed by Stingl *et al*. (2004), it suggests that the flagellates use their ectosymbionts as the energy source. Since parabasalid flagellates make lactate and hydrogen as their fermentation products (Steinbüchel and Müller, 1986), *Bacteroidales* could be feeding on the fermentation products of the flagellates.

In contrast to the *Bacteroidales*, devescovinid flagellates must have acquired the endosymbiotic “Endomicrobia” several times, independently from other flagellates. This hypothesis is supported by the fact that the close relatives of “Endomicrobia” from *Devescovina* flagellates are from several other phylogenetically unrelated flagellates. Another less parsimonious hypothesis would be that “Endomicrobia” were acquired relatively late during the evolution. This is less likely since “Endomicrobia” were present in the common ancestor of termites and the cockroach *Cryptocercus punctulatus* (Stingl *et al*., 2005). Unlike devescovinid flagellates, *Oxymonas* flagellates acquired their *Bacteroidales* ectosymbionts several times from the enormous diversity of the free-living *Bacteroidales* in the termite gut. The loss and acquisition of different *Bacteroidales* by several *Oxymonas* species indicate week/no interaction of host and symbionts. Furthermore, since some of the *Oxymonas* cells harbored very few *Bacteroidales* ectosymbionts (results of the electron microscopy), ectosymbionts may not be necessary for the survival of the host cell.

The results of the present study collectively document that the flagellate–bacteria symbioses in the termite gut are quite complex, and therefore, challenging to study the physiological basis of these symbioses.

Description of "*Candidatus* Armantifilum devescovinae"

Ar.man.ti.fi' lum. L. part. adj. armans arming; L. neut. n. filum filament; N.L. neut. n. armantifilum, an arming filament. de.ves.co.vi' nae. N.L. n. *Devescovina*, a genus of parabasalid flagellates; N.L. gen. n. devescovinae, of *Devescovina* (referring to the host genus).

Basis of assignment: filamentous rods, laterally attached on the surface of all *Devescovina* species, originally referred to as "*Fusiformis*-like rods" by Kirby (1941). So far uncultured, but form a monophyletic group based on 16S rRNA gene sequence analysis.

Experimental procedures

Termites

Termites originating from a broad geographic range were used (Table 1). *Neotermes castaneus, Cryptotermes brevis, Cryptotermes dudleyi, Cryptotermes havilandi* and *Cryptotermes longicollis* were obtained from cultures maintained at Bundesanstalt für Materialforschung und -prüfung (BAM), Berlin, Germany. *Cryptotermes secundus* was collected in a mangrove forest near Darwin, Australia (provided by Judith Korb, Regensberg). Termites were maintained in polyethylene containers on a diet of pinewood at 25 ^{0}C. Only Pseudergates were used in the experiments. Morphological identification of termites was further confirmed by sequencing their cytochrome oxidase II genes as described elsewhere (Pester and Brune, 2006).

Flagellate picking and DNA extraction from flagellates

The contents of one to three termites were suspended in Solution U (Trager, 1934). Unambiguously identified (50–200) flagellate cells were collected by micropipette (for methodological details see Ikeda-Ohtsubo *et al.*, 2007). Picked flagellates were then boiled for about 10 minutes at 95 °C. This boiled sample was directly used for PCR amplification of the SSU rRNA genes of flagellates and bacteria. Alternatively, from the gut homogenate of the same termite, flagellates were picked twice. One batch was treated as mentioned above, with the exception that SSU rRNA genes of only bacteria were amplified. Another batch was

subjected to DNA extraction by Nucleospin kit (according to manufacturer's instructions). Extracted DNA was then used for amplification of SSU rRNA genes from flagellates. The explanation for this is as follows. When we prepared 16S rRNA gene clone library from the DNA extracted by Nucleospin kit (Macherey Nagel), we obtained a high number of clones belonging to a novel phylotype of alpha-proteobacteria. This phylotype was also obtained in the clone library prepared from boiled flagellates, however, occasionally and was under-represented in the clone library. Details of this phylotype are not shown (Desai and Brune, in preparation).

PCR amplification and cloning

SSU rRNA genes of flagellates were amplified using the eukaryotic primers (Ohkuma *et al.*, 1998). PCR products were purified using minelute purification kit and were eluted in 10 µl elution buffer. Bacterial SSU rRNA genes were obtained with bacterial universal primers 27f (Edwards et al., 1989) and 1492 (Weisburg *et al.*, 1991). PCR products were ligated into a plasmid pCR2.1-TOPO and transformed into *E. coli* TOP10F' using the TOPO TA cloning kit (Invitrogen, USA) following manufacturer's instructions. White colonies (transformants) were checked for the correct-sized inserts by direct PCR using M13 primers. Correct-sized PCR products were subjected to ARDRA. For 18S rRNA gene clone libraries, 10–15 clones with the insert of right sizes were selected for ARDRA. 4–5 representatives of each ARDRA pattern were sequenced. For 16S rRNA gene clone libraries, 15–20 clones of correct insert were subjected to ARDRA. 4–5 representatives of each ARDRA pattern were sequenced. The inserts of clones were sequenced using M13 primer sets. For *Oxymonas* sp. from *N. castaneus* 26 clones were subjected to ARDRA. For *Oxymonas* spp. from *C. secundus* 27 clones were subjected to ARDRA.

Phylogenetic analysis

The SSU rRNA gene sequences obtained in this study were imported into the Silva database implemented in the ARB software package (Ludwig *et al.*, 2004). The automatic alignment of sequences was followed by the manual refinement.

The phylogenetic trees were calculated with the almost full-length SSU rRNA gene sequences (>1300 bases) using a maximum-likelihood method (AXML+fastDNAml). Phylogenetic trees were constructed using maximum-parsimony (in ARB) and maximum-likelihood methods (in PAUP version 4.0b10; Swofford, 2000). Gaps in the alignment were treated as missing data. An appropriate model of nucleotide substitution for ML analysis selected by the program MODELTEST (version 3.7; Posada and Crandall; 1998) was GTR+I+Γ. ML trees were inferred from heuristic searches under the Akaike information criterion (AIC) and a starting tree was generated by stepwise addition with ten random replicates with TBR branch swapping. The nodal supports were assessed by bootstrap analysis consisting of 100 bootstrap replicates, using the same heuristic search options. Bayesian analyses were conducted using MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). The substitution model for each alignment selected by MrModeltest (version 2.2; Nylander, 2002) was GTR+I+Γ for both *Bacteroidales* and devescovinid alignments. For the 50% majority rule consensus trees, four Markov chains were simultaneously run for 1,000,000 generations, and parameters and trees were sampled every 100 generations. The consensus tree calculated from the 10,001 trees sampled after the initial burn-in period provided estimation of posterior probabilities. The randomization test (1000 replicates) implemented in TreeMap (version 1.0a; Page, 1994) was used for testing whether the number of cospeciation events observed in the host and symbiont trees is significant ($p < 0.01$).

Whole-cell *in situ* hybridization

Hindgut contents of *N. castaneus* were fixed by incubating for 2 h at 4 °C in 3% (w/v) paraformaldehyde. The cells were washed three times 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), resuspended in PBS with an equal volume of ethanol, and stored at –21 °C. Oligonucleotide probes were designed using the probe design functions of the ARB software (Ludwig *et al.*, 2004) and checked using the Probe Match function of Ribosomal Database Project II (http://rdp.cme.msu.edu/). The newly designed probe Cy-3 labeled DVB178 and general *Bacteroidales* probe

319a were used. The newly designed probe had at two strong mismatches (in the middle) with all the database sequences. Optimal hybridization stringency was determined by varying formamide concentrations in the hybridization buffer over a range of 0 to 40% in 5% intervals at a fixed temperature of 46 °C. The hybridization was performed as described previously (Stingl and Brune, 2003), except that the ethanol series was omitted to minimize the distortion of flagellate cells. After hybridization and washing, the slides 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 covered with Citifluor (Citifluor Ltd., London, UK). Samples were inspected by epifluorescence microscopy using an Axiophot microscope (Zeiss, Jena, Germany). Unspecific probe binding was checked by simultaneous hybridization with a fluorescein-labeled EUB338 probe (Amann *et al*., 1990) and a Cy3-labeled NON338 probe (Wallner *et al*., 1993).

Scanning electron microscopy

The gut contents of four individuals of *Neotermes* sp. were fixed for 30 min in 2.5% glutaraldehyde in 0.1 M Soerensen phosphate buffer (PB; pH 7.2). After three washes for 15 min in PB the samples were fixed in 1% OsO_4 in 0.1 M SPB on ice for 1 h. The samples were washed again three times for 15 min and were pipetted into small caps covered with planktonic gauze. After dehydration in a graded series of ethanol, cells were dried with a Balzer's CPD 030, and coated with gold in a Balzer's SCD 040. Flagellates were examined using a FEI Quanta 200 ESEM.

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4 Are symbionts of flagellates responsible for nitrogen fixation in the gut of dry-wood termites (Kalotermitidae)?

Abstract

Nitrogen-poor diet of wood-feeding termites calls for a high nitrogen fixation activity by their gut bacteria, which had been demonstrated with the acetylene reduction assay. Nevertheless, identities of the nitrogen fixers have remained unknown in majority of the termite families. We studied expression of nitrogenase genes (*nifH* homologs) in four species of dry-wood termites (Kalotermitidae: *Neotermes castaneus*, *Cryptotermes longicollis*, *Kalotermes flavicollis* and *Incisitermes marginipennis*) – a group of termites known to possess the highest rates of nitrogenase activity. Terminal restriction fragment length polymorphism (T-RFLP), cloning, sequencing and phylogenetic analyses of *nifH* homologs and their mRNA transcripts demonstrated that despite a high diversity of nitrogenase genes, only a core set of four homologs is expressed, and the pattern of gene expression is specific to the species of the host termite. Based on phylogeny, two of the expressed genes belonged to *Treponema* and *Bacteroidales*. Other two genes were affiliated with the previously studied, termite-gut specific *anfH* gene and an unknown gene belonging to the Proteobacteria-Cyanobacteria group. Since *anfH* genes were obtained from the capillary picked flagellate suspensions of *Devescovina arta* and of *Snyderella tabogae*, it is strongly suggestive that the *anfH* genes belong to a symbiont of these flagellates. Finally, our data convincingly show that the active nitrogen fixers have coevolved with the host dry-wood termites.

Introduction

The 1-ul-scale hindgut of wood-feeding, phylogenetically lower termites is a structured and an efficient bioreactor with steep gradients of oxygen and hydrogen in the hindgut periphery (Brune, 1998). The hindgut is densely filled with highly diverse, anaerobic cellulolytic flagellates (Cleveland, 1923; Hungate, 1955). Nearly 90% of the phylogenetically distinct bacteria in the hindgut are associated with flagellates as ecto-

Technical assistance: Katja Meuser performed the T-RFLP analysis and sequencing of the COII genes of termites.

and endosymbionts, but the exact physiological roles of bacterial symbionts are largely unknown (for a review, see Stingl and Brune, 2006).

Since the diet of wood-feeding termites contains <0.05% nitrogen, the possibility of nitrogen fixation by the gut bacteria was suggested decades ago (Cleveland, 1925) and was later proven with the acetylene reduction assay (Benemann, 1973; Breznak *et al*., 1973). Numerous diazotrophs were subsequently isolated from termite guts (for a review, see Breznak, 2000). Of the bacterial isolates capable of fixing nitrogen, only *Treponema* spp. are abundant enough in the hindgut to be of significance for the process (Lilburn *et al*. 2002). However, *in situ* nitrogen fixation by *Treponema* spp. has not been reported thus far. In other habitats, *in situ* nitrogen fixation is routinely documented by quantifying nitrogenase-encoding mRNA, which is considered as a direct index of the enzyme activity (for a review, see Dixon and Kahn, 2004).

High diversity of homologs of *nifH*, a marker gene for the enzyme nitrogenase (for a review, see Zehr *et al*., 2004), has been reported in termite guts; the sequences of *nifH* homologs from termite guts form novel lineages and are distantly related to those of any cultured organisms (Yamada *et al*., 2007). Clustering of *nifH* homologs from the gut of several termite species, also including the sequences from the last common ancestor of termites — the wood-feeding cockroach *Cryptocercus punctulatus* — strongly indicates that nitrogen-fixing symbionts in termite guts represent a very old association of bacteria coevolving with their host insects (Yamada *et al*., 2007).

Despite a high diversity of *nifH* homologs in the gut of a dry-wood termite (Kalotermitidae) *Neotermes koshunensis*, only an alternative nitrogenase (*anfH*) is actively expressed (Noda *et al*., 1999); the closely related *anfH* genes have been found only in the gut of dry-wood termites and the roach *C. punctulatus*, suggesting that the unknown bacterium possessing the expressed *anfH* gene is specific to the gut of these insects (Yamada *et al*., 2007). Although in several bacteria, *anfH* – a paralogue of *nifH* – is differentially expressed in the absence of the molybdenum cofactor (Kessler *et al*., 1997), the *anfH* gene found in the termite gut is not inhibited by the presence of molybdenum.

A recent study reported the genome sequence of the "*Candidatus* Azobacteroides pseudotrichonymphae" – a member of the order *Bacteroidales* and an endosymbiont of a flagellate in a rhinotermitid termite – and the presence and expression of a *nifH* gene

(Hongoh *et al.*, 2008). Closely related *nifH* genes are found in termites from several other families and the roach *C. punctulatus* (Fig. 3). Nevertheless, expression of these *nifH* genes in other termites is questionable, since a different gene is expressed in the gut of the termite *N. koshunensis* (Noda *et al.*, 1999). These two studies (Noda *et al.*, 1999; Hongoh *et al.*, 2008), collectively indicate that phylogenetically distinct bacteria might be responsible for nitrogen fixation in different species of termites.

In the present study, we tried to answer the following questions: (i) Which organism possesses the *anfH* gene in Kalotermitidae? (ii) Are *anfH* genes expressed in all Kalotermitidae? (iii) Are symbionts of flagellates responsible for nitrogen fixation in the gut of Kalotermitidae? and (iv) Which bacteria fix nitrogen in Kalotermitidae? We studied the nitrogenase gene diversity (*nifH* homologs) and expression in the gut of four species of dry-wood termites (*Neotermes castaneus*, *Cryptotermes longicollis*, *Kalotermes flavicollis* and *Incisitermes marginipennis*). Additionally, in order to investigate whether symbionts of flagellates are responsible for nitrogen fixation, nitrogenase genes from the capillary-picked flagellate suspensions *Devescovina arta* and *Snyderella tabogae* were also examined.

Results

Clone libraries for nitrogenase genes from the flagellate suspensions

Clone libraries were prepared for the nitrogenase genes (homologs of *nifH*) genes in the capillary-picked flagellate suspensions of *Devescovina arta* and *Snyderella taboage* from the termites *Neotermes castaneus* and *Cryptotermes longicollis*, respectively. *S. taboage* is a calonymphid flagellate, which is abundantly represented in the gut of *C. longicollis* (Fig. 1). The deduced amino acid sequences of all the previously published sequences of *nifH* homologs from different termite species as well as major cultivated organisms were aligned against each other. *nifH* homologs from different termites fell in the previously named *anf*-methano, proteo-cyano (Proteobacteria-Cyanobacteria), termite anaerobe 1, termite anaerobe 2, termite anaerobe 3 and pseudo-*nif* groups (Fig. 2) (Ohkuma *et al.*, 1999). The similar tree topology (as shown in Fig. 2) was shown in other studies (Ohkuma *et al.*, 1999; Zehr *et al.*, 2003, Yamada *et al.*, 2007). Moreover, for these major groups, tree topology did not change in different phylogenetic methods

(neighbor joining, maximum parsimony, and different algorithms of maximum likelihood).

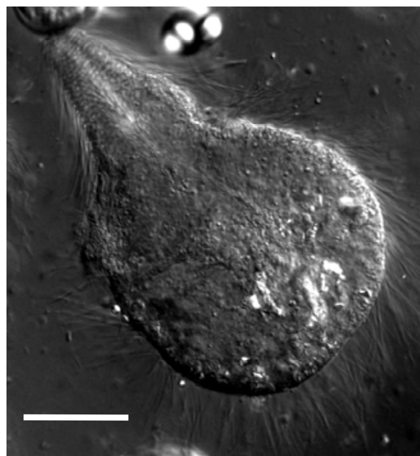

Figure 1. Differential intereference contrast micrograph of the flagellate *Snyderella tabogae* from the gut of the termite *Cryptotermes longicollis*. Scale bar, 30 μm.

PCR with universal *nif* primers (Ohkuma *et al*., 1999) for the DNA extracted from flagellate suspensions of *D. arta* and *S. tabogae* resulted in products of expected length (~470 bp). Clone libraries for both the PCR products yielded clones showing two RFLP patterns. For *D. arta* and *S. tabogae* 11 and 41 clones were screened in the RFLP analysis, respectively. 4–7 clones belonging to each RFLP pattern were sequenced (Table 1). Phylogenetic analysis showed that one of the phylotypes from both the flagellate suspension was virtually identical (99.3% sequence similarity at amino acid level) to the previously documented (Noda *et al*., 1999) *anfH* gene from the hindgut of the termite *Neotermes koshunensis*, and clustered in the previously entitled (Noda *et al*., 1999) *anf*-methano cluster (cluster I) (Fig. 2). The *anf*-methano group contains *anfH* genes from several bacteria (e.g., *Clostridium pasteurianum*, *Azotobacter vinelandii* and *Rhodobacter capsulatus*), and *nifH* genes from some methanogens (Fig. 2). The *anf*-methano cluster I contains sequences only from Kalotermitidae and the wood-feeding roach *Cryptocercus punctulatus*. Their assignment to *anfH* genes was corroborated by the culture-independent characterization of the *nif* operon by Noda *et al.* (1999). As already pointed out by Noda *et al*. (1999), it is not possible to assign these *anfH* genes to any known organism owing to the difficulty in determining the closest cultivated organism in the phylogenetic tree of *nif* genes. The *anfH* genes obtained from two

flagellate suspensions were about 10% different at the nucleotide level, but were >99.3% identical at the amino acid level.

Table 1. Different nitrogen fixation genes present in the clone libraries

Source	Clones analyzed	*anf*-methano	Proteo-cyano	Termite anaerobe 3	Termite anaerobe 2	Termite anaerobe 1	Pseudo -*nif*
Devescovina arta flagellate suspension	10	6 (1)	-	4 (1)	-	-	-
Snyderella tabogae flagellate suspension	11	7 (1)	-	4 (1)	-	-	-
Neotermes castaneus whole gut DNA	23	5 (4)	-	2 (1)	7 (3)	3 (2)	6 (5)
Cryptotermes longicollis whole gut DNA	20	14 (1)		3 (2)	2 (1)	-	1
Kalotermes flavicollis whole gut DNA	21	2 (2)	-	9 (1)	6 (1)	2 (1)	2 (1)
Incisitermes marginipennis whole gut DNA	22	-	2 (1)	-	10 (1)	-	10 (1)

Numbers in the bracket represent number of phylotypes in the particular group

The second phylotype from both the flagellate suspensions clustered (termite anaerobe group 3) (Fig. 2) with the *nifH* gene belonging to the *Bacteroidales* endosymbiont (CfPt1-2) of *Pseudotrichonympha* flagellate from the termite *Coptotermes formosanus* (Hongoh *et al*., 2008). Termite anaerobe group 3 contains sequences from only termite guts (Yamada *et al*., 2007). The identity (>99.3%, at amino acid level) of the *nifH* genes obtained from the flagellate suspensions with the *nifH* gene of CfPt1-2 clearly documents the origin of these genes from the symbiotic *Bacteroidales* of *D. arta* and *S. tabogae*.

Clone libraries of nitrogenase genes (homologs of *nifH*) from the whole gut

To check whether other termite species belonging to different genera also harbor *anfH* genes, nitrogenase gene clone libraries were prepared for the gut homogenates of *K. flavicollis* and *I. marginipennis*. Moreover, since the presence of the nitrogenase genes does necessarily mean that the nitrogenase activity is present, it was necessary to study the expression of *anfH* genes. Therefore, DNA-based and mRNA-based T-RFLP analysis was carried out in four different termite species *N. castaneus*, *C. longicollis*, *K.*

flavicollis and *I. marginipennis* (see next point for the results of T-RFLP). The selection of these termite species was based on the fact that these genera of termites represent most of the species in the termite family Kalotermitidae. In order to assign individual T-RFs to the specific genes in the DNA-based and m-RNA based T-RFLP analysis, the diversity of nitrogen fixation genes was investigated also in the hindgut of *N. castaneus* and *C. longicollis*.

Nucleic acid extracts from hindgut contents of termites were used to amply *nif* genes with the earlier mentioned primer set (Ohkuma *et al*., 1999). PCR products (~470 bp) were cloned and randomly selected 20–25 clones with the inserts of right sizes were sequenced for each termite species (Table 1). Clone libraries for all termite species showed several phylotypes of *nif* genes (Table 1). Phylogenetic analysis revealed that *nif* genes obtained from all termite species were widespread in the phylogenetic tree (Fig. 2). Sequences clustered in the previously designated (Yamada *et al*., 2007) *anf*-methano, proteo-cyano, termite anaerobe 1, termite anaerobe 2, termite anaerobe 3 and pseudo-*nif* groups. The *anfH* gene was obtained from the termites *K. flavicollis* and *C. longicollis*. *anfH* genes were not present in the clone libraries for termites *N. castaneus* and *I. marginipennis*. However, *anfH* gene was present in the flagellate suspension of *D. arta* from the termite *N. castaneus*. The absence of *anfH* genes in the whole gut library for this termite can be easily explained by the less sample size. The same could be true for the termite *I. marginipennis*.

Proteo-cyano group contains *nifH* sequences from many proteobacteria (e.g., all the members of *Rhizobiales*, *Rhodobacter* spp., etc.) and *nifH* genes from all the cyanobacteria (Fig. 2). Genes belonging to the proteo-cyano group were present only in the termite *I. marginipennis*. These genes were closely related to the *nifH* genes of *Azotobacter vinelandii* and *Klebsiella pneumoniae*, suggesting their origin from the proteobacteria. The highest diversity of *nif* genes was observed in termite anaerobe groups. Termite anaerobe group 3 contains sequences only from the termite gut. Some of the genes in the termite anaerobe group 3 clustered with the *nif* genes of termite gut isolates *Treponema primitia* and *Treponema azotonutricum* and showed high amino acid identity to the *nif* genes of these spirochetes (>99%). Therefore, it is clear that these *nif* genes come from the spirochetes in the termite gut. Genes belonging to the

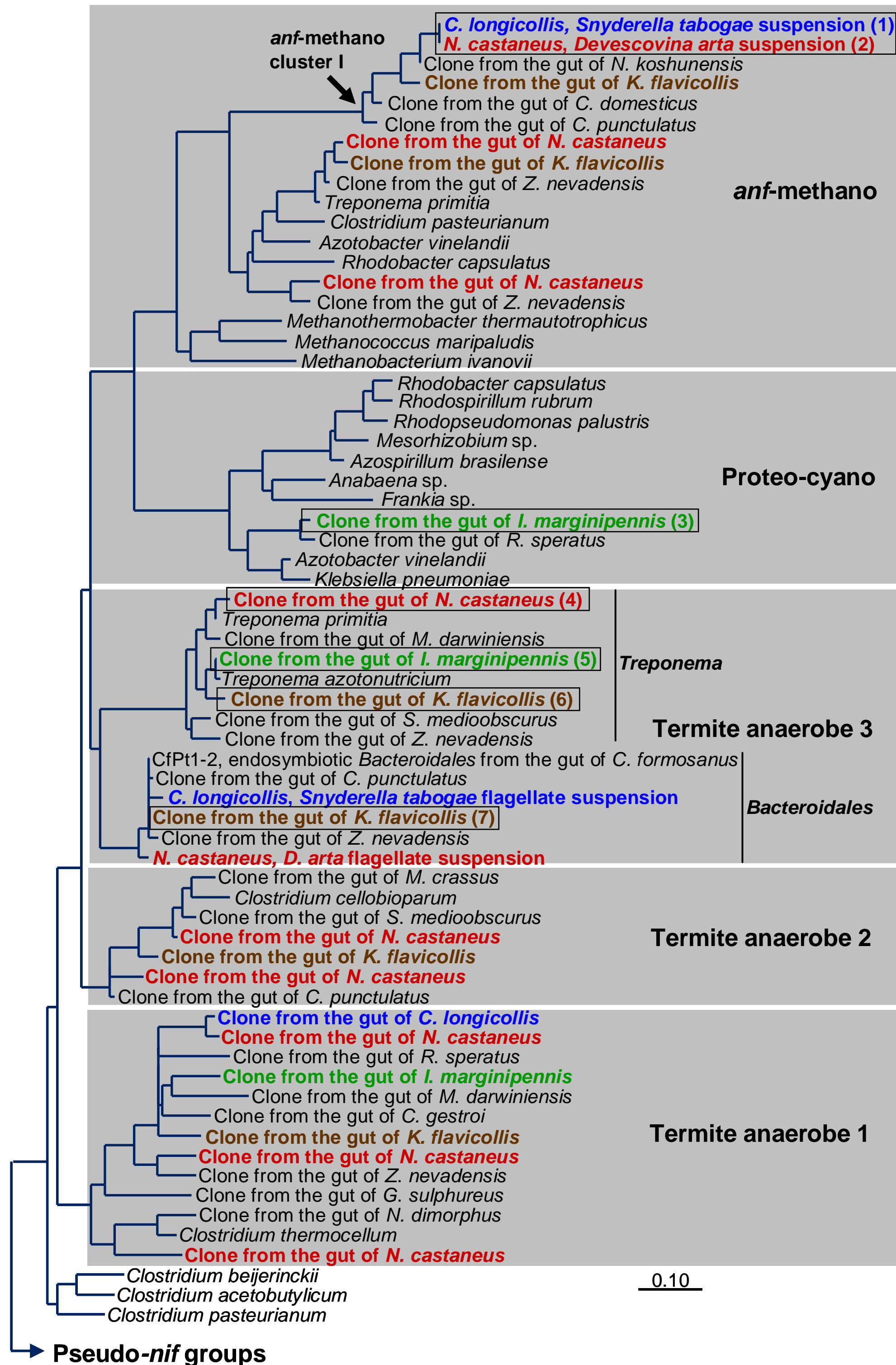

Figure 2. Maximum-likelihood tree showing phylogenetic relationships of the nitrogenase genes (homologs of *nifH*) (135 unambiguously aligned amino acids). Genes for Chlorophyll iron proteins were used as outgroup (not shown). Nomenclature of the several phylogenetic clusters

follows that of Yamada *et al.*, 2007. Colors indicate nitrogen fixation genes from the whole gut homogenate and flagellate suspensions of *Cryptotermes longicollis* (blue), *Neotermes castaneus* (red) *Kalotermes flavicollis* (brown) and *Incisitermes marginipennis* (green). Nitrogenase genes that are expressed in the mRNA based T-RFLP profiles (Figure 4) are boxed. Numbers in the bracket correspond to the peaks in the DNA-based and mRNA-based T-RFLP profiles (Figure 4). *anfH* genes obtained from flagellate suspensions of *Devescovina arta* and *Snyderella tabogae* clustered in the *anf*-methano cluster I. The bar indicates 10% sequence divergence. Pseudo-*nif* genes clustered outside all the other groups mentioned above (not shown).

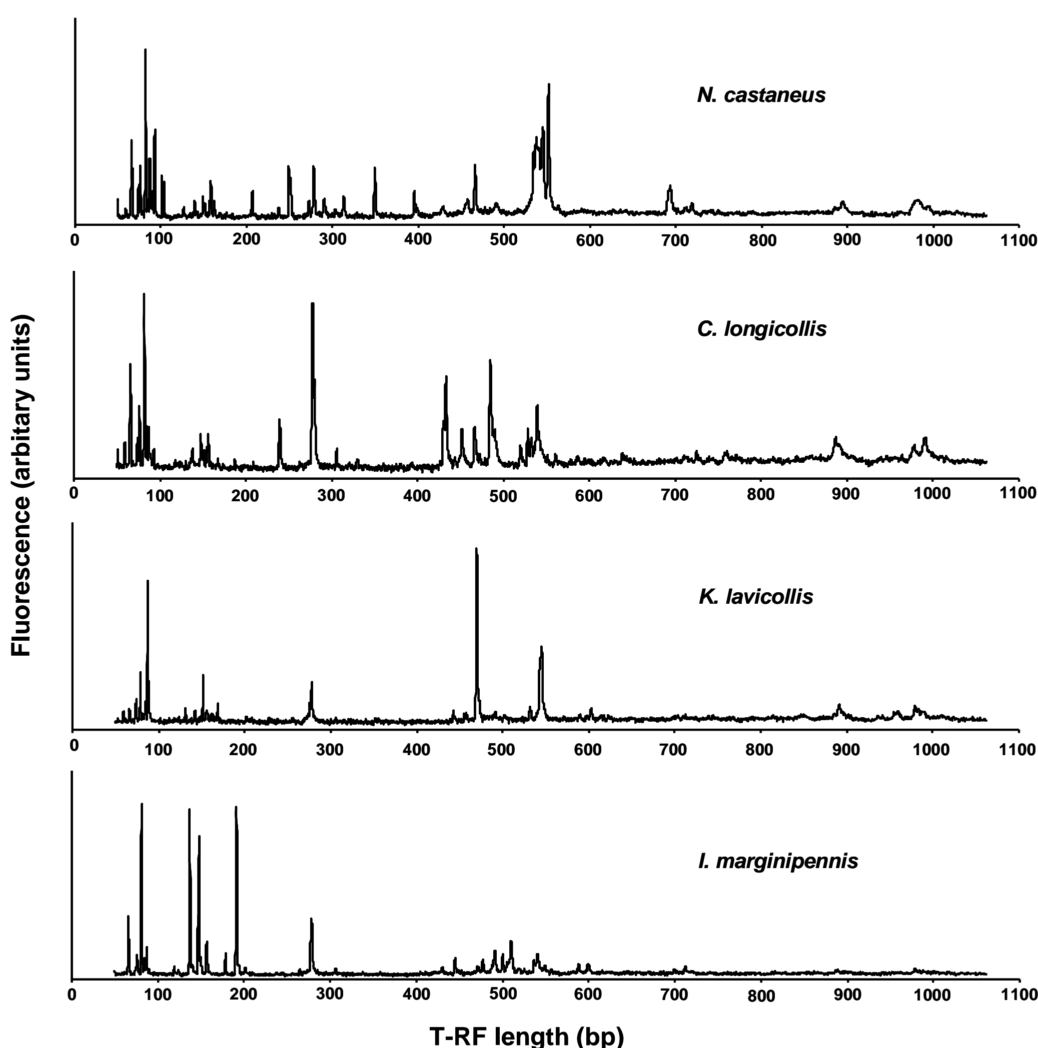

Figure 3. Terminal restriction fragment length polymorphism **(**T-RFLP) profiles of the fluorescently-labeled 16S rRNA genes amplified from the whole gut DNA of termites *Neotermes castaneus*, *Cryptotermes longicollis*, *Kalotermes flavicollis* and *Incisitermes marginipennis*. PCR products were digested with the enzyme *MspI*.

Bacteroidales were obtained in *K. flavicollis*. Termite anaerobe group 2 and 1 contains sequences from *Clostridium* spp. These two groups also contain sequences from sulfate reducers (delta-proteobacteria) and methanogenic archaea (not shown in the present tree). Except pseudo-*nif* group, all the sequences used in the phylogenetic tree (Fig. 2), including sequences obtained in this study showed conserved cysteine and arginine residues in the NifH protein, confirming their role in the process of nitrogen fixation (Dean and Jacobson, 1992). Pseudo-*nif* group, which is considered to function in some process other than nitrogen fixation is not shown in the phylogenetic tree. These genes are known to be present in the methanogenic archaea. Pseudo-*nif* genes were present in all four termite species (Table 1) and clustered outside the phylogenetic tree (Fig. 2). Genes belonging to the Pseudo-*nif* formed four clusters, as was shown in the previous study (Yamada *et al.*, 2007). The high diversity of *nifH* homologs is well correlated with the high diversity of 16S rRNA genes in these termites (Fig. 3).

Acetylene reduction assay

Since nitrogenase genes are regulated at the transcriptional and the post-translational level, before studying gene expression of nitrogen fixation genes in the four different termite species, it was necessary to examine the nitrogenase activity by the acetylene reduction assay. Gas chromatographic measurements showed production of ethylene for all termite species, confirming the presence of nitrogenase activity in the hindgut. The amount of ethylene produced by each species is listed in the Table 2.

Table 2. Rates of ethylene formation in the acetylene reduction assay determined for four species of Kalotermitidae

Termite species	**nmol C_2H_4 formed per 12 hours per 50 termites**
Neotermes castaneus	0.012
Cryptotermes longicollis	0.015
Kalotermes flavicollis	0.010
Incisitermes marginipennis	0.026

Terminal restriction fragment length polymorphism analysis of nitrogen fixation genes and their transcripts

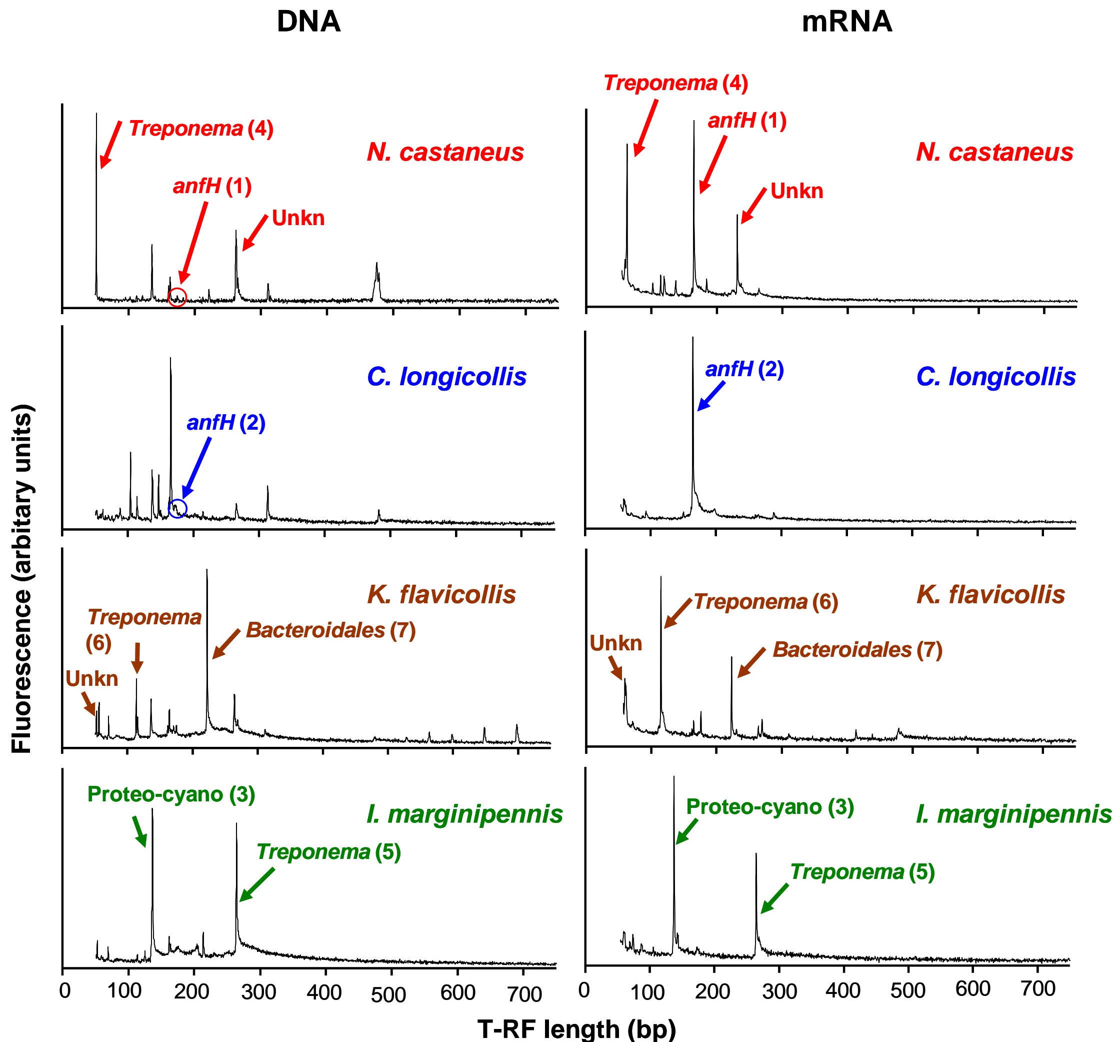

Figure 4. DNA based and mRNA-based T-RFLP profiles of nitrogen fixation genes (~470 bp) from the hindgut of termites. Four different colors indicate profiles from four different termite species: red (*Neotermes castaneus*), blue (*Cryptotermes longicollis*), brown (*Kalotermes flavicollis*) and green (*Incisitermes marginipennis*). Fluorescently-labeled PCR products were digested with the enzyme *HhaI*. Numbers in the bracket correspond to the phylogenetic positions (Figure 2) of the nitrogenase genes. The highly expressed nitrogen fixation genes which were obtained from the flagellate suspensions of *Devescovina arta* and *Snyderella tabogae*, and were identified as *anfH* genes (Noda *et al*., 1999) in the phylogenetic analysis (Figure 2) are shown as *anfH* (1) and *anfH* (2), respectively. These *anfH* genes are circled in the DNA-based T-RFLP profiles. Nitrogenase genes, which clustered in the phylogenetic tree with the previously published genes from *Treponema* spp. (Lilburn *et al*., 2002), were named as

Treponema. The expressed genes in termites *N. castaneus* and *K. flavicollis*, which could not be assigned to any phylotype are shown as unknown (Unkn).

Nucleic acid extracts from the hindguts of four termite species *N. castaneus*, *C. longicollis*, *K. flavicollis* and *I. marginipennis* were used for PCR and reverse transcription (RT)-PCR. In both the reactions, products of the expected length (~470 bp) were obtained for all termite species. These PCR products were digested with *HhaI* enzyme and were used for the T-RFLP (Fig. 2).

For *N. castaneus*, several peaks were observed in the DNA-based profiles. Based on the amino acid identity with the previously published *Treponema* sequences (Lilburn *et al*., 2002), the most abundant peak was assigned to *Treponema*. The abundance of *Treponema* is in good agreement with the abundance of this phylotype in the clone library. The peak for the *anfH* gene obtained from the flagellate suspension of *D. arta* was very small (Fig. 2, circled), suggesting that the organism carrying this gene is less abundant in the gut. In the mRNA-based profiles, three major peaks belonging to *Treponema*, *anfH* gene, and an unknown organism were observed. Interestingly, despite its less abundance in the DNA-based profile, the peak belonging to the *anfH* gene was the most abundant peak in the mRNA-based profile.

Several peaks were observed in the DNA-based profiles of *C. longicollis*. Peak belonging to the *anfH* gene obtained from the suspension of *S. tabogae* was very small (Fig. 2, circled), which again means that the organism carrying this gene is scarce in the gut. Like in the gut of *N. castaneus*, this *anfH* gene was highly expressed. In fact, this was the only expressed gene in the gut of *C. longicollis*.

DNA-based profiles of *K. flavicollis* were more complex than those of *N. castaneus* and *C. longicollis*. From about 10-12 peaks, 2 major peaks were assigned to *Treponema* and *Bacteroidales*. The abundance of these two peaks is well supported by the abundance of phylotypes representing these two peaks in the clone library, suggesting high abundance of nitrogen-fixing *Treponema* and *Bacteroidales* in the termite gut. The peak belonging to the *Treponema* was the most abundant in the mRNA-based profile of this termite. This peak was followed by the peak arising from the *Bacteroidales*. The peak for the *anfH* gene was not observed in both DNA-based and mRNA-based profiles.

Two dominant peaks were observed in the DNA-based profiles for *I. marginipennis*. These peaks were assigned to *Treponema* and a sequence in the proteo-cyano group. The abundance of *Treponema* is well supported by their abundance in the the clone libraries. Phylotype from the prote-cyano group was less abundant in the clone library. mRNA-based profiles showed that both the genes are highly expressed. No other peak was observed in the mRNA-based profiles.

Altogether, these data convincingly show that the *anfH* genes arising from the flagellate suspensions are highly expressed. *anfH* genes were not expressed in the gut of *K. flavicollis* and *I. marginipennis*. Expression of nitrogenase genes from the *Treponema* spp. provides the first evidence for the *in vivo* nitrogen fixation by spirochetes. Nitrogenase genes of spirochetes are highly expressed in *N. castaneus*, *K. flavicollis* and *I. marginipennis*. A *nifH* gene belonging to the *Bacteroidales* was expressed only in the gut of *K. flavicollis*. Finally, expression of a *nifH* gene from the proteo-cyano group in *I. marginipennis* suggests that a proteobacterium is also responsible for the nitrogen fixation in the gut of *I. marginipennis*.

Discussion

Since many species of termites feed on a nitrogen-poor diet, nitrogen fixation is an important process in the termite gut (Breznak *et al*., 1973). Despite high rates of nitrogen fixation in dry-wood termites (Kalotermitidae) (Breznak, 1973; Ohkuma *et al*., 1999), identities of the nitrogen-fixing bacteria had been unknown. In the present study, we studied nitrogenase gene (*nifH* homologs) diversity and expression in the gut of four species of dry-wood termites (*Neotermes castaneus*, *Cryptotermes longicollis*, *Kalotermes flavicollis* and *Incisitermes marginipennis*). Despite a high diversity of *nifH* homologs in all the four species of termites, only a set of four homologs was expressed. These genes belonged to *Treponema*, *Bacteroidales*, *anfH* and a gene belonging to the Proteobacteria-Cyanobacteria group. The pattern of gene expression is specific to the species of the host termite.

The nitrogen fixation gene (homologs of *nifH*) clone libraries for the picked flagellate suspensions of *Devescovina arta* and *Snyderella tabogae* yielded *nifH* genes belonging to the *Bacteroidales* (Hongoh *et al*., 2008) and *anfH* genes (Noda *et al*., 1999). The *anfH* gene originating from flagellate suspensions can be assigned to the

Bacteroidales ectosymbionts of these flagellates because (i) the "Endomicrobia" genome does not contain any nitrogen fixation genes (Hongoh *et al*. 2008), (ii) all the investigated *Devescovina* species did not harbor any other bacterial endosymbiont (iii) The *nif* operon structure of the genes (Noda *et al*., 1999) suggests that the *anfH* gene originate from a eubacterium, since archaea have a different operon structure, and (iv) Both *nifH* and *anfH* genes were present in roughly equal proportion, suggesting that they originate from the same organism.

The presence of several closely related nitrogen fixation genes in all four species of Kalotermitidae indicates coevolution of several organisms carrying these genes with the host termites. The nitrogen-fixing endosymbiont of *Pseudotrichonympha* flagellates belong to the cluster V *Bacteroidales* (Hongoh *et al*., 2008). Cluster V *Bacteroidales* are present in all species of termites and the wood-feeding cockroach *Cryptocercus punctulatus* (Noda *et al*., 2006). The closest cultivated representative of the cluster V *Bacteroidales* (Noda *et al*., 2005), *Bacteroides fragilis*, does not possess *nifH* genes. Therefore, *nifH* genes must have been acquired by the last common ancestor of the cluster V *Bacteroidales* in the common ancestor of termites and the roach *C. punctulatus*.

Similarly, spirochetal *nifH* and *anfH* genes must have been present in the common ancestor of termites and the cockroach *C. punctulatus*. At the same time, the absence of *anfH* genes in the termites of other families (Yamada *et al*., 2007), indicates loss of *anfH* genes during the termite evolution. Genes belonging to the proteo-cyano group were detected only in the gut of the termite *Incisitermes marginipennis*, which means that the organism carrying this gene was acquired later on by the termite *I. marginipennis*. Majority of the nitrogen fixation genes are present in the termite anaerobe groups 1 and 2. *Clostridium* spp. are the major cultivated members present in these group. However, the high sequence divergence between the *nifH* genes of *Clostridium* spp. and termite *nifH* genes (anaerobe groups 1 and 2) indicates that the bacteria other than *Clostridium* spp. possess these *nifH* genes.

A *nifH* gene belonging to the *Bacteroidales* was expressed in the gut of the termite *Kalotermes flavicollis*. Since this gene was very abundant at the DNA level, and ectosymbiotic *Bacteroidales* of the *Joenia* spp. (J. Strassert, personal communication) in this termite are also abundant in the gut, the expressed *nifH* gene can be assigned to

the ectosymbiotic *Bacteroidales* of the flagellates *Joenia* spp. Similarly, the endosymbiotic *Desulfovibrio* of flagellates *Trichonympha* spp. (Wienemann, 2008) are the putative carriers of the *nifH* gene expressed from the proteo-cyano group in the gut of *I. marginipennis*. Finally, the expressed gene of the *Treponema* spp. was assigned to the free-living *Treponema* in the termite gut because free-living *Treponema* are the most numerous groups of bacteria in the hindguts of wood-feeding lower termites (Paster *et al*., 1996).

The argument of the symbiotic nitrogen fixation is supported by the fact that if rates of nitrogen fixation (and the produced ammonia) are high for a non-symbiotic bacterium, the nitrogen fixation is stopped because of the post-translational modification (Dixon and Kahn, 2004). The process of nitrogen fixation consumes enormous amounts of energy (Dixon and Kahn, 2004). Therefore, it is highly probable that the symbiotic nitrogen-fixers of flagellates must be getting energy-rich compounds from the host flagellate. Finally, since 30–50% of the atmospheric nitrogen is incorporated in the termite (*Neotermes koshunensis*) tissue, it is clear that termites are also benefited by the nitrogen-fixing symbionts (Tayasu *et al*., 1998).

The results of the present study collectively suggest that the nitrogen fixation is the major physiological basis of the flagellate–bacteria symbiosis in the termite gut. Assuming that these symbiotic nitrogen-fixers will be difficult to isolate, simultaneous *in situ* localization (Pernthaler and Pernthaler, 2005; Amann and Fuchs, 2008) of the *nifH* mRNA and the SSU rRNA genes of the corresponding organisms seem necessary to corroborate the results of nitrogen fixation gene assignment to several symbionts of flagellates.

Experimental procedures

Termites

Termite species originating from a broad geographic range were used. Most of the termites are maintained as cultures at Bundesanstalt für Materialforschung und -prüfung (BAM), Berlin, Germany. *Cryptotermes secundus* was collected in a mangrove forest near Darwin, Australia. Termites were maintained in polyethylene containers on a diet of pinewood at 25 ^{0}C. Only mature termite workers were used in the experiments.

Morphological identification of termites was further confirmed by sequencing their cytochrome oxidase II genes as described elsewhere (Pester and Brune, 2006).

Acetylene reduction assay

30–70 live termites of *Neotermes castaneus*, *Cryptotermes longicollis*, *Kalotermes flavicollis* and *Incisitermes marginipennis* were used for determination of the nitrogenase activity. Termites were incubated for 12 hours in 16.8% acetylene in 10 ml serum bottles (Breznak *et al*., 1973). 0.1 ml gas sample was assayed for the production of Ethylene on the Gas Chromatograph equipped with the Flame Ionization detector.

Flagellate picking and DNA extraction from flagellates

The contents of one to three termites were suspended in Solution U. Unambiguously identified (50–200) flagellate cells were collected by micropipette (For methodological details see Ikeda-Ohtsubo *et al.*, 2007). Picked flagellates were subjected to freeze-thawing and were then boiled for about 10 minutes. DNA extraction was performed as described previously (Stingl *et al*., 2005). DNA samples were directly used for PCR amplification of SSU rRNA genes of bacteria. Extracted DNA was also used for the amplification of SSU rRNA genes from flagellates.

Phylogenetic analysis of the nitrogenase genes

Amplified *nifH* gene fragments were cloned into *Escherichia coli* cells using the TA cloning kit (Invitrogen). Clones were analyzed by RFLP using the restriction enzymes *MspI* and *HhaI* (5 U each, Promega) and grouped according to their restriction pattern. Representatives of each group were sequenced from both strands. Sequences were checked for chimera as described elsewhere (Pester and Brune, 2006) and aligned within the ARB software package (http://www.arbhome.de). A phylogenetic tree was reconstructed based on a distance matrix of deduced amino acid sequences inferred from the Dayhoff PAM 001 matrix as amino acid replacement model (Dayhoff *et al*., 1978). The tree was inferred from the distance matrix using the Fitch algorithm (Kimura, 1983) with global rearrangement and randomized input order of sequences, as implemented in ARB. Tree reconstruction, using the distance-based neighbour joining (Saitou and Nei, 1987) or the maximum-likelihood algorithm based on Dayhoff and

colleagues (1978) as implemented in ARB, resulted in dendrograms with similar topology.

Reverse transcriptase PCR of the nitrogenase mRNA

RNA extracts were digested with RQ1 RNase free DNase (1 U, Promega) according to the manufacturer's instructions. RT-PCR was performed using the Access RT-PCR System kit (Promega) in 50 µl reactions. Each reaction contained 1× reaction buffer, 1 mM MgSO4, 200 µM of each dNTP, 0.6 µM of fluorescently labeled forward *nifH* primer (Ohkuma et al., 1999), 0.6 µM of reverse *nifH* primer (Ohkuma et al., 1999), 5 U of AMV reverse transcriptase, 5 U of *Tfl* DNA Polymerase and 1 µl of the RNA extract. Thermal cycling started with reverse transcription for 45 min at 48°C, immediately followed by an initial denaturation for 2 min at 94°C, and proceeded in two phases: nine cycles of a touchdown program (30 s at 94°C, 1 min at 63°C, decreasing 1°C per cycle and 2 min at 68°C), followed by 17 cycles of a standard program (annealing temperature at 55°C). The final extension step was 7 min at 68°C. In all cases, parallel assays without AMV reverse transcriptase did not result in a PCR product, showing that the template was free of contaminating DNA. RT-PCR products were checked for the specificity of the PCR reaction by standard agarose gel electrophoresis and were further analyzed by T-RFLP.

Terminal restriction fragment length polymorphism analysis

Terminal-restriction fragment length polymorphism analysis was performed as described previously (Pester and Brune, 2006), using the nucleic acid extraction method and PCR conditions as described above, except that the forward primer was labeled with a fluorescent dye (Cy55; Operon). PCR products were digested with the restriction endonuclease *HhaI* (3 U each; Fermentas), which gave the best resolution among the different clone groups. Great care was taken to avoid over-saturated T-RF signals, which would affect relative peak heights. If necessary, samples of the restriction digestion were diluted and analyzed again. Lengths of T-RFs were calculated by comparison with molecular size markers (50–700 bp, LI-COR) and with selected *nifH* clones representing the major T-RFs, using the Gel-Pro Analyzer software (version 4.5,

MediaCybernetics). The same clones were also analyzed for pseudo-T-RF formation (Egert and Friedrich, 2003).

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5 Hydrogen partial pressures in the gut of dry-wood termites (Kalotermitidae)

Abstract

A previous study had shown that the (phylogenetically) lower termite *Reticulitermes flavipes* accumulates high amounts of hydrogen in its hindgut. It is generally assumed that anaerobic flagellate protozoa in the hindgut of lower termites produce hydrogen. However, a recently studied lower termite *Cryptotermes secundus* (Kalotermitidae) accumulated very little (~1 kPa) hydrogen. The reason behind the less hydrogen accumulation in this termite is unknown. We asked whether all the termite species of the family Kalotermitidae accumulate less hydrogen. To answer this question, hydrogen was measured in three species of Kalotermitidae *Neotermes castaneus*, *Kalotermes flavicollis* and *Incisitermes marginipennis*. Hydrogen concentration was determined both axially and radially across the termite gut with a hydrogen microsensor. The measurements revealed that *K. flavicollis* and *I. marginipennis* accumulated high amounts of hydrogen (40–50 kPa). On the other hand, *N. castaneus* accumulated much lower hydrogen (<1 kPa). High hydrogen accumulation was observed at the centre of the paunch—a region of the hindgut completely filled with flagellates. Finally, we discuss possible reasons for less hydrogen accumulation in the termite *N. castaneus*, and hypothesize that the termites harbouring large hypermastigid flagellates accumulate high amounts of hydrogen.

Introduction

Several decades ago Cleveland (1926) recognized that the digestion of lignocellulose in the gut of lower wood-feeding termites is carried out by the flagellate protozoa. These flagellates are harboured in a voluminous dilatation of the anterior hindgut known as paunch together with the several symbiotic bacteria. Hungate (1943) recognized that the H_2 is a major fermentation product of the symbiotic flagellates. Oldenson and Breznak (1985) later on demonstrated that axenic cultures of *Trichomitopsis termopsidis* fermented cellulose to hydrogen and acetate as the only detectable fermentation products. Ebert and Brune (1995) measured the H_2 and O_2 concentration in the hindgut of the termite *Reticulitermes flavipes* and showed the existence of steep H_2 gradients from the gut centre towards the epithelium. The same

study also showed that the termites treated with hyperbaric oxygen for several hours lost their flagellates and showed no H_2 accumulation. Recently, Inoue *et al*. (2007) showed that the hydrogenosome-enriched fraction of the flagellate *Pseudotrichonympha* produced hydrogen. Altogether, it is clear that symbiotic flagellates produce H_2 in the hindgut of lower termites.

Recently, Pester and Brune (2007) measured hydrogen in the hindgut of three species of lower termites *Reticulitermes santonensis* (Rhinotermitidae), *Zootermopsis nevadensis* (Termopsidae) and *Cryptotermes secundus* (Kalotermitidae). *R. Santonensis* and *Z. Nevadensis* accumulated high amounts of hydrogen (25 kPa and 70 kPa, respectively). However, *C. secundus* accumulated very little hydrogen (~1 kPa). Considering that all three species contain parabasalid flagellates, which, in principle, should make hydrogen, accumulation of little hydrogen in the gut of *C. secundus* is surprising.

What could be the reason for less hydrogen accumulation in the gut of *C. secundus*? Most likely, flagellates in the gut of *C. secundus* make less hydrogen as compared to flagellates from other two species of termites. Another less parsimonious hypothesis would be rate of hydrogen production by flagellates in *C. secundus* is higher, but rate of consumption by bacteria (homoacetogens) is extremely high as compared to other two species. To test these hypotheses, we measured hydrogen in the gut of three species of Kalotermitidae (*Neotermes castaneus*, *Incisitermes marginipennis* and *Kalotermes flavicollis*). The microsensor was used to measure hydrogen in the agarose-solidified Ringer's solution (Brune *et al*., 1995).

Results and discussion

Hydrogen microsensor measurements showed significant differences among the hydrogen partial pressures of three different termite species (Fig. 1). *I. marginipennis* showed the highest accumulation of hydrogen followed by *K. flavicollis*. Hydrogen partial pressures in the gut of *N. castaneus* were much lower (<1 kPa). For all the three termite species, highest hydrogen accumulation was observed at the centre of the hindgut paunch. The other gut regions named Crop, Midgut, Colon and Rectum accumulated very less hydrogen.

Despite harbouring anaerobic flagellates in its gut, it is an interesting question why *N. castaneus* accumulates very less hydrogen. The most parsimonious explanation is that the net amount of hydrogen production is much lower in *N.*

castaneus. Less hydrogen accumulation was also observed in the gut of Kalotermitidae *Cryptotermes secundus* (Pester and Brune, 2007). The same study showed that the rates of reductive acetogenesis were relatively higher in this termite, making it clear that some of the hydrogen is being rapidly consumed.

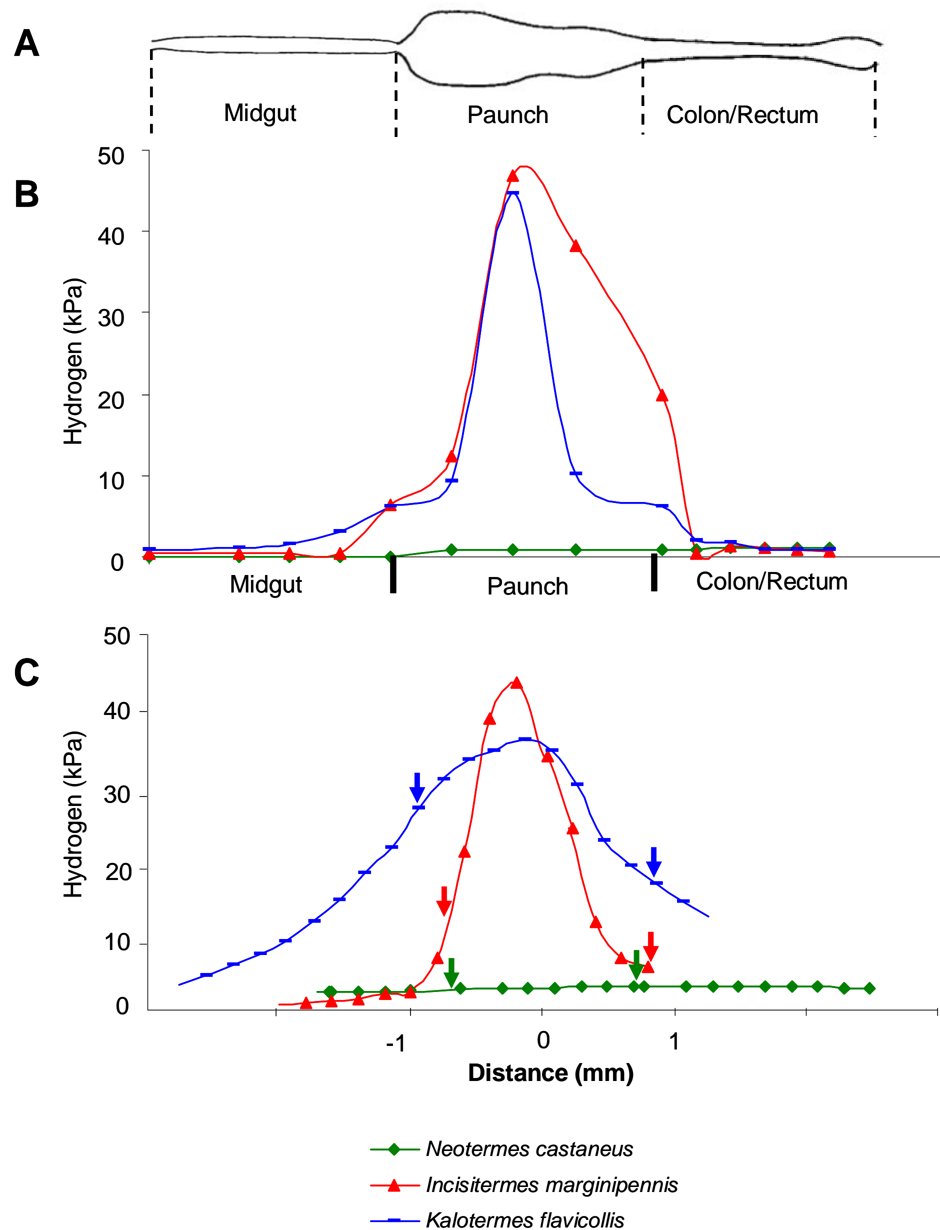

Figure 1: Axial and radial hydrogen concentration profiles (microsensor measurements) of agarose-embedded guts of Kalotermitidae *Neotermes castaneus*, *Incisitermes marginipennis*, and *Kalotermes flavicollis*. (A) Schematic diagram of the gut of lower termites (B) Typical axial profiles of the three termite species, (C) Typical radial profiles across the paunch of the three species. Arrows denote the position of the hindgut wall.

The rates of production and consumption in *C. secundus* were more balanced, resulting only in moderate accumulation of H_2. The generally accepted hypothesis of molecular H_2 as a key intermediate of lignocellulose degradation in lower termites was well supported by the recent study (Pester and Brune, 2007). The same study showed that the hydrogen is the most important electron donor in (phylogenetically) lower termites.

The H_2 accumulation might be directly correlated with the type of flagellates present in the gut of these termites. Both the termites which did not accumulate hydrogen, *N. castaneus* (this study) and *C. secundus* (Pester and Brune, 2007) do not harbor large hypermastigid flagellates (Ohkuma *et al*., 2000). In addition to two species from this study (*I. marginipennis* and *K. flavicollis*), all the other termites which have been reported to accumulate hydrogen harbour large hypermastigid flagellates. *I. marginipennis* harbours *Trichonympha* spp. (Ikeda-Ohtsubo and Brune, 2008) and *K. flavicollis* harbours *Joenia* spp. (Radek *et al*., 1992). Thus, it is suggestive that the hypermastigid flagellates make enormous amounts of hydrogen. This hypothesis needs to be tested by measuring the rates of hydrogen production by different types of flagellates.

Experimental procedures

Termites

Termites *Neotermes castaneus*, *Kalotermes flavicollis* and *Incisitermes marginipennis* were obtained from Bundesanstalt für Materialforchung (BAM) in Berlin, and were maintained in polyethylene containers on a diet of pinewood and water, except that no water was given to *I. marginipennis*. Termites were maintained in the humidity and temperature controlled chambers. Only worker termites were used for the experiments. Molecular identification of termites was carried out as described previously (Pester and Brune, 2006).

Hydrogen microsensor measurements

Hydrogen microsensor with a tip diameter of 10 μm was used. The electrode was calibrated and tested according to the Unisense manual. The experimental setup used for the hydrogen by microsensor measurement was the same as described previously (Ebert and Brune, 1997).

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6 General discussion

The present thesis opens up several interesting questions. In this chapter, general aspects of the evolutionary histories of termite–flagellate symbioses, flagellate–bacteria symbioses, and nitrogen fixation in the gut of dry-wood termites (Kalotermitidae) are discussed. Specific results are discussed in the respective chapters.

Kalotermitidae and devescovinid flagellates: cospeciation or host switching?

The dependence of lower wood-feeding termites on their cellulolytic gut flagellates for the digestion of lignocellulose is a classical example of mutualistic symbiosis (Cleveland, 1926; for review, see Breznak, 2000). Termite gut flagellates are believed to be vertically transmitted by the process of proctodeal trophallaxis—the transfer of hindgut fluids containing symbiotic flagellates (Kirby, 1937; Kirby, 1949; Honigberg, 1970; Inoue *et al.*, 2000). Kitade (2004) showed that termites of a particular family contain morphologically similar flagellates, and suggested that flagellates could have been horizontal transferred among termites of the same family. The argument of horizontal transfer was already pointed out by Andrew (1930), who stated that since flagellates can be easily transferred between termite species by mere physical contact, strict vertical transmission of flagellates is doubtful.

Recently, Noda *et al.* (2007) addressed the issue of vertical transmission of flagellates by studying the molecular phylogenies of rhinotermitid termites and their *Psuedotrichonympha* flagellates. The comparison of phylogenies revealed phylogenetic congruence between rhinotermitids and *Psuedotrichonympha*, supporting the notion of vertical transmission. On the other hand, Ikeda-Ohtsubo and Brune (2008) showed that phylogenetically unrelated *Trichonympha* species were present in the same termite, suggesting their horizontal transfer between different termite species. It is difficult to derive a conclusion about the vertical transmission of flagellates based on the contradictory results of these studies.

Based on the presence of devescovinid flagellates (morphological identification) in several termite species, Kitade (2004) showed that species of

Kalotermitidae cluster together. This clearly means that devescovinid flagellates are specific to the termite family Kalotermitidae, which implies that the last common ancestor of all Kalotermitidae possessed the common ancestor of all devescovinid flagellates. Nevertheless, it is not clear whether devescovinid flagellates strictly cospeciate with their kalotermitid hosts. Their strict cospeciation is unlikely, since two different genera like *Devescovina* and *Metadevescovina* are reported to occur in the same termite (Yamin, 1979). The present study showed that the SSU rRNA gene phylogeny of devescovinid flagellates (*Devescovina* spp., *Caduceia* sp. and *Metadevescovina* spp.) is largely incongruent with the cytochrome oxidase II gene phylogenies of host termites (Fig. 1), displaying the horizontal transfer of flagellates between termite species. At the same time, the presence of devescovinid flagellates mainly in the gut of kalotermitid termites (Yamin, 1979) suggests their specificity to the termite family Kalotermitidae. The reason for this specificity could be a unique physiological relationship of these flagellates with the host Kalotermitidae.

Devescovinid flagellates are abundantly represented in the gut of Kalotermitidae (Kirby, 1941, 1942, 1945). Twelve genera of devescovinid flagellates were reported by Kirby, which are classified mainly based on the presence of ectosymbiotic bacteria on these flagellates. To better understand the evolutionary history of Kalotermitidae and devescovinids, molecular phylogenies of several other Kalotermitidae and devescovinid flagellates need to be examined.

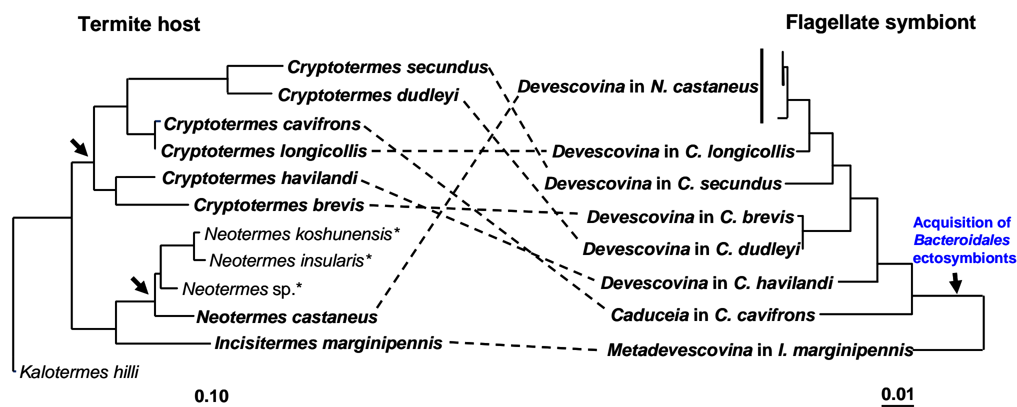

Figure 1. A tanglegram of the maximum-likelihood trees of kalotermitid termites (based on the deduced amino acid sequences of cytochrome oxidase II genes) and their devescovinid flagellates (based on SSU rRNA genes). Phylogenetic trees of termite hosts and flagellate symbionts are largely incongruent. Sequences for species with an asterisk

are from previous studies. Arrows in the termite phylogeny indicate monophyletic groups of *Cryptotermes* and *Neotermes* species. Arrow in the flagellate phylogeny indicates point of acquisition of *Bacteroidales* ectosymbionts by flagellates.

Coevolutionary history of devescovinids and *Bacteroidales* ectosymbionts

Coevolution is the process of reciprocal adaptive genetic change in two or more species (Woolhouse *et al*., 2002), which occurs due to strong "selective pressure" that each species exerts on the other (Thompson J. N., 1994). On the other hand, cospeciation is joint speciation of host and symbiont, which results from intimate and long-standing association (Baumann *et al*., 1997; Peek *et al*., 1998; Hughes *et al*., 2007; Hosokawa *et al*., 2007). Taken together, coevolution can lead to cospeciation.

Does cospeciation between devescovinid flagellates and *Bacteroidales* ectosymbionts indicate coevolution? Since endosymbionts ("Endomicrobia") of devescovinid flagellates and ectosymbionts (*Bacteroidales*) of *Oxymonas* flagellates do not show phylogenetic congruence with their host flagellates, cospeciation between devescovinids and *Bacteroidales* can be considered as a special case. Although devescovinids were transferred between different termite species, *Bacteroidales* ectosymbionts were not lost. Therefore, cospeciation between devescovinids and their *Bacteroidales* ectosymbionts strongly indicates the existence of a "selective pressure", which could be a result of host–symbiont coevolution owing to the physiological interaction. Most likely, the physiological interaction started in the last common ancestor of *Devescovina* spp. and *Caduceia versatilis* and the common ancestor of *Bacteroidales* ectosymbionts. Since the similar type of *Bacteroidales* ectosymbionts are absent on *Metadevescovina* species, and other parabasalid flagellates, the point of acquisition of *Bacteroidales* by the common ancestor of *Devescovina* and *C. versatilis* was estimated (Fig. 1). Based on the approximate rates of evolution of SSU rRNA genes (Moran *et al*., 1993), the age of this symbiosis was calculated as about 50–100 million years.

During such a long time, devescovinids and *Bacteroidales* could have become interdependent. Radek *et al.* (1996) showed that antibiotic treated *Devescovina* flagellates lose their original shape owing to the loss of ectosymbionts. This could

mean that the ectosymbionts help their host flagellates to maintain the cytoskeletal structure (missile-like shape). This observation is supported by the fact that closely related *Metadevescovina* does not show a similar shape owing to the absence of the filamentous *Bacteroidales* ectosymbionts. The missile-like shape of *Devescovina* could be beneficial for the movement in the gut. In this context, another important observation is the filamentous nature of the ectosymbionts. *Bacteroidales* endosymbionts of *Pseudotrichonympha* flagellates (Noda *et al*., 2005), which belong to the same phylogenetic cluster (V) as *Bacteroidales* ectosymbionts of devescovinid flagellates (Noda *et al*., 2006), are short rods. This could mean that the *Bacteroidales* gained filamentous form after they became ectosymbionts of devescovinid flagellates. As discussed above, the rationale behind such a change in the shape could also be to help the flagellate maintain its cytoskeletal structure.

Recently, Hongoh *et al*. (2008) published the genome of the *Bacteroidales* endosymbionts (CfPt1-2) of *Pseudotrichonympha* flagellates. Interestingly, CfPt1-2 possesses genes encoding nitrogenase (NifHDK), Mo-Fe cofactor biosynthesis proteins, *nif*-operon regulator NifA, all of which are involved in the nitrogen fixation. Using RT-PCR, these genes were shown to be highly expressed. CfPt1-2 also possessed genes for ammonium transporter, urease and urea transporter, suggesting their role in recycling of nitrogenous waste products of flagellates. On the other hand, expression profiles for nitrogen fixation genes in this study showed no expression of such genes in the gut of *Neotermes castaneus* and *Cryptotermes longicollis,* which contain *Bacteroidales* (as ectosymbionts of devescovinid flagellates). Therefore, it is clear that the *nifH* gene sof *Bacteroidales* ectosymbionts of devescovinid flagellates are not involved in the nitrogen fixation. However, like *Bacteroidales* endosymbionts (CfPt1-2), role of ectosymbionts in the biosynthesis of diverse cofactors and amino acids is highly likely. Since flagellates are oxygen sensitive, another important role of ectosymbionts could be consumption of little amounts of oxygen penetrating in the hindgut. This hypothesis is partially supported by the consumption of nanomolar amounts of oxygen by the strictly anaerobic bacterium *Bacteroides fragilis* (Baughn and Malamy, 2004). Moreover, CfPt1-2 showed high

consumption of molecular hydrogen produced by the host *Pseudotrichonympha* (Inoue *et al.*, 2007), which suggests the similar role of ectosymbionts of devescovinids. Ectosymbionts could also be benefiting from the fermentation products (e.g., lactate) of the flagellates.

To better understand the physiological basis of the symbiosis between the devescovinids and *Bacteroidales*, fermentation products of the species-pure flagellate suspensions need to be measured with a high-sensitivity method like capillary electrophoresis. Similar suspensions of flagellates can be treated with antibiotics, and change in the fermentation products can be monitored. Additionally, a cDNA library for devescovinid flagellates and *Bacteroidales* ectosymbionts could help to construct the basic metabolic network in this symbiosis. Finally, sequencing the genome of *Bacteroidales* ectosymbionts, and comparing with the genome of CfPt1-2 could help to find the additional genes involved in the symbiosis with flagellates.

Why are nitrogenase gene expression profiles species specific?

The presence of very little nitrogen in the diet of Kalotermitidae necessitates high activity of nitrogen-fixing bacteria in their gut. Breznak *et al.* (1973) provided the first experimental proof for the high rates of nitrogen fixation in the gut of Kalotermitidae. Using culture independent methods, Noda *et al.*, (1999) showed that only one nitrogen fixation gene (*anfH*) is expressed in the gut of kalotermitid termite *Neotermes koshunensis*, despite the presence of several nitrogen fixation genes. *anfH* genes are supposedly present only in the gut of Kalotermitidae, and the wood-feeding cockroach *Cryptocercus punctulatus* (Yamada *et al.*, 2007).

The interesting questions are (i) which organism is carrying these genes? and (ii) are these genes also expressed in all species of Kalotermitidae In the present study, *anfH* genes were putatively assigned to the *Bacteroidales* ectosymbionts of *Devescovina* flagellates. Expression profiles for nitrogen fixation genes in four species of Kalotermitidae (*Neotermes castaneus*, *Cryptotermes longicollis*, *Kalotermes flavicollis*, and *Incisitermes marginipennis*) revealed a unique pattern of gene expression for each Kalotermitidae, indicating nitrogen fixation activity of phylogenetically different bacteria in different species. *anfH* genes were expressed

only in the gut of *N. castaneus* and *C. longicollis*, whereas *nifH* genes of *Treponema* were expressed in *N. castaneus*, *K. flavicollis*, and *I. marginipennis*, and *nifH* genes of *Bacteroidales* were expressed only in *K. flavicollis*. Despite feeding on the same diet, and harboring nearly the same set of bacteria, why are nitrogen fixation genes of different bacteria expressed in different species of Kalotermitidae? Which factors control the expression of different genes in different species of termites?

Intriguingly, *anfH* genes were expressed only in those termites (*N. castaneus* and *C. longicollis*), which accumulated very little hydrogen (<1 kPa, hydrogen microsensor measurements). Termites that accumulated high amounts of hydrogen (*K. flavicollis* and *I. marginipennis*, >35 kPa) showed no expression of *anfH* genes. It is suggestive that *K. flavicollis* and *I. marginipennis* accumulate high amounts of hydrogen owing to the presence of hypermastigid flagellates. Hydrogen is known to be a competitive inhibitor of nitrogen reduction by nitrogenase; depending on the organism, the inhibitory constant of hydrogen typically ranges from 0.03 to 0.2 atm (Guth and Burris, 1983; Rasche and Arp, 1989). Therefore, it is tempting to hypothesize that the *anfH* genes in the termite gut are inhibited by the high amounts of hydrogen. This hypothesis can be tested by incubating *N. castaneus* and *C. longicollis* under high hydrogen partial pressure, and studying the gene expression.

Expression of *nifH* genes belonging to the *Treponema* group provided the first evidence for *in vivo* nitrogen fixation by this group of bacteria. However, no expression of *Treponema* genes in the gut of *C. longicollis* (this study), and *N. koshunesnsis* (previous study, Noda *et al*., 1999) is enigmatic. When Noda *et al*. (1999) added molybdenum in the diet of *N. koshunensis*, expression of two more genes was observed. These genes were predicted to belong to the *Treponema* phylogenetic cluster. This means that the addition of molybdenum in the diet activated the nitrogen fixation by *Treponema*. The reason behind such a change in the gene expression is difficult to explain, since *Treponema* genes are expressed in other Kalotermitidae (this study) without the addition of molybdenum.

The endosymbiotic *Bacteroidales* (CfPt1-2) fix nitrogen in the gut of subterranean termite *Coptotermes formosanus* (Hongoh *et al*., 2008). Moreover,

CfPt1-2 consumes hydrogen (Inoue *et al*., 2007), although its genome does not contain any typical hydrogenase gene (Hongoh *et al*., 2008). In the present study, nitrogen fixation genes belonging to the *Bacteroidales* were expressed in the gut of *K. flavicollis*. However, despite the presence of high number of *Bacteroidales* in *N. castaneus* and *C. longicollis*, their *nifH* genes were not expressed. Results of the present and previous studies collectively suggest that the hydrogen consumption by *Bacteroidales* could be a prerequisite for their nitrogenase activity. This means that the nitrogen fixation by *Bacteroidales* demands the presence of high amounts of hydrogen, which is present in *K. flavicollis*, but not in *N. castaneus* and *C. longicollis*. If this argument is valid, incubation of *N. castaneus* and *C. longicollis* under high hydrogen partial pressure (as mentioned above) would activate the gene expression by *Bacteroidales*.

A *nifH* gene belonging to the proteo-cyano group was expressed in the gut of *I. marginipennis*. *Trichonympha* flagellates in this termite contain endosymbionts affiliated with *Desulfovibrio*, which are highly abundant (Wienemann, 2008). DNA-based T-RFLP profiles showed that the expressed *nifH* gene from the proteo-cyano group is the most abundant *nifH* gene in *I. marginipennis*. Therefore, it would not be illogical to assign the expressed gene from the proteo-cyano group to the endosymbiotic *Desulfovibrio*. This assignment needs to be confirmed with a method like CARD-FISH (Pernthaler and Pernthaler, 2005).

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Summary

The subject of this thesis is the symbiosis between flagellates and bacteria in the gut of dry-wood termites (Kalotermitidae). In a series of studies, the evolution of devescovinid flagellates and their bacterial symbionts was elucidated, and the physiological basis of the symbiosis was investigated, with a focus on nitrogen fixation.

Devescovinid flagellates are the dominant flagellates in the gut of Kalotermitidae. Species-pure suspensions of devescovinid flagellates (*Devescovina* and *Metadevescovina* species) from a wide range of termite species in the family Kalotermitidae were isolated with micropipettes. Ribosomal RNA gene sequences of the host flagellates and their bacterial symbionts were obtained using a full-cycle-rRNA approach.

Phylogenetic analysis showed that *Devescovina* spp. present in many species of Kalotermitidae form a monophyletic group. They were consistently associated with a distinct lineage of ectosymbionts, which form a monophyletic group among the *Bacteroidales*. The well-supported congruence of their phylogenies documented strict cospeciation of flagellates and their ectosymbionts, which were temporarily classified as "*Candidatus* Armantifilum devescovinae". Nevertheless, the complete incongruence between the phylogenies of devescovinid flagellates and Kalotermitidae (COII genes) demonstrated horizontal transfer of flagellates among several species of Kalotermitidae.

The presence of filamentous "*A. devescovinae*" on the surface of *Devescovina* spp. was corroborated with scanning electron microscopy and fluorescent *in situ* hybridization. However, several *Metadevescovina* species, which form a sister group of *Devescovina* spp., did not possess *Bacteroidales* ectosymbionts. Moreover, a combination of molecular analysis and electron microscopy led to a correction of the previously overestimated diversity of *Metadevescovina* species in the gut of termite *Incisitermes marginipennis*.

In contrast to the *Bacteroidales* ectosymbionts, the endosymbionts of *Devescovina* spp., which belong to the so-called "Endomicrobia" (TG-1 phylum) and consistently colonized the cytoplasm of all flagellates of this group, were clearly polyphyletic. This suggested that they were acquired independently by each host species. The same seems to be true for the *Bacteroidales* ectosymbionts of the

Oxymonas flagellates present in several Kalotermitidae. These ectosymbionts form several distantly related novel lineages in the phylogenetic tree, underscoring the notion that evolutionary histories of flagellate–bacteria symbioses in the termite gut are complex.

Kalotermitidae are known to fix large amounts of atmospheric nitrogen, and acetylene reduction assay showed the presence of nitrogenase activity in the gut of these termites. Community fingerprinting of the nitrogenase genes (homologs of *nifH*) by T-RFLP analysis revealed that a gene encoding an alternative nitrogenase (*anfH*) of unknown origin was most highly expressed homolog in mRNA-based profiles. Cloning of the *nifH* homologs from capillary-picked suspensions of *Devescovina arta* and *Snyderella tabogae* gave strong evidence that the "*A. devescovinae*" are the putative carriers of the *anfH* gene and therefore responsible for most of the nitrogen-fixing activity in the guts of *Neotermes castaneus* and *Cryptotermes longicollis*.

Despite a high diversity of *nifH* homologs in gut homogenates, the only other homologs that were expressed belonged to *Treponema*, *Bacteroidales* (*nifH*), and the proteo-cyano group. The gene expression profiles were specific for the termites. The *anfH* genes were not expressed in termite species that accumulated large amounts of hydrogen (35–45 kPa, microsensor measurements), suggesting a repression of gene expression by high hydrogen partial pressure.

Zusammenfassung

Der Fokus dieser Arbeit beruht auf der Symbiose zwischen Flagellaten und Bakterien im Darm von Trockenholztermiten (Kalotermitidae). In einer Reihe von Versuchen konnte über molekulare Phylogenien die Evolution von devescoviniden Flagellaten und deren bakteriellen Symbionten etabliert werden. Die physiologische Grundlage der Symbiose wurde speziell hinsichtlich der Stickstofffixierung untersucht.

Devescovinide Flagellaten dominieren die Flagellatenpopulation im Darm der Kalotermitidae. Mittels Mikropipetten wurden devescovinide Flagellaten (*Devescovina*- und *Metadevescovina*-Arten) von einer Vielzahl von Termitenarten der Familie Kalotermitidae artspezifisch isoliert. Die Sequenzen der ribosomalen RNA von Wirtsflagellaten und deren bakteriellen Symbionten wurden mittels „full-cycle-rRNA“-Methode analysiert.

Phylogenetische Analysen zeigten, dass *Devescovina*-Arten, die in vielen Vertretern der Kalotermitidae vorkommen, eine monophyletische Gruppe bilden. Zudem waren *Devescovina*-Arten stets mit einer bestimmten Linie von Ektosymbionten assoziiert, die eine monophyletische Gruppe innerhalb der *Bacteroidales* bilden. Die von verschiedenen Algorithmen gut gestützte Kongruenz der Stammbäume dokumentiert eine strikte Kospeziation der Flagellaten und deren Ektosymbionten, die als *Candidatus* „Armantifilum devescovinae“ vorläufig klassifiziert wurden. Dennoch demonstriert die Inkongruenz zwischen den Phylogenien der devescoviniden Flagellaten und Kalotermitidae (COII-Gene) den horizontalen Transfer von Flagellaten innerhalb vieler Spezies der Kalotermitidae.

Die Identität von „A. devescovinae“ mit den filamentösen *Bacteroidales* auf der Zelloberfläche von *Devescovina*-Arten konnte mittels Rasterelektronenmikroskopie und Fluoreszenz-*in-situ*-Hybridisierung bestätigt werden. Dabei stellte sich heraus, dass einige *Metadevescovina*-Arten, welche eine Schwesterngruppe zu den *Devescovina*-Arten bilden, keine ektosymbiontischen *Bacteroidales* besitzen. Weitere molekulare Analysen und elektronenmikroskopische Untersuchungen führten zu einer Revision der vormals überschätzten Diversität von *Metadevescovina*-Arten im Darm der Termite *Incisitermes marginipennis*.

Neben den *Bacteroidales*-Ektosymbionten besitzen Flagellaten der Gattung *Devescovina* auch Endosymbionten. Diese gehören zu den “Endomicrobia” (TG1-Phylum) und konnten im Zytoplasma aller Arten dieser Gruppe nachgewiesen

werden. Im Gegensatz zu den Ektosymbionten sind die “Endomicrobia” polyphyletisch, was vermuten lässt, dass sie von den jeweiligen Wirtsarten unabhängig erworben wurden. Dasselbe Szenario scheint auch für die *Bacteroidales*-Ektosymbionten der *Oxymonas*-Flagellaten zu gelten, die in vielen Kalotermitidae vorkommen. Diese Ektosymbionten bilden mehrere entfernt verwandte Entwicklungslinien im Stammbaum der *Bacteriodales*, was die Ansicht bestärkt, dass die Flagellaten–Bakteriensymbiosen im Termitendarm entwicklungsgeschichtlich komplex sind.

Trockenholztermiten fixieren in großen Mengen Luftstickstoff, und mittels Acetylenreduktionstest konnte Nitrogenaseaktivität im Darm dieser Termiten nachgewiesen werden. Fingerprint-Analysen der Nitrogenase-Gene (*nifH*-Homologe) mittels T-RFLP-Methode zeigten, dass ein für eine alternative Nitrogenase kodierendes Gen (*anfH*) unbekannten Ursprungs das am stärksten exprimierte Homolog in mRNA-basierten Profilen darstellte. Eine Klonierung der *nifH*-Homologe aus mittels Mikropipetten gesammelten Suspensionen von *Devescovina arta* und *S. tabogae* brachte deutliche Hinweise, dass „A. devescovinae“ der Träger dieser *anfH*-Gene ist und somit für den Großteil der Stickstoff fixierenden Aktivität im Darm von *Neotermes castaneus* und *Cryptotermes longicollis* verantwortlich ist.

Obwohl die Darmhomogenate eine hohe Diversität von *nifH*-Homologen beherbergen, wurden neben den erwähnten *anfH*-Genen nur wenige weitere Homologe exprimiert. Phylogenetische Analyse ergab eine Zugehörigkeit zu *Treponema*, *Bacteroidales* (*nifH*) und der Proteo-Cyano-Gruppe. Die Genexpressions-Profile waren charakteristisch für die jeweilige Termitenart. Die *anfH*-Gene wurden nicht in Termiten exprimiert, die große Mengen an Wasserstoff akkumulierten (35–45 kPa, Mikrosensorenmessungen), was auf eine Repression der Genexpression durch hohe Wasserstoff-Partialdrücke hindeutet.

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Appendix

Attached manuscript

Phylogenetic diversity of 'Endomicrobia' and their specific affiliation with termite gut flagellates

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'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 rRNA genes of the flagellate and the symbionts, which originated from suspensions of individual flagellates isolated by micropipette. Each flagellate harboured 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 hosts. Interestingly, the 'Endomicrobia' sequences obtained from flagellates belonging to the genus *Trichonympha* formed a monophyletic group, suggesting co-speciation 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 & 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). We have demonstrated previously that the 'Endomicrobia' clones retrieved from *Reticulitermes santonensis* represent intracellular symbionts of flagellate protists (as previously proposed by Ohkuma *et al.*, 2001), and documented that the two major gut flagellates of this termite, *Trichonympha agilis* and *Pyrsonympha vertens*, each harbour 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 & Lee, 2000; Yamin, 1979). Phylogenetic studies using small-subunit (SSU) rRNA and other molecular markers have confirmed the presence of two distinct phylogenetic lineages, i.e. Oxymonadida and Parabasalidea (Dacks *et al.*, 2001; Gerbod *et al.*, 2002; Stingl & Brune, 2003; Ohkuma *et al.*, 2005). Although little is known about the metabolic functions of termite gut flagellates (Brune & 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 & 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 & 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 harbouring various gut

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Abbreviations: FISH, fluorescence *in situ* hybridization; SSU, small subunit.

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequence data reported in this paper are AB297984–AB298082, AB326107, AB326370–AB326383, AM747388 and AM747389.

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flagellates (Stingl *et al.*, 2005) collectively suggest a strong connection between the phylogenetic diversity of the symbionts 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 test this hypothesis, we phylogenetically analysed SSU rRNA genes of the major flagellates and their symbionts in the termite *Hodotermopsis sjoestedti* and in selected flagellates of five other termite species.

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* came 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 (Bio 101, Savant Instruments) 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 three to seven hindguts were suspended in Solution U (Trager, 1934) and diluted to a density of approximately 10 flagellate cells μl^{-1}. Aliquots (20 µl) of the diluted suspension were placed in the wells of a 10-well Teflon-coated glass slide (Erie Scientific Company). Flagellate cells were sorted by morphology (Radek *et al.*, 1992; Tamm, 1999; Brugerolle & 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 15 µl sterile PBS and washed by at least three transfers into fresh PBS-containing wells. Approximately 100 cells were finally resuspended 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), following the manufacturer's instructions. The extracted DNA was finally eluted with 30 µl distilled water and used as a template for PCR reactions.

PCR amplification. Flagellate SSU rRNA genes were amplified using universal eukaryotic primers as described by 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), following the manufacturer's instructions. Clones with a flagellate SSU rRNA gene insert and clones with an '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 *Msp*I and *Alu*I, and subjected to electrophoresis on a 3 % agarose gel. The clones were sorted according to their restriction patterns, and two to ten 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 neighbour-joining and maximum-parsimony methods implemented in ARB. Shorter sequences were added using the ARB parsimony tool. Chimaeric 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 described previously (Stingl & 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 non-specific probe binding in the same suspension. For each probe, hybridization stringency was optimized by testing formamide concentrations over a range of 0–50 %.

RESULTS

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

Clone libraries of SSU rRNA genes amplified from *H. sjoestedti* whole-gut DNA extract with 'Endomicrobia'-specific primers contained more than 10 distinct monophyletic lineages of 'Endomicrobia'. To test whether individual phylotypes 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; Fig. 1b, c). 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.

The eukaryotic SSU rRNA gene libraries prepared from the *Eucomonympha* and *Dinenympha* suspensions each contained a single phylotype (Table 1). In the library from the *Eucomonympha* suspension, the obtained sequence was virtually identical to that of clone HsL3 recovered in a clone library of a mixed flagellate population of *H.*

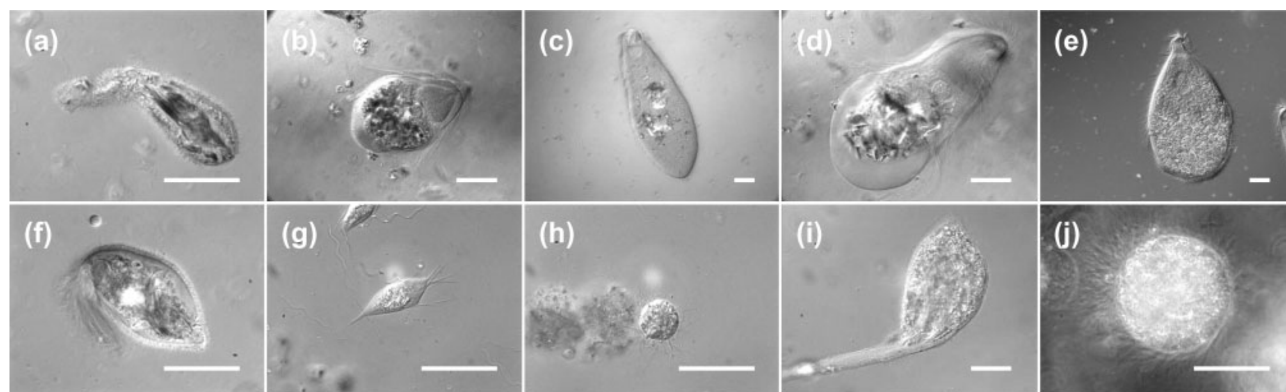

Fig. 1. Light micrographs of ten flagellate species used in this study: *Dinenympha* sp. (a), *Trichonympha* sp. (b) and *Eucomonympha* sp. (c) from *H. sjoestedti*; *Trichonympha* sp. (d) from *Z. nevadensis*; *Deltotrichonympha* sp. (e) from *M. darwiniensis*; *Joenia* sp. (f) from *K. flavicollis*;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

sjoestedti (Ohkuma *et al.*, 2000), corroborating the tentative assignment of this clone to the genus *Eucomonympha*. 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

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

Termite species (family)	Flagellate suspension (Order*)	Flagellate phylotype (accession no.)	Closest relatives (accession no.)	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* (Christamonadida)	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.

Dinenympha. The *Trichonympha* suspension yielded three different phylotypes of eukaryotic SSU rRNA genes, whose sequences were virtually identical to those of the three *Trichonympha* phylotypes previously obtained from this termite by Ohkuma *et al*. (2000).

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 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.

'Endomicrobia' in representative flagellates from other termites

Using the same strategy, we phylogenetically analysed the host flagellates and 'Endomicrobia' in the flagellate suspensions prepared from five other termites, which represent seven additional flagellate genera of parabasalids and oxymonadids (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 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) obtained from a clone library of the same termite and assigned to the flagellate genus *Foaina* by other authors (Gerbod *et al*., 2000).

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 almost-full-length (>1400 bp) SSU rRNA gene sequences obtained in this and previous studies clearly

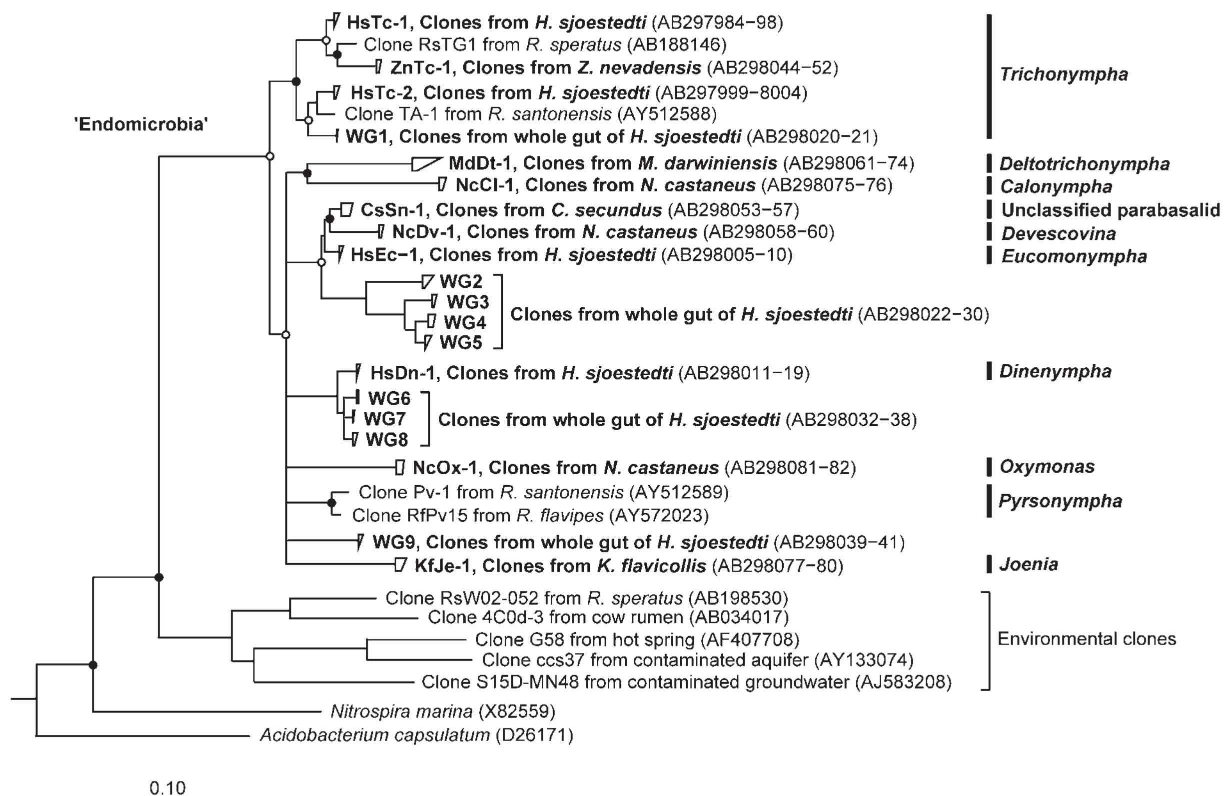

Fig. 2. Phylogenetic tree of 'Endomicrobia' and selected environmental clones in the TG-1 phylum, based on SSU rRNA gene sequences. The core tree (maximum-likelihood) was constructed from almost-full-length sequences (>1300 bp). Tree topology was tested by neighbour-joining and parsimony analysis with bootstrapping (DNAPARS, 1000 replicates). Marked nodes have bootstrap values of >95 % (●) and >50 % (○), nodes not supported by all analyses are shown as multifurcations. 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.

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 with 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 fluorescence *in situ* hybridization (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 the design of specific probes covering each phylotype. Therefore, we designed a set of oligonucleotide probes that covered the phylotypes in question (Table 2).

Simultaneous FISH was conducted with a fluorescein-labelled bacterial probe and a Cy3-labelled '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 the location of 'Endomicrobia' on the cell surface or within the nucleus of the host.

Since the morphotypes of certain flagellates (*Dinenympha* spp. in *H. sjoestedti* and *Joenia* sp. 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). 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.

DISCUSSION

The results of this study corroborate that 'Endomicrobia' are host-specific intracellular symbionts of termite gut flagellates. Each of the flagellates investigated harboured a unique phylotype of 'Endomicrobia', which supports 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 analysed in this study invariably harboured 'Endomicrobia'. Together with the phylotypes that remain to be assigned to a particular host, 'Endomicrobia' represent the symbionts of up to 24 parabasalid and oxymonadid species, and probably more in view of the presence of '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

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

Probe name	Target*	Sequence (5′–3′)†	Formamide concn (%)
TG1End1023T1	'Endomicrobia' phylotypes ZnTc-1, HsTc-1, RsTG1	GCTGACTCCCTTGCGGGTCA	20–50
TG1End1027	Most 'Endomicrobia' lineages (including HsDn-1)	CTCTGCTAACTCCCTTGCGG	40
TG1End1023	Some 'Endomicrobia' lineages (including HsEc-1)	ACTAACTCCCTTGCGGGTCA	20‡
TG1-TriG1-Hsj	Symbiont HsTc-1 of *Trichonympha* sp. HsTcA	TTGGTCCAGAAGACTGCTT	20
TG1-Joe-Kf	Symbiont KfJe-1 of *Joenia* sp. KfJeA	GCTAACTCTCTTGCGAGTCA	20
TG1-Dev-Nca	Symbiont NcDv-1 of *Devescovina* sp. NcDvA	GCATAGGACCACAGTTTGGC	20
Flg-Dine-Hsj	*Dinenympha* sp. HsDnA of *H. sjoestedti*	GCTTTTTGAGGCGGCTAT	35
Flg-Tricho1-Hsj	*Trichonympha* sp. HsTcA of *H. sjoestedti*	GCTAGATTTCAAGATAGTCT	10
Flg-Euc-Hsj	*Eucomonympha* sp. HsEcA of *H. sjoestedti*	AAACCTCCAGACCACGCT	10‡
Flg-Joe-Kfl	*Joenia* sp. KfJeA of *K. flavicollis*	GCTAGGTTGCACACTAGTGG	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; www.microbial-ecology.net/probebase/).
‡Not optimized.

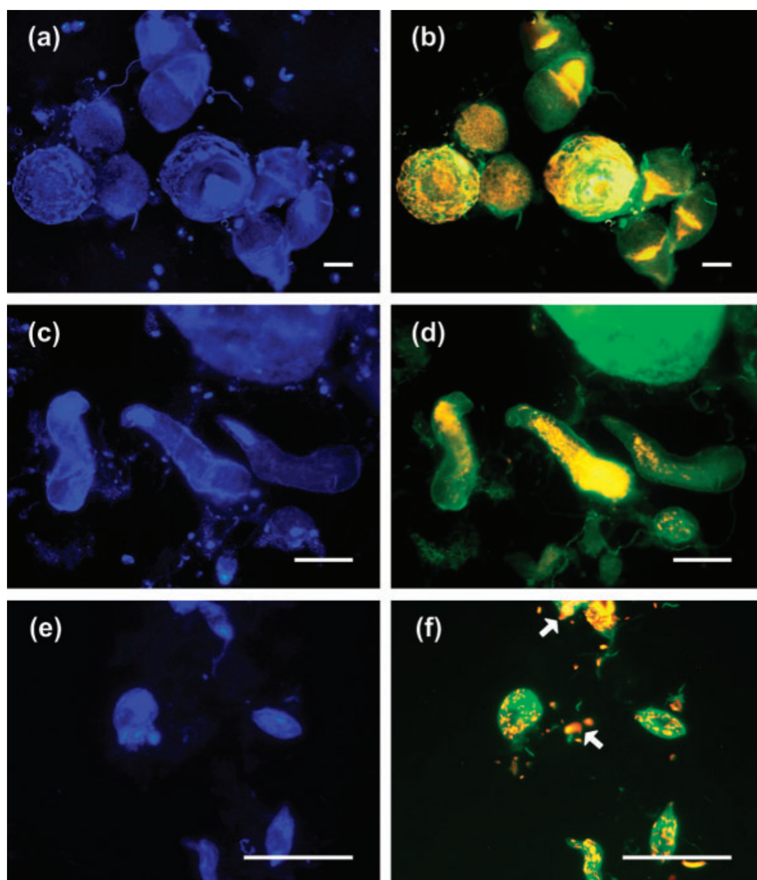

Fig. 3. Epifluorescence micrographs of hindgut preparations of *Z. nevadensis* (a, b), *H. sjoestedti* (c, d) and *Devescovina* from *N. castaneus* (e, f). The preparations were stained with DAPI (a, c, e) and simultaneously hybridized with the fluorescein-labelled (green) probe EUB338 and the Cy3-labelled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

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 under-represented 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 co-speciation between the partners (Page & Charleston, 1998). This would imply that each of the extant *Trichonympha* flagellates harbours a specific lineage of 'Endomicrobia' inherited by vertical transmission from their common ancestor – an issue that cannot be resolved based on the current dataset. Conversely, it is 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*) harbour '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.*, 2007). 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. The host flagellates may also 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, 1981; Odelson & 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.

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