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.

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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.

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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*, *Kaloterms 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'-GATTCTACTTCATGTG-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 *MspI* and *AluI*, 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 fastDNAmI), 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: *Dinonympha* 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 *Dinonympha* suspension, the sequence

showed 94 % identity to that of a *Dinonympha* 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 (%)†
<i>Hodotermopsis sjoestedti</i> (Termopsidae)	<i>Trichonympha</i> (Trichonymphida)	HsTcA (AB326107)	<i>Trichonympha</i> sp. HsL5 from <i>H. sjoestedti</i> (AB032233)	99.9
		HsTcB (AB326371)	<i>Trichonympha</i> sp. Hs8 from <i>H. sjoestedti</i> (AB032229)	99.5
		HsTcC (AB326373)	<i>Trichonympha</i> sp. HsS9 from <i>H. sjoestedti</i> (AB032239)	99.6
	<i>Eucomonympha</i> (Trichonymphida)	HsEcA (AB326375)	<i>Eucomonympha</i> sp. HsL3 from <i>H. sjoestedti</i> (AB032231)	99.0
	<i>Dinonympha</i> (Oxymonadida)	HsDnA (AB326376)	<i>Dinonympha</i> sp. OS1 from <i>R. hesperus</i> (AB092933)	94.0
		ZnTcA (AB326378)	<i>Trichonympha</i> cf. <i>collaris</i> from <i>Z. angusticollis</i> (AF023622)	95.2
<i>Zootermopsis nevadensis</i> (Termopsidae)	<i>Trichonympha</i> (Trichonymphida)	MdDtA (AB326380)	<i>Deltotrichonympha operculata</i> from <i>M. darwiniensis</i> (AJ583379)	99.5
<i>Mastotermes darwiniensis</i> (Mastotermitidae)	<i>Deltotrichonympha</i> (Christamonadida)	KfJeA (AB326381)	Gut symbiont Kf5 from <i>K. flavicollis</i> (AF215857)	98.7
<i>Kaloterms flavicollis</i> (Kalotermitidae)	<i>Joenia</i> (Cristamonadida)	NcDvA (AM747389)	<i>Devescovina</i> sp. D16 from <i>N. jouteli</i> (X97974)	98.9
<i>Neotermes castaneus</i> (Kalotermitidae)	<i>Devescovina</i> (Cristamonadida)	NcClA (AM747388)	<i>Calonympha</i> sp. B14 from <i>N. jouteli</i> (X97976)	98.5
	<i>Calonympha</i> (Cristamonadida)	NcOxA (AB326383)	<i>Oxymonas</i> sp. Nk_U08 from <i>N. koshunensis</i> (AB092931)	94.5
	<i>Oxymonas</i> (Oxymonadida)	CsSnA	Unclassified parabasalid from <i>C. brevis</i> (AF052699)	96.1
<i>Cryptotermes secundus</i> (Kalotermitidae)	Unclassified parabasalid (Cristamonadida)			

*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 oxymonads (Table 1). Again, we were able to assign the eukaryotic SSU rRNA gene sequences obtained from each flagellate suspension to the identical or similar respective genera, whose SSU rRNA gene sequences have been 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 <i>Trichonympha</i> sp. HsTcA	TTGGTCCAGAAGACTGCTT	20
TG1-Joe-Kf	Symbiont KfJe-1 of <i>Joenia</i> sp. KfJeA	GCTAACTCTTGCGAGTCA	20
TG1-Dev-Nca	Symbiont NcDv-1 of <i>Devescovina</i> sp. NcDvA	GCATAGGACCACAGTTTGCC	20
Flg-Dine-Hsj	<i>Dinenympha</i> sp. HsDnA of <i>H. sjoestedti</i>	GCTTTTGGAGGCGGCTAT	35
Flg-Tricho1-Hsj	<i>Trichonympha</i> sp. HsTcA of <i>H. sjoestedti</i>	GCTAGATTCAAGATAGTCT	10
Flg-Euc-Hsj	<i>Eucomonympha</i> sp. HsEcA of <i>H. sjoestedti</i>	AAACCTCCAGACCACGCT	10‡
Flg-Joe-Kfl	<i>Joenia</i> sp. KfJeA of <i>K. flavicollis</i>	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 hind-gut 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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