Microhabitat-specificity of the hindgut microbiota in higher termites

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Summary

Termites are a group of eusocial insects in the superorder *Dictyoptera*, believed to have evolved from a lineage of ancient cockroach-like ancestors 150 million years ago. They play an important role in the breakdown of dead plant material, with the help of microorganisms harboured in the gut. The termites can be classified into flagellate-harbouring lower termites and flagellate-free higher termites. In comparison to the lower termites, the higher termites have undergone immense phylogenetic and dietary diversification, that has led to major changes in their gut structure. This diversification in the host is reflected in differences in their gut communities.

To understand how host phylogeny and diet help shape bacterial communities in higher termites, I conducted an extensive pyrosequencing-based community survey of the gut communities of the major higher termite subfamilies, *Macrotermitinae, Termitinae*, and *Nasutitermitinae.* First, I constructed clone libraries and calculated phylogenetic trees for relevant bacterial taxa found in a variety of higher termites. The node information in these trees was used to provide a robust phylogenetic backbone for the accurate taxonomic assignment of the shorter pyrosequences. The analysis revealed that phylogenetically related termites in general, have similar community structure. However, one of the wood-feeding termites showed a greater similarity in gut community structure to other wood-feeders, in spite of not being phylogenetically related to them. The results suggest that although host phylogeny appears to be the major driving force in the determination of gut community membership, host diet can significantly contribute to community structure.

However, far from being a homogenous environment, the higher termite gut is a highly structured habitat and shows the presence of spatially separated and physicochemically distinct compartments. Conditions unique to each compartment, play a significant role in shaping distinct compartment-specific communities. I used pyrotag sequencing to conduct an in-depth analysis of the communities of gut compartments from termites belonging to the families *Termitinae* and *Nasutitermitinae*. I found that homologous compartments from closely related termites are more similar in their community structure than adjacent compartments from the same termite. Based on our results, we hypothesize that similar ecological conditions such as increased alkalinity in the anterior gut, drive community structure in the gut compartments, and are reflected in overall hindgut community structure as well.

The paunch (or P3 compartment) is the most voluminous of all hindgut compartments in wood-feeding higher termites, and is densely colonized by bacteria. Studies have shown that cellulase activity in the hindgut is particle-associated and possibly of bacterial origin. By fractionation of particles in the paunch lumen, using density-dependent centrifugation, I was able to show that the fraction enriched in wood fibers contributes substantially to the total cellulase activity in the hindgut. Using pyrosequencing, I examined the bacterial communities associated with the wood fibers in two wood-feeding members of the *Nasutitermitinae.* The results revealed the presence of a distinct cellulolytic fiber-associated community, primarily composed of the phyla TG3, *Fibrobacteres* and *Spirochaetes*. This fiber-associated community appears to have filled the niche for cellulose digestion, vacated by the flagellates.

Lastly, the gut wall in termites is one of the major habitats in the gut, and home to an endospore-forming filamentous bacterium called '*Candidatus* Arthromitus'. Due to the lack of a cultured isolate, the

phylogenetic identity of 'Arthromitus' was disputed, and often confused with similar filamentous bacteria from mammalian guts. Phylogenetic analysis of picked filaments reveals '*Candidatus* Arthromitus' to be a diverse clade of bacteria, found widely among arthropods, that is distinct from the segmented filamentous sequences recovered from mammalian guts.

Zusammenfassung

Die Termiten sind eine Gruppe eusozialer Insekten in der Superordnung *Dictyoptera* und haben sich vermutlich vor 150 Millionen Jahren aus einer Linie Schaben-ähnlicher Vorfahren entwickelt. Sie spielen beim Abbau toter Pflanzenmasse eine wichtige Rolle, wobei sie von in ihrem Darm lebenden Bakterien unterstützt werden. Man unterscheidet zwischen den Flagellaten beherbergenden niederen Termiten und den Flagellaten-freien höheren Termiten. Im Gegensatz zu den niederen Termiten vollzogen die höheren Termiten eine immense phylogenetische und diätische Diversifizierung, die grundlegende Veränderungen ihrer Darmstruktur zur Folge hatte. Diese Diversifizierung im Wirt tritt in Unterschieden der Darmmikrobiota zu Tage.

Um zu verstehen, wie Phylogenie und Ernährungsweise des Wirtes die bakteriellen Gemeinschaften in höheren Termiten formen, führte ich eine extensive Pyrosequenzierung-basierte Vergleichsstudie der Darmgemeinschaften in den wichtigsten Unterfamilien der Termiten, *Macrotermitinae, Termitinae* und *Nasutitermitinae*, durch. Zunächst konstruierte ich Klonbibliotheken und berechnete phylogenetische Bäume für relevante bakterielle Taxa aus verschiedenen höheren Termiten. Die Knotenpunkt-Informationen aus diesen Bäumen dienten als robustes phlylogenetisches Rückgrat für die korrekte taxonomische Zuordnung der kürzeren Pyrosequenzen. Die Analyse ergab, dass phylogenetisch verwandte Termiten im Allgemeinen eine ähnliche Gemeinschaftsstruktur aufweisen. Eine der holzfressenden Termiten zeigte jedoch eine größere Ähnlichkeit zu anderen holzfressenden Arten, ohne mit ihnen phylogenetisch verwandt zu sein. Die Ergebnisse legen nahe, dass die Phylogenie des Wirtes zwar die wesentliche treibende Kraft bei der

Festlegung der Darmgemeinschaft darstellt, die Ernährungsweise des Wirtes jedoch signifikant zur Gemeinschaftsstruktur beitragen kann.

Der Darm der höheren Termiten, mitnichten eine homogene Umgebung, bildet ein hoch strukturiertes Habitat und weist örtlich getrennte und physiochemisch verschiedene Kompartimente auf. In jedem Kompartiment bestimmen einzigartige Bedingungen die spezifische Gemeinschaft. Mittels Pyrotag-Sequenzierung führte ich eine ausführliche Analyse der Kompartiment-spezifischen Gemeinschaften in Termiten der Familien *Termitinae* und *Nasutitermitinae* durch. Ich fand heraus, dass homologe Kompartimente nah verwandter Termiten einander ähnlicher waren als benachbarte Kompartimente derselben Termite. Basierend auf unseren Ergebnissen vermuten wir, dass ähnliche ökologische Bedingungen, wie beispielsweise erhöhte Alkalinität im Vorderdarm, die Gemeinschaftsstruktur in den Darmkompartimenten beeinflussen und ebenfalls in der Gemeinschaftsstruktur des Dickdarms insgesamt reflektiert werden.

Der Pansen (oder P3-Kompartiment) ist volumenmäßig der größte Dickdarmabschnitt in holzfressenden höheren Termiten und ist vollständig mit Holzfasern und Bakterien gefüllt. Über den Beitrag von Bakterien zum Celluloseverdau ist jedoch wenig bekannt. Durch Fraktionierung der Partikel im Lumen des Pansen mittels Dichtegradientenzentrifugation konnte ich zeigen, dass die in Holzfasern angereicherte Fraktion substantiell zur gesamten Cellulase-Aktivität beitrug. Mittels Pyrosequenzierung untersuchte ich die mit den Holzfasern assoziierten bakteriellen Gemeinschaften in zwei holzfressenden Vertretern der *Nasutitermitinae*. Die Ergebnisse offenbarten die Anwesenheit einer eindeutig cellulolytischen, Faser-assoziierten Gemeinschaft, die vor allem aus den Phyla TG3, *Fibrobacteres* und *Spirochaetes* bestand. Diese Faser-assoziierte Gemeinschaft scheint die von den Flagellaten verlassene Nische des Celluloseverdaus zu besetzen.

Die Darmwand der Termiten ist eines der wichtigsten Habitate des Darms und beherbergt ein Endosporen-bildendes filamentöses Bakterium namens “*Candidatus* Arthromitus”. Mangels eines kultivierten Isolates wurde die phylogenetische Identität von 'Arthromitus' angezweifelt und oft mit ähnlichen filamentösen Bakterien aus Säugetierdärmen verwechselt. Phylogenetische Analyse einzeln ausgewählter Filamente zeigte *Candidatus* Arthromitus als einen diversen Stamm von Bakterien, der unter Arthropoden weit verbreitet ist und sich deutlich von segmentierten filamentösen Sequenzen aus Säugetierdärmen unterscheidet.

Chapter 1

General Introduction

1.1 Termites

Termites (Isoptera) are eusocial insects comprise over 2750 species in 285 genera and are believed to have evolved from a lineage of ancient cockroach-like ancestors, 150 million years ago (Engel et al. 2009; Inward et al. 2007; Legendre et al. 2008). They play a major role in the decomposition of dead plant matter, ranging from non-humified wood to more humified material like humus (Donovan et al. 2001). In order to degrade such recalcitrant material, they depend on microbial symbionts harboured in their hindgut (Brune & Ohkuma 2011).

Based on the presence or absence of hindgut flagellates, they can be divided into phylogenetically lower and higher termites, respectively. The lower termites are paraphyletic, comprised of many families (Engel et al. 2009)(See Figure 1.1). The flagellate-free higher termites constitute a monophyletic taxon (family *Termitidae*) that contains 70% of all termite species. Unlike the lower termites that only feed on wood, the higher termites show a greater diversity in their feeding behaviour (Table 1.1) (Donovan et al. 2001).

Table 1.1 | Diversity of feeding-groups found in termites (summarized from the results of Donovan et al., 2001)

Taxon	Dietary specializations
Lower Termites (multiple families)	wood, wood/litter interface, Grass
Higher Termites (*Termitidae*)	
Macrotermitinae	fungal hyphae, wood, wood/litter interface
Apicotermitinae	wood/soil interface, soil
Termitinae	soil, wood/soil interface, wood
Nasutitermitinae	wood, soil, wood/litter interface, grass, epiphytes, litter

1.2 The termite gut: basic design in higher termites

The termite gut could be divided into three major regions – the foregut, midgut and the hindgut. The hindgut is the most easily discernible of the regions and is packed to capacity with microbial symbionts. All lower termite families share this basic hindgut design and characteristically lack any significant compartmentalization (Noirot 1995; Noirot 2001). As opposed to lower termites (Figure 1.1), the higher termites show a remarkable diversity in hindgut structure, ranging from a very primitive (lower termite-like gut) structure observed among *Macrotermitinae* to highly derived and compartmentalized guts in the advanced subfamilies of higher termites like the *Termitinae* and *Nasutitermitinae* (Noirot 2001).

Additionally, during their evolutionary transition from lower termites, all higher termites have lost their intestinal flagellates, and have come to possess an entirely prokaryotic gut community (Brune & Ohkuma 2011).

1.3 The higher termite hindgut: a complex collection of microhabitats

1.3.1 Diet and host evolution as a driver for gut microhabitats

Through 50 million years or so of termite evolution, the higher termites have adapted to a number of ecological niches, and have come to display a remarkable diversity in both gut anatomy and physiology (Noirot 2001; Engel et al. 2009). Previous studies in the guts of higher termites have shown that the gut community structure of termites reflects the phylogeny of the host, and closely-related termites share more bacterial lineages in common than distantly-related ones (Schmitt-Wagner et al. 2003; Hongoh et al. 2005; Hongoh, Ekpornprasit, et al. 2006; Warnecke et al. 2007). However, it has also been observed that minor

modifications to the diet of a wood-feeding termite, *Nasutitermes takasagoensis* are reflected in significant changes in the gut community structure (Miyata et al. 2007). Nevertheless, the evolutionary radiation of the higher termites has resulted in the diversity in community structure, through the modification of existing (e.g. gut compartmentalization in soil-feeding termites) as well as the introduction of new microhabitats (e.g. the surface of wood fibers in higher wood-feeding termites). We are still far from understanding the extent to which host diet and phylogeny contribute to changes in the gut community structure of higher termites.

1.2.2 Hindgut compartments as microhabitats

Hindgut compartmentalization goes hand-in-hand with differences in many physiological parameters (Bignell & Eggleton 1995) and is hence a strong structuring agent for the communities, in both soil-feeding (Friedrich et al. 2001; Schmitt-Wagner et al. 2003) and wood-feeding higher termites (Köhler et al. 2012). This makes the compartments one of the most spatially defined of all microhabitats. In comparison to the fungus-cultivating *Macrotermitnae*, advanced subfamilies of higher termites such as the *Termitinae* and *Nasutitermitinae* show much more pronounced hindgut compartmentalization. This trend of increased compartmentalization in *Termitinae* and *Nasutitermitinae* also correlates well with extreme differences in the pH between the compartments (Bignell & Eggleton 1995; Brune & Kühl 1996; Köhler et al. 2012).

Gut pH in many insects, has been shown to be a parameter under host control, and significantly affects, among other things, the regulation of enzymatic reactions, solubilization of food, and microbial community structure (Harrison 2001). The compartments in the anterior hindgut of most higher termites are characterized by high alkalinity and has been shown by previous studies to harbour a bacterial community that is quite

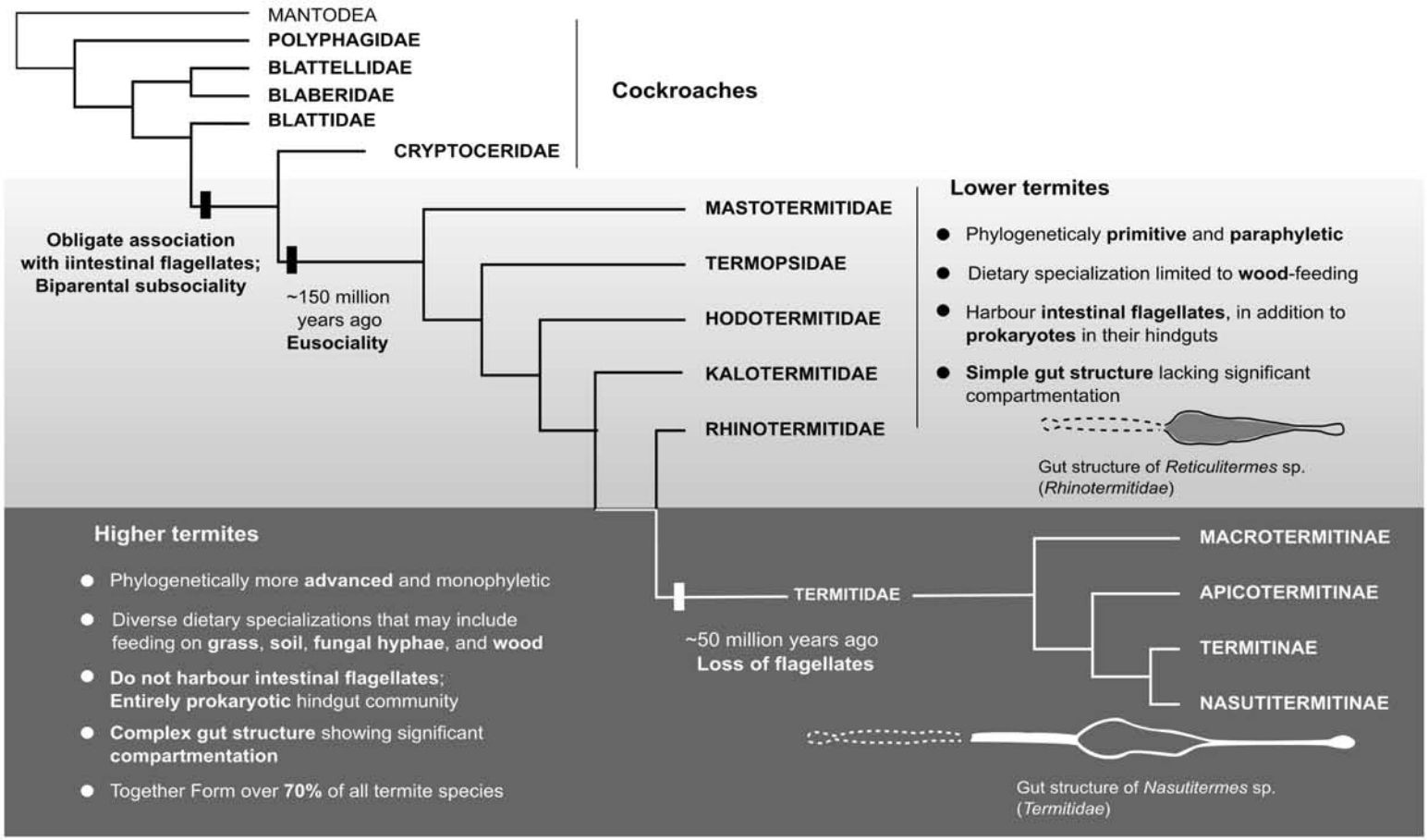

Figure 1.1 | Cladogram showing the major families/subfamilies of the termites, highlighting the differences between the lower and higher termites

distinct from other compartments (Brune 1998; Schmitt-Wagner et al. 2003; Köhler et al. 2012). However, a detailed comparative community analysis of hindgut compartments from different higher termites is still lacking.

1.2.3 The gut wall as a microhabitat

The hindgut is also characterized by steep radial gradients in oxygen and hydrogen resulting in the formation of an anoxic center and a microoxic periphery near the gut wall (Brune et al. 1995; Ebert & Brune 1997; Köhler et al. 2012). The most prominent and conspicuous member of the gut wall-associated community, is a segmented filamentous bacterium of unknown phylogenetic affiliation called “Arthromitus” (Leidy 1849). Since its discovery in termite guts, many studies claimed to have resolved the identity of Arthromitus. It has also been conveniently suggested that Arthromitus is growth stage of *Bacillus cereus* (Margulis et al. 1998). Bacteria within the *Clostridiaceae* with similar filamentous morphology that colonize the gut walls of mammals have also been called “Arthromitus” (Snel et al. 1995), despite having no phylogenetic affiliation to clones obtained from termite guts (Yang et al. 2005; Hongoh et al. 2005). The true phylogenetic identity of “Arthromitus” still remains unknown.

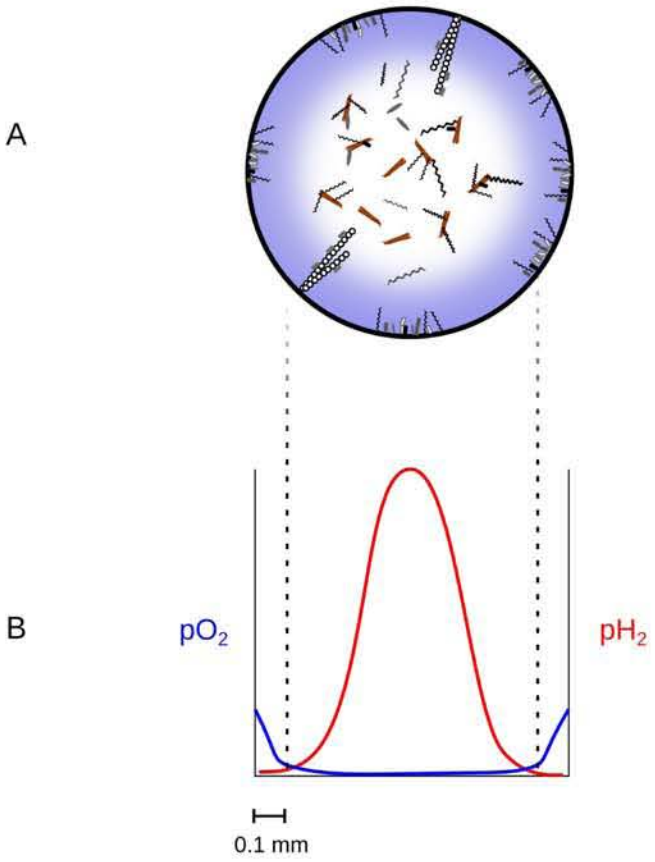

Figure 1.2 | Schematic cross section (**A**) of an agarose-embedded P3 compartment of *Nasutitermes corniger*, illustrating the presence of wood fibers (brown structures) and *Spirochaetes* (squiggles) at the anoxic center (white region), and *Arthromitus* filaments attached to the microoxic wall. Radial profiles (**B**) of O_2 and H_2 partial pressure (Scheme based on data from Köhler et al., 2012).

1.2.4 Wood fibers as important surface microhabitats in higher wood-feeding termites

It is well established that anaerobic environments involving the degradation of plant fiber (e.g. rumen, landfill sites), are often characterized by the presence of distinct fiber-associated bacterial communities that aid in fiber-digestion. Some of the best studied of these cellulolytic communities reside in the bovine rumen, and typically contain members of phyla such as the *Fibrobacteres*, *Firmicutes* and the *Spirochaetes* (Koike et al. 2003; Pandya et al. 2010). Analogous to the rumen, the hindgut of lower termites is also characterized by cellulose

breakdown, and cellulolytic flagellates phagocytize the wood particles for digestion (Brune & Ohkuma 2011) thereby rendering them less available for colonization by bacteria.

Following the loss of flagellates in the ancestor of the higher termites, several species, within the subfamilies *Termitinae* and *Nasutitermitinae*, are believed to have secondarily evolved the ability to feed on wood (Donovan et al. 2001; Köhler et al. 2012). They are marked by the abundance of the phyla, *Fibrobacteres*, *Spirochaetes*, and TG3 (Hongoh, Deevong, et al. 2006; Köhler et al. 2012), that are distantly-related to those found in the rumen. Tokuda et al., (2007) observed that a significant proportion of the cellulase activity in the hindgut of *Nasutitermes takasagoensis*, is associated with insoluble particles in the lumen, suggesting that the enzymes responsible could be bacterial cell-bound cellulases (Tokuda & Watanabe 2007). More circumstantial evidence for bacterial involvement in cellulose digestion, comes from the assignment of metagenomic fragments encoding putative cellulases to *Fibrobacteres* and *Spirochaetes* (Warnecke et al. 2007; He et al. 2013). The question of the existence of a fiber-associated community in higher wood-feeding higher termites has not yet been addressed.

1.4 Aims of this investigation

1. **Host-specificity of bacterial communities in higher termites**

   The flagellate-free higher termites constitute a highly diverse monophyletic taxon, and contain a host-specific prokaryotic gut communty (Hongoh et al. 2005; Hongoh et al. 2006; Schmitt-Wagner et al. 2003; Köhler et al. 2012). However, our understanding of the forces that shape these communities, is relatively poor. I conducted a comprehensive survey of nine higher termites from the major subfamilies and feeding guilds, using high-throughput 454 pyrosequencing of 16S rRNA genes. The reference database used in a previous study (Köhler et al. 2012) was further expanded to include more full-length Sanger sequence 16S rRNA clones from previously unsampled higher termites. Phylogenetic trees were constructed for bacterial groups that were critical to comparative studies with higher termites, to improve the taxonomic affiliation at the genus-level.

2. **Compartment-specificity of bacterial communities in higher termites**

   The hindgut of higher termites is highly compartmentalized and characterized by steep axial gradients in pH and these extreme differences are reflected in fundamental differences in the community structure between compartments of the same termite (Schmitt-Wagner et al. 2003; Köhler et al. 2012). Little however is known about how similar conditions shape communities in the homologous compartments. To answer these questions, I carried questions, I carried out comparative analysis on the bacterial communities associated with hindgut compartments from five termite species from the two major subfamilies, *Nasutitermitinae* and *Termitinae*, using 454 pyrosequencing.

3. Fiber-associated bacterial community in higher termites

The hindgut community of higher wood-feeding termites share many bacterial phyla, with fiber-associated communities encountered in the rumen (Hongoh et al. 2006; Warnecke et al. 2007; Koike et al. 2003). A large proportion of the cellulase activity in the hindgut of *N. takasagoesnsis*, associated with the particulate fraction (Tokuda et al. 2007), further suggests the presence of a cellulolytic fiber-associated community in the hindgut of higher termites. To investigate this probability, I developed a density-dependent sorting method for separating the wood fibers from luminal contents. Using this method, I characterized the bacterial community in the higher termites *N. corniger* and *N. takasagoensis,* and measured the cellulase activity associated with the fiber fraction.

4. The phylogenetic identity of Arthromitus

The gutwall is a microoxic microhabitat, common to all termites, and is home to a distinct bacterial community (Yang et al. 2005). The most easily recognizable member of this community is a segmented filamentous bacterium "Arthromitus" (Leidy 1849; Marguilis et al. 1990), whose phylogenetic identity is not known. Using DNA amplified from picked filaments, I constructed phylogenetic trees based on the 16S rRNA gene for Arthromitus, and its epibionts, belonging to *Bacteroidales* Cluster V.

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Chapter 2

Manuscript in preparation

Host-specific bacterial communities in higher termites

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Contributions: AM and AB designed the study; AM and TK dissected the termites and extracted the DNA; TK contributed the original reference database; AM constructed the clone libraries, conducted the phylogenetic analysis, set up the required bioinformatic pipelines for processing and analyzing pyrosequencing data, and evaluated the data; AM prepared the draft.

2.1 Abstract

The termites, comprised of phylogenetically lower and higher termites, play an important role in the breakdown of plant material in tropical systems. The *Termitidae* (higher termites) are the most diverse of all termite families are characterized by their dependence on an entirely prokaryotic hindgut community. The higher termites are characterized by significant variation in gut structure and dietary specializations that could include wood, grass and soil. Previous studies suggest that closely-related higher termites with the same dietary specialization possess similar gut communities. However, we still lack a deeper understanding of the role played by host diet and phylogeny in the evolution of gut microbial communities in the higher termites. We conducted a comprehensive analysis using pyrosequencing to comparatively investigate the gut communities of nine termites belonging to three different subfamilies, *Macrotermitinae*, *Termitinae* and *Nasutitermitinae*, and spanning multiple lifestyles including species feeding on fungi, soil, grass and wood. We expanded the database to include more full-length 16S rRNA sequences from novel termite taxa. In addition, we constructed phylogenetic trees and used the node information to provide an improved phylogenetic framework for the assignment of pyrosequences. Our results show that the gut bacterial communities have co-diversified with their higher termite hosts. Both host diet and phylogeny appear to play a role in determining community structure; while the effect of diet is more apparent at broader taxonomic levels of the community, the effect host phylogeny is obvious only at higher taxonomic resolution.

2.2 Introduction

Termites play a crucial ecological role in tropical ecosystems in the decomposition of plant material (Bignell et al. 1997). The primitive “lower” termites comprise multiple families and essentially feed on only wood, and achieve this through a dependence on intestinal cellulolytic flagellates. On the other hand, phylogenetically “higher” termites, despite their diversity, are a monophyletic taxon (family *Termitidae*) and are characterized by the absence of gut flagellates and instead possess a completely prokaryotic community (Brune & Ohkuma 2011).

The higher termites are the most numerically and ecologically dominant family within the termites and show a wide variety of dietary specializations that can include wood, grass and soil (Eggleton & Tayasu 2001), and have been classified into various feeding groups, based on gut content analysis (Donovan et al. 2001). These differences in diet are reflected in the diversity of their hindgut structure, ranging from simple lower termite-like hindguts of fungus-cultivating *Macrotermitinae*, to the highly compartmentalized hindguts of the soil-feeding members of the *Termitinae* (Noirot 2001).

This phylogenetic and dietary diversity in the group is reflected in the differences in their gut microbiota (Schmitt-Wagner et al. 2003; Hongoh, Ekpornprasit, et al. 2006; Hongoh et al. 2005; Köhler et al. 2012). Close relatives belonging to similar feeding groups, have been shown to harbour phylogenetically related bacterial members (Hongoh et al. 2005; Köhler et al. 2012; Schmitt-Wagner et al. 2003). On the other hand, minor changes in diet have also been shown to cause shifts in the relative abundances of bacterial phyla, in a wood-feeding termite, *Nasutitermes takasagoensis* (Miyata et al. 2007). The role played by host phylogeny and diet, in the structuring of gut bacterial communities higher termites is still

largely unclear. Most community surveys in higher termite guts have been limited to clone libraries (Hongoh et al. 2005; Warnecke et al. 2007; Hongoh, Deevong, et al. 2006), and offer limited information on less-abundant lineages. By increasing the detection limit, deep sequencing approaches such as 454 pyrosequencing, overcome many of the disadvantages of clone libraries.

The current study examines communities of nine higher termites from the major subfamilies, *Macrotermitinae*, *Termitinae* and *Nasutitermitinae*. Termite guts are colonized by many novel bacterial lineages that are rarely encountered elsewhere (Hongoh 2010). We also expanded the database used by Köhler et al. (2012), by incorporating more near-full-length 16S rRNA genes from novel termites, and by calculating phylogenetic trees to provide the taxonomic framework necessary to accurately bin the shorter pyrosequences to genus-level clades. This approach significantly increased taxonomic assignment of pyrosequences to critical bacterial groups that were not represented in generic databases, provided by the Ribosomal Database Project (RDP) (rdp.cme.msu.edu/) and the Silva group (www.arb- silva.de).

2.3 Methods and Materials

2.3.1 Termites and dissection

All specimens were either from laboratory-reared colonies, or collected in the wild (see Table 2.1 for details). The hindgut sample from *Nasutitermes corniger* has been previously analysed, as part of another study (Köhler et al. 2012). Individuals were dissected using sterile fine-tipped forceps. Specimen were identified by partial sequencing of the cytochrome oxidase II gene.

2.3.2 DNA extraction and purification

DNA from compartment-pools from 10 to 20 individuals was extracted using a previously described bead-beating protocol (Paul et al., 2012). The final pellet was dissolved in 50 µL of elution buffer (MinElute PCR Purification Kit; Qiagen, Germany) and quantified fluorimetrically (Qubit; Invitrogen, USA).

2.3.4 Phylogenetic curation of the reference database

Sequence alignment and taxonomic classifications were done using a manually curated reference database of near-full-length 16S rRNA gene sequences, based on the Silva non-redundant database (Pruesse et al. 2007). The reference database used for taxonomic assignment of the pyrotag sequences to genus-level clusters, was composed publicly available sequences from insect guts and other gut environments. In an attempt to further improve the phylogenetic coverage of the reference database, we constructed clone libraries from the hindguts of termites, *Trinervitermes* sp., *Ophiotermes* sp., and *Cubitermes* sp., using the protocol described in a previous study (Schauer et al. 2012).

Table 2.1 | Sample information

Termite	Family/Subfamily	Feeding guild	Gut section	Treatment/ Maintenance	Origin
Nasutitermes corniger	*Termitidae/Nasutitermitinae*	wood-feeding	Hindgut	Freshly dissected	Rudolf H. Scheffrahn[a]
Nasutitermes takasagoensis	*Termitidae/Nasutitermitinae*	wood-feeding	Hindgut	Freshly dissected	Gaku Tokuda[b]
Trinervitermes sp.	*Termitidae/Nasutitermitinae*	grass-feeding	Hindgut	Freshly dissected	JKUAT, Kenya
Microcerotermes sp.	*Termitidae/Termitinae*	soil-feeding	Whole gut	Ethanol-stored	JKUAT, Kenya
Cubitermes sp.	*Termitidae/Termitinae*	soil-feeding	Hindgut	Freshly dissected	Kakamega forest reserve, Kenya
Ophiotermes sp.	*Termitidae/Termitinae*	soil-feeding	Whole gut	Ethanol-stored	Kakamega forest reserve, Kenya
Macrotermes bellicosus	*Termitidae/Macrotermitinae*	fungus-feeding	Hindgut	Freshly dissected	BAM[c]
Macrotermes sp.	*Termitidae/Macrotermitinae*	fungus-feeding	Whole gut	Ethanol-stored	JKUAT, Kenya
Odontotermes sp.	*Termitidae/Macrotermitinae*	fungus-feeding	Whole gut	Ethanol-stored	Kajiado, Kenya
Reticulitermes santonensis.	*Rhinotermitidae/ Rhinotermitinae*	wood-feeding	Hindgut	Freshly dissected	Forêt de la Coubre, France

[a] … Laboratory colony maintained at the University of Florida, (USA)

[b] … Termites collected from Iriomote Island (Japan)

[c] … Laboratory colony maintained at the Bundesanstalt für Materialforschung und -prüfung (BAM) (Germany)

This core of aligned sequences along with the node information from the guide tree in the SILVA database was used for the hierarchical classification of the pyrosequences obtained from the termites. Sanger sequences in the database were added to their respective core trees by the quick add marked species feature in ARB, and the node information was assigned to each sequence as its taxonomy. This node information for the sequences was used to identify and assign taxonomies to pyrotag sequences. Phylogenetic trees were constructed wherever necessary, with sequences in the database for bacterial groups, where phylogenetic resolution was lacking. Maximum Likelihood trees were constructed for *Fibrobacteres* and TG3 based on sequences constructed with the phyML implementation in ARB, using the General Time Reversal (GTR) model. For the *Treponema* I cluster, core tree of 80 sequences was constructed using the FastTree program (Price et al. 2009), and reimported into ARB.

Classification success at various taxonomic levels was tested using a test dataset of 1000 pyrotag sequences derived from 5 diverse termite species (subsamples of 200 sequences from each termite).

2.3.3 Pyrotag sequencing

The 16S rRNA genes in the samples were amplified using the primers 343F and 753R (Köhler et al. 2012) and pyrosequenced as described previously (Köhler et al. 2012). The pyrosequences were classified by comparison to sequences in the reference database.

2.3.4 Sequence analysis

Sequences were extracted in multifasta format from the standard flowgram files, along with their quality scores. Sequences were processed with *mothur* (Schloss et al. 2009), and quality-trimmed to only include sequences of 200 bases or greater in length, and denoised using the

“pre.cluster” command implemented in mothur, with the goal of removing sequences that could have arisen due to errors inherent to pyrosequencing (Huse et al. 2010). Sequences containing ambiguities, or homopolymeric regions of greater than ten bases in length, were eliminated. The pyrotag sequences were aligned using the mothur aligner and taxonomic information for each sequence was assigned, upto the genus-level, using the *Naïve Bayesian Classifier* implemented in the *mothur* software with confidence cutoff value of 60.

In order to look at the pattern of distribution of phylotypes within genus-level clades, at higher taxonomic resolution, sequences belonging to *Treponema* Ia were selected and clustered using a sequence similarity of 90% to form operational taxonomic units (OTU). Representatives from OTUs were selected for the construction of a maximum likelihood tree using FastTree (Price et al. 2009) with the General Time Reversal (GTR) model.

2.3.5 Statistical analyses

Sequences were normalized by random subsampling of 3000 sequences per sample. A phylogenetic tree for all sequences, constructed using FastTree, was used as input for calculation of the Unifrac metric. The Unifrac metric is a pair-wise estimate of the cumulative phylogenetic distance between the lineages from different communities (Lozupone & Knight 2005), and measures the fraction of the total branch length that is unique to each sample in a pair. A random Monte Carlo-based permutation test (with 1000 iterations) was used to test if the distance between two communities is greater than what could be expected by chance alone. For the weighted Unifrac analysis, hierarchical clustering was also performed, and the R package pvclust (Suzuki & Shimodaira 2006) was used to test the uncertainty in the clustering of the communities by multiscale bootstrap resampling. Confidence values are reported as

Approximately Unbiased (AU) p-values (Suzuki & Shimodaira 2006).

The clustering of the samples was then visualized with non-metric multidimensional scaling (NMDS) using the *vegan* package (Oksanen et al. 2008) in the R software suite.

To identify the taxa contributing the most to the community dissimilarities, a Principal Component Analysis (PCA) of the occurrence and abundance of genus-level taxa was done, followed by ordering of the taxa based on component loadings.

2.4 Results

2.4.1 Clone libraries and phylogenetic analysis

Clone libraries were constructed with near-full-length 16S rRNA sequences from *Trinervitermes* sp., *Ophiotermes* sp. and *C. ugandensis*, to expand the diversity covered by the database, and to increase its resolving power at genus level, for classification of the pyrosequences. For *Trinervitermes* sp., 170 clones were selected for sequencing; 8 were found to be chimeric. The remaining clones that were incorporated into the reference database, revealed a phylum-level distribution, similar to that observed in the 454 pyrosequencing library for the same sample (See **Figure S1.1**). Slight differences were observed for minor groups, due to differences in primer coverage, and sequencing depth. A total of 96 clones were unidirectionally-sequenced from *Ophiotermes* sp. and *C. ugandensis.* Only novel phylotypes (those previously not observed by us), were sequenced in the opposite direction. A total 52 sequences from *C. ugandensis*, and 24 sequences from *Ophiotermes* sp., were incorporated into the reference database.

Phylogenetic analysis of the *Treponema* I cluster (Ohkuma et al. 1999) revealed the sequences to fall into six clusters (**Figure 2.1**). Although the branching order between the clusters showed some local differences between algorithms, the clusters themselves were highly reproducible. Cluster Ia consists of all the treponemes isolated from termite guts – *Treponema primitia*, *Treponema azotonutricium*, *Treponema isoptericolens*, in addition to clones from both lower and higher termites. Some sequences formed well-supported clusters containing representatives from a narrow host range, such as *Treponema* Ib and Id. The analysis also showed clusters Ic and If to be exclusively composed of sequences from higher termites, particularly lignocellulose-feeders, such

as *Microcerotermes* sp., *Nasutitermes* spp., and *Trinervitermes* sp. Deeper analysis of the *Treponema* If cluster shows that the sequences fall into sub-clusters, which correspond to host phylogeny.

We reanalyzed the phylogeny of subphylum 2 of *Fibrobacteres*, and TG3 phylum, in the context of recent sequence data that has been generated since their discovery in higher termites (Hongoh, Deevong, et al. 2006; Hongoh et al. 2005). In both *Fibrobacteres* subphylum 2 and TG3, a large proportion of the clones from *Nasutitermes* sp. (Warnecke et al. 2007) clustered with sequences reported from the congeneric species *Nasutitermes takasagoensis* (Hongoh, Deevong, et al. 2006) (**Figure 2.2**). We were unable to detect the presence of *Fibrobacteres* subphyum 2 in the clone library of *Trinervitermes*, suggesting that it probably did not occur in all *Nasutitermitinae*. We were however, able to identify one TG3 sequence from *Trinervitermes* that clustered with other sequences *Nasutitermes* spp. In both TG3 and *Fibrobacteres* subphylum 2, clones from *Nasutitermitinae* and *Microcerotermes* spp. fell into distinct clusters.

Apart from *Treponema* I, *TG3* and *Fibrobacteres* subphylum 2, the phylogeny of other groups was also analyzed to improve the identification of critical bacterial taxa in the pyrosequencing libraries (See **Table 2.3** for details).

2.4.2 Database improvement

Due to the addition of reference sequences from termites, the curated reference database performed much better, in comparison to other generic databases provided by RDP and Silva. Table 2.2 summarizes the results of the classification success obtained with a We classified a test dataset, containing 1000 pyrosequences, using the naïve bayesian classifier in combination with different databases. The node information from the phylogenetic trees greatly increased the classification

success for many critical genus-level clades (Table 2.3).

Table 2.2 | Percentage of 16S rRNA pyrosequences binned to different taxonomic levels by comparison of a test dataset* against different sequence databases

Taxonomic level	RDP[a](%)	Silva[b](%)	Termite DB[c](%)
Phylum	90.7	93.8	99.3
Order	82.6	84.4	95.5
Family	72.4	74.0	92.0
Genus	42.1	59.0	84.8

* … A set of 1000 pyrotag sequences from five termite species (200 sequences subsampled from each pyrotag library)

[a] … RDP version 9 released in March 2012

[b] … Silva reference database and taxonomy based on the SSURef (v102) database (See Supplementary methods for details on databases)

[c] … The latest version of the reference database (03.2013) used in this study

2.4.3 Pyrotag analysis

The V3 – V4 region of the bacterial 16S rRNA genes were amplified using primer sequences bearing sample-specific barcodes. After quality trimming, the sequences (7000 – 14000) were classified upto the genus-level. The samples differed in the taxonomic composition, with 101 – 197 genus-level taxa per sample. Using a sequence dissimilarity of 0.03, we were also able to identify 1080 – 1560 operational taxonomic units (OTUs), in the different samples.

2.4.4 Community similarities among the higher termites

Community similarity was analysed using the unweighted and weighted Unifrac metrics. The Unifrac significance test showed that the phylogenetic differences observed with both metrics was significant ($P < 0.001$). Ordination analysis of unweighted Unifrac distances (**Figure 2.3**), revealed that termites from the same subfamily that had the same dietary specialization clustered together.

Table 2.3 | Relative abundance of six major phyla, identified in a test dataset* of 16S rRNA pyrosequences. Vertical bars represent bacterial groups that are taxonomically unresolved in the generic databases like RDP and silva.

Phylum-level	Genus-level	RDP[a](%)	silva[b](%)	Termite DB[c](%)
Bacteroidetes	Arthropod cluster[#]	0	0	1.7
	COB P4 1 cluster	0	0	1
	Alistipes 1[#]	2.7	2.8	2.4
	Alistipes 2[#]			1.9
	others			0.1
Elusimicrobia	*Endomicrobium*	0	0	2.7
Fibrobacteres	Termite Cluster II[#]	0	0	1.5
	Fibrobacter	1.8	0	0
Firmicutes	*Enterococcus*	0.6	0.5	0
	Candidatus Arthromitus[#]	0	0	2.9
	Gut Cluster 1[#]	0	0	1.7
	Gut Cluster 2[#]	0	0	2
	uncultured 65	0	0	1.3
	uncultured 23	0	0	2.1
	uncultured 30	0	0	1
	Bilophila	0.8	0.4	0
Spirochaetes	*Spirochaeta*	0.5	0.2	0.4
	Treponema Ia[#]	25.7	0	14.8
	Treponema Ic[#]		20.5	8.3
	Treponema If[#]			8.9
	Treponema II[#]		0	3.8
	others		14.4	0
TG3[#]	Termite Cluster I[#]	0	0	1
Total		32.1	38.8	59.5

* … A set of 1000 pyrotag sequences from five termite species (200 sequences subsampled from five pyrotag libraries)

[#] … Classification nodes introduced in the current study

[a] … RDP version 9 released in March 2012

[b] … Silva reference database and taxonomy based on the SSURef (v102) database (See Supplementary methods for details on databases)

[c] … The latest version of the reference database (03.2013) used in this study

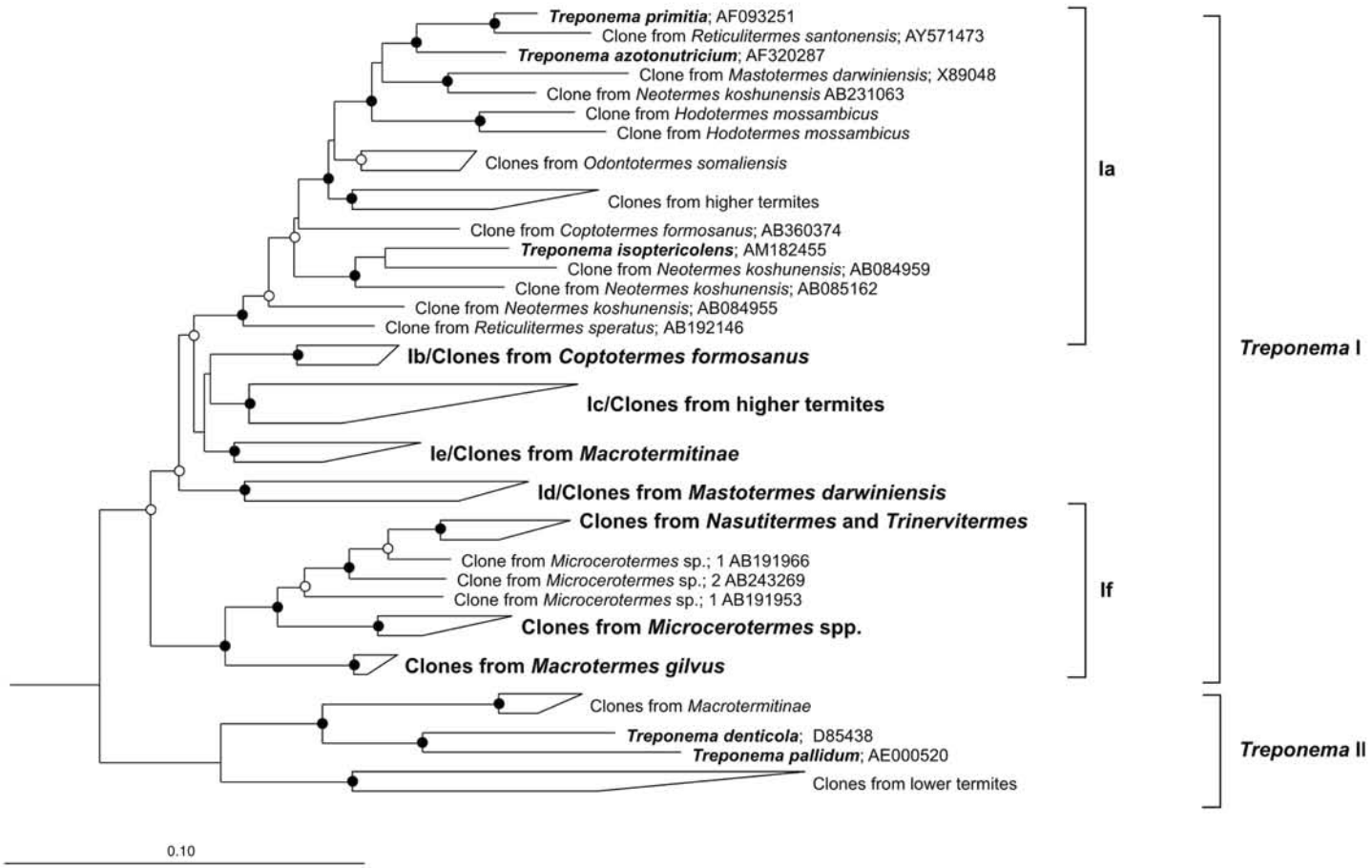

Figure 2.1 | Maximum Likelihood tree detailing the phylogenetic diversity among the termite gut treponemes. Circles indicate bootstrap values above 90% (●) and 75% (○).

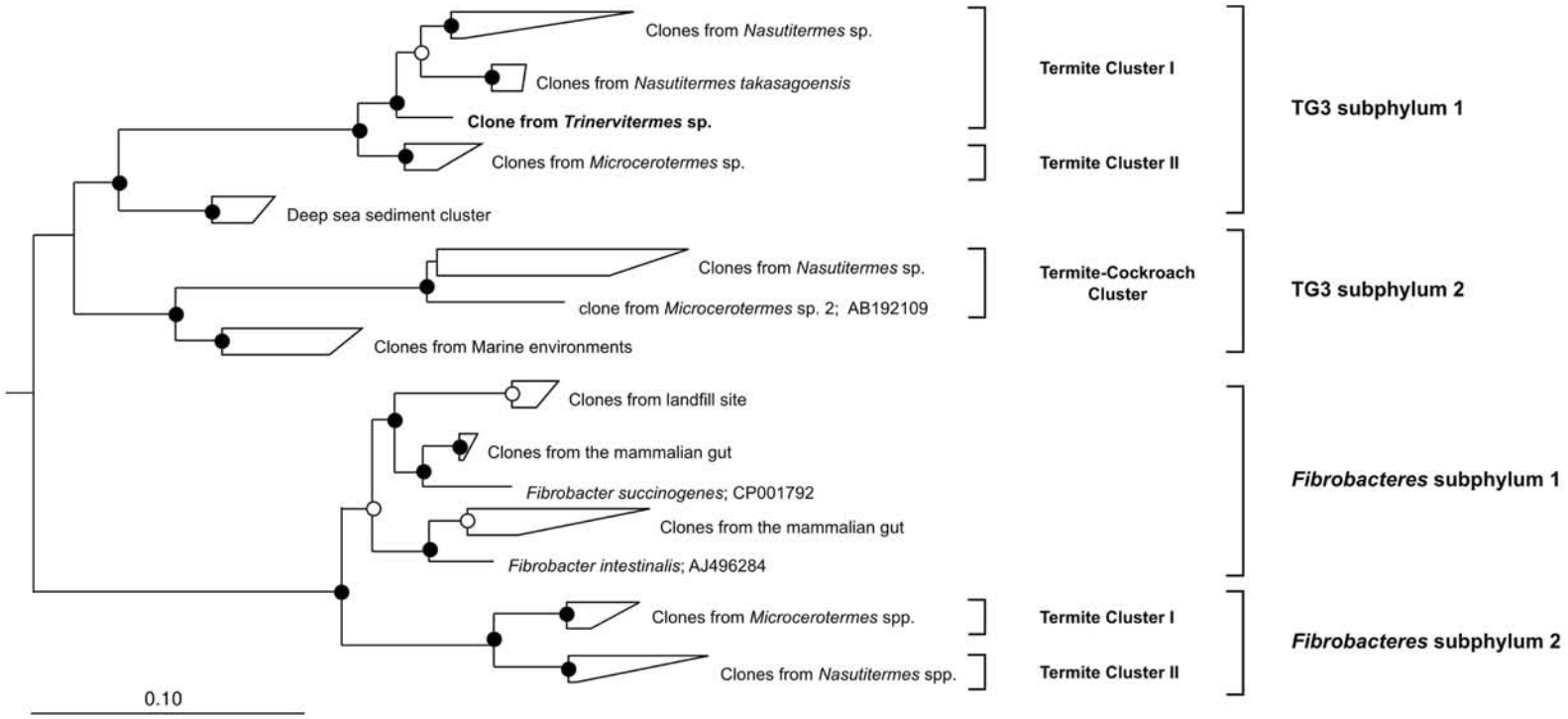

Figure 2.2 | Maximum Likelihood tree detailing the phylogenetic diversity among the termite gut treponemes. Circles indicate bootstrap values above 90% (●) and 75% (○).

The wood-feeding *Microcerotermes* sp. showed a greater similairty in community structure to other wood-feeding members in the *Nasutitermitinae*, than its closer phylogenetic relatives.

The general pattern of similarity observed in the weighted Unifrac analysis was similar to what was observed with the unweighted analysis (**Figure 2.4**); however, the samples clustering observed was better resolved due to the importance given to lineage abundance. Three major clusters could be identified in the ordination analysis. The hierarchical cluster analysis revealed these clusters to be well-supported (AU > 90%; See methods for details). The fungus-cultivating *Macrotermitinae* were found to cluster together, and so did the soil-feeding *Termitinae. Microcerotermes* sp. was clustered with the grass-feeding and wood-feeding members of the *Nasutitermitinae* (AU = 100%).

2.4.3 Differences in community membership at the phylum-level

Analysis of the taxa contributing to the observed clustering of communities, revealed major differences already at phylum level (**Figure 2.5**). *Bacteroidetes* was observed in high abundance (23 – 38%) in the *Macrotermitinae*, in comparison to the other subfamilies. A genus-level analysis reveals *Alistipes* 1 and *Alistipes* 2, to be primarily responsible for the abundance of the phylum. Among the soil-feeding Termitinae, the *Firmicutes* form the most dominant phylum, forming as much as 70% of the total community of *Ophiotermes sp*.

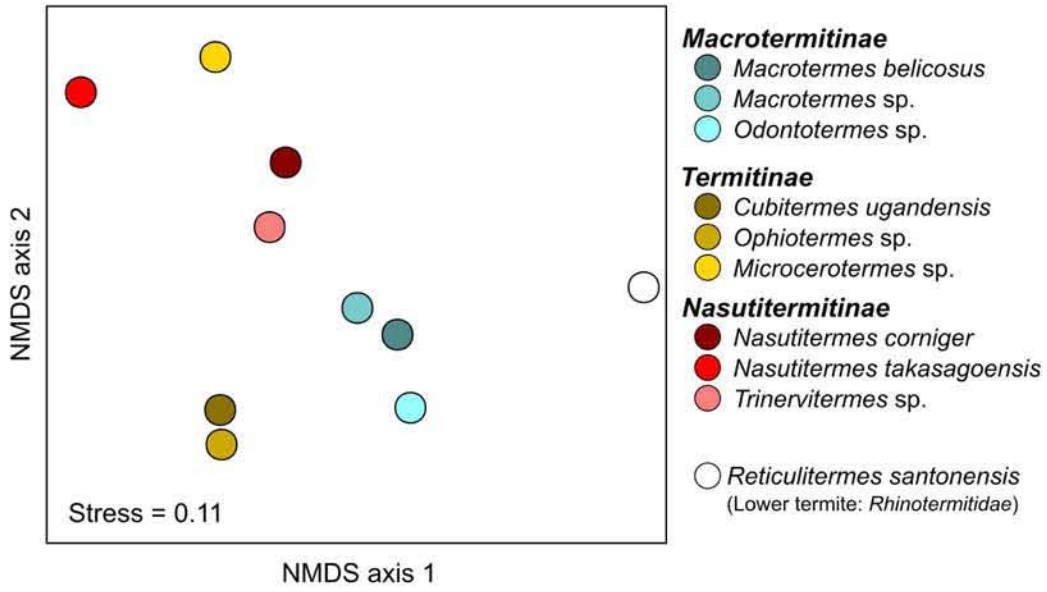

Figure 2.3 | Non-metric multi-dimensional scaling plot based on unweighted Unifrac distances, showing the clustering of gut communities from higher termites

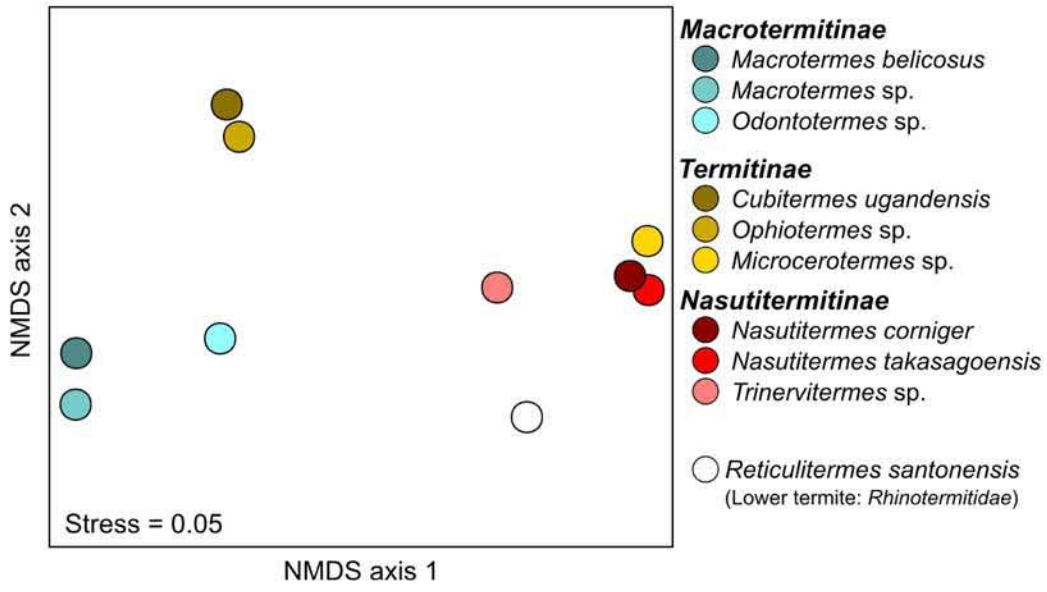

Figure 2.4 | Non-metric multi-dimensional scaling plot based on unweighted Unifrac distances, showing the clustering of gut communities from higher termites

A distinctive feature among all lignocellulose feeders that we investigated, was the abundance of *Spirochaetes*. This phylum formed around 70% of the total community in *N. takasagoensis*, *N. corniger*, and *Microcerotermes* sp., and around 50% in *Trinervitermes* sp.

Other phyla found specifically among the wood-feeding termites included the TG3 phylum, and the *Fibrobacteres* (subphylum 2). *Microcerotermes* sp. and *Nasutitermes corniger* showed a high abundance of both phyla, whereas *N. takasagoensis* was only found to harbour only TG3 in high abundance. In the grass-feeding *Trinervitermes* sp, both TG3 and *Fibrobacteres* were present but were considerably depleted, in comparison to the wood-feeders.

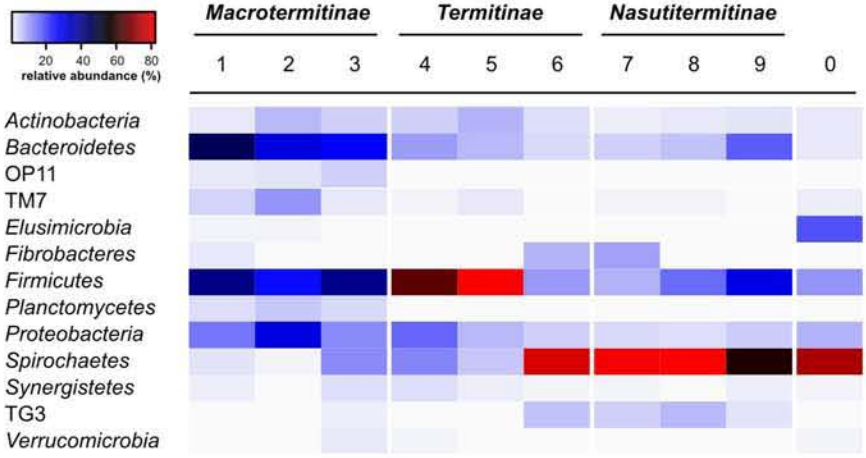

Figure 2.5 | Relative abundances of various bacterial groups in the guts of *Macrotermes bellicosus* (1), *Macrotermes* sp. (2), *Odontotermes* sp. (3), *Cubitermes ugandensis* (4), *Ophiotermes* sp. (5), *Microcerotermes* sp. (6), *Nasutitermes corniger* (7), *Nasutitermes takasagoensis* (8), *Trinervitermes* sp. (9), and Reticulitermes santonensis (0)

Although the *Fibrobacteres*, *Spirochaetes* and TG3 were either absent or barely present in the soil-feeders and fungus-feeders,

Odontotermes sp. (*Macrotermitinae*), was found to harbour *Spirochaetes* and TG3 at comparatively higher proportions.

2.4.4 Differences in community membership at the genus-level

We looked at the contribution of bacterial taxa at the higher resolution, we compared the community composition at genus-level **(Figure 2.6)**. At this resolution, we observed clear differences in the pattern of distribution many taxa even where the community composition appeared to be similar at broader taxonomic levels (**Figure 2.5**).

Among the *Macrotermitinae*, many taxa in the phyla *Bacteroidetes* and the *Proteobacteria*, showed considerable differences. *Alistipes* 1 and 2 that together form about 25% of the community of both *Macrotermes* spp., compared to about 10%., in *Odontotermes* sp. Similarly, *Arcobacter* was also found to be comparatively higher in the *Macrotermes* spp. However, *Odontotermes* sp. was found to harbour lineages within *Treponema* I in comparatively higher abundance than in the other *Macrotermitinae.*

Interestingly, the *Macrotermitinae* were found to share many lineages from the *Firmicutes* in common with the soil-feeding *Termitinae,* including "*Candidatus* Arthromitus" and uncultured 65. However, the distribution between the soil-feeding *Termitinae,* of genus-level clades was observed to be quite similar.

Using the updated phylogenetic framework (**Figures 2.1** and **2.2**) in the reference database, we were able to significantly improve classification especially for the abundant phyla (**Figure 2.5**) that commonly encountered in wood-feeding higher termites. This allowed us to identify critical differences between genus-level taxa within the phyla *Fibrobacteres*, the TG3 and the *Spirochaetes*.

Among the *Nasutitermitinae*, these differences are most clearly

illustrated by the distribution of genus-level clades in *Treponema* I (**Figure 2.6**). *Treponema* Ic was found to be abundant in both *N. corniger* (32%) and *N. takasagoensis* (48%) in comparison to *Trinervitermes (*11%). In *Treponema* If was also observed in high abundance both in *N. corniger* (23%) and *Trinervitermes* sp. (20%) in comparison to *N. takasagoensis* (5%). Interestingly, the distribution of clusters within *Treponema* I observed in the wood-feeding *Microcerotermes* sp. was very similar to that observed in the wood-feeding *Nasutitermitinae.* This could suggest that their abundances are possibly determined by the diet of the host.

Despite the similarity in the co-occurrence of *Fibrobacteres* and TG3 among the wood-feeding species, our analysis at the genus-level reveals an almost exclusive association of certain clades with a particular subfamily of higher termites. *N. corniger* and *N. takasagoensis* were both found to be preferentially associated with Termite Cluster II of *Fibrobacteres*, and Termite Cluster I of TG3. The opposite was found to be true for *Microcerotermes,* which was found to be preferentially associated with Termite Cluster I of *Fibrobacteres*, and Termite Cluster II of TG3. The association, albeit at low abundance, of *Ophiotermes* sp. with Termite Cluster II of TG3 is further indicative of a subfamily-specific selection of one genus-level taxon over another.

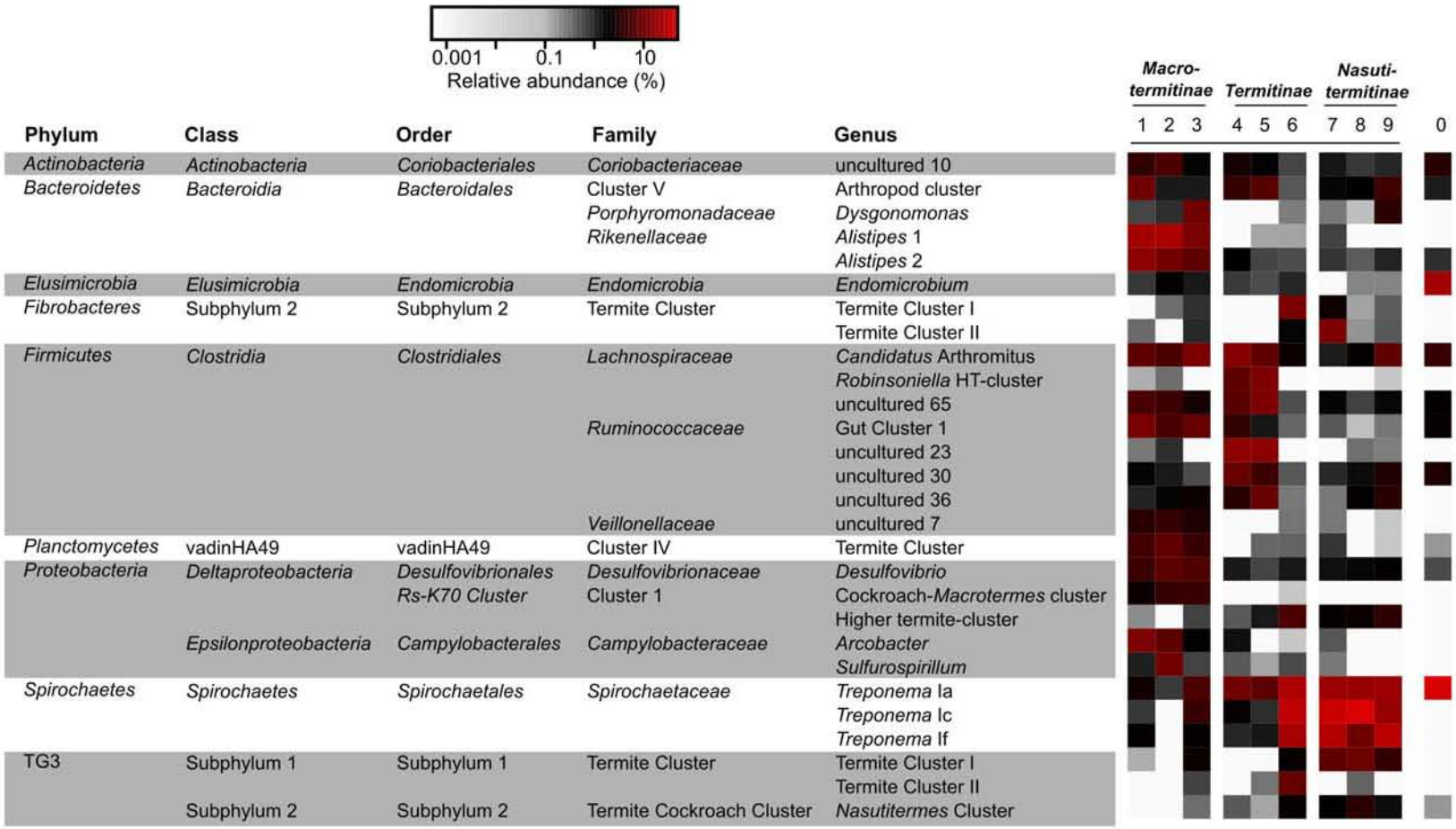

Figure 2.6 | Relative abundances of various bacterial groups in the guts of *Macrotermes bellicosus* (1), *Microtermes* sp. (2), *Odontotermes* sp. (3), *Cubitermes ugandensis* (4), *Ophiotermes* sp. (5), *Microcerotermes* sp. (6), *Nasutitermes corniger* (7), *Nasutitermes takasagoensis* (8), *Trinervitermes* sp. (9), and *Reticulitermes santonensis* (O).

The tree in **Figure 2.7** allows for a more detailed analysis of the relative contribution of each taxon to the observed overall phylogenetic or dietary signal. We observed that the phylogenetic structure within each genus-level taxon contributes significantly to the overall pattern of similarity.

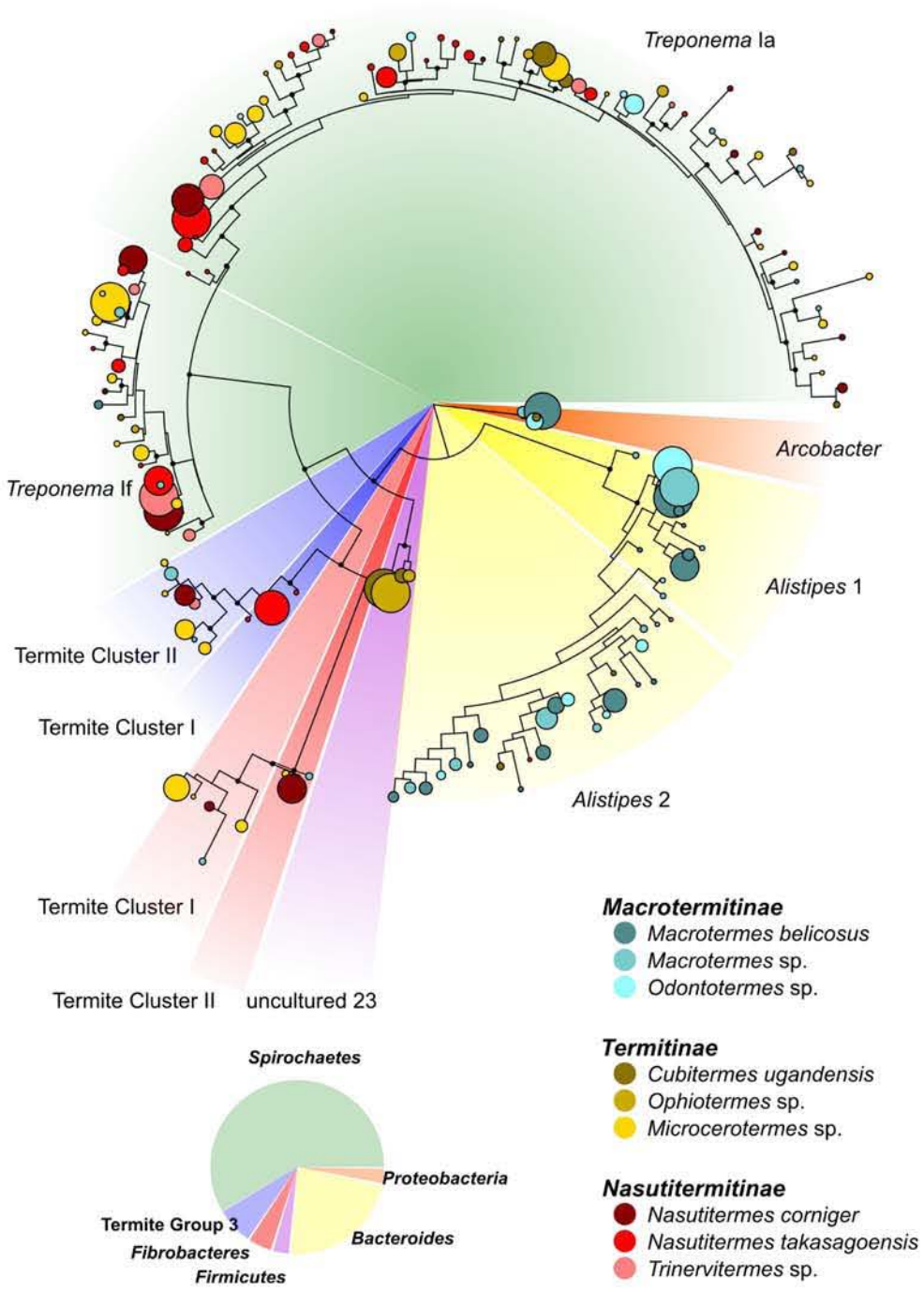

Figure 2.7 | A phylogram of select OTUs (defined at 90% sequence similarity) along with their taxonomic affiliation. The size of the circular nodes represent the relative abundance of that OTU within its genus-level affiliation. The black circles on the internal nodes represent bootstrap support (> 75%)

2.5 Discussion

Previous community studies have shown that phylogenetically-related termites harbour gut communities of similar composition (Hongoh et al. 2005; Warnecke et al. 2007; Köhler et al. 2012). Most of these studies have been limited to the comparison of very closely related termites that also specialize on the same diet. Moreover phylogenetically distant higher termites have independently evolved certain dietary specializations and also share many similarities in gut community structure (Hongoh, Ekpornprasit, et al. 2006; Köhler et al. 2012). This makes it difficult to address the relative contribution of host phylogeny and diet to the structuring of gut communities in higher termites.

In order to make informative comparisons between the higher termites, we sampled species from multiple subfamilies and feeding-groups. Our results clearly show the impact of host phylogeny and diet on the assembly of bacterial communities in hindguts of higher termites.

Among the subfamilies in the higher termites, *Macrotermitinae* is considered to be the most phylogenetically basal group (Inward et al. 2007), and is the only subfamily that has evolved a mutualistic association with fungi (*Termitomyces* spp.). The results from our Unifrac analyses clearly demonstrate the similarity in community structure among the three investigated members of *Macrotermitinae*. Moreover, the communities associated with the *Macrotermes* spp. are more similar to each other than to the community of *Odontotermes* sp. Although we could observe a clustering of Sanger-sequenced clones by host phylogeny (not shown) our results indicate that most of the observed community differences between the two genera, come more from differences in the relative abundance of bacterial lineages, such as the abundance of *Spirochaetes* and TG3 in *Odontotermes* sp. in comparison to the *Macrotermes* spp.

Macrotermes spp. primarily consume plant material that has been

de-lignified in the fungal combs, for more efficient use of cellulose (Hyodo et al. 2003). On the other hand, the *Odontotermes* spp. appear to be consuming the fungus itself as a source of nourishment (Hyodo et al. 2003). This fundamental difference in the role played by the fungal symbionts could also be responsible for the difference in bacterial community structure.

In comparison to the *Macrotermitinae*, the more advanced subfamilies of higher termites, are characterized by much greater nutritional diversification. It has been suggested in these subfamilies, that dietary specializations such as wood-feeding and soil-feeding have evolved independently along different lineages of termites.

We observed considerable similarity in community structure among members of the *Nasutitermitinae*, especially with respect to the distribution of *Spirochaetes*, and TG3. This has been previously noted for *N. corniger* and *N. takasagoensis* (Köhler et al. 2012). Although previous studies have found a higher abundance (Hongoh, Deevong, et al. 2006) of *Fibrobacteres* in *N. takasagoensis*, we could detect the phylum at a relative abundance of just under 1% in our pyrosequencing library. This low abundance could be attributed to differences between batches of termites. Most of the clones in the clone library of *Trinervitermes* sp. cluster with wood-feeding relatives in the *Nasutitermitinae*. However, classification of the pyrosequences reveals that *Trinervitermes* sp. also shares a significant similarity in community structure with its wood-feeding relatives. The overlap in community composition observed between the wood-feeding members of *Nasutitermitinae* and grass-feeding *Trinervitermes* sp. could hence be explained by a combination of both host phylogeny and similarity in diet.

The impact of host diet on the community structure, in our study, was most apparent in the *Termitinae*. Our analyses included two soil-

feeding members (*Cubitermes ugandensis* and *Ophiotermes* sp.), and one wood-feeding member (*Microcerotermes* sp.). Although a member of *Termitinae*, the gut community structure of *Microcerotermes* sp. is completely unlike that of *C. ugandensis* and *Ophiotermes* sp. Furthermore, Unifrac analyses revealed *Microcerotermes* sp. to cluster with other wood-feeders in *Nasutitermitinae.* The fact that these phyla are either absent or barely present in the soil-feeding relatives of *Microcerotermes* sp. (*C. ugandensis* and *Ophiotermes* sp.) further suggests that the distribution of lineages at broad taxonomic levels in higher termites is strongly influenced by diet.

The contribution of host phylogeny to gut community structure only becomes apparent at higher taxonomic resolution. This is illustrated by the preferential enrichment of different genus-level clusters (e.g. those found within *Fibrobacteres* and TG3), in *Microcerotermes* sp. and *N. corniger*. Furthermore, this signal of host phylogeny was also observed at the OTU level for groups like *Treponema* Ia (**Figure 2.7**), where phylogenetically related termites were found to possess a greater abundance of phylogenetically-related OTUs.

Based on our analysis, we conclude that both host diet and phylogeny are responsible for the overall structure of gut bacterial communities in higher termites; however, their effects are visible at different levels of taxonomic resolution. The sampling depth of pyrosequencing allowed us to detect certain members in the higher termites that were previously not detected in clone libraries due to undersampling. This suggests that many bacterial groups have been transmitted from generation to generation by trophallaxis, and have been evolving throughout the diversification of their hosts. However, the relative abundance of these bacterial taxa is restricted by environmental factors such as the dietary specialization of the host. Understanding the influence

of host diet and phylogeny ultimately depends on our ability to detect differences in the 'rare biosphere' (Sogin et al. 2006) in the guts of various higher termites. Generic databases like RDP and Silva, typically lack close representatives of sequences commonly found in insect guts (Köhler et al. 2012). Our study also highlights the underlying need for using a combination of deep sequencing with a manually-curated phylogenetic framework of full-length sequences in subsequent surveys of termite gut communities.

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Chapter 3

Manuscript in preparation

Compartment-specific bacterial communities in higher termites

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Contributions: AM and AB designed the study; AM and JN dissected the termites and extracted the DNA; KM prepared the pyrosequencing libraries and identified the termites by COII-sequencing, AM set up the required bioinformatic pipelines for processing pyrosequencing data, and evaluated the data; AM prepared the draft.

3.1 Abstract

The higher termite gut is a highly structured habitat and shows the presence of spatially separated compartments. The differences in physicochemical parameters between the compartments, most importantly pH, play an important role in shaping the local community structure. Most of the previous community studies on compartments have been limited to the study of very closely related termites. However, a deeper understanding of how these communities evolved could only be gained by the comparison of compartments from more distantly-related termites. Here, we apply 16S rRNA gene pyrosequencing to investigate the bacterial communities in the hindgut compartments of five different termite species, belonging to the subfamilies *Termitinae* and *Nasutitermitinae*. Our results show that despite the co-diversification of the gut community with the termite host, bacterial communities in homologous gut compartments are more phylogenetically related to each other than to consecutive gut compartments in the same termite. We hypothesize that similar ecological conditions such as increased alkalinity in the anterior gut have played a significant role in the structuring of communities in the gut compartments of higher termites.

3.2 Introduction

Termites possess the ability to digest lignocellulose in various stages of humification (Donovan et al. 2001). In order to accomplish this, they harbour a dense community of obligate microbial symbionts in their enlarged hindguts (Brune & Ohkuma 2011). The primitive lower termites are characterized by the presence of flagellates in their hindguts. The more evolutionarily derived higher termites (family *Termitidae)* lack these symbiotic flagellates, and are instead characterized by a completely prokaryotic community. The higher termite hindgut represents a highly structured habitat consisting of spatially separated and functionally distinct gut compartments (Noirot 2001) that differ significantly in physicochemical parameters such as redox potential and pH (Brune & Kühl 1996; Köhler et al. 2012).

The anterior hindgut of the advanced higher termite subfamilies *Nasutitermitinae* and *Termitinae* is characteristically alkaline (Bignell & Eggleton 1995). In the wood-feeding *Nasutitermes* spp. (*Nasutitermitinae*) the P1 compartment is mildly alkaline at pH 10, followed by the P3 compartment that is close to neutral pH (Brune et al. 1995; Köhler et al. 2012). In the soil-feeding *Cubitermes* sp. (*Termitinae*), the overall hindgut pH is significantly greater with the homologous P1 compartment being hyperalkaline at pH 12 and the consecutive P3 compartment being slightly less alkaline at pH 10 (Brune & Kühl 1996). Although the significance of this alkalinity in the hindguts of higher termites is not fully understood, the steep differences in pH are reflected in the bacterial community structure of the compartments (Schmitt-Wagner et al. 2003; Thongaram et al. 2005; Köhler et al. 2012).

Most comparative analyses of bacterial communities in higher termite guts have been limited to the study of whole guts (Hongoh,

Deevong, et al. 2006; Hongoh, Ekpornprasit, et al. 2006). Fewer studies have focused on the bacterial communities associated with the functionally and physicochemically distinct microhabitats within individual gut compartments.

The current study uses 16S rRNA pyrosequencing to compare hindgut compartments of five termites, *Nasutitermes corniger* (subfamily *Nasutitermitinae*), *Trinervitermes* sp. (subfamily *Nasutitermitinae*), *Amitermes* sp., *Cubitermes* sp. (subfamily *Termitinae*), and *Ophiotermes* sp. (subfamily *Termitinae*). It expands on a previous investigation which involved an intra-specific comparison of the compartment communities in *N. corniger* (Köhler et al. 2012). We analyzed the taxonomic composition of the communities at genus-level by comparing the short pyrosequences with a taxonomically curated reference database containing Sanger sequences from different termite hosts (Chapter 2). Additionally, we also compare the phylogenetic structure among the bacterial communities in the homologous compartments of the higher termites, for a better understanding of the evolutionary trends contributing to the diversification of compartment communities.

3.3 Methods and Materials

3.3.1 Termites and dissection

All specimens were either from laboratory-reared colonies, or collected in the wild (see Table 3.1 for details). The hindgut sample from *Nasutitermes corniger* has been previously analysed, as part of another study (Köhler et al. 2012). Individuals were dissected using sterile fine-tipped forceps. Specimen were identified by partial sequencing of the cytochrome oxidase II gene.

3.3.2 DNA extraction and purification

DNA was extracted from pooled compartments from 10—20 individuals using a previously described bead-beating protocol (Paul et al., 2012). The final pellet was dissolved in 50 µL of elution buffer (MinElute PCR Purification Kit; Qiagen, Germany) and quantified fluorimetrically (Qubit; Invitrogen, USA).

3.3.3 Pyrotag sequencing

The 16S rRNA genes in the samples were amplified using the primers 343F and 753R (Köhler et al. 2012) and pyrosequenced as described previously (Köhler et al. 2012). The sequences obtained were classified against the previously described reference database (Köhler et al. 2012) with modifications from the current study.

3.3.4 Sequence analysis

FASTA-formatted sequences, with the accompanying quality scores were extracted from the standard flowgram files (sff). The sequences were processed using mothur (Schloss et al. 2009), and quality trimmed to only include pyrosequences of at least 200 bases length, and denoised using

Table 3.1 | Sample information

Termite	Family/Subfamily	Feeding group	Gut sections	Treatment/ Maintenance	Origin
Nasutitermes corniger	*Termitidae/Nasutitermitinae*	wood-feeding	P1, P3, P4, P5	Freshly dissected	Rudolf H. Scheffrahn[a]
Trinervitermes sp.	*Termitidae/Nasutitermitinae*	grass-feeding	P1, P3, P45	Freshly dissected	JKUAT, Kenya
Cubitermes sp.	*Termitidae/Termitinae*	soil-feeding	P1, P3, P4, P5	Freshly dissected	Kakamega forest reserve, Kenya
Ophiotermes sp.	*Termitidae/Termitinae*	soil-feeding	P1, P3, P4, P5	Ethanol-stored	Kakamega forest reserve, Kenya
Amitermes sp.	*Termitidae/Termitinae*	wood-interface feeding	P1, P3, P4, P5	Freshly dissected	JKUAT, Kenya

[a] … Laboratory colony maintained at the University of Florida, (USA)

the “pre.cluster” command implemented in *mothur*, with the goal of removing sequences that could have arisen due to errors inherent to pyrosequencing (Huse et al. 2010). Sequences containing any ambiguities, or homopolymer-stretches greater than 10 bases in length, were eliminated. A reference database containing aligned near-full-length sequences (Chapter 2), was used for alignment and classification of the shorter pyrosequences (Köhler et al. 2012). The taxonomic information for pyrotag sequences was assigned, upto the genus-level, using the *Naïve Bayesian Classifier* (Wang et al. 2007) implemented in the *mothur* software suite (Schloss et al. 2009), by comparison to full-length quality sequences in the reference database, with a confidence threshold of 60%.

3.3.5 Phylogenetic and statistical analyses

Sequences were normalized by random subsampling of 1500 sequences per sample. A phylogenetic tree for all sequences, constructed using FastTree (Price et al. 2009), was used as input for Unifrac analysis. The Unifrac metrics are pair-wise estimates of the cumulative phylogenetic distance between different communities (Lozupone & Knight 2005; Lozupone et al. 2011). The unweighted metric considers only presence or absence of lineages, while the weighted metric also factors abundance into its calculation of community relatedness. Both calculations were conducted using the Unifrac implementations in *mothur.* A random Monte Carlo-based permutation test (with 1000 iterations) was used to test if the distance between two communities is greater than what could be expected by chance alone. The clustering of the samples was then visualized with non-metric multidimensional scaling (NMDS) using the vegan package (Dixon 2003; Oksanen et al. 2008) in the R software suite. For the weighted Unifrac analysis, hierarchical clustering was also performed, and the R package *pvclust* (Suzuki & Shimodaira 2006) was used to test the uncertainty in the clustering of the communities by multi-scale bootstrap

resampling. Confidence values are reported as Approximately Unbiased (AU) p-values (Suzuki & Shimodaira 2006).

For a phylogenetic analysis of the core community, genus-level taxa observed in all the investigated P1 and P3 compartments were selected. The sequences belonging to these core taxa were clustered into OTUs based on a sequence-similarity criterion of 90%. OTUs that formed more than 1% of the core community in each sample, were selected from the construction of the phylogenetic tree, using the Maximum Likelihood implementation in FastTree with the general time reversal (GTR) model.

The Morisita-Horn similarities between the different communities, were calculated for a classification-dependent comparison of the taxonomic composition, and visualized using the *arcdiagram* plug-in for the *igraph* package (Csardi & Nepusz 2006) using the R statistical software package.

3.4 Results

3.4.1 Pyrotag analysis

For each compartment, the extracted DNA was used to amplify the V3–V4 region of the bacterial 16S rRNA gene with PCR primers bearing sample-specific barcode sequences. After quality trimming, the sequences (10,000 – 22,000) were classified upto the genus-level using a manually-curated reference database (See Chapter 2). Each sample differed considerably in the taxonomic composition, with the number of genus-level taxa discovered ranging from 88 to 176 taxa. Using a sequence dissimilarity of 0.03, we were also able to identify 618–3974 operational taxonomic units (OTUs), in the different samples.

3.4.2 Taxonomic composition of the communities in the different compartments

Differences in community structure between the gut compartments of the termites are already apparent at phylum-level (Figure 3.1). The *Spirochaetes* formed the most dominant phylum associated with the lignocellulose-feeding *Nasutitermes corniger* and *Trinervitermes* sp. They were found to be particularly abundant in the P3 compartments of both termites. Some other phyla such as the *Fibrobacteres* and TG3 phylum, were only observed in high abundance, in *N. corniger*.

The *Firmicutes* dominated the community composition of the soil-feeding termites, *Ophiotermes* sp., *Cubitermes* sp. and *Amitermes* sp., being most abundant in the P1 compartments. It was interesting to note a consistent association of *Elusimicrobia* to the P4 and P5 gut compartments of the three *Termitinae* members, the highest relative abundance of which was observed in *Ophiotermes* sp.

Many of the lineages within the *Firmicutes*, were encountered in much greater abundance among the *Termitinae*, particularly in the alkaline P1 compartments. Some taxa such as uncultured 23 and uncultured 24 clusters (family *Ruminococcaceae*), which are highly abundant in *Termitinae,* but not in the compartments of the *Nasutitermitinae*.

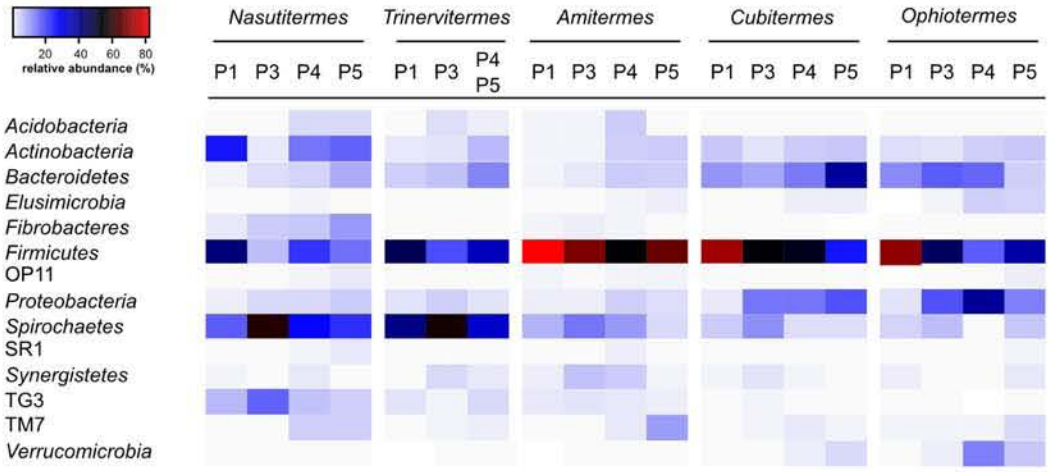

Figure 3.1 | Relative abundances of bacterial phyla in the hindgut compartments of *Nasutitermes corniger* (N), *Trinervitermes* sp. (T), *Amitermes* sp. (A), *Ophiotermes* sp. (O), and *Cubitermes ugandensis* (C), respectively.

On the other hand, certain other genus-level taxa were observed to be highly abundant only among the *Nasutitermitinae* e.g. *Treponema* Ia, Ic and If. Other groups such as the TG3 phylum and subphylum 2 of the *Fibrobacteres* were not observed to be as abundant in *Trinervitermes* sp., as in *Nasutitermes* sp.

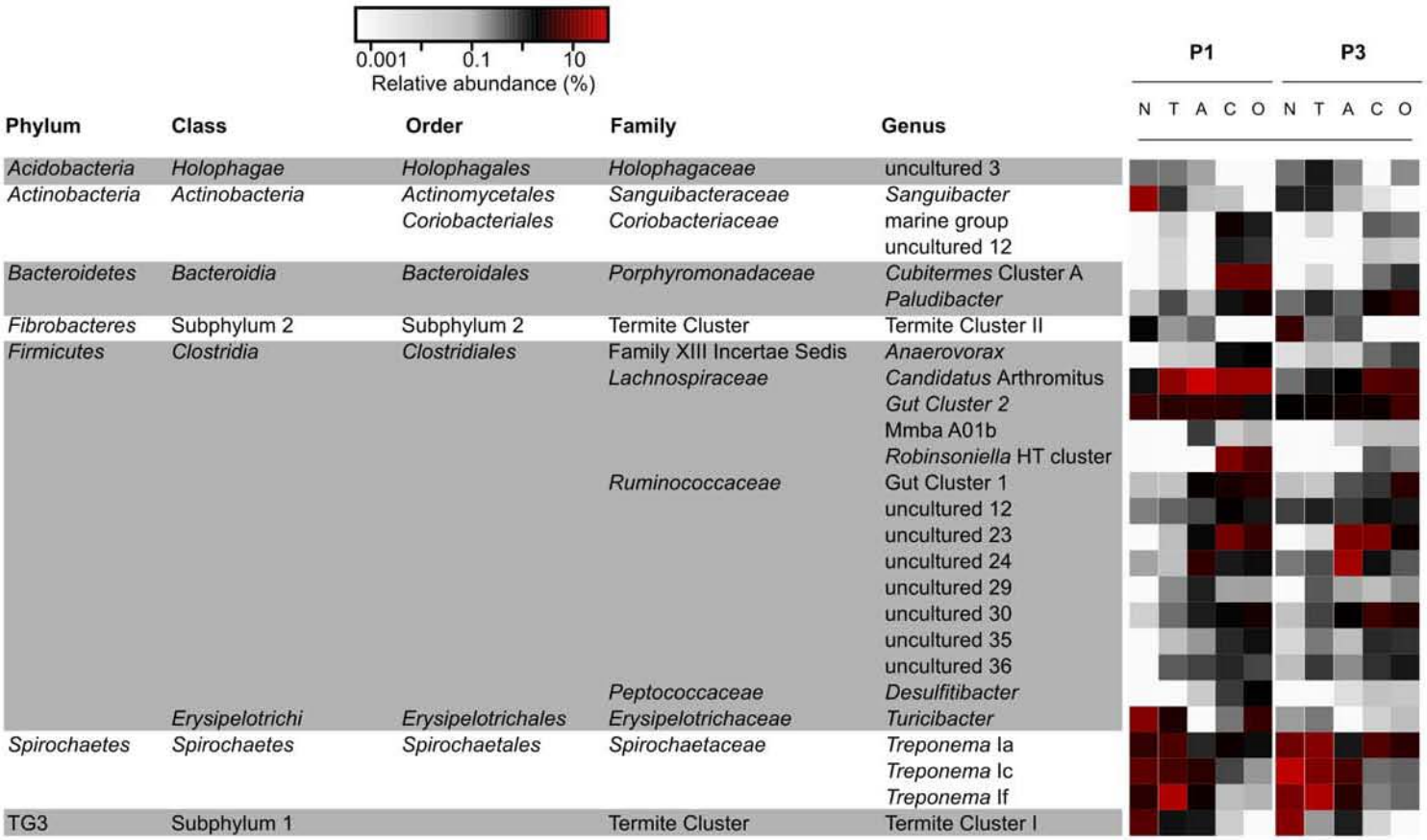

Figure 3.3 | A comparison of the relative abundances of genus-level bacterial groups from the homologous P1 and P3 hindgut compartments of *Nasutitermes corniger* (N), *Trinervitermes* sp. (T), *Amitermes* sp. (A), *Ophiotermes* sp. (O), and *Cubitermes ugandensis* (C), respectively.

3.4.2 Bacterial community shared between compartments

We analyzed the degree of community similarity at genus-level, between the different compartments, by calculating pairwise similarities based on the Morisita-Horn metric (Figure 3.3). Intra-specific comparisons between the compartments showed that a large proportion of the bacterial community in any given termite, is shared between the compartments.

In *N. corniger* and *Trinervitermes* sp., the intra-specific comparisons of hindgut compartments yielded higher similarity values than inter-specific comparisons between homologous compartments. For the homologous compartments of *N. corniger* and *Trinervitermes* sp., a higher similarity was observed for the P3 (Morisita-Horn similarity of 0.67) and the P4/P45 compartments/regions (0.69), but not for the alkaline P1 regions (0.35). Interestingly, the P1 compartment of *Trinervitermes* sp. shows much higher Morisita-Horn similarity values (0.5) with the P1 compartment of a phylogenetically more distant termite, *Amitermes* sp. This is also reflected in the fact that the core bacterial community shared by all P1 compartments forms only 25% of the total P1 community in *N. corniger* (Figure 3.3).

Morisita-Horn similarity values for homologous compartments were higher for the members of the *Termitinae*, particularly between *C. ugandensis* and *Ophiotermes* sp., demonstrating the highest similarity values for the pairwise comparisons between their P1 compartments (0.91) and P3 compartments (0.71). Although, the P1 compartment of *Amitermes* sp. also showed a high degree of similarity with the homologous compartments from *C. ugandensis* (0.63) and *Ophiotermes* sp. (0.69), the P3 compartments were observed to be more dissimilar.

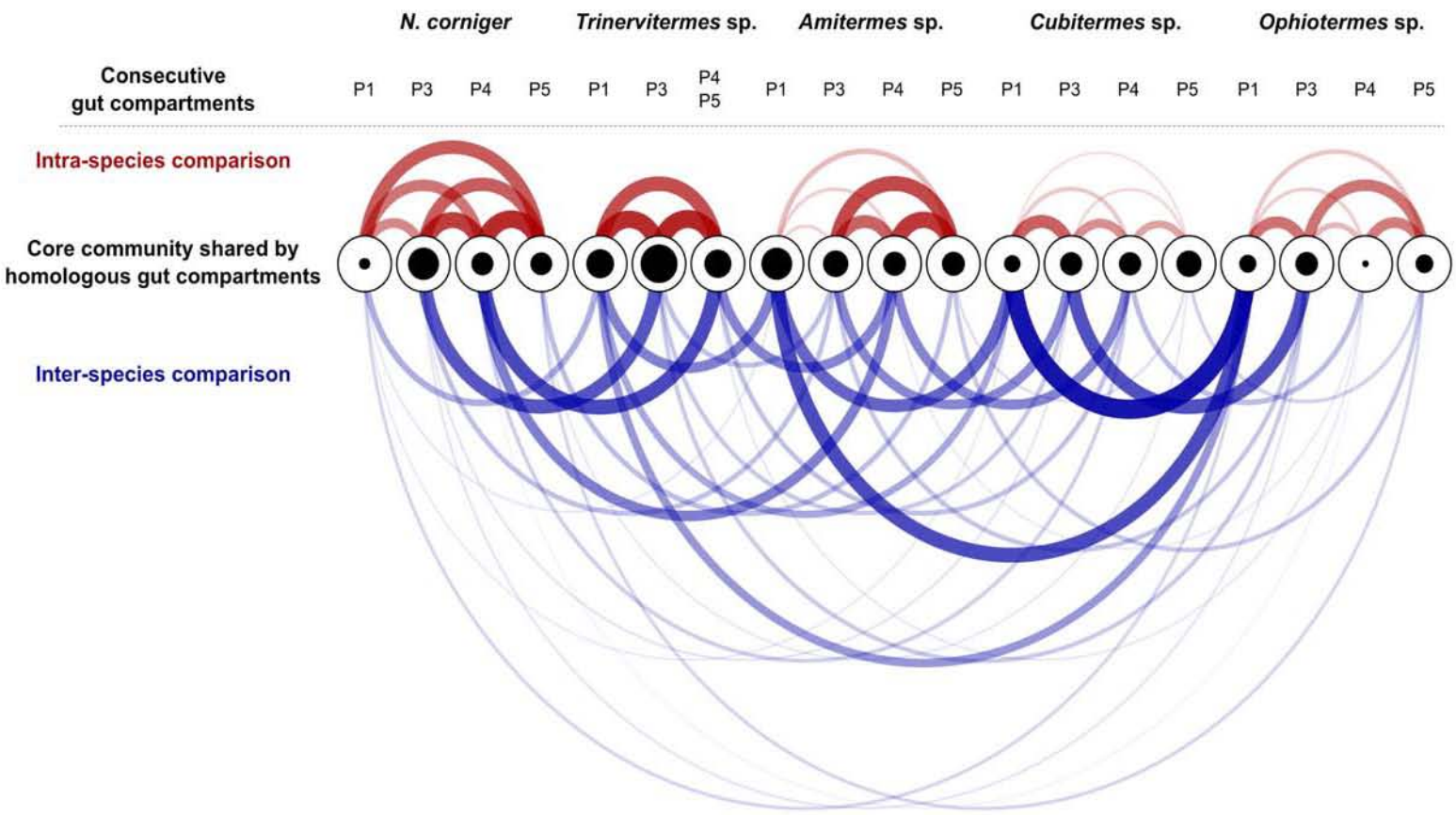

Figure 3.3 | Pairwise comparisons of the community structure, based on the Morisita-Horn metric, among the different compartments. The weights of edges are proportional to the similarity for the pair of nodes connected by the edge. The size of the circles at the center of the nodes represent the total relative abundance of the core bacterial taxa shared by the homologous compartments.

3.4.3 Phylogenetic relatedness between compartment communities

The Unifrac metrics – unweighted and weighted, were used to estimate the overall phylogenetic relatedness of bacterial lineages in the communities. Unweighted Unifrac similarities between the communities, visualized using NMDS (**Figure 3.4**), show a clear host-specific clustering of the hindgut compartments for all termites, with the exception of *Cubitermes ugandensis* and *Ophiotermes* sp.

The weighted Unifrac analysis (Figure 3.4), indicated the homologous compartments of different termites to be more similar in their community structure. This compartment-specific clustering of communities was more significant in case of the *Termitinae* (AU = 95% for the P1 compartments; AU = 90% for the P3 compartments) than for the *Nasutitermitinae* (AU = 75% for the P1 compartments; AU = 93% for the P3 compartments). No significant clustering could be observed for the posterior hindgut compartments P4 and P5, and were eliminated for this analysis.

3.4.4 Phylogenetic analysis of the core taxa shared by all anterior gut compartments

We identified genus-level taxa that occurred in all anterior hindgut compartments. These core taxa were found to constitute 24 – 67% of the compartment communities, depending on the sample. The Maximum Likelihood tree constructed with representative sequences of 137 dominant core OTUs (Figure 3.6) reveals the phylogenetic contribution of each of these bacterial lineages to the pattern of similarity observed among the anterior gut compartments (P1 and P3) (Fig 3.5).

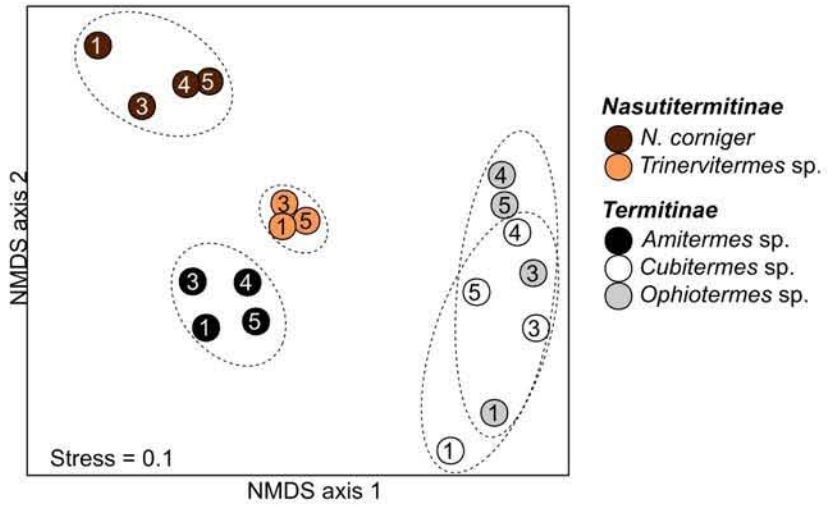

Figure 3.4 | Non-metric multi-dimensional scaling plot based on unweighted Unifrac distances, showing the clustering of gut communities from different hindgut compartments. The numbers in the circles indicate different hind gut compartments (P1, P3, P4 and P5). Compartments from the same termite species are encircled by ellipses.

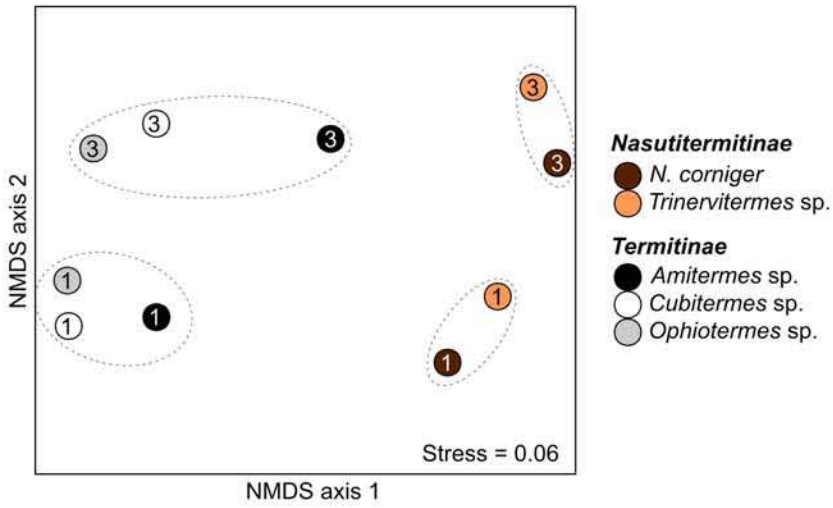

Figure 3.5 | Non-metric multi-dimensional scaling plot based on unweighted Unifrac distances, showing the clustering of gut communities from different hindgut compartments. The numbers in the circles indicate different hind gut compartments (P1, P3, P4 and P5). Homologous compartments are encircled by ellipses.

Among the core taxa, most OTUs could be taxonomically assigned to the phyla, *Firmicutes* and *Spirochaetes*. Most of the OTUs belonging to *Treponema* Ia, Ic and If grouped in host-specific clusters, containing sequences from both compartments.

On the other hand, the clustering of OTUs within taxa such as '*Candidatus* Arthromitus' (family *Lachnospiraceae*) and Gut Cluster 1 (family *Ruminococcaceae*), suggests that OTUs from homologous compartments of different termites were more phylogenetically related to each other than OTUs from other compartments of the same termite.

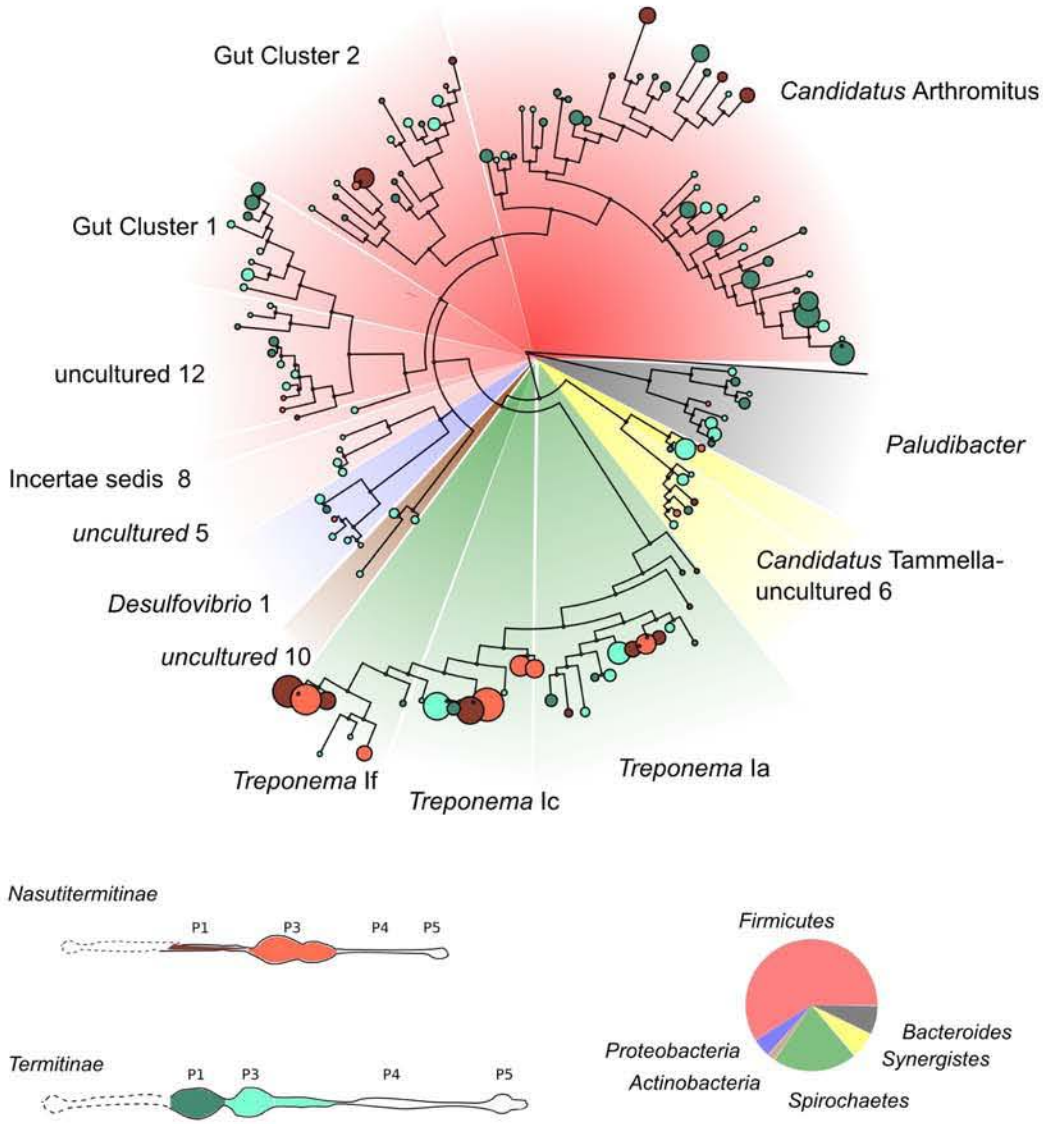

Figure 3.6 | Maximum Likelihood phylogenetic tree of representative OTUs within genus-level taxa shared by the P1 and P3 compartments of the termites, *Nasutitermes corniger*, *Trinervitermes* sp., *Amitermes* sp., *Ophiotermes* sp., and *Cubitermes ugandensis*.

3.5 Discussion

The anterior hindgut compartments of higher termites are characterized by steep axial variations in pH, that are reflected in considerable differences in the composition of their bacterial communities. We have shown that the bacterial communities associated with homologous compartments in the anterior hindgut are very similar in phylogenetic structure.

Several pioneering studies have explored the differences in the gut communities associated with the alkaline P1 compartment and the consecutive P3 compartment in different subfamilies of higher termites (Schmitt-Wagner et al. 2003; Thongaram et al. 2005; Köhler et al. 2012). However, few studies have attempted to compare the overall phylogenetic structure of homologous hindgut compartments from different termites. A previous study used Terminal Fragment Length Polymorphism (T-RFLP) to show that communities in homologous compartments of closely-related species can be more similar than communities in consecutive compartments of the same termite. However, community fingerprinting methods like T-RFLP lack the taxonomic resolution required to compare the phylogenetic structure of communities.

In our comparisons we used two approaches : a phylogeny-dependent approach using the Unifrac metrics (Lozupone & Knight 2005), and a classification-dependent approach, in which we used a highly-curated, taxonomically-classified reference database for classifying pyrosequences to the genus level.

The classification-based analysis reveals that homologous compartments among the *Termitinae* display a high level of similarity in community composition. Among the *Nasutitermitinae*, Morisita-Horn values indicated the P3 compartments of *N. corniger* and *Trinervitermes*

sp. to be highly similar, but the same trend could not be observed for the P1 compartments. Because comparisons with traditional ecological indices rarely take into account the phylogenetic structure of the communities (Martin 2002), our approach included both the unweighted and the weighted Unifrac metrics (Lozupone & Knight 2005; Lozupone et al. 2011). The unweighted Unifrac analysis is based on presence/absence of lineages, while the weighted Unifrac analysis also considers abundance of lineages.

The host-specific clustering observed for *Amitermes* sp., *N. corniger* and *Trinervitermes* sp., with the unweighted Unifrac analysis shows that a large proportion of the lineages are shared among compartments of the same termite. On the other hand, the compartment-specific clustering observed in *Ophiotermes* sp. and *C. ugandensis* indicates that a larger proportion of lineages are shared between homologous gut compartments from different termites than consecutive compartments in the same termite. The weighted Unifrac analysis showed that when abundance is incorporated into the comparison, the clustering indicates that *Nasutitermitinae* and *Termitinae* differ in the distribution of abundant deep-branching bacterial lineages. Furthermore, within each subfamily of termites, the phylogenetic structure of homologous compartments from different termites, is more similar than the consecutive compartments of the same termite.

The abundance of *Spirochaetes* in lignocellulose-feeding termites like *Trinervitermes sp.* and *N. corniger* can be correlated with diet (Chapter 2). On the other hand, the soil-feeding *Termitinae* are characterized by a greater abundance of *Firmicutes* (Chapter 2). In line with this argument, as a wood-soil interface feeder, *Amitermes* sp. shows an association with phyla that are abundant in wood-feeders and soil-feeders. We suggest that the separation observed in the weighted Unifrac analysis between the

two subfamilies of termites can be explained by differences between the two groups of termites at broader taxonomic levels.

Alkalinity in gut compartments is commonly encountered among the higher termites, especially in the advanced subfamilies like the *Nasutitermitinae* and *Termitinae*. While among the *Nasutitermitinae*, this alkaline zone covers only the P1 compartment (pH 10) (Bignell & Eggleton 1995; Brune et al. 1995; Köhler et al. 2012), in highly compartmented hindguts of the soil-feeding *Termitinae*, it includes both the P1 (pH 12) and the consecutive P3 (pH 10) compartments(Bignell & Eggleton 1995; Brune & Kühl 1996). We found these alkaline zones in both subfamilies to be marked by the strong presence of *Clostridia.* Although by no means a trait common to all clostridia, many clostridial isolates display an adaptation to alkaline conditions (Horikoshi 1999). Our results suggest that pH acts as an environmental filter to select for bacterial taxa that are better at coping with such high levels of alkalinity.

Although the differences between consecutive compartments regions could be explained at broader taxonomic levels, the clustering of homologous compartments of termites in the weighted Unifrac analysis is indicative of differences at finer scales of community composition.

Certain genus-level taxa such as '*Candidatus* Arthromitus' and were clearly more preferentially associated with the P1 compartments. '*Candidatus* Arthromitus' is a gut wall-associated segmented filamentous bacterium belonging to the family *Lachnospiraceae* (Chapter 5). Being a sessile bacterium attached to the gut wall, the selective pressure to adapt to extreme environmental conditions could be expected to be higher for '*Candidatus* Arthromitus'. The phylogenetic relatedness of OTUs belonging to homologous compartments from different termites (**Figure 3.6**) highlights the contribution of these bacterial members to the overall relatedness observed among the homologous compartments.

On the basis of the trends observed in our comparisons, we conclude that homologous compartments in higher termites show significantly greater phylogenetic overlap among related species of termites than among consecutive compartments of the same termite. This phylogenetic conservatism could be explained by physicochemical and functional similarities between the homologous hindgut compartments. Moreover, the sorting of bacterial lineages that are more adapted to alkaline and hyper-alkaline conditions in the P1 compartment could have played an important role in the development of habitat-specific communities.

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Chapter 4

Submitted

The fiber-associated cellulolytic bacterial community in wood-feeding higher termites comprises Fibrobacteres, Spirochaetes, and members of the TG3 phylum

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Contributions: AM and AB designed the study; electron microscopy was carried out by JFHS; AM performed all other lab experiments, and designed the bioinformatic pipelines for processing and analyzing pyrosequencing data; GT provided termites and contributed to the discussion; AM and AB wrote the manuscript.

4.1 Abstract

The cellulolytic flagellates in the hindgut of primitive lower termites phagocytose the wood fibers for cytoplasmic digestion. In contrast, the advanced higher termites, possess an entirely prokaryotic community and the absence of flagellates makes the wood fibers available as a potential site for bacterial colonization. However, little is known about this abundant microhabitat and about the existence or composition of a fiber-associated bacterial community. Here we investigated the composition of this community using density gradient centrifugation to separate the wood fibers and their associated bacteria, from those occurring free in the lumen of the termite hindgut. Assays of this fiber-fraction revealed that it comprises a substantial portion of the cellulase activity in the P3 region of the hindgut of *Nasutitermes corniger*. T-RFLP and pyrosequencing analysis of 16S rRNA genes demonstrate a consistent association of *Fibrobacteres* subphylum 2, the TG3 phylum and certain lineages of *Treponema* I, to the wood fibers in the guts of both *Nasutitermes corniger* and *Nasutitermes takasagoensis.* This work suggests that the wood fibers represent an abundant surface-microhabitat supporting a highly specific fiber-associated community. It also lends support to the hypothesis that, in response to the loss of cellulolytic flagellates, certain bacterial groups that were previously competitively excluded, took over the role of cellulose digestion in the hindgut of higher termites.

4.2 Introduction

Termites digest lignocellulose with the help of their symbiotic gut microbiota (Brune & Ohkuma 2011). In primitive lower termites, this is achieved through an obligatory association with a diverse population of cellulolytic flagellates that reside in their hindguts (Breznak & Brune 1994), which complete the breakdown of ingested wood particles initiated by endogenous cellulases secreted by the salivary glands (Watanabe et al. 1998). While cellulolytic flagellates are present in all but one termite family, they are absent in the more derived higher termites (family *Termitidae*). The loss of the flagellates appears to have triggered dietary diversification of higher termites, which led to the development of novel feeding guilds such the humus-feeding termites (Bignell et al. 1997) and substantial changes in their exclusively prokaryotic gut microbiota (Brune & Ohkuma 2011).

While members of the subfamily *Macrotermitinae* efficiently digest wood and plant litter through a unique mutualism with a basidiomycete fungus, the breakdown of lignocellulosic substrates in members of other subfamilies is unclear. It has been suggested that *Nasutitermes* species rely entirely on endogenous cellulases, with little or no contribution of cellulase activity from the bacterial communities in their hindguts (Hogan et al. 1988; Slaytor 1992). This claim was supported by the observation that in comparison to lower termites, the soluble hindgut cellulase activity, released into the supernatant after tissue homogenization, was almost negligible in *Nasutitermes takasagoensis (Tokuda et al. 2004)*. This notion was subsequently dispelled when substantial cellulase activities were detected in the previously uninvestigated pellet-extract of the hindgut homogenate in *N. takasagoensis* (Tokuda et al. 2005). Further examination of this pellet-extract of *Nasutitermes takasagoensis* and *Nasutitermes walkeri* (Tokuda & Watanabe 2007) suggested that this

particle-associated activity originated from either cell-bound or exclusively fiber-bound enzymes.

Numerous glycosyl hydrolase genes encoding putative cellulases were encountered in the metagenomic fragments from the enlarged P3 compartment of *Nasutitermes* sp. (Warnecke et al. 2007) and subsequently, *Amitermes wheeleri* (He et al. 2013). By taxonomic binning, the majority of the genes were assigned to the phyla *Fibrobacteres* and *Spirochaetes* (Warnecke et al. 2007), which are abundantly represented in the hindgut of wood-feeding higher termites (Hongoh et al. 2006; Köhler et al. 2012). The overrepresentation of clostridial cohesins and dockerins in *Amitermes wheeleri*, and a cohesin analog in *Fibrobacteres* in *Nasutitermes* sp., allude to the possibility of these glycosyl hydrolases to be cell-associated. Fibrolytic members in the rumen like *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* are characterized by extracellular multi-enzyme complexes that greatly increase the efficiency of fiber degradation and allow them to firmly attach to plant fiber (Flint et al. 2008). These ruminal fiber-associated communities have been extensively studied for their central role in plant fiber degradation. In contrast, apart from some preliminary ultrastructural evidence (Tokuda et al. 2005), nothing is known about the existence or composition of such a community in higher termites, or about its contribution to the particle-associated cellulase activity in the hindgut.

In ruminants, the fiber-associated community has been studied by separating the relatively large feed particles from the liquid by simple filtration (Koike et al. 2003; Brulc et al. 2011). This is not possible in the case of termites because the grinding action of mandibles and gizzard break down the wood to particles that overlap in size with the larger hindgut bacteria (Tokuda et al. 2012). Therefore, we developed a new method that separates wood fibers from luminal fluids based on

differences in buoyant density. This allowed us to differentiate between the contribution of fiber-associated and merely cell-associated acitivities to cellulose hydrolysis in the hindgut of *N. corniger*. Using T-RFLP analysis and 454-pyrosequencing of 16S rRNA genes, we investigated diversity and structure of the bacterial communities associated with the wood fibers and compared it to the unattached bacterial populations in the hindgut of *Nasutitermes corniger* and *Nasutitermes takasagoensis*.

4.3 Materials and Methods

4.3.1 Scanning Electron Microscopy

The gut contents from the P3 regions of ten individuals of *Nasutitermes* sp. were fixed for 30 min in 2.5% glutaraldehyde in 100 mM sodium phosphate buffer (pH 7.2). After three rinses with the same buffer (15 min), the samples were post-fixed on ice for 1 h in 1% OsO_4 in buffer, and washed again three times (15 min). The samples were then pipetted into small cups covered with planktonic gauze and dehydrated in a graded series of ethanol. After drying using a Balzer CPD 030, the gut contents were coated with gold in a Balzer SCD 040. The samples were examined with a FEI Quanta 200 ESEM.

4.3.2 Sample preparation

Nasutitermes corniger individuals were from a laboratory-reared colony (Scheffrahn laboratory, University of Florida). *Nasutitermes takasagoensis* was collected on Iriomote island, Japan. Only intact hindguts from worker termites were used for all the preparations. Pools of five individuals were used for the measurements of cellulase activities, unless mentioned otherwise.

4.3.3 Percoll-based density dependent purification of the fiber-fraction

Percoll is a silica-based self-forming gradient material that has been widely used for the isolation of viable cells and cellular components (Pertoft 2000). It shows little variation with osmolality with variation in gradient, and can easily be made isotonic for use. Due to the relative inertness of Percoll, it has been known to be possible to retrieve enzymatic activities from a gradient run (C. E. Neat et al. 1981; Pertoft 2000).

The concentration of Percoll and conditions for centrifugation were standardized in preliminary experiments. A working solution of Percoll was made by making the Percoll solution isotonic by mixing 1 part of 10X phosphate-buffered saline (PBS) with nine parts of Percoll (GE life sciences), as per the manufacturer's guidelines. An 83% working Percoll solution (prepared in 1X PBS) was then used for the experiments. P3 luminal fluid from ten individuals was pooled in 100 µL 1X PBS and separated using 2 mL of the gradient solution of Percoll in 2mL microcentrifuge tubes. All steps upto the pooling were done on ice. The tubes were than centrifuged at 4 ºC at 20,000 g in an Eppendorf centrifuge. The procedure yields 2 distinct well-separated bands.

The fractions were observed using bright field and phase-contrast microscopy. Wood fibers were observed by staining the fractions with an equal volume of Toluidine blue O, a stain commonly employed to visualise lignin in histochemistry. Bacterial cells were observed by phase contrast microscopy.

The fiber-free fraction was a translucent band of cells cushioned at the top of the gradient, and was collected using a syringe from the top of the tube. The bottom of the tube was punctured with a syringe and the brown band enriched in wood fibers was withdrawn. Both fractions were washed with three volumes of 1X PBS, and the particles pelleted by centrifugation (20,000 g at 4 ºC) in order to remove residual Percoll. This step was repeated three times and the pelleted contents were resuspended in 100 µL of 1X PBS for DNA extraction, or 100 µL protease inhibitor solution (EDTA-free, Roche Molecular Biochemicals) for the cellulase assay described previously by Tokuda et al., (2007).

DNA content was used as a proxy for the distribution of biomass in the two fractions and the amount of lignins was used as proxies for the fiber content. DNA content was measured using a dye binding assay

specific for double stranded DNA (Qubit, Invitrogen), and lignin was extracted (Zimmer 1999), and determined by measuring the absorbance after incubation with phloroglucinol.

4.3.4 Preparation of the crude enzyme extracts and assay of cellulolytic activity

Enzyme extracts were prepared according to procedure of Tokuda et al. (2007). Briefly, the guts were pooled in 100 µL protease inhibitor solution and sonicated as described above for the assay of activity in the hindgut and the P3 segment. The debris was then pelleted by centrifugation. The supernatants from this step were collected and were called crude-extracts. The pellet was washed thrice with 100 µL protease inhibitor solution, and finally resuspended in 100 µL CelLytic-B (Sigma-Aldrich). The samples were vortexed for 15 s to release membrane-bound enzymes. After a ten minute incubation on ice, the tubes were centrifuged to pellet the debris; the supernatant was used as pellet-extract.

4.3.5 DNA extraction and T-RFLP analysis

Samples from the P3 luminal contents and the Percoll fractions were used for DNA extraction using the method proposed by Zhou et al. (1996) with some modifications. Briefly, the samples collected for DNA extraction (as described above) were resuspended in 1 mL of 1X PBS, followed by the addition of 675 µL of extraction buffer [100 mM Tris-HCl, 100 mM Na_3PO_4, 100 mM Na_2EDTA, 1.5 M NaCl, 1% cetyltrimethylammonium bromide (CTAB) (pH 8.0)], and 75 µL of 20% SDS. The suspension was transferred into a 2 mL bead-beating vial, containing 0.5 g heat-sterilized by zirconia/silica beads (0.1 mm diameter), and homogenized in a FastPrep-24 cell disruptor (MP Biomedicals, Germany). The homogenate was then incubated on a heating block for 1 hour with periodic end-to-end inversion of the tubes. The rest of the

protocol was followed as described in the original protocol (Zhou et al., 1996), and DNA was precipitated with 0.6 volume of isopropanol.

The bacterial community structure for both *N. corniger* and *N. takasagoensis,* in the fiber-fraction, fiber-free fraction, and the total P3 luminal contents was studied using Terminal Fragment Length Polymorphism (T-RFLP). The appropriate restriction enzyme that would help for maximal resolution of the bacterial community members was carefully chosen based on in-silico surveys using the TRF-cut add-on (Ricke et al. 2005) for the ARB software package.

PCR products obtained from genomic DNA, isolated from each of the above mentioned samples, using forward FAM-labeled primer U341F (5'-CCTACGGGRSGCAGCAG-3') (Baker 2003) and reverse primer 1390R (5'-ACGGGCGGTGTGTACAA-3') (Thongaram et al. 2005), were purified as described previously, and digested with the restriction enzyme *Taq*I (at 65 ºC for 4 hours). The digests were then analysed as described previously on an automated sequence analyzer (Schauer et al. 2012).

4.3.7 454-Pyrosequencing of the 16S rRNA genes

The 16S rRNA genes in the samples were amplified using the primers 343F and 753R (Köhler et al. 2012) and pyrosequenced as described previously (Köhler et al. 2012). The sequences obtained were classified against the previously described reference database (Köhler et al. 2012) with modifications from the current study.

4.4 Results

4.4.1 Distribution of cellulase activity in the hindgut

The cellulase activity in the hindgut of *N. corniger* released by homogenization accounts for about 10% of the total activity in the hindgut **(Table 4.1)**. The activity released by sonication was higher, but more than half of the total activity remained in the pellet and was liberated only by detergent treatment (CelLytic B), indicating that the majority of the activity is particle-associated. Disproportionately high particle-associated activities were also previoulsy observed for *N. takasagoensis* (Tokuda & Watanabe 2007). When we repeated the experiments with *N. takasagoensis*, we were able to confirm this finding, except that the values for absolute activities were lower than those previously reported (Tokuda & Watanabe 2007). In both termite species, most of the cellulase activity in the hindgut was confined to the enlarged hindgut paunch (P3 compartment; **Table 4.1**).

4.4.2 Density-dependent enrichment of wood fibers from the P3 lumen

The size of most wood particles in the hindgut paunch of *N. corniger* ranged between 10 µm and 50 µm; only a few measured more than 100 µm in length (**Figure 4.1**). The average particle size of wood fibers in the hindgut paunch (25 ± 18 µm) was significantly smaller than that in the crop (124 ± 69 µm) and the midgut (128 ± 66 µm). Most wood particles were densely colonized by various types of filamentous or spiral-shaped bacterial cells (**Figure 4.2**). It was not possible to separate the bacteria attached to the wood particles from the unattached bacteria by filtration because the size of the wood particles overlapped with that of the larger bacteria in the luminal fluid.

Table 4.1 | Cellulase activity in the hindgut of *Nasutitermes* species released into the supernatant by homogenization or sonication and by detergent extraction of the sonicated pellet. Values are in units[a] per gram of termite

Species	Compartment	Homogenization[b]	Sonication[c]	Detergent treatment[d]	Total[e]	Particle-associated fraction[f] (%)
N. corniger	Hindgut	0.023 ± 0.009	0.069 ± 0.024	0.141 ± 0.025	0.210 ± 0.049	89
	P3	0.017 ± 0.008	0.099 ± 0.004	0.122 ± 0.022	0.221 ± 0.026	92
N. takasagoensis	Hindgut[g, h]	0.014 ± 0.053[g]	0.039 ± 0.02[h]	0.058 ± 0.012[h]	0.097 ± 0.032[g]	86
	Hindgut	n.d.	0.028 ± 0.005	0.026 ± 0.005	0.054 ± 0.010	82
	P3	n.d.	0.029 ± 0.004	0.020 ± 0.008	0.049 ± 0.012	80

[a] … One unit of enzyme activity is defined as the amount of enzyme required to release 1 µmol of reducing sugar equivalents per minute from microcrystalline cellulose. Values are means of three homogenates (five termites each) ± standard error

[b] … Cellulase activity released into the assay buffer after pestle homogenization, and represents particle-free activity

[c] … Cellulase activity released upon sonication of the sample

[d] … Cellulase activity released upon detergent treatment of the post-sonication pellet with CelLytic B

[e] … Sum of the activities released by Sonication and Detergent treatment

[f] … Cellulase activity that remains after subtraction of the particle-free activity from the total activity

[g] … Results from Tokuda et al., 2005

[h] … Results from Tokuda and Watanabe (2007)

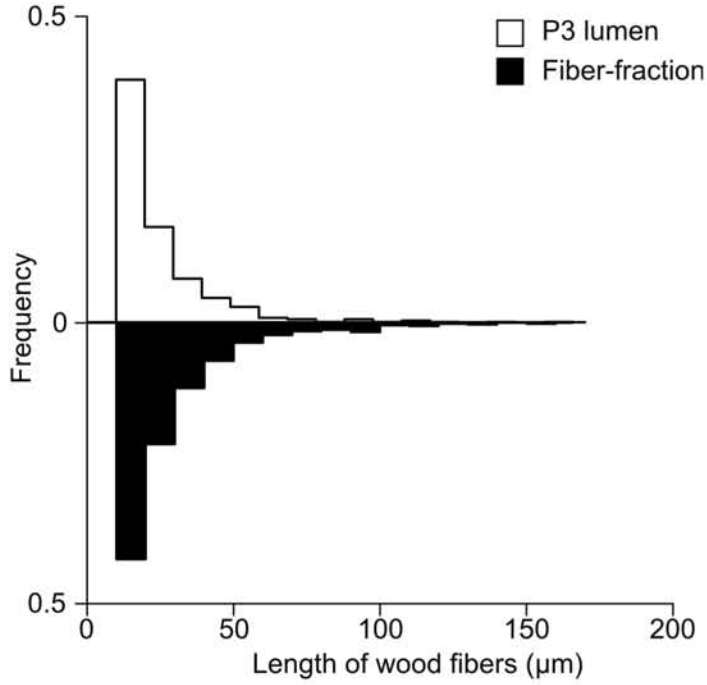


Figure 4.1 | Histogram comparison of the length distribution of wood fibers in the P3 fluid (top) and fiber fraction obtained from *Nasutitermes corniger*

However, fractionation of the luminal content by buoyant density using Percoll-gradient centrifugation yielded two well-separated bands, with the bulk of the wood particles concentrated near the bottom of the tube ("fiber fraction") and a turbid zone free of visible particles close to the meniscus ("fiber-free fraction"; **Figure 4.3A**).

The fiber fraction contained 83% of the lignin and 28% of the DNA in the sample, whereas the fiber-free fraction contained only 17% of the lignin but 68% of the DNA in the P3 lumen, indicating that a substantial part of the gut microbiota in the P3 contents is associated with wood particles (**Figure 4.3B**). Microscopic inspection of Toluidine Blue-stained preparations by bright-field and phase-contrast microscopy confirmed that the fiber fraction consisted mostly of wood particles and few unassociated cells, whereas the fiber-free fraction contained many suspended bacteria

and only few smaller wood particles. The size distribution of wood-particles in the fiber fraction was virtually identical to that in the P3 fluid (**Figure 4.1**), which indicates that the separation procedure is not biased against wood particles of a particular size.

When we collected the wood particles and microbial cells in the different fractions by centrifugation and determined the cellulase activities released by sonication and detergent treatment, we recovered 45% of the cellulase activity in the original sample in the fiber fraction and 40% of the activity in the fiber-free fraction (**Figure 4.3C**). The proportion of the activity that was released only after detergent treatment was significantly higher in the fiber fraction (ANOVA, $P < 0.05$).

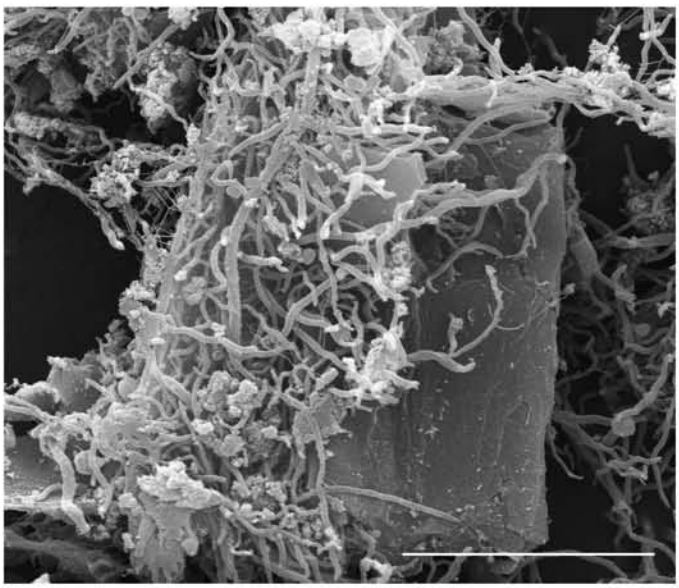


Figure 4.2 | Electron micrograph of bacterial cells adhering to wood fibers in the hindgut of *Nasutitermes corniger*. Bar represents a length of 10 µm.

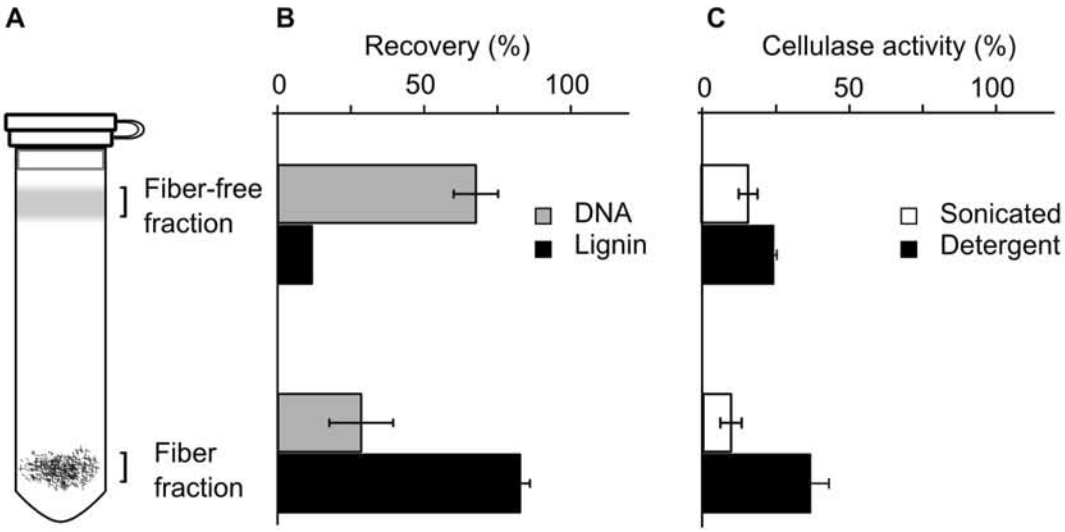


Figure 4.3 | Density-dependent sorting of wood particles in the gut of N. corniger. **A.** Fractionation of "fiber-free" and "fiber" fractions using Percoll. **B.** Relative distribution of lignin and DNA between the fiber and fiber-free fractions
C. Relative distribution of cellulase activity in the P3 luminal fluid between the two fractions.

T-RFLP analysis of the samples revealed that the bacterial community in the luminal contents was unevenly distributed between the two fractions. In all replicates, two of the major peaks in the luminal fluid (144 and 400 bp) were recovered almost exclusively from the fiber fraction, whereas the dominant T-RF (618 bp) was present in both fractions (**Figure 4.4**). Using the predicted T-RFs of the sequences from clone libraries (Hongoh et al., 2006), we tentatively identified them as TG3 phylum, *Fibrobacteres*, and *Spirochaetes*.

When we fractionated the P3 luminal fluid of *N. takasagoensis* in the same manner, the resulting T-RFLP profiles were similar to those of *N. corniger*, except that the peak representing *Fibrobacteres* subphylum 2

was quite small already in the luminal fluid (**Figure 4.4**). Again, the dominant T-RF of 618 bp was recovered from both fractions, representing many unresolved phylotypes in the *Treponema* I lineage.

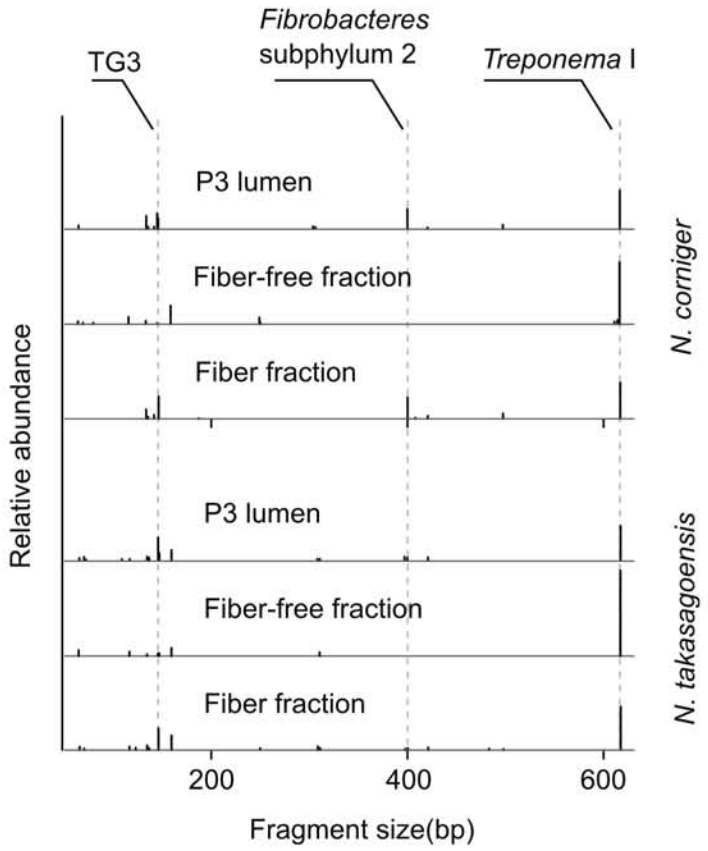


Figure 4.4 | T-RFLP profiles (TaqI digestion) of the bacterial communities associated with the P3 lumen, the fiber-free and fiber fractions from *N. corniger* (top) and *N. takasagoensis* (bottom)

4.4.3 Pyrotag analysis

To increase the taxonomic resolution of the community analysis, we amplified the V3–V4 region (about 450 bp) of the 16S rRNA genes in luminal fluid, fiber and fiber-free fractions of the two termite species and analyzed them by 454 pyrotag sequencing. The sequences (5,000–10,000

reads per sample) were classified against a comprehensive database that included all publicly available 16S rRNA sequences obtained from insect guts. This allowed to identify groups that cannot be resolved with generic databases. These lineages included termite-specific lineages in the TG3 phylum and the *Fibrobacteres* (Köhler et al. 2012).

We further improved the classification of the diverse phylotypes in the *Treponema* I lineage that were not resolved by the T-RFLP analysis by adding numerous, previously unpublished sequences to the near-full-length sequences available in public databases (see Chapter 2; **Figure 2.1**).Classification yielded around 300 genus-level taxa in total, and between 33 and 154 for each sample. The numbers of operational taxonomic units (OTUs, 3% sequence dissimilarity) in the respective samples were 2–10 times higher, indicating additional diversity at species level (Supplementary Table S4.1).

Ordination analysis showed that the bacterial communities associated with the fiber- and fiber-free fraction of *N. corniger* differed strongly from each other and from the luminal content (**Figure 4.5**). Also in the case of *N. takasagoensis*, fiber and fiber-free fraction clustered separately but the fiber fraction was not significantly separated from the luminal content. With both termites, the fractions obtained from three replicate preparations were highly similar.

Closer inspection of the taxonomic composition of the respective communities confirmed the distinct differences between fiber and fiber-free fractions of both termites (**Figure 4.6**). In *N. corniger*, there was a strong enrichment of two termite-specific clusters of the *Fibrobacteres* (40% vs. 4% mean relative abundance in fiber and fiber-free fraction, $P < 0.05$) and the TG3 phylum (12.1% vs. 2.7%, $P < 0.05$), indicating an association with the wood particles. This is in agreement with the results of the T-RFLP analysis, which was done with the same samples.

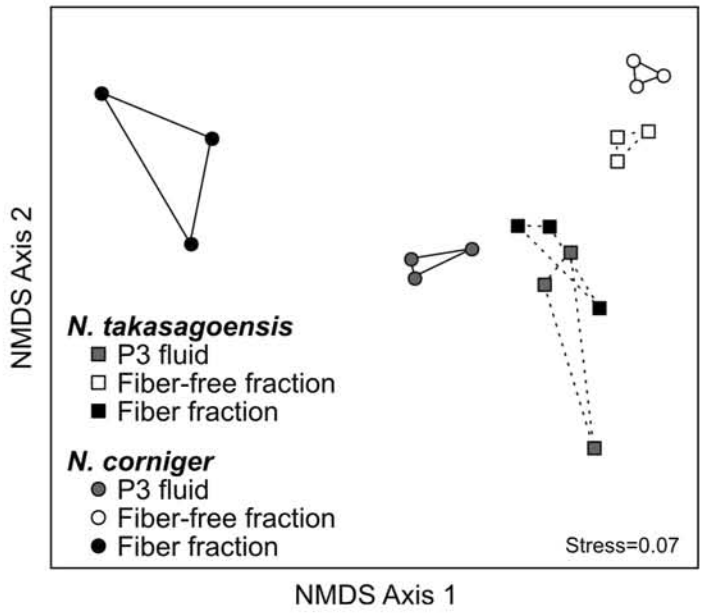


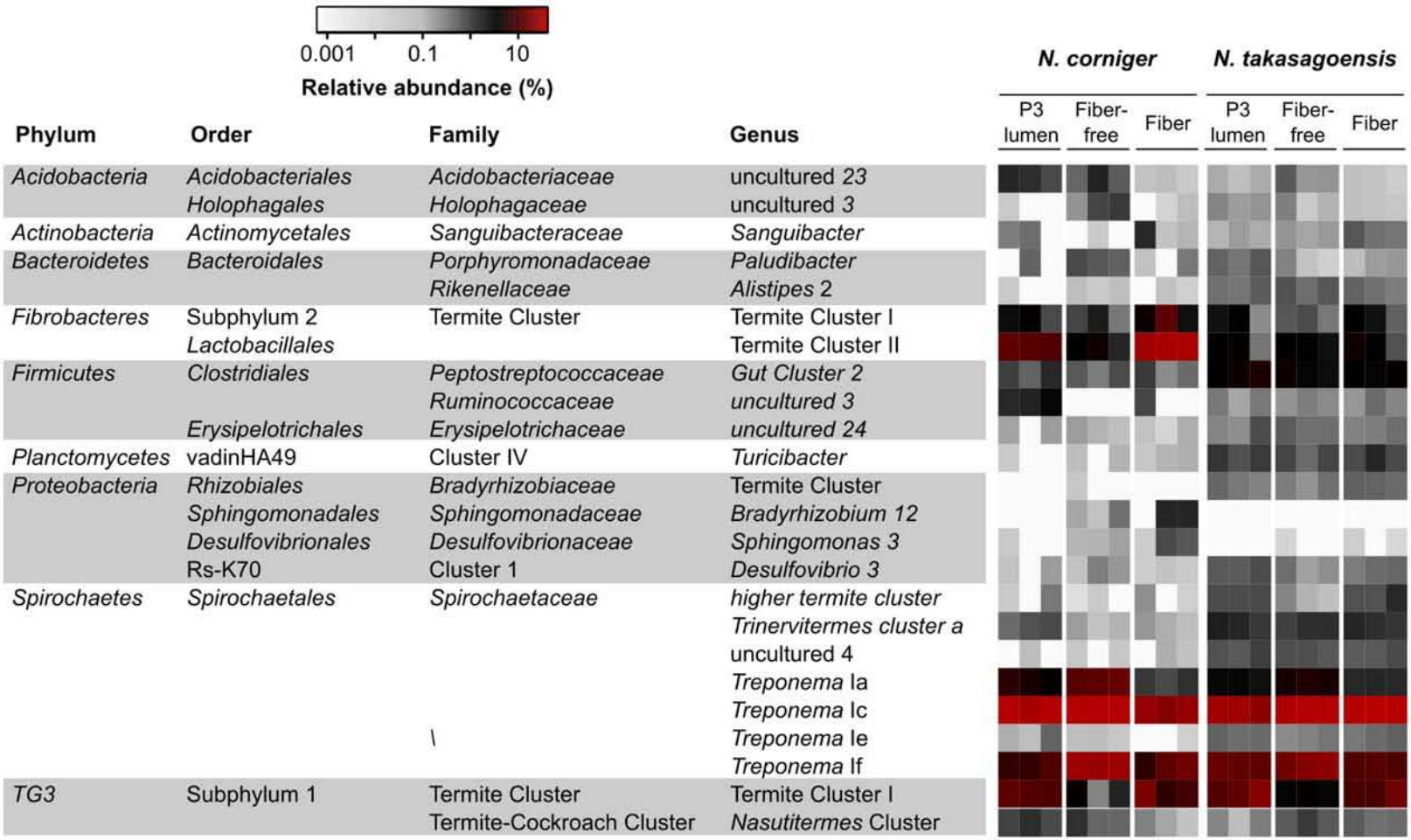
Figure 4.5 | NMDS ordination of the community-dissimilarity between three replicates each of the P3 luminal contents, the fiber and the fiber-free fractions respectively, from *N. corniger* and *N. takasagoensis.*

In *N. takasagoensis*, the number of reads in the luminal fluid assigned to the TG3 phylum was higher than that of *Fibrobacteres*. Again, the relative abundance of the TG3 phylum was higher in the fiber than in the fiber-free fraction (13.4% vs. 4.5%, $P < 0.05$), corroborating the results of the T-RFLP analysis, which in this case was even based on a different batch of termites. Interestingly, the relative abundance of *Fibrobacteres* in the pyrotag analysis was higher than in the T-RFLP analysis of *N. takasagoensis*, adding to the notion that the fraction of *Fibrobacteres* in *N. takasagoensis* varies between batches (see also Köhler et al., 2012). In contrast to *N. corniger*, the difference in abundance of *Fibrobacteres* between fiber and fiber-free fraction was not significant.

The enrichment of *Spirochaetes* in the fiber-free fraction was the same as in *N. corniger*. While the reads could be classified to *Treponema*

Ia and Ic, two well supported apical groups (*Treponema* Ib and Ic) consisting exclusively of sequences from higher termites were clearly enriched in the fiber-free fraction (12.8% and 31.1% in *N. corniger*, and 5.5% and 23.3% in *N. takasagoensis*). The situation was different in the case of *Treponema* Ib. This lineage was the most abundant group in both the luminal fluid and in the fiber-free fraction of both termites (37% in *N. corniger*, and 33.3% in *N. takasagoensis*). They were also highly represented in the fiber fraction of *N. corniger* and formed the most abundant group in the fiber fraction of *N. takasagoensis* (36.9%).

By contrast, the *Spirochaetes* (*Treponema* I lineage), which represent the most abundant group in the luminal fluid, decreased in abundance in the fiber fraction and increased in the fiber-free fraction (see below). Similar trends were observed in several other groups, e.g., the *Acidobacteria* and the *Bacteroidetes*.



Phylum	Order	Family	Genus
Acidobacteria	*Acidobacteriales*	*Acidobacteriaceae*	uncultured *23*
	Holophagales	*Holophagaceae*	uncultured *3*
Actinobacteria	*Actinomycetales*	*Sanguibacteraceae*	*Sanguibacter*
Bacteroidetes	*Bacteroidales*	*Porphyromonadaceae*	*Paludibacter*
		Rikenellaceae	*Alistipes* 2
Fibrobacteres	Subphylum 2	Termite Cluster	Termite Cluster I
	Lactobacillales		Termite Cluster II
Firmicutes	*Clostridiales*	*Peptostreptococcaceae*	*Gut Cluster 2*
		Ruminococcaceae	*uncultured 3*
	Erysipelotrichales	*Erysipelotrichaceae*	*uncultured 24*
Planctomycetes	vadinHA49	Cluster IV	*Turicibacter*
Proteobacteria	*Rhizobiales*	*Bradyrhizobiaceae*	Termite Cluster
	Sphingomonadales	*Sphingomonadaceae*	*Bradyrhizobium 12*
	Desulfovibrionales	*Desulfovibrionaceae*	*Sphingomonas 3*
	Rs-K70	Cluster 1	*Desulfovibrio 3*
Spirochaetes	*Spirochaetales*	*Spirochaetaceae*	*higher termite cluster*
			Trinervitermes cluster a
			uncultured 4
			Treponema Ia
			Treponema Ic
		\	*Treponema* Ie
			Treponema If
TG3	Subphylum 1	Termite Cluster	Termite Cluster I
		Termite-Cockroach Cluster	*Nasutitermes* Cluster

Figure 4.6 | A comparison of the relative abundances of genus-level bacterial groups from the P3 lumen, fiber-free and the fiber fractions from *N. corniger* and *N. takasagoensis*, respectively.

4.5 Discussion

This study establishes the existence of a distinct fiber-associated bacterial community in wood-feeding higher termites of the genus *Nasutitermes*. Using a newly developed method to separate wood-fibers and associated microorganisms from unattached cells in the gut fluid, we showed that the wood fibers are specifically colonized by members of *Fibrobacteres*, *Spirochaetes*, and the TG3 phylum. The fiber fraction also contained a substantial fraction of the cellulase activity in the hindgut of *N. corniger*, which further suggests that a substantial proportion of the cellulolytic bacteria are attached to the wood fibers.

First evidence for the presence of a fiber-association of cellulolytic bacteria was provided by Tokuda et al. (2005), who found that the majority of the cellulolytic activity in the hindgut *N. takasagoensis* was trapped in the pellet; it was released into the supernatant only by subsequent sonication and detergent-treatment (Tokuda & Watanabe, 2007). We obtained similar results for *N. corniger*, where the particle-associated activity amounted to almost 90% of the total cellulase activity in the hindgut. After separation of the luminal contents of the P3 region, the particle-associated cellulase activity was almost equally distributed among the fiber fraction and the fiber-free fraction, indicating that the particle-associated activity is in part contributed by bacteria in the gut fluid, and in part by bacteria (or enzymes) associated with the wood particles. The particle-associated activity in the fiber-free fraction clearly points to the activity being cell-bound, and could potentially be from originally fiber-associated bacteria that spontaneously detached from the wood fibers during the density gradient centrifugation step. The origin of the particle-associated cellulolytic activity in the fiber-fraction, however, could be either cell-associated or from exclusively fiber-bound enzymes.

The consistent association of certain bacterial groups with the fiber-fractions clearly suggests to the colonization of the wood fibers by a specialized fiber-associated bacterial community in both *N. corniger* and *N. takasagoensis*. This is most obvious in several lineages from the TG3 phylum and the *Fibrobacteres*. The phylum *Fibrobacteres* is so far defined by only two cultured representatives, *Fibrobacter succinogenes* and *Fibrobacter intestinalis,* which are important cellulose degraders in herbivore guts. While these isolates cluster with numerous uncultivated bacteria from mammalian guts and other environments (subphylum 1), the clones from the guts of Nasutitermes species and other termites fall into a separate, well-supported cluster (subphylum 2; Hongoh et al. 2006). Genes encoding GHF9 cellulases have been detected in the metagenome of *Nasutitermes* sp. detected and taxonomically binned to the phylum *Fibrobacteres.* In a follow-up proteomic study (Burnum et al. 2011), these genes were also shown to produce active proteins with predicted cellulase activity. Given this circumstantial proof, the high relative abundance of *Fibrobacteres* in the fiber fractions, especially in the context of the high levels of particle-associated cellulase activity, points to similar crucial roles for *Fibrobacteres* subphylum 2 in the attachment and hydrolysis of insoluble plant material in flagellate-free wood-feeding higher termites.

It is possible that a similar case is represented by the fiber-associated lineages of the TG3 phylum, but functional predictions are even more difficult in the absence of any cultured representatives. The candidate phylum is defined by 16S rRNA sequences that were derived almost entirely from termite guts (Hongoh et al. 2006). They co-occur in high abundance with Fibrobacteres subphylum 2 in wood-feeding higher termites of the genus *Microcerotermes* (Hongoh et al. 2006) and *Nasutitermes* (Hongoh et al. 2006, Warnecke et al. 2007 , Köhler et al. 2012); some authors even group them with the *Fibrobacteres* (Warnecke et al. 2007; He et al. 2013).

A third major bacterial group associated with the fiber fraction comprises several lineages of the *Treponema* I cluster (Ohkuma et al. 1999). This cluster includes only sequences the termite guts, including the three cultured representatives of this lineage – *Treponema primitia* (Graber et al. 2004), *Treponema azotonutricium* (Graber et al. 2004), and *Treponema isoptericolens* (Dröge et al. 2008). Representatives of *Treponema* I are abundant in both lower and higher termites; in *N. corniger* and *N. takasagoensis*, they represent as much as 70% of the hindgut community (Köhler et al. 2012).

We further investigated the phylogenetic grouping within the *Treponema* I lineage and upon analysis, observed many well-supported clusters (Fig 2.1, Chapter 2). We incorporated this taxonomic information into the 16S rRNA reference database, in order to improve the taxonomic assignment of pyrosequences belonging to *Treponema* I. This allowed us to form better hypotheses about the different functional roles that these lineages could be playing in the termite gut.

The fact that in both *N. corniger and N. takasagoensis*, the sub-lineages, *Treponema* Ia, Ic, Ie, and If (Chapter 2) were observed in the fiber as well as the fiber-free fractions indicates that they may not be explicit members of the fiber-associated assemblage. On the contrary, cluster Ia clearly appears to be consistently associated with the fiber-free fraction. Although closely related, the cultured relatives appear to have distinct physiological and nutritional capabilities (Graber & Breznak 2004; Graber et al. 2004), and hence could be hypothesized to occupy distinct microniches within the termite gut.

The only cultivated *Treponema* I representatives also belong to Cluster Ia and although they are not known to be cellulolytic, some have been found to be able to utilize di- and oligosaccharide breakdown products of cellulose (Dröge et al. 2008). It is also interesting to note that

all the tested enzyme activities for these breakdown products were cell-associated (Dröge et al. 2008), and could partly contribute to the cell-associated cellulase activity that was observed in both the fiber as well as the fiber-free fraction. On the other hand, metagenomic analysis from *Nasutitermes* spp. allude to the presence of numerous treponemal genes that encode putative cellulases belonging to various CAZy families (Warnecke et al., 2007; He et al., 2013). It seems plausible to hypothesize the metabolically diverse population of treponemes in wood-feeding higher termites, includes numerous lineages, some of which appear to have the genomic potential to be primary cellulose degraders, while others may rely on the products of cellulose depolymerisation from other cellulolytic organisms. The motility of the treponemes, in addition to their genomic capability to effectively respond to a chemotactic signal (Warnecke et al., 2007), precludes the need to associate with wood fibers for nourishment.

The SEM images of the microbial consortia associated with the wood particles indicate that the fiber-associated communities of *N. corniger* and *N. takasagoensis* contain similar morphotypes of spirilloid bacteria. This is in agreement with the abundance of spirochetes in the fiber-associated communities, but many of them may also represent the fiber-associated lineages of *Fibrobacteres* and the TG3 phylum. Hongoh et al. (2006) reported that the bacterial cells in gut homogenates of *N. takasagoensis* that hybridized with FISH probes specific for *Fibrobacteres* and TG3 sequences obtained from termite guts had spirilloid morphology. Although these cells were not associated with the wood fibers, their relative abundance was similar to the proportion of both groups in a clone library of the entire gut homogenate (Hongoh et al., 2006), which suggests that they became detached during the fixation procedure. We found it extremely difficult to directly observe cells on the wood particles because of the strong autofluorescence of lignin. However, using a more indirect method, we have been able to show these bacterial lineages to be

dominant members of the fiber-associated communities in the termite species we investigated.

The higher termite gut is a highly structured environment with numerous microhabitats (Brune & Ohkuma 2011). In the absence of phagocytic flagellates, the wood fibers in the hindgut of higher termites are colonized by a specific fiber-associated bacterial community. Given the consistent association of certain bacterial members to wood fibers in the *Nasutitermes* spp., it would be interesting to conduct a comparative investigation of the fiber-associated members in other wood-feeding species from subfamilies other than *Nasutitermitinae*. Although the exact roles of the members of this community remain to be clarified, our results show that a sizeable portion of the previously particle-bound activity in higher termites is also associated with the wood fibers, confirming the cellulolytic role the bacterial community plays in flagellate-free higher termites.

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Chapter 5

'Candidatus Arthromitus' revised: segmented filamentous bacteria in arthropod guts are members of *Lachnospiraceae*

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Contributions: CLT and AB designed the study; CLT, TW and RV picked the *Arthromitus* filaments for MDA analysis; CLT conducted PCR and constructed clone libraries; RV conducted the FISH experiments; AM conducted the phylogenetic analysis for the Sanger sequences and analyzed pyrosequencing data; CLT and AB wrote the manuscript.

5.1 Abstract

The name *Arthromitus* has been applied collectively to conspicuous filamentous bacteria found in the hindguts of termites and other arthropods. First observed by Joseph Leidy in 1849, the identity of these filaments has remained contentious. While Margulis and colleagues declared them to be a life stage of *Bacillus cereus* (PNAS 95:1236–1241, 1998), others have assumed them to belong to the same lineage as the segmented filamentous bacteria (SFB) from vertebrate guts, a group that has garnered much attention due to their unique ability to specifically modulate their host's immune response. Both SFB and *Arthromitus* from arthropod guts were grouped under provisional name "*Candidatus* Arthromitus" as they share a striking similarity in terms of their morphology and close contact to the host gut wall. While SFB form a distinct lineage within the family *Clostridiaceae*, the identity of *Arthromitus* remains elusive. Using whole-genome amplification of single *Arthromitus* filaments capillary picked from termite guts and fluorescence in situ hybridization of 16S rRNA with group-specific oligonucleotide probes, we show that *Arthromitus* represent a monophyletic lineage within the family *Lachnospiraceae*. This "*Arthromitus* cluster" consists of sequences derived exclusively from the guts of those arthropod species where *Arthromitus* filaments are typically present on the gut wall. We propose to reserve "*Candidatus* Arthromitus" for the members of this cluster, as it comprises the filaments that were originally described by Leidy. For the SFB from vertebrate guts, we propose the provisional name "*Candidatus* Savagella" in honor of the American gut microbiologist Dwayne C. Savage, who was the first to describe this important bacterial group.

5.2 Introduction

Arthromitus is the collective name for conspicuous, segmented filamentous bacteria commonly found on the gut wall of certain arthropods. Based on their distinctive cell morphology, the presence of putative endospores, and their characteristic mode of attachment, members of the genus *Arthromitus* were first described in 1849 by Joseph Leidy in the guts of millipedes and later also in the intestinal tracts of termites and cockroaches (Leidy, 1849; 1881). However, the taxonomic position of *Arthromitus* was never clarified.

An attempt by Margulis et al. (1998) to cultivate *Arthromitus* assumed that the putative endospores were heat resistant and that cells could be grown on conventional laboratory media. Not surprisingly, they readily isolated several strains of the ubiquitous bacterium *Bacillus cereus* from boiled gut homogenates of a range of arthropods including termites and sow bugs (*Porcellio scaber*) and, inasmuch as some of their isolates were morphologically similar to *Arthromitus* as described by Leidy, they concluded that *Arthromitus* was simply a life stage of *B. cereus*. However, they made no effort to correlate colony counts of *B. cereus* with direct microscopic counts of *Arthromitus*, nor did they confirm that their isolates were genotypically identical to the *Arthromitus* filaments observed in situ. There is also the unsettling enigma that – although *Arthromitus* filaments are quite abundant in termite guts – sequences related to *B. cereus* are completely absent from all 16S-rRNA-based inventories of termite gut microbiota (e.g., Hongoh et al. 2003; 2005; Schmitt-Wagner et al. 2003; Yang et al. 2005). In fact, the only reports on the presence of *B. cereus* in termite guts have originated solely from cultivation-based studies (Margulis et al. 1998; Thayer, 1976; Kuhnigk et al. 1995). This type of approach is known to provide a strongly biased and limited view of microbial diversity, missing many microorganisms whose presence in

insect guts is evidenced only by cultivation-independent molecular approaches.

A few years prior to the report by Margulis et al. (1998), Snel et al. (1995) had already used the provisional name "*Candidatus* Arthromitus" for the segmented filamentous bacteria (SFB) colonizing the intestinal tract of vertebrates. Although the SFB from vertebrate guts share morphology, ecological niche, and mode of attachment with *Arthromitus*, they had been clearly identified as members of the *Clostridiaceae* (Snel et al. 1994). While nothing is known about the function of *Arthromitus* in arthropod guts, SFB are considered important members of the mammalian gut microbiota and play a critical role in host immune function (reviewed in Ivanov & Littman 2010).

We therefore re-investigated the identity of *Arthromitus* in arthropods, focusing on subterranean termites of the genus *Reticulitermes* − not only because of considerable knowledge on the symbiotic digestion in these termites, including a comprehensive inventory of the diversity and community structure of their gut microbiota (Hongoh et al. 2003; Yang et al. 2005), but also because Leidy had extensively studied *Arthromitus* in a member of this genus (Leidy, 1881). To avoid all cultivation bias, we used a full-cycle rRNA approach (Amann et al. 1995), which included: (i) whole genome amplification of single *Arthromitus* filaments capillary picked from hindguts, (ii) phylogenetic analysis of 16S rRNA genes, and (iii) fluorescence in situ hybridization (FISH) with specifically designed oligonucleotide probes.

5.3 Methods and Materials

5.3.1 Animals

Reticulitermes santonensis was collected in the Forêt de la Coubre near Royan, France. *Zootermopsis nevadensis* was collected from the Angeles National Forest in California, USA. All termites were maintained in the laboratory on a diet of pine wood and water. *Blaberus giganteus* cockroaches were maintained in the laboratory and fed chicken feed (Gold Plus, Versele-Laga, Deinze, Belgium). Sow bugs (*Porcellio scaber*) and millipedes (*Tachypodoiulus niger*) were collected in Marburg, Germany. Rose chafer larvae (*Pachnoda marginata*) were purchased from a commercial breeder (b.t.b.e. Insektenzucht, Schnürpflingen, Germany). Insects were identified through sequence analysis of cytochrome oxidase subunits I and II. *Arthromitus* filaments were identified by phase-contrast microscopy of gut homogenates.

5.3.2 Micromanipulation of single bacterial filaments

Termite hindguts were dissected and homogenized in solution U (Trager, 1934) (1 gut per 100 µl) to prevent lysis of the gut flagellates. A 10 µl aliquot of gut homogenate was placed in one well of a ten-well Teflon slide. Filaments of *Arthromitus* were identified using an inverted microscope, and individual filaments were captured using a micropipette attached to a micromanipulator. Each captured filament was aspirated into another well containing 20 µl of sterile solution U. This procedure was repeated three more times to remove any extraneous bacteria in the sample. Finally, individual filaments were aspirated separately into sterile 0.2 ml PCR tubes and frozen at –20 °C.

5.3.3 DNA extraction and whole genome amplification

Approximately 10 mg of sterile 0.1 mm glass beads were added to a 0.2 ml tube containing a single *Arthromitus* filament. Cells were lysed by vortexing for 10 min. Microscopy confirmed that this method was sufficient to lyse *Arthromitus* filaments in the sample. Cell lysate (1 µl) containing DNA was used as template for multiple displacement amplification (MDA) using the Repli-g UltraFast Mini Kit (Qiagen) according to the manufacturer's instructions, with the exception that the incubation time was increased to 16 h as described previously to allow sufficient amplification of DNA from a single cell (Woyke et al. 2010).

5.3.4 PCR

MDA products were diluted 1:25 in water and 2 µl was used as template for PCR. 16S rRNA genes were amplified using the primer pair 27f and 1492r (Lane et al. 1985). Each 50 µl PCR reaction contained 1× PCR reaction buffer, 2.5 mM MgCl2, 1 U of Taq DNA polymerase (Invitrogen), 50 µM deoxynucleoside triphosphates mix, 0.3 µM of each primer, and 0.8 mg ml−1 bovine serum albumin. The program used was as follows: initial denaturation (94 °C for 3 min), followed by 35 cycles of denaturation (94 °C for 20 s), annealing (48 °C for 20 s), and extension (72 °C for 50 s), with a final extension (72 °C for 7 min). PCR products were sequenced on an automatic sequence analyzer (ABI 3130, Applied Biosystems).

5.3.5 Phylogenetic analysis

16S rRNA gene clone libraries were constructed from capillary-picked suspensions of filaments collected from the guts of the termite *R. santonensis* and the millipede *T. niger*. PCR products were purified using the MinElute kit (Qiagen) and cloned using the pGEM-T easy vector kit

(Promega) according to the manufacturer's instructions. Positive clones were amplified with M13 vector primers, and insert size was checked on a 1% agarose gel. Clones were sequenced and phylogenetic trees were constructed using the ARB program package.

5.3.6 Pyrotag sequencing

Relative abundances of the *Arthromitus* cluster and the *B. cereus* group among the bacterial gut microbiota of various insects were assessed using 454 pyrotag sequencing. DNA extracted from the guts of *Nasutitermes takasagoensis*, Trinervitermes geminatus, Hodotermes mossambicus, Coptotermes niger, *R. santonensis*, *Z. nevadensis*, *Cryptocercus punctulatus*, *Blatta orientalis*, *Shelfordella lateralis*, *Eurycotis floridiana*, and *Pachnoda ephippiata* was amplified using primers 343Fmod (TAC GGG WGG CWG CA) and 784Rmod (GGG TMT CTA ATC CBK TT) targeting the V3–V4 region of the bacterial 16S rRNA gene. Both primers had an additional, sample-specific 6-bp barcode at the 5' end. Adaptor ligation, subsequent amplification, and pyrosequencing (454 GS FLX with Titanium technology, Roche) were done by a commercial service (GATC Biotech, Konstanz, Germany). The sequences were classified against a manually curated reference database containing representative sequences of the *Arthromitus* cluster, using the mothur software suite (version 1.15.0; Schloss et al. 2009) with a confidence threshold of 60%. The complete dataset will be published in a different context.

5.3.7 FISH

Oligonucleotide probes were designed with the Probe_Design tool, and probe specificity was tested using the function Probe_Match, both implemented in ARB. Probe Lachno758 (CCC CAC GCT TTC GTG ACT, 20–30% formamide) was specific for most of the sequences present in the *Arthromitus* cluster, while probe Lachno1215 (CAC GTG TGT TGC CCA

AGA, 20% formamide) was specific for four subgroups of the *Arthromitus* cluster, including one of the MDA sequences (**Figure 5.2**). Other probes targeted *B. cereus* (ATG CAG TTC AAA ATG TTA TCC GG, modified from Liu et al. 2001), the *Bacteroides-Porphyromonas-Prevotella* subgroup (CFB935: CCA CAT GTT CCT CCG CTT GT, 45% formamide) (Daly et al. 2003; Zijnge et al. 2010), and all bacteria (EUB338; Amann et al. 1990). Non-specific binding was excluded by the use of a nonsense probe (NON338) (Wallner et al. 1993). FISH was performed as previously described (Stingl & Brune 2003). Hybridization of the probes was performed at 48 °C, with the exception of CFB935, which was hybridized at 50 °C (Zijnge et al. 2010).

As a positive control for the FISH assay of *B. cereus*, an isolate was obtained using the method described by Margulis et al. (1998). Briefly, termite gut homogenate was heat treated for 10 min at 95 °C and aerobically cultured on nutrient agar. Identity was confirmed through sequencing of the 16S rRNA gene as outlined above.

5.4 Results

5.4.1 Filament morphology

The hindgut wall of *Reticulitermes santonensis* was colonized by three morphotypes of *Arthromitus*: thick filaments (1.5 µm in diameter) that bore oblong endospores along the entire filament (**Figure 5.1A**), and thinner filaments (0.8−1 µm in diameter) with round endospores in pairs along the entire length of the filament (**Figure 5.1B**) or with oval endospores located at the distal end (**Figure 5.1C**). These morphotypes resembled in morphology and sporulation patterns the original observations made by Leidy with *Reticulitermes flavipes* (Leidy 1881), a species considered synonymous with *R. santonensis* (Austin et al. 2005).

We also noted morphological differences among the filaments adhering to the gut wall of other arthropod species. The hindgut of *Zootermopsis nevadensis* harbored four distinct morphotypes: thick filaments (2–2.5 µm in diameter) with oblong spores (**Figure 5.1D**) that resembled a morphotype present also in *Zootermopsis angusticollis* (Margulis et al. 1990), thinner filaments (0.8–1 µm in diameter) with pairs of smaller round spores (**Figure 5.1E**), and other filaments that consisted of long cells (up to 10 µm in length) or had a beaded appearance (**Figure 5.1F,G**). Filaments present in the julid millipede *Tachypodoiulus niger* were thin (1 µm in diameter) and had oval spores (**Figure 5.1H**). We also found *Arthromitus*-like filaments colonizing the hindgut of rose chafer larvae (*Pachnoda marginata*). They were thin (0.8–1 µm in diameter) and had oval spores spaced more distantly (3–6 µm) along the filament (**Figure 5.1I**). The gut of the sow bug *Porcellio scaber* contained an abundance of large, single and paired, rod-shaped bacteria, but contrary to the report of Margulis et al. (1998), we did not observe any *Arthromitus*-like filaments.

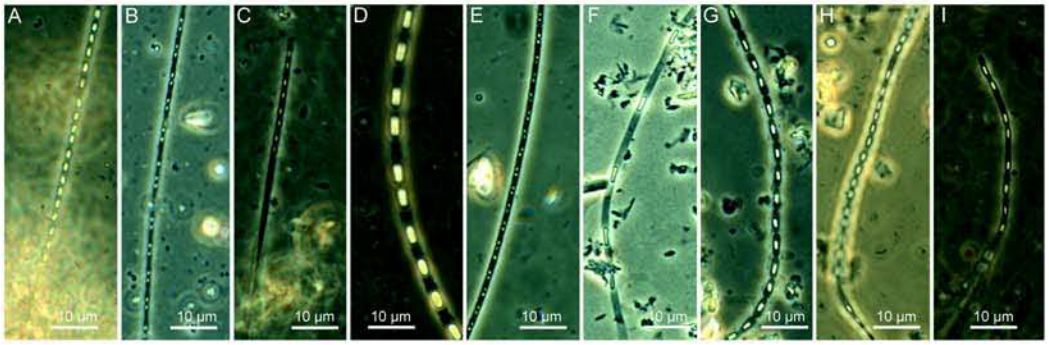

Figure 5.1 | Phase-contrast micrographs of the different morphotypes of *Arthromitus* observed in the hindguts of termites (**A–C**: *Reticulitermes santonensis*, **D–G**: *Zootermopsis nevadensis*), a millipede (H: *Tachypodoiulus niger*), and a scarab beetle larva (I: *Pachnoda marginata*).

5.4.2 Clone libraries of filament suspensions

From hindgut homogenates of *R. santonensis*, we picked individual *Arthromitus* filaments using glass capillaries, an inverse microscope, and a manual micromanipulator. Picked filaments were pooled into four filament suspensions, each containing approximately 20 filaments. DNA was extracted from each suspension, and 16S rRNA genes were PCR-amplified, cloned and sequenced. Phylogenetic analysis of the clone libraries revealed that 9 of 29 randomly selected clones (**Table S5.1**) belonged to the family *Lachnospiraceae* and fell into a cluster that consists exclusively of clones from the guts of termites and other arthropods, hereafter referred to as the *Arthromitus* cluster (**Figures 5.2 & S5.1**). Most of the remaining clones fell into the radiation of termite-associated clones within the *Bacteroidales* (**Figures 3 & S5.2**). Phylogenetic analysis indicated that most clustered within *Bacteroidales* Termite Cluster V (Ohkuma et al. 2002), a group that also contains many ectosymbionts of termite gut flagellates (Noda et al. 2006; 2009; Desai et al. 2010).

Examination of the *Arthromitus* cluster indicated that it contained

sequences obtained from the gut microbiota of a range of arthropods, including termites from 12 genera, a cockroach, a millipede, and a scarab beetle larva (**Figure 5.2**). The clones from *Reticulitermes* (this study) fell into two subgroups (*Reticulitermes* clusters I and II) together with other clones previously obtained from termites of this genus.

5.4.3 Multiple displacement amplification of single filaments

To further establish the identity of the filaments, we isolated single *Arthromitus* filaments from hindgut homogenates of *R. santonensis* using the same micromanipulation technique as above. To obtain a sufficient amount of template for PCR amplification of the 16S rRNA genes and direct sequencing, we amplified the genomic DNA of single filaments using multiple displacement amplification (MDA). As most *Arthromitus* filaments observed in situ have adherent prokaryotes on their surface (see below), it was not altogether surprising that only two of ten MDA products were sufficiently pure to yield unambiguous 16S rRNA gene sequence reads (**Table S5.1**). The sequences fell into the same subgroups (*Reticulitermes* clusters I and II) within the *Arthromitus* cluster of *Lachnospiraceae* as the clones from the filament suspensions (**Figures 5.2 & S5.1**). The other MDA products yielded ambiguous 16S rRNA gene sequence reads, but did reveal the presence of *Porphyromonadaceae*.

5.4.4 FISH of *Arthromitus*

To confirm that the 16S rRNA genes in the *Arthromitus* cluster indeed originated from the filaments, we completed the full-cycle rRNA approach using FISH. Two probes targeting different regions of the 16S rRNA were specifically designed for this purpose: a general probe (Lachno758) that matched most of the clones in the *Arthromitus* cluster, and a specific probe (Lachno1215) that matched several subgroups within this cluster, including *Reticulitermes* cluster I (**Figure 5.2**).

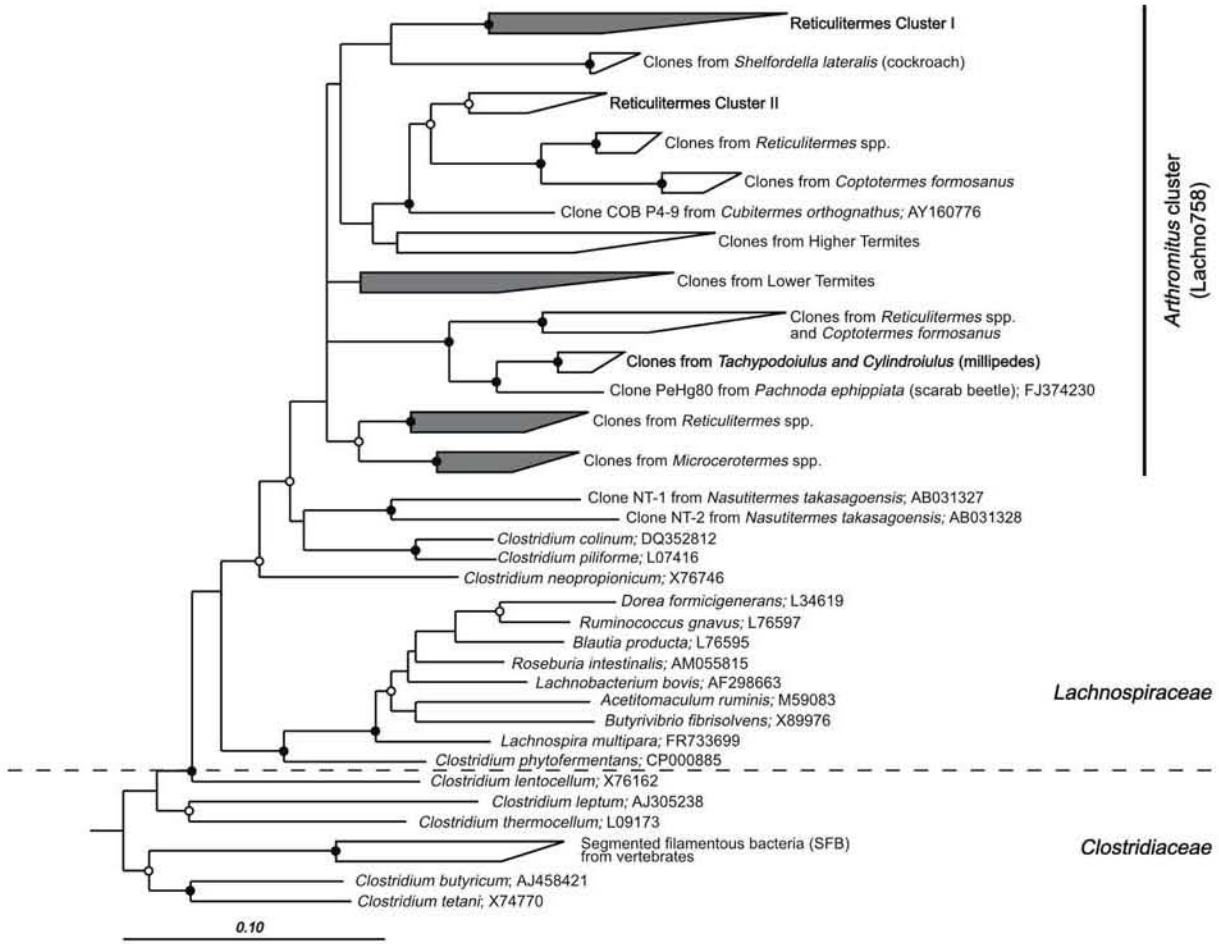

Figure 5.2 | Maximum-likelihood tree outlining the *Arthromitus* cluster, its position within the *Lachnospiraceae*, and its distant relationship to the segmented filamentous bacteria (SFB) in the *Clostridiaceae*. Sequences targeted by probe Lachno758 are indicated by the vertical bar; clusters that are targeted by probe Lachno1215 are shaded in gray. Clusters that contain clones obtained in this study are marked in bold. Circles indicate bootstraps values above 95% (●) and 70% (○).

While the majority of filaments in gut homogenates of *R. santonensis* hybridized with the general Lachno758 probe, only a subset hybridized with the more specific Lachno1215 probe (**Figure 5.4A–D**). All filaments that hybridized with either probe had *Arthromitus*-like morphology.

The surface of *Arthromitus* filaments is known to be colonized by ectosymbiotic bacteria (Leidy, 1881; Margulis et al. 1990). In view of the abundance of *Porphyromonadaceae* in the 16S clone libraries obtained from the filament suspensions, particularly of *Bacteroidales* Cluster V (comprising ectosymbionts of termite gut flagellates), it seemed likely that *Arthromitus* filaments are associated with bacteria from this group. Therefore, we conducted FISH using a previously published oligonucleotide probe (CFB935) that targets most bacteria in the *Bacteroides-Porphyromonas-Prevotella* subgroup, including the clones obtained in this study. In gut homogenates of *R. santonensis*, the probe CFB935 hybridized with bacteria colonizing the surface of the *Arthromitus* filaments (**Figure 5.4E**), which indicated that the *Porphyromonadaceae* sequences in the clone libraries stem from a "contamination" of the filament suspensions by ectosymbionts of *Arthromitus*.

5.4.5 *Arthromitus* in other arthropods

Capillary-picked filament suspensions from the gut of the millipede *Tachypodoiulus niger* yielded sequences affiliated with both the *Arthromitus* cluster and the family *Porphyromonadaceae* (**Table S5.2**). The closest relative of the clones in the *Arthromitus* cluster was a clone previously obtained from the gut microbiota of the millipede *Cylindroiulus fulviceps* (**Figure S5.1**) (Knapp et al. 2010).

The *Arthromitus* filaments observed in the hindguts of the termite *Z. nevadensis* (see above) and the cockroach *Blaberus giganteus* (Feinberg

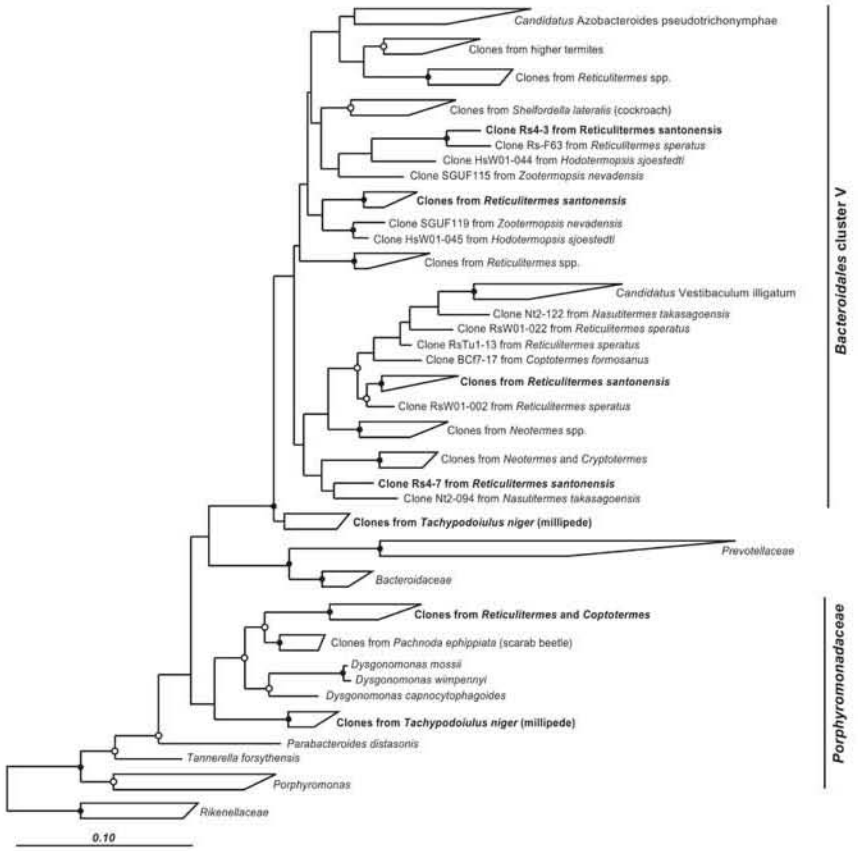

Figure 5.3 | Maximum-likelihood tree outlining the phylogenetic position of putative ectosymbionts of *Arthromitus* filaments within the order *Bacteroidales*. Sequences were obtained from filament suspensions from the termite *Reticulitermes santonensis* (JN653012–23) and the millipede *Tachypodoiulus niger* (JN653034–38). Clones obtained in this study are marked in bold. Circles indicate bootstraps values above 95% (●) and 70% (○). Refer to Figure S5.2 for accession numbers.

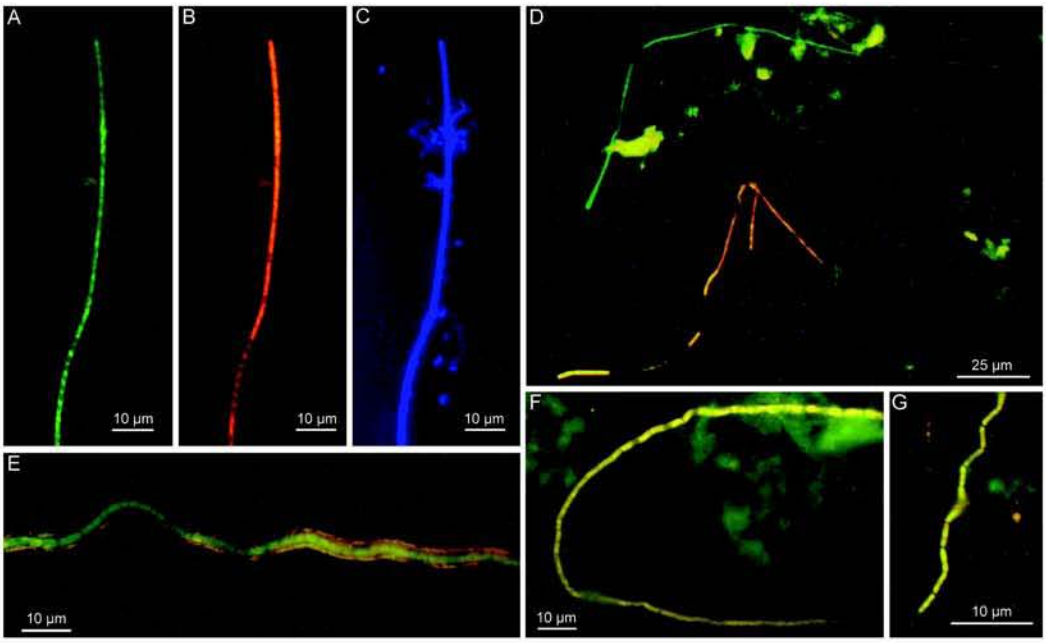

Figure 5.4 | Epifluorescence micrographs of hindgut preparations of the termites *Reticulitermes santonensis* (**A–E**) and *Zootermopsis nevadensis* (**F**), and the cockroach *Blaberus giganteus* (**G**). **A–C**: *Arthromitus* filaments hybridized with probe EUB338 (**A**), probe Lachno758 (**B**), and stained with DAPI (**C**). **D**: Hindgut homogenates hybridized with probes Lachno758 (fluorescein, green) and Lachno1215 (Cy3, red); cells hybridizing with both probes are yellow. **E**: *Arthromitus* filaments with ectosymbionts, hybridized with probes CFB935 (Cy3, red) and EUB338 (fluorescein, green); cells hybridizing with both probes are yellow. **F–G**: Filaments from *Z. nevadensis* (**F**) and *B. giganteus* (**G**) hybridized with probes Lachno758 (Cy3, red) and EUB338 (fluorescein, green); cells hybridizing with both probes are yellow.

et al. 1999) also hybridized with the general probe (Lachno758) for the *Arthromitus* cluster (**Figure 5.4F,G**).

5.4.6 *Arthromitus* is not *Bacillus cereus*

Using the method described by Margulis et al. (1998), we isolated an aerobic, rod-shaped, endospore-forming bacterium from the gut of *R. santonensis*. It did not possess the filamentous morphology typical of *Arthromitus* and had a 16S rRNA sequence (JN653057) that was 99.2%

similar to that of *B. cereus*. An oligonucleotide probe specific for the *B. cereus* group (Bac394) hybridized to this isolate, both in pure culture and after spiking gut homogenates of *R. santonensis*. However, unspiked gut homogenate contained only very few cells that hybridized with this probe. By contrast, none of the numerous *Arthromitus* filaments present in the homogenates hybridized with probe Bac394. We estimated the relative abundance of bacteria in the *Arthromitus* cluster and in the *B. cereus* group using 16S rRNA pyrotag libraries of the bacterial gut microbiota from a wide range of insect species, including cockroaches, termites, and a scarab beetle larva. Each library contained between 6,129 and 25,173 sequences. While the relative abundance of *Arthromitus* sequences ranged from 0.35% to 3% of the sequences in the libraries (**Figure 5.5**), sequences in the *B. cereus* group were completely absent in 7 of the 11 libraries, including those from *Z. nevadensis* and *R. santonensis*, and were just above the detection limit (0.005–0.07%) in the three remaining libraries.

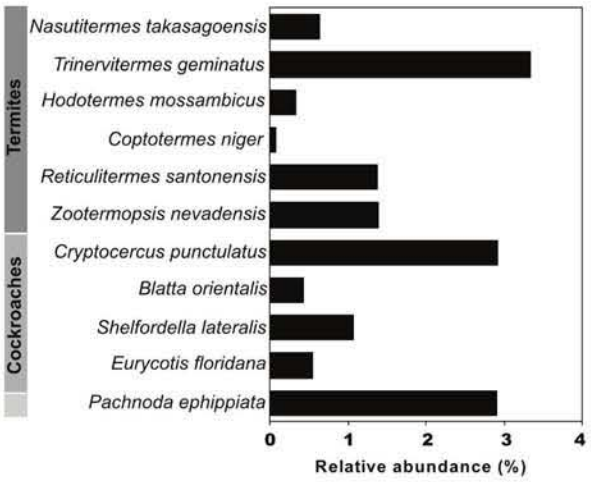

Figure 5.5 | Relative abundance of sequences belonging to the *Arthromitus* cluster in 454 pyrotag libraries of the bacterial community in the guts of termites, cockroaches, and the larva of the scarab beetle *Pachnoda ephippiata*.

5.5 Discussion

Our results indicate that the endospore-forming filaments present in guts of termites, cockroaches, and other arthropods, described by Leidy as members of the genus *Arthromitus* (Leidy 1849; 1881), represent a lineage of uncultivated bacteria within the *Lachnospiraceae*, distantly related to Clostridium piliforme. They are definitely not a life-stage of *Bacillus cereus*, as asserted by Margulis and colleagues (1998). While strains of the ubiquitous, endospore-forming *B. cereus* can be readily isolated from termite gut homogenates (and from almost any natural material) by using the procedures of Margulis et al. (1998), the present study is yet another illustration of the pitfall that may accompany assignment of an identity to cells in situ without some sort of cultivation- and morphology-independent (i.e., molecular) procedure to confirm such an assignment.

Quantification of *Arthromitus* cluster sequences in the 16S-rRNA-based pyrosequencing survey of bacterial diversity in insect guts showed that such sequences are always present, whereas sequences from the *B. cereus* group were not even detectable in most insects, e.g., in *Z. nevadensis* and *R. santonensis*, where *Arthromitus* filaments are readily observed. It seems safe to conclude that *B. cereus* is a minority inhabitant of the termite gut or, more likely, an allochthonous transient. Similarly, we were unable to observe any filaments in that matched Leidy's description of *Arthromitus* in the gut of the sow bug *Porcellio scaber*, although Margulis and colleagues (1998) reportedly isolated *B. cereus* from this habitat. This observation fits with absence of sequences from the *Arthromitus* cluster in a previously published library of 16S rRNA clones from the gut of this species (Lapanje et al. 2010), whose gut wall has been shown to be colonized by bacteria belonging to the phylum Tenericutes (Kostanjsek et al., 2007).

Sequences from the *Arthromitus* cluster have been previously noted as abundant and possibly authochthonous members of the gut microbiota of many termites (Hongoh et al., 2003; Schmitt-Wagner et al., 2003; Hongoh et al., 2005; Tokuda et al., 2000). Some of the sequences in the cluster were obtained specifically from the hindgut wall (Yang et al., 2005; Nakajima et al., 2005), the niche occupied by *Arthromitus*. Our study shows that members of this cluster are present also in other arthropods (**Figures 5.2 & 5.5**) and include many taxa in which *Arthromitus* has been previously described, such as termites of the genera *Coptotermes*, *Reticulitermes*, *Zootermopsis*, and *Incisitermes* (Leidy, 1881; Margulis et al. 1998; Breznak & Pankratz, 1977), blattid cockroaches (Bracke et al. 1979), and millipedes (Leidy, 1849). The presence of the *Arthromitus* cluster in the gut of scarab beetle larvae (Egert et al. 2003) agrees with our observation of *Arthromitus*-like filaments attached to the gut wall of *Pachnoda marginata* (**Figure 5.1l**).

Arthromitus filaments have been observed also in other termites and cockroaches *[Pterotermes occidentis* (To et al. 1980) and *Blaberus gigantus* (Feinberg et al. 1999)], but the gut microbiota of these species remains to be characterized by molecular methods. Notably, the presence of *Arthromitus* seems to be restricted to termites and cockroaches (*Dictyoptera*), scarab beetle larvae (*Scarabaeidae*), and millipedes (*Diplopoda*) – intriguingly, these are the only terrestrial arthropods that produce methane (Hackstein & Stumm 1994; Brune 2010). A better understanding of the basis of this phenomenon may also provide clues to the function of *Arthromitus*.

The morphology of *Arthromitus* filaments differs between host species, and even individual guts usually contain more than one morphotype (**Figure 5.1**) (Leidy 1881; Margulis et al. 1990). A similar situation has been observed for SFB in the mammalian intestine

(Blumershine & Savage, 1978; Phillips et al. 1978), and it has been debated whether these different morphological forms merely constitute different developmental stages of the same species (Blumershine & Savage 1978; Ferguson & Birch-Andersen 1979). The clear host specificity of the clones in the *Arthromitus* cluster and the presence of several phylotypes of *Arthromitus* filaments in the gut of *R. santonensis* (**Figures 5.2 & 5.4**) suggest that multiple species of *Arthromitus* are inhabiting the same ecological niche.

The presence of rod-shaped ectosymbionts on the surface of *Arthromitus* has been already observed by Leidy (1881). Not all filaments are colonized, and the location of the ectosymbionts on filaments and also their mode of attachment vary (Margulis et al. 1990; Breznak & Pankratz 1977; Leadbetter & Breznak 1996). Leadbetter & Breznak (1996) reported that *Arthromitus* filaments in *Reticulitermes flavipes* are methanogens. In our study of *R. santonensis*, we found evidence that the *Arthromitus* filaments are colonized by uncultivated bacteria from the *Bacteroidales* cluster V, which agrees with the report of Nakajima and colleagues (2006) for the filaments in *R. speratus*. Members of *Bacteroidales* cluster V are known to be specific ectosymbionts of gut flagellates (Noda et al. 2006; 2009; Desai et al. 2010).

There are striking similarities between the *Arthromitus* filaments of arthropods and the SFB of vertebrates. They share the segmented morphology of the filaments and the close contact to the gut wall of their respective host (Davis & Savage 1974; Chase & Erlandsen 1974). While the function of *Arthromitus* in arthropod guts remains unknown, SFB are known to play a crucial role in host immune function through the coordination of T cell responses, including the differentiation of T helper (Th17) cells and the induction of immunoglobulin A (Talham et al. 1999; Gaboriau-Routhiau et al. 2009; Ivanov et al. 2009). The close

morphological resemblance of the two groups prompted Snel and colleagues (1995) to group all of these prokaryotes under the collective epithet, "*Candidatus Arthromitus*". However, SFB form a distinct lineage among the *Clostridiaceae*, which is in conflict with the newly established phylogenetic position of *Arthromitus* within the *Lachnospiraceae*.

Revised description of "*Candidatus* Arthromitus"

The genus name "*Arthromitus*" was first used by Leidy to describe prokaryotic filaments in the guts of termites and millipedes (Leidy, 1849; 1881). This name was later applied also to similar bacterial filaments in the gut of vertebrates (i.e., SFB), although this lineage within the *Clostridiaceae* does not contain a single sequence derived from arthropod guts. Since *Arthromitus* and SFB do not form a monophyletic group, we propose to reserve the provisional name "*Candidatus* Arthromitus" for the members of the *Arthromitus* cluster in the *Lachnospiraceae*, which comprises the filaments of arthropods that were originally described by Leidy. For the SFB from vertebrate guts, we propose the provisional name "*Candidatus* Savagella" in honor of the American gut microbiologist Dwayne C. Savage, who was the first to describe this important bacterial group in the ileum of rodents

5.6 References

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Chapter 6

General Discussion

The present study explored the structure of bacterial communities associated with the major microhabitats in the hindguts of higher termites. For all the studies detailed in this thesis, a manually-curated reference database was created that provided a comprehensive coverage of bacterial groups relevant to termite gut research. Phylogenetic trees were constructed and node information from the tree was used to provide a classification scheme wherever required. Since, classification success of pyrosequences clearly depends on the presence of closely-related representative reference sequences (Werner et al. 2012), the database was updated with full-length Sanger sequences from novel termites, whenever possible.

This section provides an overview of the major outcomes from the research described in the thesis. Specific results have been discussed in detail in their respective chapters.

The evolutionary history of termites can be defined by three key events which led to significant shifts in their community structure: 1) The acquisition of flagellates by the ancestor of all termites (Nalepa et al. 2001); 2) The loss of flagellates in the higher termites (Brune & Ohkuma 2011); 3) The dietary diversification in higher termites (Donovan et al. 2001).

6.1 Flagellates as microhabitats in lower termites

It is clear that in the hindgut of lower termites, the flagellates are in charge of most of the niches, and act as a major microhabitat for prokaryotes inhabiting the gut (Brune & Ohkuma 2011). This mutualism that is based on the dietary dependence of lower termites on flagellates have produced a highly complex tripartite-symbiosis between the termite,

the flagellates and the bacterial community (Brune & Stingl 2006). This degree of mutualism in many lower termites has caused a majority of the community to be associated with flagellates, as ectosymbionts or as endosymbionts (Ohkuma 2008). Moreover, the bacterial community structure in lower termites can be significantly altered through starch feeding caused by changes in flagellate community structure (Ikeda-Ohtsubo et al. 2010). These reasons make flagellates a crucial determinant of bacterial community structure in lower termites. A certain degree of 'phylogenetic continuity' between generations (Nalepa et al. 2001) is ensured by the vertical transmission of symbiotic flagellates (and their bacterial and archaeal symbionts) through trophallaxis. This is reflected in the high degree of congruence observed between “host-symbiont” phylogenies (Ikeda-Ohtsubo & Brune 2009; Desai et al. 2009), and dominant coevolving bacterial lineages will significantly contribute to an overall pattern of coevolution of the gut bacterial community with its lower termite host.

The loss of the flagellate population in the higher termites marks the second major event in termite evolution (Engel et al. 2009) that saw the loss of a major microhabitat, and also the extinction of the flagellate-associated prokaryotic symbionts. This loss of lineages is illustrated by the low abundance of the *Endomicrobia* in most higher termites, in comparison to the lower termite *Reticulitermes santonensis* (**Figure 2.6; Chapter 2**).

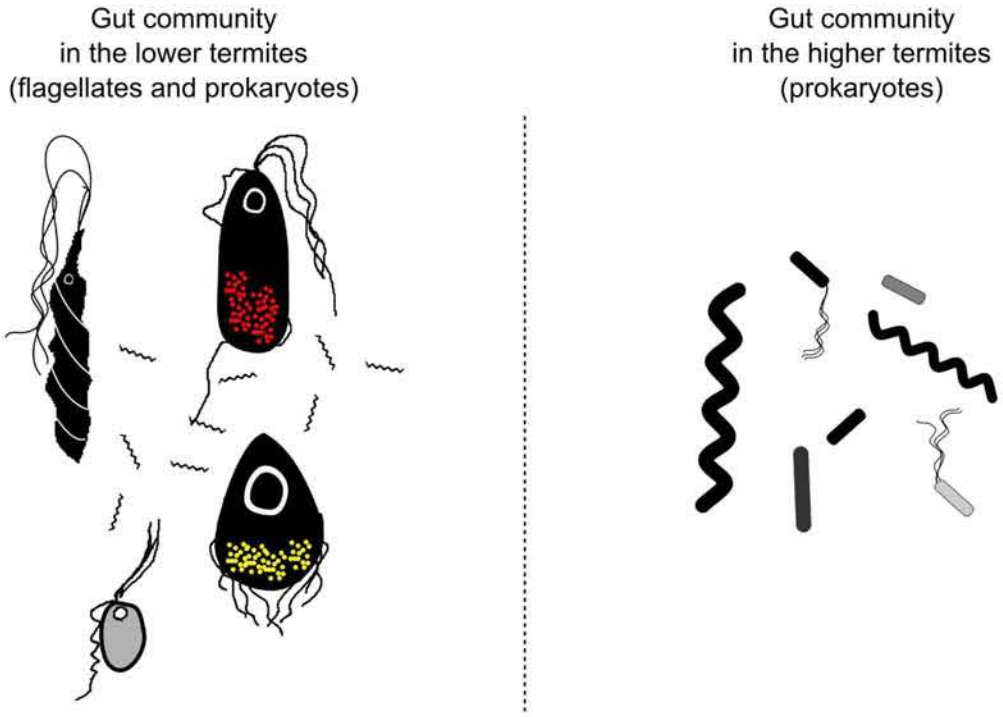

Figure 6.1 | The difference in community structure of the gut microbiota in lower termites and higher termites

This loss of flagellates also created new opportunities for bacterial lineages to fill niches left behind by the flagellates e.g. members of the phyla *Fibrobacteres, Spirochaetes* and TG3 (**Figure 2.6; Chapter 2**). This period also featured other biological and physiological changes on the host side including an increase in dietary diversification and gut compartmentalization in more recent higher-termite-subfamilies such as the *Termitinae* and the *Nasutitermitinae* (Noirot 2001).

6.2 Diet and phylogeny as determinants of community structure

Due to the metabolic co-dependency between higher termites and their symbionts, their continued mutualistic relationship must be ensured

by vertical transmission of gut microbiota from generation to generation through a process called proctodeal trophallaxis (Nalepa et al. 2001) (evidenced by the detection of hindgut bacterial lineages in the crop of *Nasutitermes corniger* (Köhler et al. 2012)). In some very strict mutualisms, involving fewer partners, perfect congruence between host-client can be observed (Desai et al. 2009; Moran et al. 2008). However, the evolution of an entire community is governed by numerous multi-specific interactions, where a simple congruency in the relationship between the host and the gut community may not be demonstrable. Some of the factors that affect the co-diversification of a gut community with the host have been collectively dubbed by authors as “legacy effects” (Rawls et al. 2006; Ley et al. 2008), and include the effect of host phylogeny and environment. These “legacy effects” when combined with “gut habitat effects” (that include factors related to physiology, gut anatomy, diet composition etc.) (Rawls et al. 2006) can further affect the pattern of co-diversification between the host and the gut community.

In order to differentiate between the relative contribution of these effects, we examined the community structures of nine higher termites from different phylogenetic groups and possessing different dietary specializations (**Chapter 2**).

Among the major subfamilies of higher termites, the *Macrotermitnae* form the most phylogenetically basal group and are characterized by a mutualistic relationship with a fungus (*Termitomyces* spp.) (Brune & Ohkuma 2011). Fungal hyphae are grown in 'fungal combs' that essentially consist of fecal matter from the termites, where foraged wood, grass and litter is broken down (Brune & Ohkuma 2011). *Odontotermes* species are largely wood-foragers who also maintain fungal combs, but consume the fungi as a source of nourishment (Hyodo et al. 2003). On the other hand, members of *Macrotermes* do not consume the

fungus, and instead use them solely to predigest the lignocellulosic material to increase the efficiency of cellulose digestion (Hyodo et al. 2003). In our study (**Chapter 2**), these dietary differences appeared to be reflected in the distribution of bacterial lineages between *Macrotermes* spp. and *Odontotermes* sp. Additionally, *Odontotermes* sp. harboured higher abundances of *Fibrobacteres*, TG3 and *Treponema* Ic, in higher than *Macrotermes* spp. This potential diet-related shift in the bacterial community could be in response to the presence of undigested wood particles in the gut of *Odontotermes* sp.

In comparison to *Macrotermitinae,* the subfamilies *Termitinae* and *Nasutitermitinae* are more diverse in dietary specializations. In our study (**Chapter 2**), wood-feeding *Microcerotermes* sp. (family *Termitinae)* and *N. corniger* (family *Nasutitermitinae*) showed an almost identical community composition at the phylum-level (**Figure 6.2**) that primarily included *Fibrobacteres*, TG3 and *Spirochaetes* [previously reported in *Nasutitermes* spp. (Hongoh et al. 2006; Köhler et al. 2012) as well as *Microcerotermes* (Hongoh et al. 2006)]. Metagenomic evidence suggests that *Fibrobacteres* and *Spirochaetes* may be playing an important role in cellulose digestion (Warnecke et al. 2007). Moreover, the abundance of these phyla in higher termites that have independently evolved the wood-feeding behaviour (Donovan et al. 2001), indicates a diet-based distribution.

The effect of host phylogeny, however, was visible only upon analysis at more resolved taxonomic levels. 16S rRNA clone libraries from *Trinervitermes* sp. (**Chapter 2**) confirmed that most of the clones showed a phylogenetic affiliation to other clones from *Nasutitermitinae*.

Furthermore, classification analysis (**Chapter 2**) revealed that pyrosequences from all *Nasutitermitinae* showed a preferential

association with the cluster “Termite Cluster I” (subphylum 1 of TG3) (**Figure 2.6; Chapter 2**). On the other hand, *Microcerotermes* sp. (subfamily *Termitinae*) shows a specific enrichment of “Termite Cluster II”. It is important to note that this association was not exclusive, as both *N. takasagoensis* and *Microcerotermes* sp. showed the presence of both Termite Clusters. Since both *Microcerotermes* sp. and *N. takasagoensis* belong to the same diet group, it is unlikely to be an effect of diet. Furthermore, the detection of Termite Cluster II also in *Ophiotermes* sp. (subfamily *Termitinae),* further suggests this to be signal of host phylogeny.

The phylogenetic clustering of OTUs from *Treponema* Ia (**Supplementary figure S2.3**) derived from phylogenetically related termites indicates that the imprint of host phylogeny can be seen even beyond the genus-level.

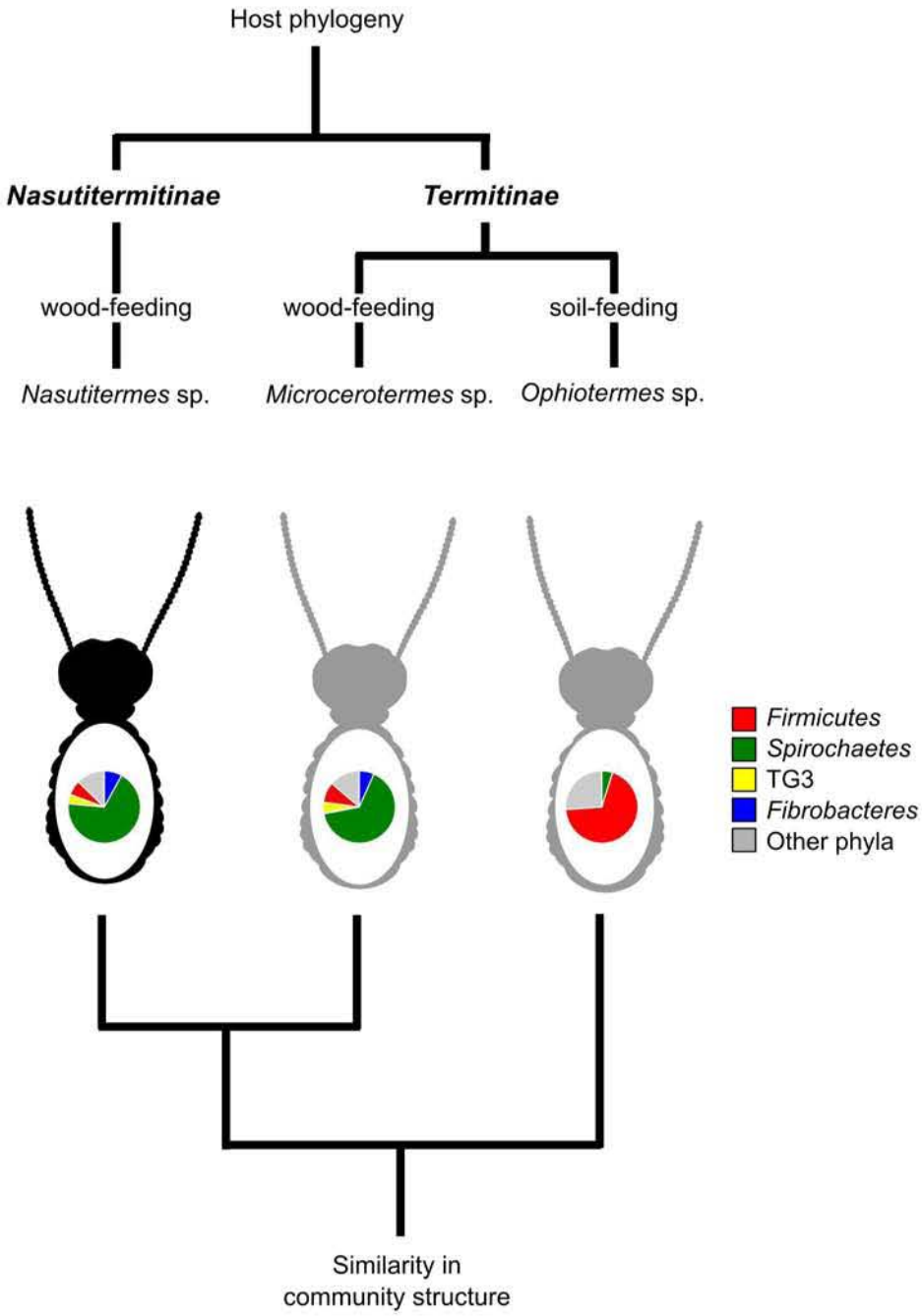

Figure 6.2 | Phylum-level similarities observed in community structure between wood-feeding termites, *Microcerotermes* sp. and *Nasutitermes* sp., in spite of belonging to different subfamilies.

6.3 Gut alkalinity as a determinant of community structure

In case of the advanced subfamilies of higher termites such as the *Termitinae* and *Nasutitermitinae*, nutritional diversification appears to have been accompanied by hindgut compartmentalization (Noirot 2001) and an increase in gut alkalinity (Bignell & Eggleton 1995).

Mildly alkaline values can be encountered in the *Nasutitermes* spp. (Brune et al. 1995; Köhler et al. 2012), but the highest pH values are found in the guts of soil-feeding members in the *Termitinae* (Brune & Kühl 1996; Bignell & Eggleton 1995). Although the reason for this elevation in gut pH of these soil-feeders is unknown, it appears to be a phylogenetic trait for the *Cubitermes* group. (subfamily *Termitinae),* because neither phylogenetically-related wood-feeders (Brune et al. 1995; Bignell & Eggleton 1995) nor soil-feeding *Nasutitermitinae* (Bignell & Eggleton 1995) show such high pH values.

Comparison of gut community structure in the current study clearly shows major differences at the phylum-level between the alkaline gut regions of the soil-feeders and wood-feeders of *Termitinae* (**Figure 6.3; Figure 3.1, Chapter 2**). Both soil-feeding *Termitinae* members showed a generally higher abundance of *Firmicutes*. This selective enrichment has been previously observed for other *Cubitermes* spp. (Schmitt-Wagner et al. 2003). Interestingly, although the whole hindgut of wood-feeding *Nasutitermitinae* members showed a low abundance of these groups (**Chapter 2**), their respective alkaline gut compartments showed an enrichment of the same lineages observed in the *Cubitermes* spp. (**Chapter 3**; Köhler et al. 2012).

Most interesting of these lineages was '*Candidatus* Arthromitus', a

segmented filamentous organism that is found attached to the gut wall of many arthropods (**Chapter 5**). Phylogenetic analysis of OTUs clearly revealed that this genus contributes significantly to the overall phylogenetic similarity observed among homologous gut compartments (**Figures 3.5 and 3.6, Chapter 3**).

Interestingly, the distribution of *Firmicutes* in the alkaline P1 compartments of the *Nasutitermitinae* resembles the distribution observed in the alkaline P3 compartment of the *Termitinae.* It has also been observed that the alkaline gut sections in other higher termites (Thongaram et al. 2005) share many phylogenetically related clostridial lineages [including '*Candidatus* Arthromitus' (**Figure 5.5, Chapter 5**)] with other alkaline gut environments from phylogenetically unrelated insects such as *Pachnoda* beetle larva midgut (Egert et al. 2003). Moreover, it has been observed that the alkalinity in the anterior gut coincides significantly with a drop in the density of bacteria in *Cubitermes* spp. (Schmitt-Wagner et al. 2003) as well as *N. corniger*.(Köhler et al. 2012)

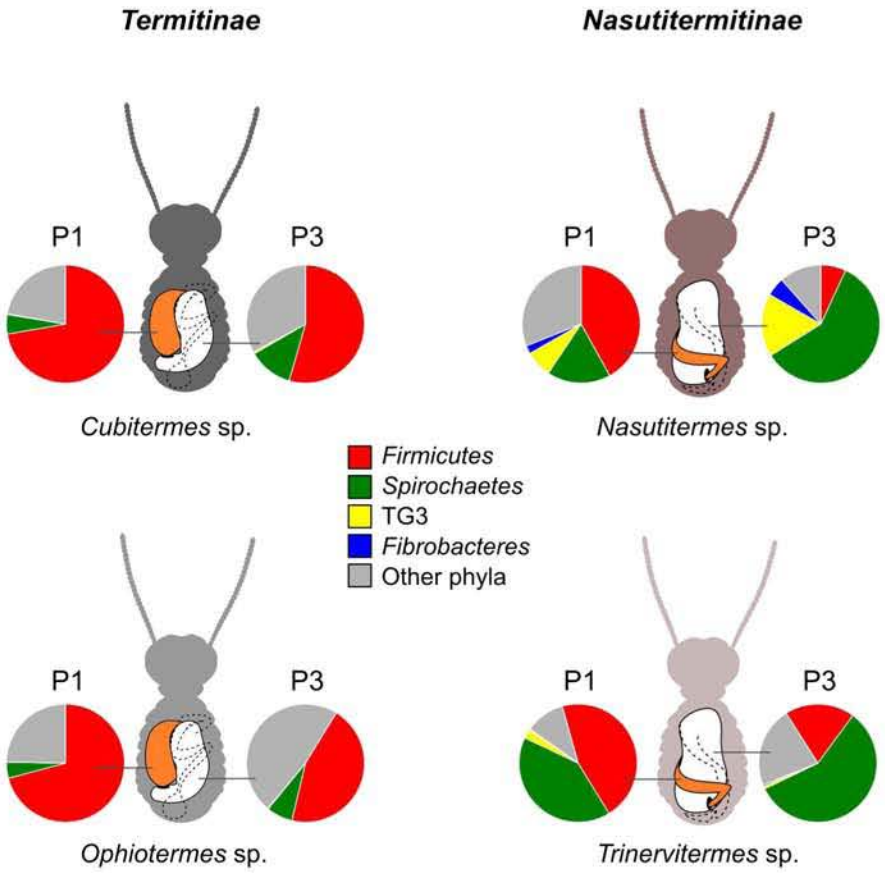

Figure 6.3 | Community structure at phylum-level for the P1 and P3 hindgut compartments in two soil-feeding termites and two wood-feeding higher termites

It has been shown in soil environments, that pH can significantly impact community structure at the phylum-level (Lauber et al. 2009). Our analysis strongly suggests that pH could have a similar effect as a strong structuring agent of communities in the alkaline regions of the hindgut in higher termites. This could explain the general drop in bacterial density, and an increase in the relative abundance of putatively alkali-tolerant/alkaliphilic lineages.

6.4 Wood fibers as a microhabitat and the role of treponemes in higher termites

In case of the wood-feeding high termites, a diet of wood can directly influence community structure through the development of a fiber-associated community (**Chapter 2; Chapter 4**). The results of the community analysis of the fiber-fraction and the measurements of cellulase activity shed new light on our understanding of how an alternative mechanism of cellulose digestion may have evolved in the hindgut of higher termites.

In the wood-feeding *Nasutitermitinae* (represented by *N. corniger* and *N. takasagoensis*) the bacterial phyla closely associated with the wood fibers include the *Fibrobacteres*, TG3 and the *Spirochaetes* (**Figure 6.4; Figure 4.6, Chapter 4**). The augmentations made to the classification scheme in the reference database (**Table 2.3, Chapter 2**) significantly improved our ability to identify and discern between critical bacterial groups, which were highly abundant in wood-feeding higher termites. The detection of TG3 in *Reticulitermes santonensis* for instance, shows that some of the lineages we found to be abundant in higher wood-feeding termites, were present already in the ancestor of higher termites, and not likely to have been gained from the environment.

Most of the *Spirochaetes* observed in the termites belong to the *Treponema* I lineage (**Chapter 2**) (Ohkuma et al. 1999). We observed distinct patterns in the distribution of genus-level clades within *Treponema* I. The groups relevant to our discussion include Treponema Ia, Ic and If (**Chapter 2**).

Spirochaetes have been implicated to be the major organisms involved in reductive homoacetogenesis in lower termite guts (Pester &

Brune 2006). Phylogenetic analysis showed that most of the *Treponema* I sequences from lower termites fell into the *Treponema* Ia cluster. Additionally, *Treponema* Ia have also been shown to be associated with flagellates (Noda et al. 2003; Yang et al. 2005). Additionally, majority of the isolates from the termite gut also cluster in *Treponema* Ia. The isolates include the homoacetogenic isolate *Treponema primitia* (Graber & Breznak 2004; Graber et al. 2004), the non-homoacetogenic, nitrogen-fixing isolate *Treponema azotonutricium* (Graber et al. 2004) and the non-homoacetogenic *Treponema isoptericolens* (Dröge et al. 2008). Although none of the isolates from *Treponema* Ia are capable of utilizing cellulose as sole carbon source, *Treponema isoptericolens* and *Treponema azotonutricium* possess the ability to utilize the disaccharide cellobiose (Dröge et al. 2008). This points a significant functional variation within the cluster.

It has been suggested that this rise in the diversity of spirochetes in the higher termites, could be an ecological response to fill niches vacated by flagellates in the ancestors of the higher termites (Ballor & Leadbetter 2012). Genus-level analysis of the pyrosequencing data from higher termites suggests that although *Treponema* Ia was consistently associated with all termites (**Figure 2.6, Chapter 2**), clusters Ic and If were observed to be specifically enriched in lignocellulose-feeding higher termites, indicative of a diet-related enrichment of these clades. This point is further illustrated by the low abundance of these lineages in soil-feeders (**Figure 2.6, Chapter 2**).

Without an isolate, it would be difficult to address the exact role played by the *Treponema* Ic and If in the fiber-associated community. Cellulose digestion is a complex process and efficient hydrolysis in insect guts require the joint action of enzymes with different activities (Watanabe & Tokuda 2010).

In metagenomic studies many cellulase genes (belonging to, GHF 1, 2, 3, 5 and 13) could be assigned to the *Spirochaetes* (by taxonomic binning) (Warnecke et al. 2007; Burnum et al. 2011). This suggests that they may have a role in the breakdown of cellulose in higher termites. In our analysis, *Treponema* Ia was observed to be associated only with the fiber-free fraction, while *Treponema* Ic and If were found to be dominant groups in both the fiber-fraction and the fiber-free fraction (**Figure 4.6, Chapter 4**). Both fractions were also associated with considerable amounts of cellulase activity (**Figure 4.3, Chapter 4**). This lends further support to the argument for their possible involvement in breakdown of cellulose.

The interactions between fiber-associated bacteria in ruminants has been studied to a much greater depth, and it has been shown that non-cellulolytic bacteria contribute greatly to the function of fiber digestion. The interactions between the cellulolytic and non-cellulolytic bacteria could involve cross-feeding of fermentation products, oligomers/monomers derived from cellulose or even maintenance of suitable conditions (such as pH) for the cellulolytic bacteria (See Flint 1997; Koike & Kobayashi 2009, and references within). For instance, it has been shown that non-cellulolytic fiber-associated *Treponema bryantii* has a pronounced effect on cellulose digestion when co-cultured with cellulolytic *Fibrobacter succinogenes* (Kudo et al. 1987). These studies suggest that a member does not necessarily have to be cellulolytic to be a part of an important fiber-associated community and a similar role could also be played by the treponemes in the higher termite gut.

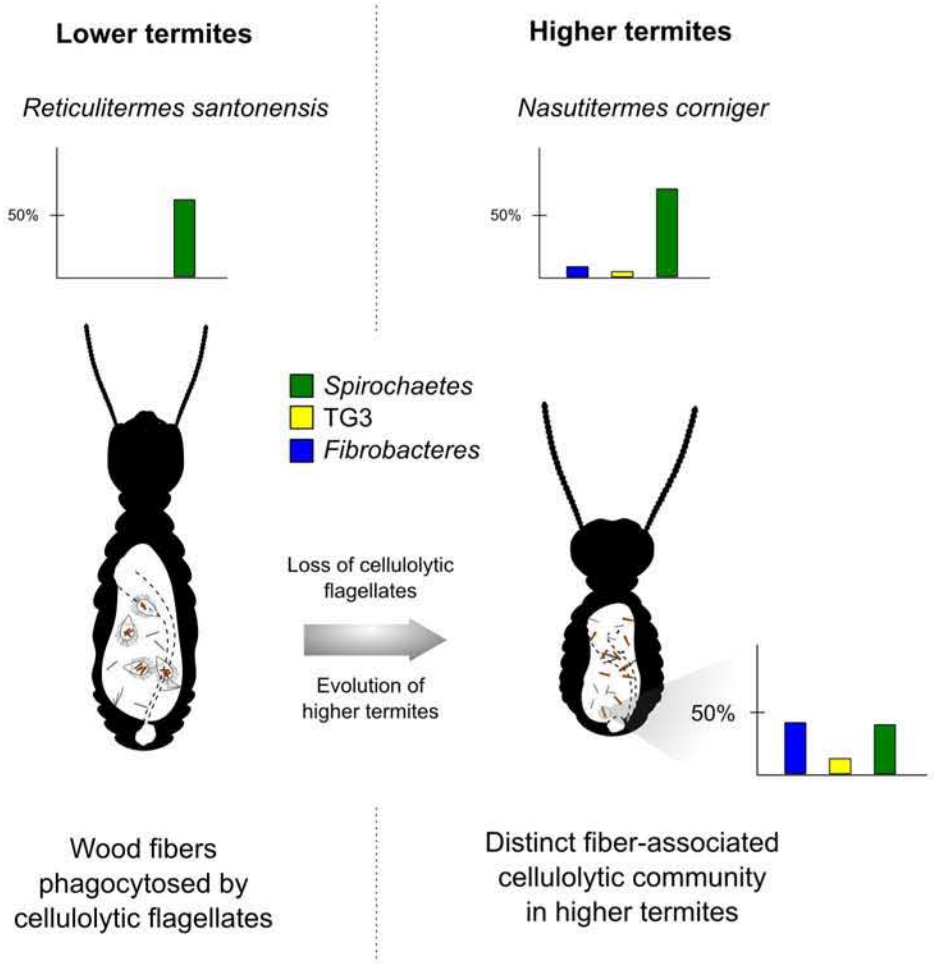

Figure 6.4 | A scheme depicting the rise in the abundance of fiber-associated bacterial phyla in higher wood-feeding termites after the loss of flagellates

6.5 Concluding remarks and future perspectives

The evolution and radiation of the higher termites, following the loss of flagellates, points to an important period in their evolution, marked by extensive dietary diversification. This diversity in higher termites is reflected in the variation observed in hindgut structure. Morphological and physicochemical differences in the gut, have resulted in an extremely structured environment with many qualitatively distinct microhabitats.

Understanding the structure and function of the communities associated with each of the microhabitats, is crucial to fully appreciate the complexity of bacterial communities in the higher termite gut.

The present study was designed to shed light on some of the forces that could have contributed to shaping community structure in the higher termites. Our study demonstrates that gut communities co-diversify with the host in response to many factors that contribute to major changes in the gut environment. The community changes at the microhabitat-level contribute to the differences observed between whole gut communities.

In summary, we studied some of the major microhabitats in the guts of higher termites. Based on our results, we have discussed the importance of host phylogeny, gut alkalinity and diet as driving forces for defining community structure. However, many questions still remain unanswered. For instance, although the major subfamilies of higher termites were covered in the study, almost nothing is know about the bacterial or archaeal community structure in the soil-feeders in the subfamily *Apicotermitinae* and *Nasutitermitinae*. A detailed survey of the gut microbial communities of these subfamilies, may even answer questions on the independent acquisition of the same feeding strategies in multiple lineages of termites.

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Supplementary Material

Supplementary tables

Table S2.1 | Summary statistics of pyrosequencing libraries constructed for the higher termites

Termite	Gut section	Number of reads after quality filtration	OTUs (at 97% similarity)	Classification at phylum level (%)	Classification at order level (%)	Classification at genus level (%)
Macrotermes sp.	whole gut	2285	576	99.8	85.5	71.6
Macrotermes bellicosus	hindgut	9670	1384	99.4	89.6	79.4
Odontotermes sp.	whole gut	4623	1079	98.9	91.7	80.6
Cubitermes ugandensis	hindgut	1642	860	98.6	95.6	71.1
Ophitoermes sp.	hindgut	7136	1563	99.8	95.7	75.3
Microcerotermes sp.	whole gut	28172	3570	99.2	97.2	91.5
Nasutitermes corniger	hindgut	2986	625	99.0	96.4	93.0
Nasutitermes takasagoensis	hindgut	13901	2739	98.9	96.6	90.8
Trinervitermes sp.	hindgut	21509	3179	99.8	98.3	91.7

Table S3.1 | Summary statistics of pyrosequencing libraries constructed for the higher termite gut compartments

Termite	Gut section	Number of reads after quality filtration	OTUs (at 97% similarity)	Classification at phylum level (%)	Classification at order level (%)	Classification at genus level (%)
C. ugandensis	P1	4498	1276	99.2	96.3	82.7
	P3	27575	6000	99.6	96.6	72.7
	P4	9486	2162	99.7	96.0	72.9
	P5	17309	977	99.6	96.1	85.4
Ophiotermes sp.	P1	4244	847	96.7	92.3	74.5
	P3	30700	4400	99.4	96.4	63.8
	P4	7738	847	99.5	96.5	51.4
	P5	12929	1145	99.0	96.1	60.2
Trinervitermes sp.	P1	9980	1179	99.7	97.5	80.6
	P3	10709	1369	99.8	98.1	91.3
	P45	2570	751	99.7	96.3	85.3
N. corniger	P1	8379	1414	99.3	91.5	70.4
	P3	24029	3437	99.3	97.4	93.6
	P4	25957	3713	99.1	90.0	76.1
	P5	3270	798	99.0	89.2	76.2
Amitermes sp.	P1	10679	1238	99.6	97.4	78.6
	P3	16684	2075	99.4	97.5	77.4
	P4	14989	1954	99.1	92.0	78.1
	P5	20805	2322	99.6	89.2	72.4

Table S4.1 | Summary statistics of pyrosequencing libraries constructed from the fractionation of P3 fluids from *N. corniger* and *N. takasaogensis*

	Sample	Replicate	Number of reads after quality filtration	OTUs (at 97% similarity)	Classification at phylum level (%)	Classification at order level (%)	Classification at genus level (%)
N. corniger	P3 fluid	1	4998	496	99.4	97.4	94.7
		2	1773	312	99.1	96.6	93.0
		3	904	180	99.1	97.8	91.7
	Fiber-free	1	29630	915	99.8	99.4	97.4
		2	6506	324	99.9	99.5	97.3
		3	4947	227	100	99.5	97.6
	Fiber	1	11261	607	99.8	99.3	96.9
		2	11930	286	99.9	99.7	96.3
		3	11067	312	99.8	99.6	97.4
N. takasagoensis	P3 fluid	1	4110	1362	98.1	95.5	87.6
		2	7474	1926	98.3	95.7	87.5
		3	7439	2133	97.9	94.8	85.9
	Fiber-free	1	8722	2188	98.4	96	87.5
		2	7491	1580	98.6	96.9	89.7
		3	5788	1375	98.7	96.4	88.0
	Fiber	1	4561	1162	98.4	96.1	88.2
		2	1976	636	98.2	96	87.7
		3	10208	2273	98.2	96	88.8

Table S5.1 | Sequences of 16S rRNA genes obtained from clone libraries of capillary picked *Arthromitus* filaments and after multiple-displacement amplification (MDA) of single filaments from the hindgut of *Reticulitermes santonensis*.

Clone library	Clone number	Taxonomic affiliation	Accession number
Clone library 1	Rs1-1	*Spirochaetaceae*	JN653024
	Rs1-2	*Porphyromonadaceae*	JN653012
	Rs1-3	*Porphyromonadaceae*	JN653013
	Rs1-4	*Lachnospiraceae*	JN653028
Clone library 2	Rs2-1	*Ruminococcaceae*	JN653025
	Rs2-2	*Lachnospiraceae*	JN653003
	Rs2-3	*Porphyromonadaceae*	JN653014
	Rs2-4	*Lachnospiraceae*	JN653004
	Rs2-5	*Porphyromonadaceae*	JN653015
Clone library 3	Rs3-1	*Lachnospiraceae*	JN653005
	Rs3-2	*Lachnospiraceae*	JN653006
	Rs3-3	*Lachnospiraceae*	JN653029
	Rs3-4	*Spirochaetaceae*	JN653026
	Rs3-5	*Porphyromonadaceae*	JN653016
	Rs3-6	*Bacteroidetes*	JN653027
	Rs3-7	*Lachnospiraceae*	JN653030
	Rs3-8	*Lachnospiraceae*	JN653007
	Rs3-9	*Lachnospiraceae*	JN653031
	Rs3-10	*Lachnospiraceae*	JN653008
Clone library 4	Rs4-1	*Porphyromonadaceae*	JN653017
	Rs4-2	*Lachnospiraceae*	JN653009
	Rs4-3	*Porphyromonadaceae*	JN653018
	Rs4-4	*Porphyromonadaceae*	JN653019
	Rs4-5	*Porphyromonadaceae*	JN653020
	Rs4-6	*Porphyromonadaceae*	JN653021
	Rs4-7	*Porphyromonadaceae*	JN653022
	Rs4-8	*Lachnospiraceae*	JN653010
	Rs4-9	*Porphyromonadaceae*	JN653023
	Rs4-10	*Lachnospiraceae*	JN653011
Single filaments	MDA1	*Lachnospiraceae*	JN653001
	MDA2	*Lachnospiraceae*	JN653002

Table S5.2 | Clone library of 16S rRNA genes obtained from capillary picked *Arthromitus* filaments from the gut of *Tachypodoiulus niger*.

Clone number	Taxonomic affiliation	Accession number
M1-4	*Lachnospiraceae*	JN653032
M1-6	*Clostridiaceae*	JN653039
M1-7	*Porphyromonadaceae*	JN653034
M1-9	*Rhizobiales*	JN653040
M1-10	*Ruminococcaceae*	JN653041
M1-18	*Clostridiaceae*	JN653042
M1-19	*Enterobacteriaceae*	JN653043
M1-21	*Lachnospiraceae*	JN653044
M1-24	*Bacteroidaceae*	JN653045
M1-26	*Clostridiaceae*	JN653046
M1-27	*Bacteroidaceae*	JN653047
M1-28	*Desulfovibrionaceae*	JN653048
M1-29	*Clostridiaceae*	JN653049
M1-30	*Porphyromonadaceae*	JN653035
M1-31	*Clostridiaceae*	JN653050
M1-32	*Lachnospiraceae*	JN653051
M1-34	*Veillonellaceae*	JN653052
M1-35	*Clostridiaceae*	JN653053
M1-36	*Porphyromonadaceae*	JN653036
M1-37	*Rhizobiales*	JN653054
M1-40	*Lachnospiraceae*	JN653033
M1-41	*Verrucomicrobia*	JN653055
M1-42	*Porphyromonadaceae*	JN653037
M1-43	*Porphyromonadaceae*	JN653038
M1-45	*Desulfovibrionaceae*	JN653056

Supplementary figures

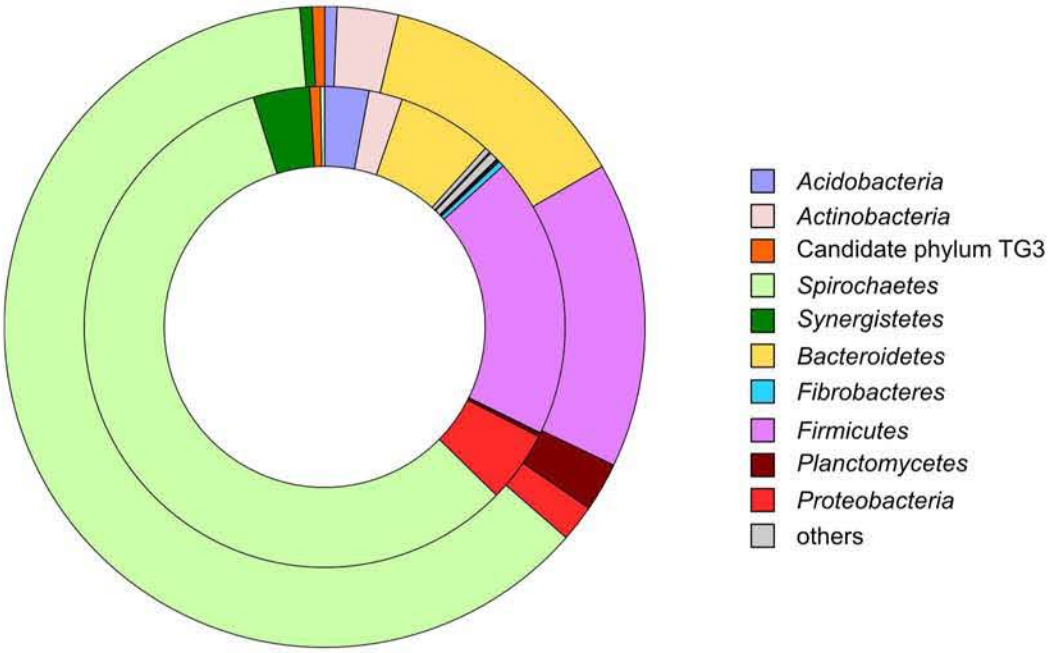

Figure S2.1 | Distribution of major phyla in the 16S rRNA clone library (inner circle) and Pyrosequencing library (outer circle) constructed from the P3 compartment of *Trinervitermes* sp.

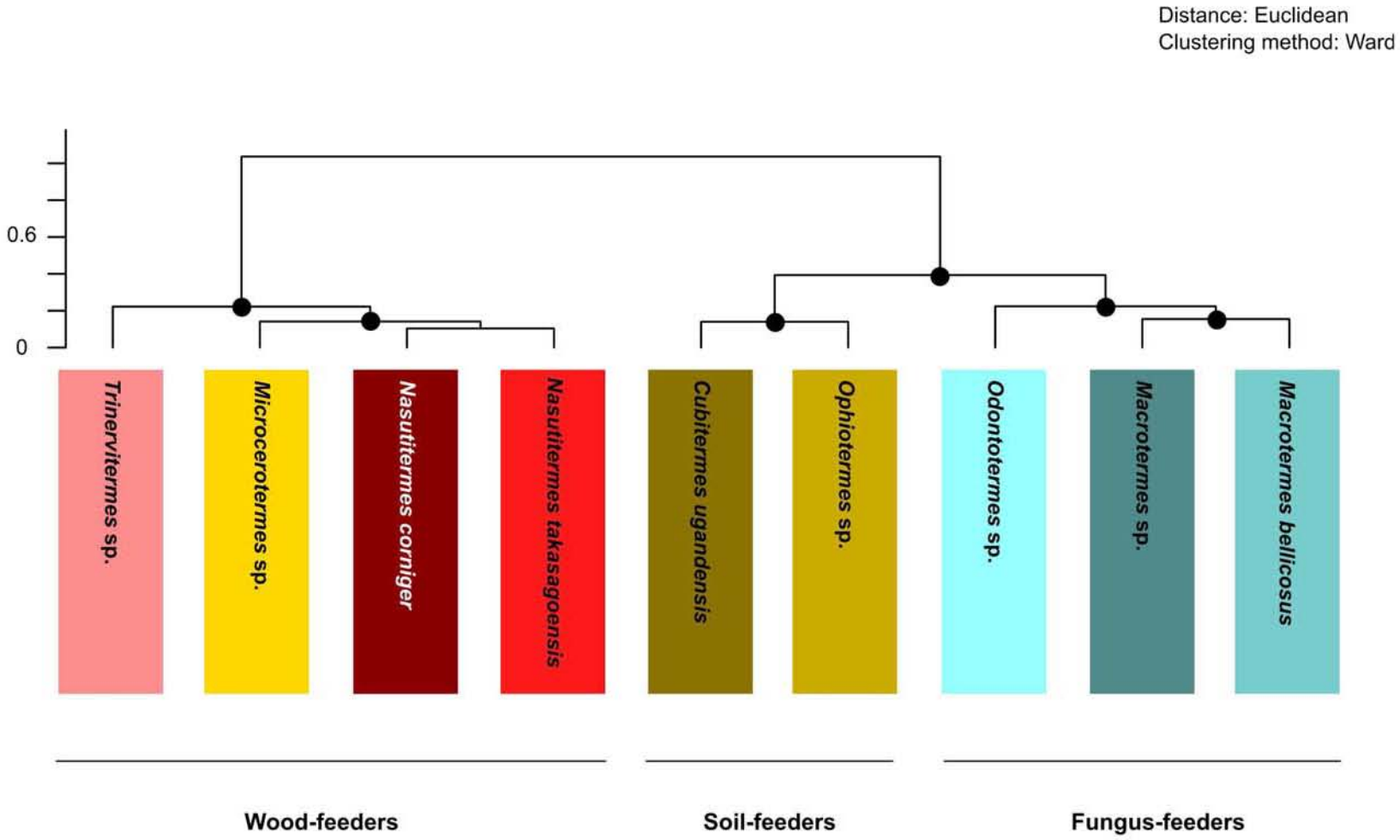

Figure S2.2 | Hierarchical cluster analysis of (weighted) Unifrac distances among the hindgut bacterial communities from higher termties. Internal nodes represent confidence values (AU p-values) of greater than 85% (●) and 70% (○); See Figure S2.3 for a more detailed colour code.

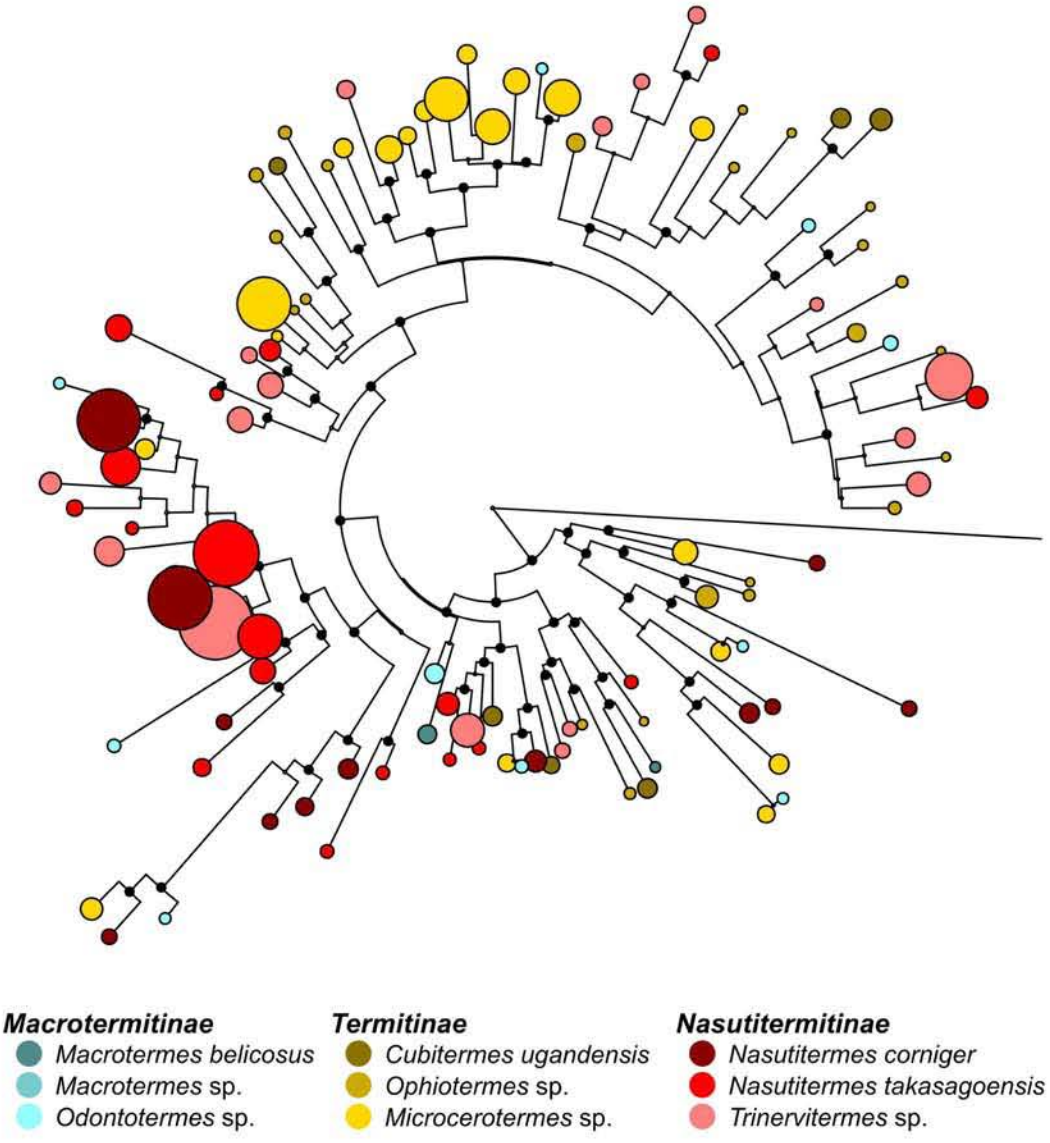

Figure S2.3 | Maximum Likelihood tree showing the phylogenetic relatedness of OTUs (clustered at 95% similarity) from Treponema Ia. Internal nodes represent bootstrap values of greater than 85% (●).

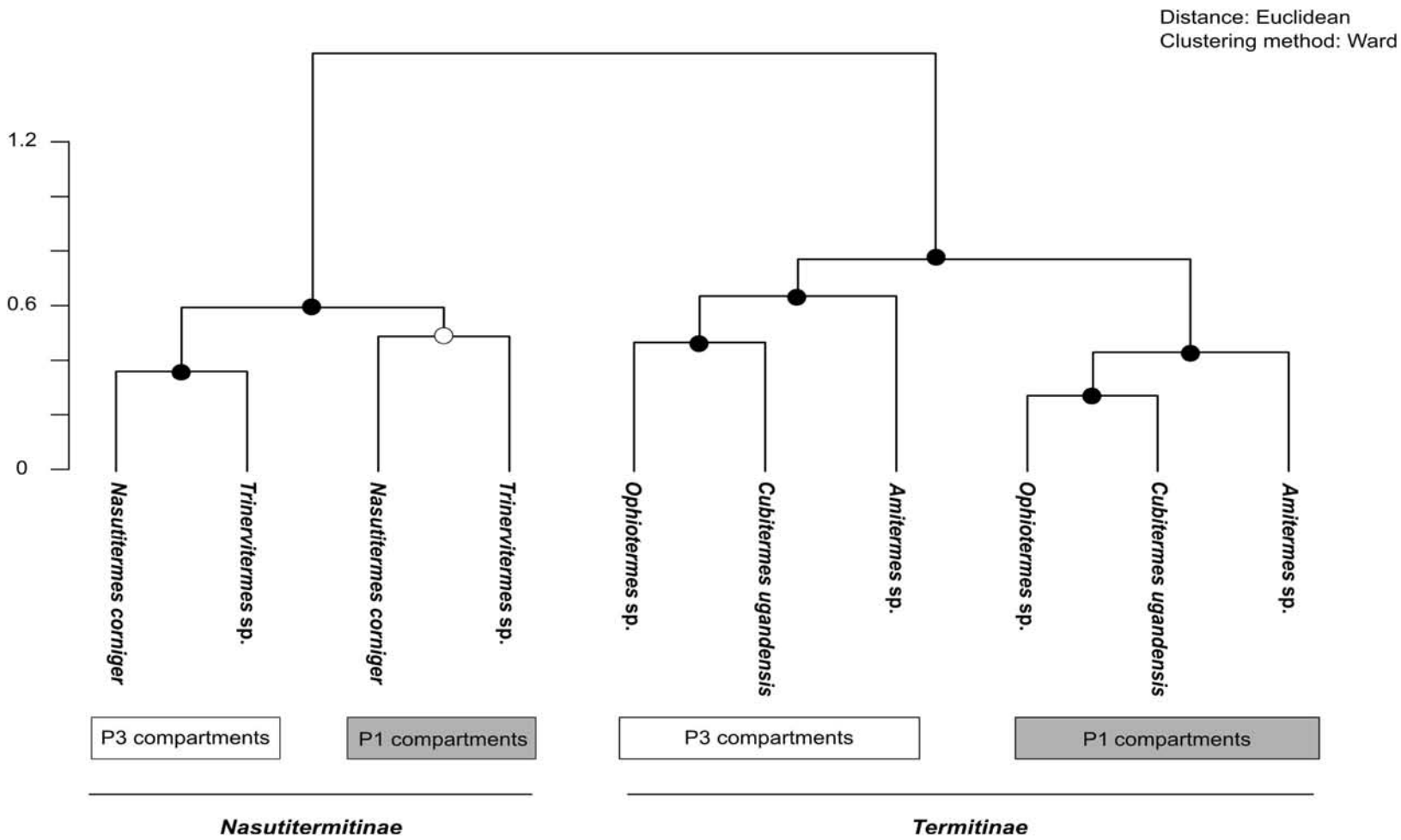

Figure S3.1 | Hierarchical cluster analysis of (weighted) Unifrac distances among the bacterial communities from different higher termite hindgut compartments. Internal nodes represent confidence values (AU p-values) of greater than 85% (●) and 70% (○).

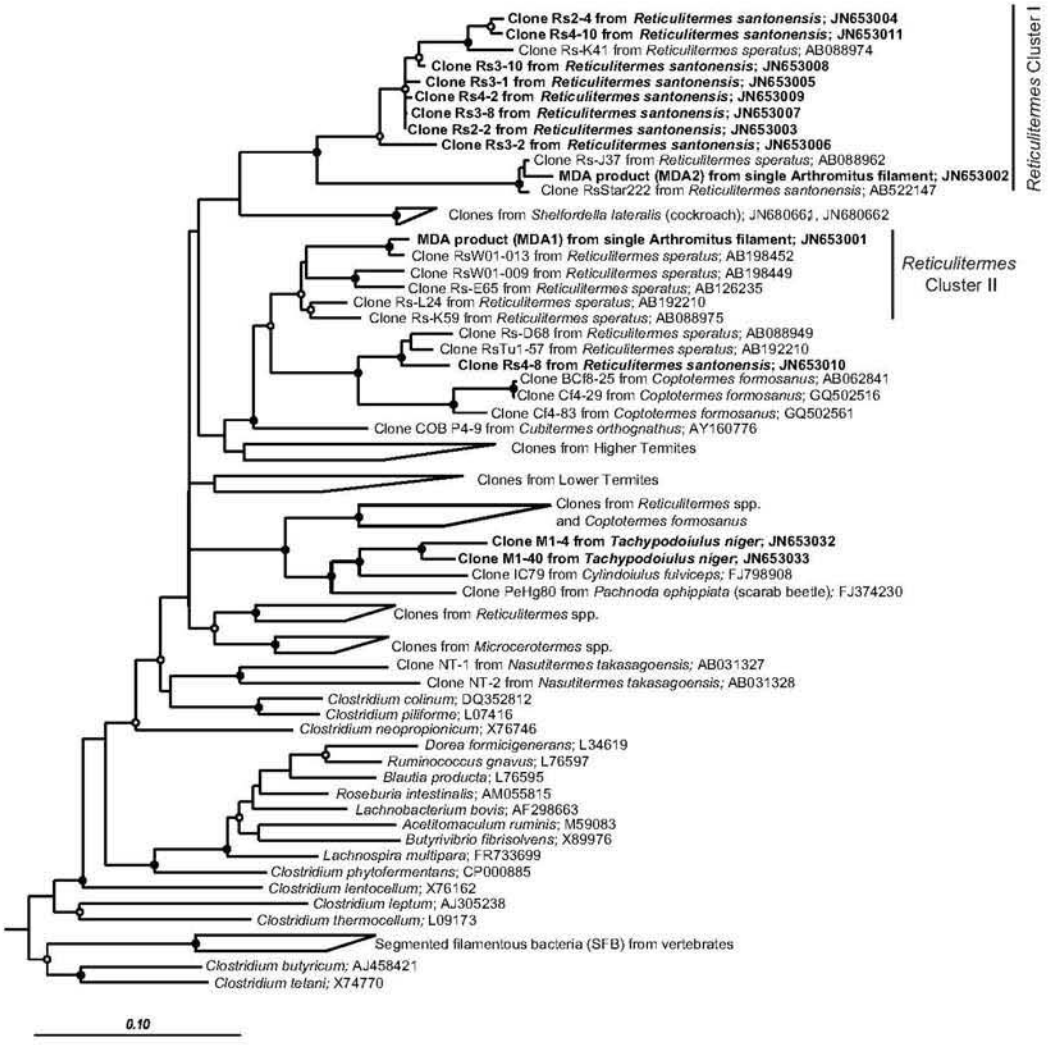

Figure S5.1 | Maximum-likelihood tree detailing the *Arthromitus* cluster, its position within the family *Lachnospiraceae*, and its distant relationship to the segmented filamentous bacteria (SFB) in the family *Clostridiaceae*. Clones obtained in this study are marked in bold. Circles indicate bootstraps values above 95% (●) and 70% (○)

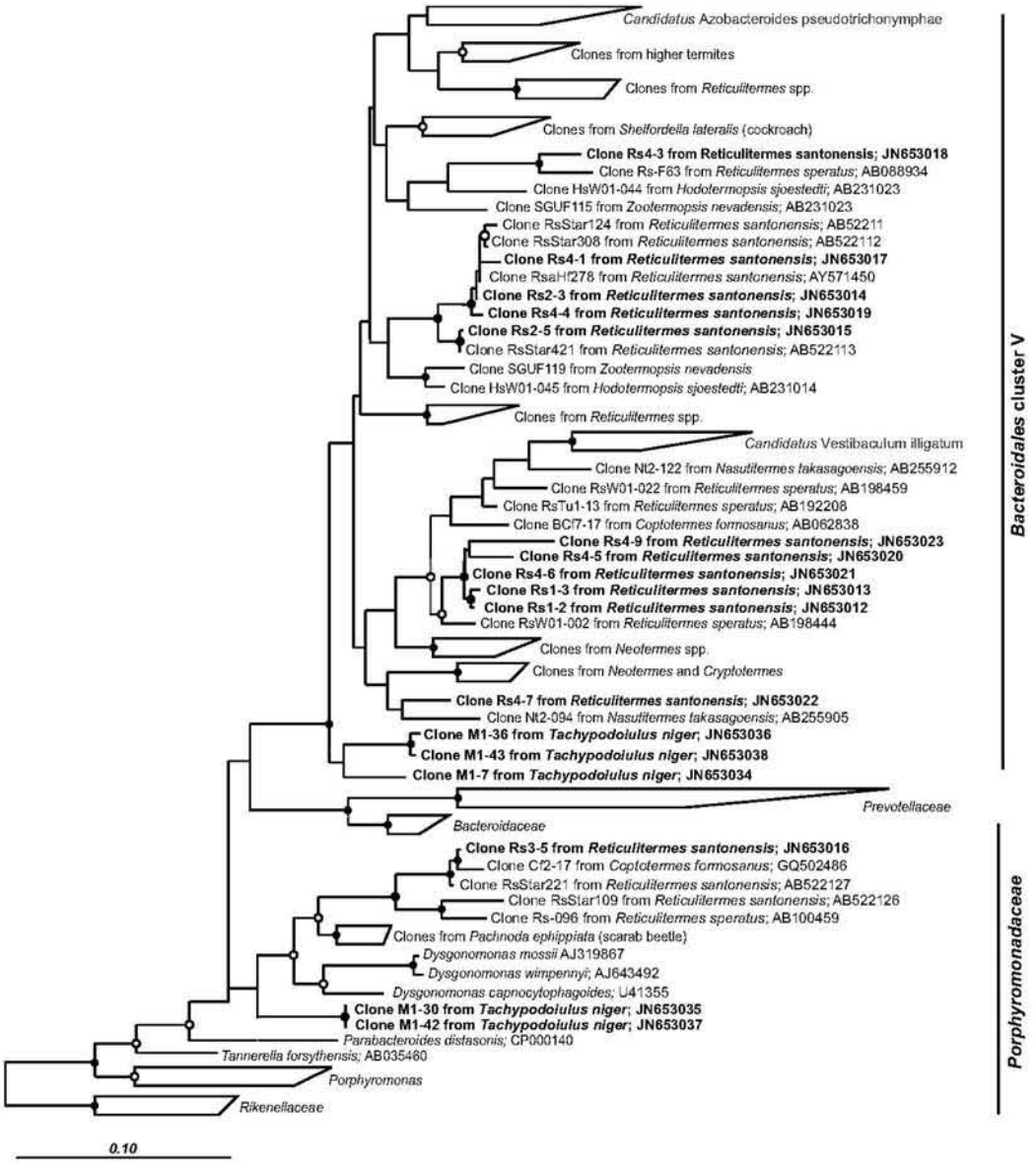

Figure S5.2 | Maximum-likelihood tree detailing the phylogenetic position of putative ectosymbionts of *Arthromitus* filaments within the *order Bacteroidales*. Sequences were obtained from filament suspensions from the termite *Reticulitermes santonensis* (JN653012-23) and the millipede *Tachypodoiulus niger* (JN653034-38). Clones obtained in this study are marked in bold. Circles indicate bootstraps values above 95% (•) and 70% (○).

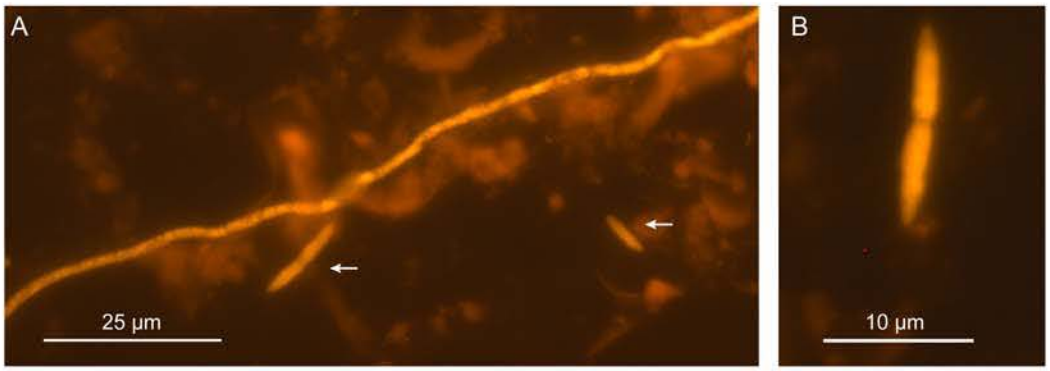

Figure S5.3 | Epifluorescence micrographs of hindgut preparations of the termite *Reticulitermes santonensis*, indicating the presence of rod-shaped bacteria with pointed ends that also hybridized with both probes specific for the *Arthromitus* cluster. (A) Lachno758 (Cy3-labelled) showing the rod-shaped bacteria (indicated by arrows) in comparison with an *Arthromitus* filament and (B) a rod-shaped bacterium hybridizing with Lachno1215 (Cy3-labelled). They may represent early division stages of germinated *Arthromitus* spores.

Supplementary files

Interactive MS Excel spreadsheets containing relative abundance of the bacterial lineages at different taxonomic levels for Chapters 2, 3 and 4 can respectively be found at

http://www.termites.de/downloads/546865736973/FileS2.1.xlsx

http://www.termites.de/downloads/546865736973/File**S3.1.xlsx**

http://www.termites.de/downloads/546865736973/File **S4.1.xlsx**

List of Abbreviations

16S rRNA	small subunit bacterial rRNA
AU	Approximately Unbiased p-values
CTAB	Cetyltrimethylammonium bromide
EDTA	Ethylenediaminetetraacetic acid
FISH	Fluorescent in situ Hybridization
GTR	General Time Reversible Model of evolution
NMDS	Non-metric Multidimensional Scaling
OTU	Operational Taxonomic Unit
P1	First proctodeal hindgut compartment
P3	Third proctodeal hindgut compartment
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
Pyrosequencing	454 pyrosequencing/pyrotag sequencing
RDP	Ribosomal Database Project
SDS	Sodium dodecyl sulphate
T-RF	Terminal Restriction Fragment
T-RFLP	Terminal Restriction Fragment Length Polymorphism
TG3	Candidate phylum Termite Group 3

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Chapter 2

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Chapter 3

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Chapter 4

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Chapter 5

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Pledge

I certify that the present thesis entitled:

“Microhabitat-specificity of hindgut microbiota in higher termites”

was carried out without any unlawful means. This work has never been submitted before in this or in a similar format to any other university and has not been used before any examination.

Marburg, July 2013

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