The bacterial gut microbiota of wood- and humus-feeding termites: Diazotrophic populations and compartment-specific response of bacterial communities to environmental factors

Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)

am Fachbereich Biologie der Philipps-Universität Marburg

vorgelegt von

Wanyang Wang

aus Tianjin, China

Universitätsstadt Marburg, 2017

Die Untersuchungen zur folgenden Arbeit wurden von Juli 2014 bis Dezember 2017 am Max-Planck-Institut für Terrestrische Mikrobiologie in Marburg unter der Leitung von Prof. Dr. Andreas Brune durchgeführt.

Vom Fachbereich Biologie der Philipps-Universität Marburg als Dissertation angenommen am:

Erstgutachter: Prof. Dr. Andreas Brune

Zweitgutachter: Prof. Dr. Wolfgang Buckel

Tag der Disputation: 20.02.2018

Die in dieser Dissertation beschriebenen Ergebnisse sind in folgenden Publikationen veröffentlicht bzw. zur Veröffentlichung vorgesehen:

Li, H. [†]**, Wang, W.** [†]**, Nonoh, J., Sillam-Dussès, D., and Brune A.** Environmental factors of the gut compartments in humivorous higher termites. (in Vorbereitung).

Wang, W., Li, H., Meuser, K., and Brune, A. Effect of diet and gut environment on community structure in higher termites of different feeding groups (in Vorbereitung).

Wang, W., Dietrich, C., Meuser, K., Lampert, N., Mikaelyan, A., and Brune, A. Abundance and diversity of nitrogen-fixing bacteria in termite and cockroach guts (in Vorbereitung).

[†] Beide Autoren trugen gleichermaßen zu dieser Arbeit bei.

Summary

The subject of this thesis is the influence of the microenvironment on the symbiosis between higher termites and their intestinal bacteria. The gut environmental factors pH, hydrogen partial pressure, redox potential and nitrogen pool size were measured. Bacterial gut community structure from each highly compartmentalized gut section was investigated. Furthermore, one specific function, nitrogen fixation, was comparatively analyzed in lower termites, higher termites and cockroaches.

Hydrogen partial pressure, pH and redox potential in the gut compartments of humus- and soil-feeding termites were measured using microsensors. The size of the entire bacterial communities in each compartment was determined by 16S rRNA gene copies in qPCR. The diets of humus- and soil-feeders are nitrogen-rich, so the pool size of ammonia, nitrite and nitrate were also quantified by colorimetric assay.

Higher termites have adapted to utilize diverse lignocellulosic diets in various stages of humification, like wood, humus and soil. The high alkalinity in the anterior hindgut of humus- and soil-feeding termites may play an important role in the digestion of proteins and polypeptides. Our comprehensive determination of physicochemical parameters reinforce the hypothesis that intestinal microenvironments are evolutionarily adapted to diet-related differences.

The analysis of bacterial diversity by amplicon sequencing (Miseq) of 16S rRNA genes underscored that the community structure of intestinal bacteria in each gut section is influenced by multiple environmental factors like pH, hydrogen and host dietary substrate. The gut bacteria in homologous compartments of hindguts of humus- and soil-feeders showed similarity even when the hosts were from different subfamilies. In wood- and grass- feeding termites, dominating gut microbiota were from *Actinobacteria*, *Fibrobacteres* and *Spirochaetes*. On the other hand, abundant genera were from *Bacteroidetes*, *Spirochaetes* and *Firmicutes* in humus- and litter-feeding termites. This suggests that they make essential contributions to the digestive processes.

Nitrogen supply should also influences the composition of the microbiota in termite guts, especially in wood-feeding termites, where diazotrophy is of major importance. From the study of nitrogen metabolism in different gut sections, the high concentrations of ammonia, nitrite and nitrate were found in the gut of humus- and soil-feeding termites not in wood-feeding termites. This phenomenon associated with the intake of the termites. For the wood

feeders, they rely on a nitrogen-limiting diet with a high carbon to nitrogen ratio. They need some strategies to overcome this difficulty. Nitrogen fixation of symbiotic gut bacteria helps them in nitrogen nutrition supply.

Quantification of nitrogen fixing populations was carried at DNA level by qPCR, using the *nifH* gene as a molecular marker. After normalized by 16S rRNA gene copy numbers, the ratio of *nifH* to 16S rRNA gene copy numbers was less than 0.15 in all termite species studied. Nevertheless, this surprisingly low proportion of diazotrophs is sufficient to account for the nitrogen fixation rate of the termites. It is supported by the nitrogen fixation ability measured by acetylene reduction assay of *Treponema* isolates from *Zootermopsis angusticollis* and live *Zootermopsis* sp.

The bacterial symbionts of flagellate protists contribute to the nitrogen fixation in lower termites. Especially in Kalotermitidae, the abundant *nifH* genes which clustered with *nifH* genes from flagellate symbionts are consistent with the cospeciation of flagellates and lower termites. Nitrogen fixed by the endosymbiont can be converted to more valuable nitrogenous compounds such as amino acids and supplied directly for protein synthesis of the protist. This asset allows the protist to grow stably and independently, and ensures that the host termite maintains the essential cellulolytic protists. In wood-feeding higher termites, flagellates are lost and the diazotrophs in the gut link with fiber-associated bacteria. This was verified by comparative analysis of *nifH* genes in amplicon libraries and annotated metagenomes.

Apart from flagellate symbionts, another interesting *nifH* subcluster is in Group IV. The verified diazotroph with only *nif* genes encoding Group IV nitrogenase revealed potential functional *nifH* subgroup in previously unfunctional Group IV. *Endomicrobium* cluster is abundant in Kalotermitidae, Termopsidae and Cryptoceridae. This is the first analysis of the diazotrophic communities in termite gut which take into account the potential diazotrophs with functional *nifH* in Group IV.

Zusammenfassung

Thema dieser Arbeit ist der Einfluss der Mikroumgebung auf die Symbiose zwischen höheren Termiten und ihren Darmbakterien. Die intestinalen Umweltfaktoren pH, Redoxpotential, Wasserstoffpartialdruck sowie die Stickstoff-Poolgrößen wurden gemessen. Die Struktur der bakteriellen Darmgemeinschaft wurde separat in jedem Abschnitt des stark kompartimentierten Darms untersucht. Darüber hinaus wurde die spezifische Funktion der Stickstofffixierung in niederen Termiten, höheren Termiten und Schaben untersucht.

Wasserstoffpartialdruck, pH, und Redoxpotential in den Darmabschnitten von humus- und bodenfressenden Termiten wurden mit Mikrosensoren gemessen. Die Größe der gesamten bakteriellen Gemeinschaften in den einzelnen Darmabschnitten wurde erfasst, indem die 16S-rRNA-Genkopienzahl durch qPCR bestimmt wurde. Da die Diät von Humus- und Bodenfressern sehr stickstoffreich ist, wurden weiterhin die Poolgrößen von Ammoniak, Nitrit und Nitrat durch einen kolorimetrischen Assay quantifiziert.

Höhere Termiten haben sich daran angepasst Lignocellulose in verschiedenen Stadien der Humifizierung, wie Holz, Humus, oder Boden umzusetzen. Die hohe Alkalinität im vorderen Enddarm von humus- und bodenfressenden Termiten spielt möglicherweise eine wichtige Rolle bei der Verdauung von Proteinen und Polypeptiden. Unsere umfassenden Messungen der intestinalen physikochemischen Parameter bestärken die Hypothese, dass die intestinale Mikroumgebung evolutionär an ernährungsbedingte Unterschiede angepasst ist.

Die Ergebnisse der Amplicon-Sequenzierungen (Miseq) unterstrichen, dass die Gemeinschaftsstruktur der Darmbakterien in jedem Darmabschnitt durch zahlreiche Umweltfaktoren wie pH, Wasserstoffpartialdruck und Ernährung des Wirts beeinflusst wird. Homologe Kompartimente des Enddarms von Humus- und Bodenfressern beherbergten ähnliche Bakterien, unabhängig von der Unterfamilie des Wirts. In holz- und grasfressenden Termiten bestand die Darmmikrobiota hauptsächlich aus *Actinobacteria*, *Fibrobacteres* und *Spirochaetes*. In humus- und detritusfressenden Termiten waren hingegen vor allem Gattungen aus *Bacteroidetes*, *Spirochaetes* und *Firmicutes* vertreten. Das deutet darauf hin, dass sie essentiell zum Verdauungsprozess beitragen.

Stickstoffverfügbarkeit sollte ebenfalls die Zusammensetzung des Darmmikrobioms von Termiten beeinflussen. Das gilt vor allem für den Darm von Holz-fressende Termiten, wo Diazotrophie besonders wichtig ist. Der Darm von humus- und bodenfressenden Termiten wies hohe Konzentrationen von Ammoniak, Nitrit und Nitrat auf, nicht jedoch der von

holzfressenden Termiten. Dieses Phänomen hängt mit der Ernährung der Termiten zusammen. Holzfresser sind aufgrund des hohen C/N-Quotienten ihrer Diät Stickstoff-limitiert. Sie benötigen Strategien um diese Schwierigkeit zu überwinden. Stickstofffixierung durch symbiotische Darmbakterien hilft ihnen bei der Stickstoffversorgung.

Die Quantifizierung der Stickstoff fixierenden Populationen erfolgte auf DNA-Ebene durch qPCR des molekularen Markergens *nifH*. Normalisiert nach 16S-rRNA-Genkopiezahlen betrug das Verhältnis von *nifH* zu 16S-rRNA-Genkopien weniger als 0.15 in allen untersuchten Spezies. Nichtsdestotrotz reicht dieser überraschend geringe Anteil an Diazotrophen aus, um die Stickstofffixierungsrate von Termiten zu decken, und steht im Einklang mit den Fixierungsraten (mittels Acetylen-Reduktions-Assay bestimmt) von *Treponema*-Isolaten aus *Zootermopsis angusticollis* und lebenden *Zootermopsis* sp.

Die bakteriellen Symbionten von Flagellaten tragen zur Stickstofffixierung in niederen Termiten bei. Insbesondere in Kalotermitidae stimmen die abundanten *nifH*-Gene, die mit *nifH*-Genen von Flagellatsymbionten clustern, mit der Kospeziation von Flagellaten und niederen Termiten überein. Der durch den Endosymbionten fixierter Stickstoff kann in wertvollere stickstoffhaltige Verbindungen wie Aminosäuren umgewandelt und direkt der Proteinsynthese des Protisten zugeführt werden. Dies ermöglicht dem Protisten, stabil und unabhängig zu wachsen, und stellt sicher, dass die Termite die essentiellen cellulolytischen Protisten beibehält. Weiterhin wurde durch vergleichende Untersuchungen von *nifH*-Genen in Amplikonbibliotheken und annotierten Metagenomen verifiziert, dass holzfressende höhere Termiten ihre Flagellaten und die mit Faser-assoziierten Bakterien verbundenen Diazotrophen wieder verloren haben.

Neben Flagellaten-Symbionten repräsentieren Sequenzen aus der *nifH*-Gruppe IV einen weiteren interessanten Subcluster. Das bisher als nicht funktionell beschriebene *nifH*-Homolog IV beinhaltet mit *Endomicrobium proavitum* mindestens einen aktiven Diazotrophen, der ausschließlich ein *nifH*-Gen der Gruppe IV besitzt. *Endomicrobium*-verwandte *nifH*-(IV)-Sequenzen, sind abundant in Kalotermitidae, Termopsidae und Cryptoceridae. Dies ist die erste Analyse der diazotrophen Gemeinschaften im Termitendarm, die auch potentielle diazotrophe Mikroorganismen mit *nifH* aus Gruppe IV berücksichtigt.

Contents

Chapter 6

Chapter 1

Introduction

1.1 The phylogeny of termites

Termites (Order: Isoptera) are regarded as an epifamily of cockroaches (Blattodea), with wood-feeding cockroaches of the genus *Cryptocercus* (family Cryptocercidae) as closest relatives (Lo and Eggleton, 2011) (Fig. 1.1). The combined clade of termites and *Cryptocercus* is sister to Blattidae (Inward et al., 2007). Termites also have several subfamilies which differ from diets and lifestyle (Eggleton and Tayasu, 2001). Although there are some arguments on problematic groups, recent studies agree on the order in Fig 1.1 (Lo and Eggleton, 2011). Across the termite subfamilies, the majority of termites feed on humus , soil and dead plant material, including dead wood, leaf litter and dry grass (Bignell and Eggleton, 2000).

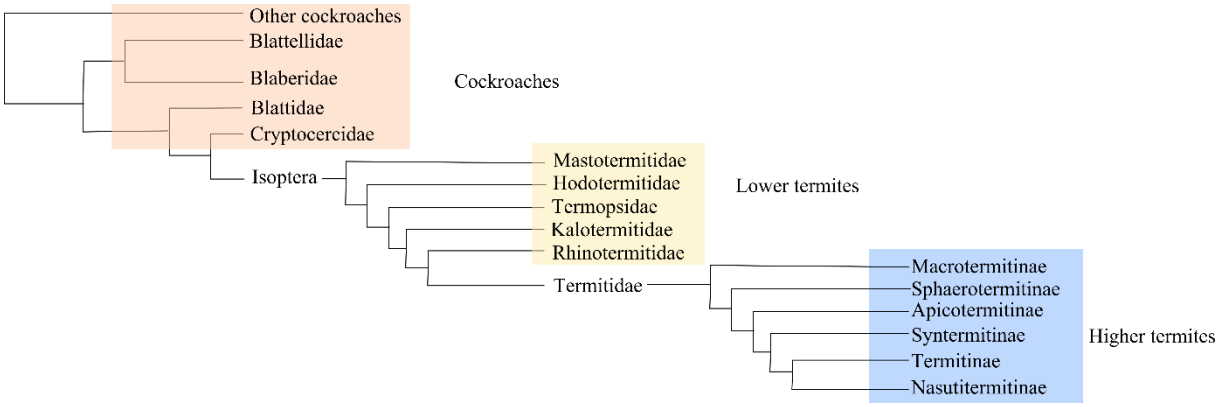

Figure 1.1: Phylogenetic tree of termites and close relative cockroaches (modified from Inward et al., 2007).

1.2 The diet of termites

Termites have the typical feature of feeding on lignocellulosic plant materials at various stages of humification. In the global environment, termites act as distributors of terrestrial decomposers of biomass. Termites play an important role in the global biogeochemical cycles and ingest 50%–100% of the dead plant biomass in tropical ecosystems (Bignell and Eggleton, 2000). The composition of the diet differs between lower termites and higher termites. Lower termites mainly rely on wood, while higher termites ingest a wide range of materials including soil (e.g., *Cubitermes* spp.), grass (e.g., *Trinervitermes* spp.), wood (e.g., *Nasutitermes* spp.) and plant litter/fungus (e.g., *Macrotermes* spp.)(Donovan et al., 2001).

Termites digest lignocellulose in various stages of humification, ranging from sound wood to soil organic matter and contribute substantially to the carbon and nitrogen cycling in

rainforest and savanna ecosystem (Bignell, 2006; Jones and Eggleton, 2010). While the evolutionarily 'lower' termites harbor cellulolytic flagellates in their hindgut, all 'higher' termites (family Termitidae) lost their gut flagellates and show a largely prokaryotic gut microbiota (Brune, 2014; Brune and Dietrich, 2015). Unlike lower termites, which feed almost exclusively on wood, higher termites may thrive on sound wood or dry grass, herbivore dung, decaying wood or plant litter in different stages of humification, or even soil organic matter (Bignell, 2010; Donovan et al., 2001). This dietary diversification is considered an explanation of the evolutionary and ecological success of higher termites, which represent more than 85% of all termite species described to date (Krishna et al., 2013).

Since lignocellulose is a nitrogen-poor substrate, lower termites have an abundant amount of carbon source while the supply of nitrogen is quite limiting. To solve this dilemma, the symbiosis of flagellates is established in lower termites besides a preference for fungi-colonized lignocellulosic substrates that have a decreased C-to-N ratio and proctodeal trophallaxis. The microbes function by recycling of uric acid and fixing nitrogen. Although the nitrogen-fixing activity of lower termite hindgut has been discovered about forty years ago and a number of nitrogen-fixing bacteria have been studied from then on, the bacteria who related to the main nitrogen-fixing activity in the hindgut are still undiscovered (Brune and Ohkuma, 2011).

Although the food of termite is wood in common sense, termites have a diversity of feeding substrates across different subfamilies. One important substrate is soil. Apicotermitinae, the *Capritermes* clade of oriental soil-feeding Termitinae (including *Cubitermes* group) and the *Subulitermes*- and other clades of soil-feeding Nasutitermitinae feed on it (Brauman et al., 2000). The syntermitine species includes grass/litter-feeders, intermediate feeders, and humus-feeders (Inward et al., 2007). Soil is higher humified compared with wood, so it has less energy. However, it is a widely distributed resource (Eggleton and Tayasu, 2001). This may explain the fact that about 1100 species of soil-feeding termites have been described which consist of 50% of current known termite species. Soil-feeding termites are dominant termites' group in Africa and Asia tropical forests (Brauman et al., 2000). In tropical ecosystems, termite mound soils constitute an important soil compartment covering around 10% of African soils (Fall et al., 2007) and to impact C mineralization by decomposition to roughly the same extent as all mammalian herbivores and natural fires. The percentage is between 2 and 5% of the CO_2 flux to the atmosphere from all terrestrial sources (Bignell, 2006).

1.3 Soil feeding termites

The advantage of soil-feeding is low energetic cost, minimal competition for resources and avoidance of predators (Bignell, 1994).There are two kinds of soil-feeding termites: one is "genuine" soil-feeders, who feed widely in the soil profile with little selection, while the "wood/soil interface feeders" utilize organic litter material such as old rotting wood (Brauman et al., 2000). The true soil-feeders ingest soil including higher proportions of soil organic matter and silica, and lower proportions of recognizable plant tissue than in other groups' resource (Bignell and Eggleton, 2000).

The term humus was introduced to refer the dark-colored organic material in soil (De Saussure, 1804). Later studies characterized element composition of humic substances and assigned them to a defined chemical formula and structure (Berzelius, 1839). In the recent years, the use of cutting-edge techniques (e.g. FTIR (Fourier-transform infrared spectroscopy), ESR (Erythrocyte sedimentation rate), pyrolysis-GC-MS (Pyrolysis–gas chromatography–mass spectrometry), NMR (Nuclear magnetic resonance), etc.) revealed more details about the composition of humic substances and the processes occurring during humification, leading to the modern understanding of humic substance formation and composition: humic substances contain various aromatic subunits, peptides, amino acids, carbohydrates, which are original from cell wall of bacteria and fungal in addition to the degradation of organic residues of plant and microbes (Miltner et al., 2012; Piccolo, 1996; Stevenson, 1994).

One essential part in the soil for soil-feeding termites is soil organic matter (SOM). Although it is merely about less than 10% of the mineral soil, soil organic matter provide more than 90% of N (Stevenson, 1994). In principle, it can be classified into two parts: one is the nonhumic fraction, like polysaccharides, proteins, sugars and amino acids; the other is the highly stable humic fraction which is around 70% in SOM (Schulten, 1995). In the presence of termites, the highly stable humic fraction like microbial biomass, peptides, and cellulose can be degraded and mineralized (Rong and Brune, 2001; Rong, 2000; Rong and Brune, 2005). The hypothetical products (e.g., amino acids and sugars) can be utilized either by the host or by microbes in the anoxic hindgut compartments (Schmitt-Wagner and Brune, 1999; Schmitt-Wagner et al., 2003; Tholen and Brune, 1999). The final products are short-chain fatty acids (e.g., acetate) that can be mineralized and assimilated by the host.

Termites inhabit approximately 75% of the terrestrial soil surface and, depending on the species, consume the dietary substrates along the humification gradient of the dietary substrates. More than half of all termite genera are considered humivorous (Bignell and Eggleton, 1995; Noirot, 1992) and consume large amounts of soil (Okwakol, 1980; Wood, 1988). Through NMR techniques examination of soil source and feces, the carbohydrate decrease through the gut passage and the increase of humic-acid-to-fulvic-acid ratio in the feces showed the influences of termites on soils (Garnier-Sillam and Harry, 1995). Soil feeding termites' influences range from physical effects to changes in the chemical properties of soil organic matter (e.g., disturbance of soil profiles, changes in soil texture and structural stability, nature and distribution of soil organic matter, C/N ratios) (Lobry de Bruyn and Conacher, 1990; Wood, 1988; Brussard and Juma, 1996.).

In humus feeders, the pronounced gut compartmentalization and the highly alkaline pH values in the anterior hindgut compartments play an important role in the digestion of stabilized humus components in intake soil (Bignell and Eggleton, 1995; Brune and Kühl, 1996).

Due to the decrease of C: N ratio along with the humification process, soil-feeding termites are limited by energy, not by the N content of their diet (Eggleton and Tayasu, 2001). Previous feeding studies in soil feeders, *Cubitermes* spp., shows that they prefer to digest and mineralize the peptidic component of humic model compounds (Ji et al., 2001). In soil particles, 20% of the organic carbon is peptidic carbon (Knicker et al., 2000). So there is not surprising to see that the soil feeding termites have proteolytic activities which can mineralize the peptidic component of synthetic humic acids. The most active part of the proteolytic activity is M/ms section. The pretreatment of the enzyme in different pH value shows that the gut extraction has higher proteolytic activity compared with commercial enzymes like subtilisin and trypsin (Ji and Brune, 2005). The alkaline condition in anterior gut enables the release of amino acids from humic acids and extraction of organic matter from the soil (Brune, 1998; Kappler and Brune, 1999; Swift and Posner, 1972). In addition, the proteolytic activity in gut extracts possesses a high tolerance toward humic acids, especially under alkaline conditions (Ji and Brune, 2005). The ammonia concentration shows that mineralization starts from anterior gut at a low level while peptide mineralization has a high rate in the hindgut section. While the anterior gut mineralized amino acids usually under anaerobic conditions, the mineralization process is greatly stimulated by anaerobic conditions in the P1 and P3 gut sections (Ngugi et al., 2011). A ^{15}N trace experiment shows that an anaerobic oxidation of ^{15}N-labelled ammonia to nitrite in P4 section of *Cubitermes*

ugandensis. After incubation homogenate with ^{15}N-labelled ammonia, labelled nitrite accounts for 14% of the recovered nitrite pool. No nitrification and anamox activities are detected (Ngugi and Brune, 2012).

1.4 Higher termite gut environments

Compared with the lower termites, higher termites have a highly differentiated compartment gut associated with their diversity diet. The degree of compartmentalization increases with the humification of the substrate (Bignell and Eggleton, 1995). The guts of higher termites (family Termitidae) are divided into foregut (crop), midgut, mixed segment and hindgut, which consists of several consecutive segments: ileum (P1), enteric valve (P2), colon (P3 and P4) and rectum (P5; Noirot, 2001). With the exception of the fungus-cultivating Macrotermitinae, higher termites show a dilation not only of the P3 (similar to the strongly dilated P3 of lower termites) but also of the P1 and P4 which are very short and narrow in lower termites, respectively (Noirot, 1995).

Although there are several parts of termites' intestinal tract, hindgut is a major site for digestion and absorption, which represents the great diversity of major gut microorganism and may contribute as much as 40% of the weight of termite (Schulz et al., 1986; Slaytor et al., 1997). The central part of hindgut is anoxic, while the outer zone contains oxygen influx from the outer environment. The highest density of hydrogen appears in the center of the hindgut. On the other hand, the outer space contains the lowest hydrogen concentration (Ebert and Brune, 1997).The hydrogen distribution results from anaerobic flagellates and microbes serve in fermentation, methanogenesis and reductive acetogenesis. Fig. 1.2 shows symbiotic relationships in the nutrition utilization. To adapt to more diverse diets, the gut of higher termites is more elongated and complicated compared with lower termites (Fig. 1.3). Therefore, there are more diverse habits for gut microbes in higher termites.

Among the most notable adaptations of higher termites to a humivorous lifestyle are the extensive digestive modifications of their intestinal tracts, both from the anatomical and physicochemical perspective (Bignell, 2010; Eggleton, 2011). Some lineages of higher termites even became true soil feeders that thrive exclusively on the humic substances of mineral soil (Donovan et al., 2001; Eggleton and Tayasu, 2001). Feeding experiments with labeled model compounds have shown that *Cubitermes* spp. enable to mobilize recalcitrant humus constituents, in particular preferentially digested peptidic or other nitrogenous residues

derived from microbial biomass, but do not mineralize polyphenols (Ji et al., 2000; Ji and Brune, 2006). Therefore, the peptides derived from microbial biomass in soil organic matter are probably a major dietary resource of true soil feeders. The presence of an even more pronounced elongation, dilated and extreme alkalization (to >pH 12) of the anterior hindgut, which had long been assumed to unlock the polyphenols in soil diet (Brune and Kühl, 1996). Although humivorous termites have a strong impact on nitrogen metabolism in tropical soils (Krishna et al., 2013), the nitrogen transformation processes in their guts have remained unclear.

In soil-feeding, humus-feeding, wood-feeding higher termites and lower termites, each gut compartment has different physicochemical conditions (Bignell and Eggleton, 1995; Ebert and Brune, 1997; Köhler et al., 2012; Schmitt-Wagner and Brune, 1999). In soil-feeding and humus-feeding termites, the gut content shows extremely high alkalinity in P1 (Bignell and Eggleton, 1995). In the wood-feeding *Nasutitermes* spp., the pH trend along the gut axial is similar except the highest pH value is smaller than soil feeders (Köhler et al., 2012). In lower termites, the range of pH is limited to the slightly acidic condition (Bignell and Eggleton, 1995). The high alkaline condition may play a role in the digestion of humus or lignocellulose as a pretreatment before the digestion in the posterior hindgut. Hydrogen accumulation results from hydrogen production like microbial fermentations of carbohydrates and hydrogen consumption like methane formation and homoacetogenesis. The most complicated situation is in the soil feeding Termitinae. H_2 partial pressure is high in mixed segment and P3 (Schmitt-Wagner and Brune, 1999). In wood-feeding higher termites *Nasutitermes* spp., P3 accumulates high partial pressure of H_2 (Köhler et al., 2012). In wood-feeding lower termite *Reticulitermes flavipes*, hydrogen accumulation is strongest within the anterior hindgut and correlation with large acetate pool, anoxic and a low redox potential (Ebert and Brune, 1997).

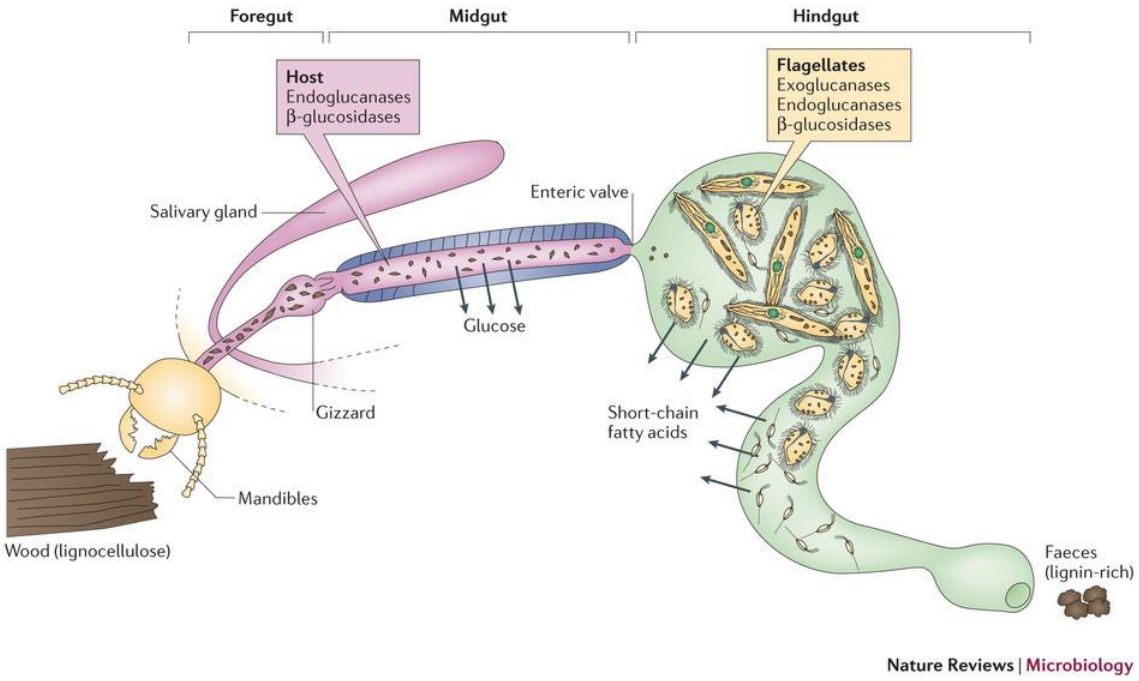

Figure 1.2: Gut structure of lower termites and lignocellulose digestion in lower termites. In the foregut, the mandibles produce the wood particles which are further digested by enzymes from the salivary glands and comminuted by the muscular gizzard. In the midgut, glucose is resorbed via the epithelium. The partially digested wood particles are phagocytized by cellulolytic flagellates, which hydrolyze the remaining polysaccharides using cellulases and hemicellulases. The short-chain fatty acids are resorbed by the host, and the lignin-rich residues are defecated. (Brune, 2014)

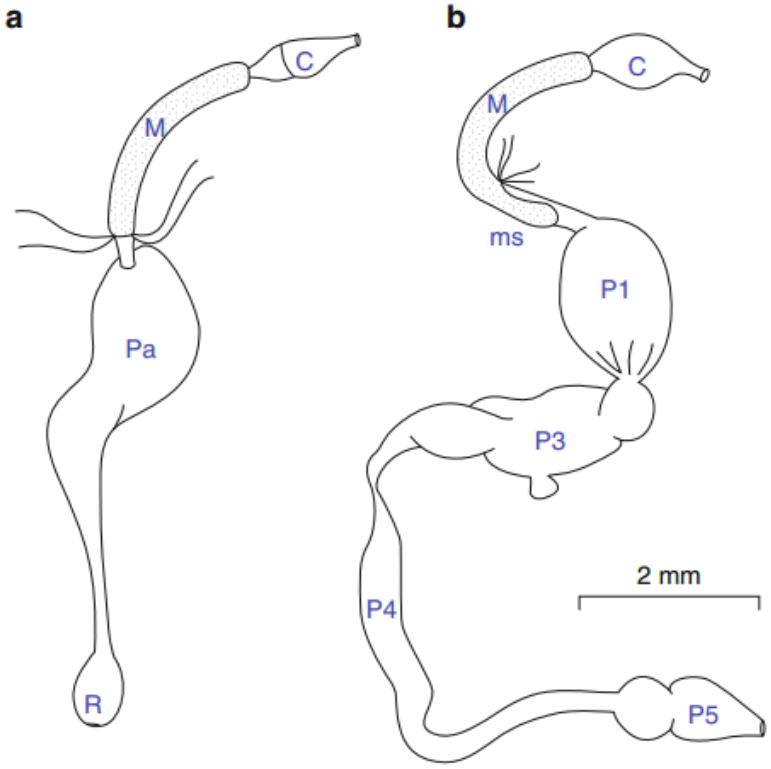

Figure 1.3: Gut structure of termites. a, the gut structure of a lower termite (*Reticulitermes*); b, the gut structure of a higher termite (*Cubitermes*) with highly compartmentalized hindgut (Brune, 2013) C: crop, M: midgut, ms: mixed segment, Pa: Paunch, R: Rectum, P1 to P5: the proctodeal hindgut compartments.

1.5 Gut community structure in termites

In the termites gut, intestinal microbiota help hosts in the digestive process. In the study of cockroaches and termites, the community structure shared similarity with major host phylogeny (Dietrich et al., 2014). Higher termites have adapted to diverse food sources in different stages of humification. Therefore, diet becomes an essential driving force to form the intestinal bacterial composition in higher termites (Mikaelyan et al., 2015a). Despite the similarity explained by diet, a detailed study showed that community structure in major homologous compartments of hindguts in each species clearly converged (Mikaelyan et al., 2016).

Spirochaetes are quite abundant in wood-feeding termites, while they are low in fungus-cultivating and humus-feeding termites (Dietrich et al., 2014; Makonde et al., 2013; Otani et al., 2014). In lower termites, *Elusimicrobia* composes a large proportion of the bacterial community (Dietrich et al., 2014) because *Candidatus* Endomicrobium lineages are

endosymbionts of certain flagellates (Ikeda-Ohtsubo and Brune, 2009). In wood-feeding higher termites, the fiber-associated members of *Fibrobacteres* and the candidate phylum TG3 are abundant (Dietrich et al., 2014; Hongoh et al., 2006; Mikaelyan et al., 2016).

Interestingly, several humus-feeding lineages started to exploit diets of relative decreasing humification. The gut content analyses of characteristic fiber-digesting lineages TG3 (Termite cluster III), *Fibrobacteres* (Termite cluster I) and *Spirochaetes* (*Treponema* clusters Ic and If) showed that these termites consume a greater proportion of plant material and/or wood fibers than the true soil feeders, suggesting the considerable differences in the degree of humification in their lignocellulosic diets (Sleaford et al., 1996; Donovan et al., 2001). In addition, there is evidence that the composition and functional role of the bacterial gut microbiota differ between different diet groups, with a higher abundance of *Firmicutes* in humus and soil feeders opposed to a prevalence of putatively fiber-degrading *Fibrobacteres* and *Spirochaetes* in wood and grass feeders (Mikaelyan et al., 2015a). Moreover, it also has been shown that the microbiota differs fundamentally between the different hindgut compartments of wood- and humus-feeding higher termites (Mikaelyan et al., 2016). However, the physicochemical conditions in the hindgut of higher termites have been studied only in a few representatives of different subfamilies: soil-feeding Termitinae (Brune and Kühl, 1996; Schmitt-Wagner and Brune, 1999), wood-feeding Nasutitermitinae (Köhler et al., 2012), and fungus-cultivating Macrotermitinae (Li et al., 2012); the microenvironmental conditions in humus feeders have remained entirely unexplored.

Up to now, there are studies about whole communities in the guts of termites showing the various phylogenetic patterns in the gut microbiota from different host subfamilies (Dietrich et al., 2014; Mikaelyan et al., 2015a). When more comprehensive coverage of termites from different diet groups is studied, diet acts as the primary determining factor of gut microbiota in higher termites. In a study with increasing resolution in the major hindgut compartments, the community structure of homologous compartments of the major feeding groups clearly converged (Mikaelyan et al., 2016).The current information about gut microenvironment and molecular study of gut microbiota are mostly from different studies. The direct linkage of gut microbiota and the gut environment from the same termite species of humus and soil feeders are still lacking. Last but not least, sufficient numbers of host species are needed to get a solid dataset for identifying and differentiating the environmental drivers.

1.6 Nitrogen fixation in termites

The nitrogen content in diet of termites increases from wood to soil. Since nitrogen is an essential element for the formation of nucleic acids and proteins, gut microbiota in termites feeding on sound wood recycle of uric acid and fix nitrogen to fulfill the nitrogen requirement with an N-poor diet. Nitrogen fixation carried by their symbionts in the gut can play an important role for nutrition supply (Benemann, 1973; Breznak et al., 1973; Breznak 2000).

Nitrogenases are the essential enzymes which function in nitrogen fixation process. The nitrogenase structural genes and other *nif*-specific genes should function well for the maturation of structural component. Although the gene encoding the nitrogenase structural components are not catalytically competent before active by other nitrogen-specific gene products, *nifH* which encode Fe protein subunit is a prerequisite for nitrogen fixation (Dean et al., 1992). Moreover, the *nifH* gene is conserved in among diverse nitrogen-fixing microorganism (Young, 1992). *nifH* has been used as a molecular marker in many phylogenetic studies.

Phylogenetic analyses of *nifH* gene sequences have revealed five primary clusters of genes homologous to *nifH* (Fig. 1.4). Group I consists of aerobic nitrogen fixers including *Proteobacteria, Cyanobacteria, Frankia,* and *Paenibacillus*. Group II is generally thought of as the alternative nitrogenase cluster because it contains sequences from FeFe and FeV nitrogenases which differ from the conventional FeMo cofactor-containing nitrogenase. Group III consists of anaerobic nitrogen fixers from Bacteria and Archaea including, for instance, the *Desulfovibrionaceae, Clostridia*, *Spirochaetes*, and *Methanobacteria*. Group IV contain sequences that are paralogs of *nifH* and which are not involved in nitrogen fixation except *Endomicrobium proavitum* (Zheng et al., 2016). Most *nifH* paralogues in Group IV do not function in nitrogen fixation but other processes like photopigment biosynthesis and electron transport (Young, 2005). Group V contains the subunits of protochlorophyllide reductase and chlorophyllide reductase in photosynthetic pigment biosynthesis(Raymond et al., 2004).

The nitrogen fixation in termites was verified by acetylene reduction assay and ^{15}N labeled nitrogen fixation into biomass of termites (Benemann, 1973; Bentley, 1984; Breznak and Canale-Parola, 1973). In lower termites, the diet is not so diverse and N nutrition rich like in higher termites, and the majority of lower termites feed on wood. The termite tissues contain 8-13 % nitrogen of ash-free dry weight and their C/N ratio is about 4-12 (Matsumoto, 1976).

They feed on an N-limiting diet which only contains 0.1-0.7% nitrogen in most woody tissues based on dry weight and the C/N ratio is about 70-430 (Yoneda et al., 1977). Therefore, termites must be able to effectively acquire and retain nitrogen to solve nitrogen limiting and C/N balance problem (Holt and Lepage, 2000). Nitrogen fixation rates reflect differences in diet, such that xylophagous termites have higher nitrogen fixation rates relative to detritus feeders (Breznak, 2000). Therefore, nitrogen fixation is a very important N supply for xylophagous termites. In *Neotermes koshunensis*, isotopic studies showed that symbiotic nitrogen fixation accounts for between 30 and 60% of the whole nitrogen content (Tayasu et al., 1994).

The major players catalyzing nitrogen fixation in termite guts have not yet been identified. Only a few diazotrophic bacteria have been isolated from termite guts, like *Enterobacter agglomerans* in *Coptotermes formosanus* (Potrikus and Breznak, 1977), *Treponema azotonutricium* and *Treponema primitia* in *Zootermopsis angusticollis* (Graber et al., 2004), *Citrobacter freundii* in *Mastotermes darwiniensis*, *Coptotermes lacteus*, and *Nasutitermes exitiosus* (French et al. , 1976). Most of the isolated diazotrophic bacteria represent only a small percentage of the nitrogen-fixing microbes in termite guts (Breznak, 2000; Brune, 2013). The only exception is spirochetes, which can account for up to 50% in *Nasutitermes lujae* (Lilburn et al., 2001; Paster et al., 1996).

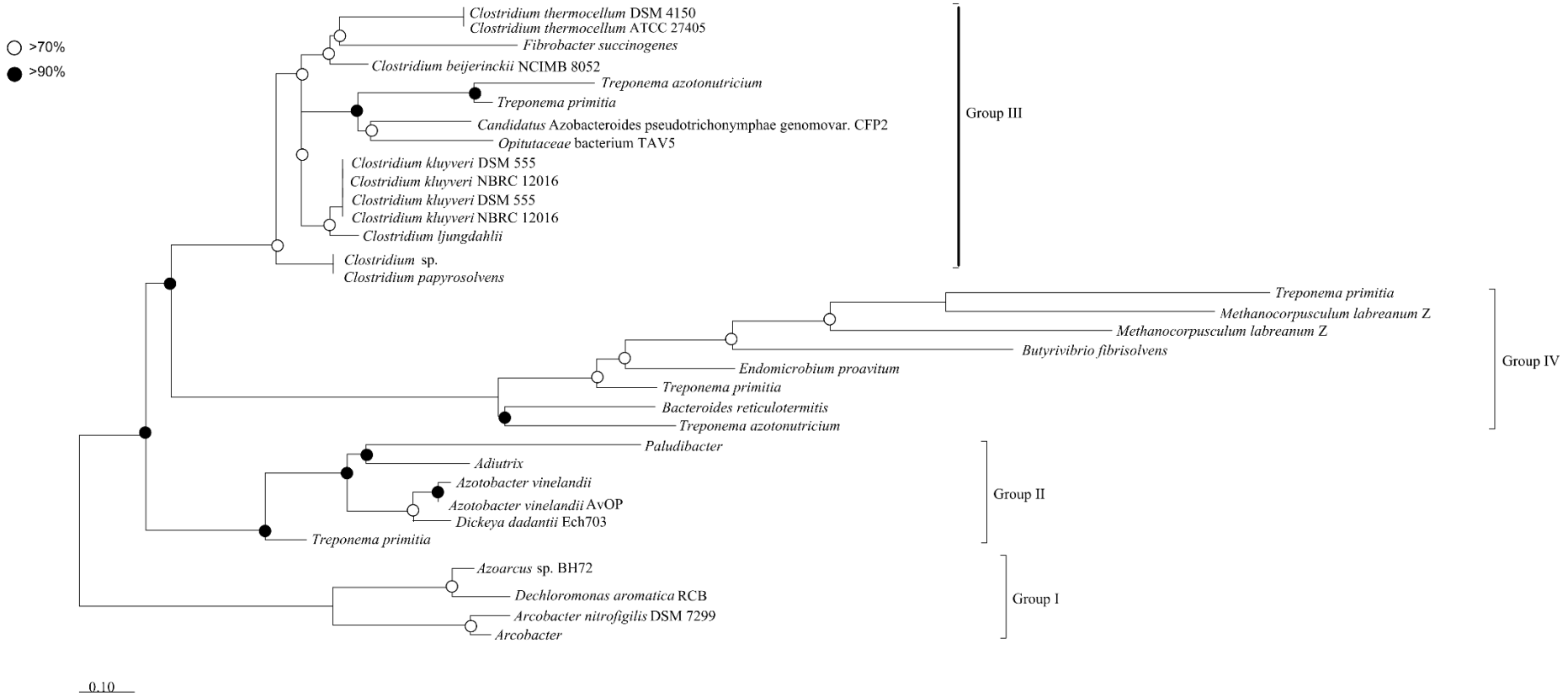

Figure 1.4: Overview of four phylogenetic groups composed of *nifH* homologs found in complete genomes. The maximum-likelihood tree was calculated by deduced amino acid sequences. The local-bootstrap support values were analyzed from 1000 resampling. Nodes are labelled with black circles for local-bootstrap support values > 90% and white circles for values > 70%. Scale bar represents 0.10 substitutions per position.

Culture-independent molecular methods provide a broader view of nitrogen fixation in termites. The enzyme nitrogenase catalyzes nitrogen fixation and comprises the components *nifH*, *nifD* and *nifK*. The *nifH* gene codes for the dinitrogenase reductase enzyme (Rubio and Ludden, 2002) and is widely used as marker gene of nitrogen fixation. In this way, Ohkuma *et al.* (1996) and Kudo *et al.* (1998) identified the diversity of nitrogen-fixing symbionts in the hindgut of the lower termite *Reticulitermes speratus* (Rhinotermitidae). Ohkuma *et al.* (1999) found that *nifH* groups in six lower termites and three higher termites were similar within each termite family but different across termite families. The results suggest an evolutionary trend reflecting the diazotrophic habitats in the symbiotic community. Yamada *et al.* (2007) analyzed *Cryptocercus* and various termite families, Rhinotermitidae, Mastotermitidae, Termopsidae, Kalotermitidae and Termitidae and came to the conclusion that the phylogenetic diversity of *nifH* gene links to host phylogenetic position and lifestyle. However, in spite of the diversity of the *nifH* sequences in the gut microbial community, some termite species exhibit only a low level of nitrogen fixation activity (Ohkuma et al., 1999). There are five major groups of *nifH* homologs described. The previous studies of termite gut microbiota were focused on Group I to III because Group IV was previously regarded as nonfunctional. However, *Endomicrobium proavitum*, which actively fixes nitrogen, only has a Group IV *nifH* gene (Zheng et al., 2016).

An important evolution event of lower termites is the acquisition of flagellates. Although there are some occasional losses of flagellates and their horizontal transfer across different termite families, the co-speciation of flagellate and hosts are characteristic for each termite species (Inoue et al., 2000; Lo and Eggleton, 2011; Noda et al., 2012). The symbiosis of flagellate protists and lower termites enhance the nutrition supply of hosts like cellulose digestion and nitrogen fixation (Brune, 2012; Hongoh, 2011). The flagellate symbionts in different dry-wood termites shape the nitrogen-fixing community (Desai and Brune, 2011). More and more flagellate symbionts were discovered to play a role in nitrogen fixation in *Cryptocercus* and termites (Hongoh et al., 2008; Ohkuma et al., 2015; Tai et al., 2016; Zheng et al., 2016). In the study of dry wood-feeding termites, the ectosymbionts of flagellates *Devescovina arta* have an important role in nitrogen fixation (Desai and Brune, 2011).

Unlike soil or humus feeders, wood feeders lack nitrogen in their diet. To overcome the difficulty, they rely on nitrogen fixation of their prokaryotic gut microbiota. Although nitrogen fixation in termites has been discovered for more than forty years (Breznak et al., 1973), the study of nitrogen fixation communities in termite guts was limited by the molecular

biology method. Many studies are based on clone library which results in a limitation of taking sufficient samples to represent the whole communities. Meanwhile, the understanding of nitrogen fixation of flagellate symbionts in lower termites gut and the discovery of a functional *nifH* gene in Group IV also should be considered.

1.7 Aim of this thesis

In this study, we combined microsensor measurements of physicochemical conditions (pH, hydrogen partial pressure and redox potential) with an assessment of microbial density and the concentrations of inorganic N species in individual gut compartments of hitherto unstudied representatives of several humus- and soil-feeding lineages (*Embiratermes neotenicus, Labiotermes labralis, Palmitermes impostor* and *Amitermes* sp.) and compare them to existing datasets. The environmental condition we measured in this study was also determined the gut microbiota in each gut section (C, M, ms, P1, P3, P4 and P5) from two humus feeders and one soil feeders using Miseq of 16S rRNA genes and combination of taxonomy-based and phylogeny-based approaches. The previous sequencing data from major hindgut compartments (P1, P3, P4 and P5) of termites feed on lignocellulose in different stages of humification (Mikaelyan et al., 2016 and unpublished data) were also involved in the analysis process. A broader selection of gut homologous sections from more hosts representing different subfamilies of higher termites improved our identification of the major drivers of community structure in individual gut compartments among diet, host phylogeny and homologous gut compartments.

While inorganic N pool sizes were measured in higher termites, N nitrogen fixation was studied in lower termites. Although nitrogen fixation in termites has been discovered for more than forty years, the study of nitrogen fixation communities in termite guts was based on clone library which results in a limitation of taking sufficient samples to represent the whole communities. Here, we use Illumina Miseq to explore diverse *nifH* sequences across eight phylogenetic families of wood-feeding termites and cockroaches, and qPCR to quantify the *nifH* gene and 16S rRNA gene distribution. Meanwhile, the understanding of nitrogen fixation of flagellate symbionts in lower termites gut and the discovery of a functional *nifH* gene in Group IV also should be considered. Therefore, we also considered *nifH* genes from capillary-picked suspensions of the flagellate symbionts. Despite the amplicon sequencing, the relationship of nitrogen fixation bacteria and diets and phylogeny of hosts was also studied by

the abundance of *nifH* genes in annotated metagenomes from major gut section (P1, P3 and P4) in higher termites.

In order to understand the community structure and diversity of whole gut microbiota and diazotrophs, my PhD study was structured with the following objectives:

1. **Environmental factors of the gut compartments in humivorous termites**
   With different diet, termites gut developed different compartment environments. Previous studies have shown typical feature in soil-feeding termites, like high alkaline condition in the hindgut. However, the hosts are limited in the true soil feeder and humus feeders are still uncovered. Due to the rich nitrogen in the soil, the ammonia concentration in various gut sections is also relatively abundant. In this thesis, I aim to study the microenvironments in two humus feeders and one true soil feeders from hydrogen production, redox potential, pH and ammonia concentration.
2. **Population structure of the gut compartments in humivorous termites**
   There are already researches about whole termite guts and several gut sections in hindguts. But the gut communities in every gut sections and their interactions of microenvironments still need further study. I aim to discover the microbial communities in various gut compartments in humivorous termites and the linkage of microenvironments.
3. **Nitrogen fixation bacteria in gut communities across cockroaches, higher and lower termites**
   In wood feeders, nitrogen supply from gut microbes is more important than termites feed on a nitrogen-rich diet. In the study, we examined intestinal microbial *nifH* genes in cockroaches and termites feed on various diets across eight phylogenetic families by amplicon sequencing and quantitative method (qPCR). I aim to study the diazotroph in the gut, particularly potential diazotroph with the similarity of flagellate symbionts. I also study the abundance of *nifH* genes metagenomes from major gut section (P1, P3 and P4) in higher termites to study the relationship of nitrogen fixation bacteria and host diet and phylogeny.

Chapter 2

Materials and methods

Termites and sample preparation

The termites were collected together with the nest material and carried in plastic containers to our laboratory, where they were maintained together with soil from the vicinity of their mounds. All measurements were made within one month of termite collection. For all the experiments described in this study, only the worker caste termites were used. Species identification was confirmed by sequence analysis of the gene encoding cytochrome oxidase subunit 2 (COII; for accession numbers, see Table 2.1) using the same protocols of our previous studies (Pester and Brune, 2006; Austin et al., 2004; Liu and Beckenbach, 1992).

Termites (worker caste) were dissected with fine-tipped forceps within a few days of arrival in the laboratory in Marburg. For each termite species, each gut compartments, comprising the crop, the midgut, mixed segment and the four major hindgut compartments (P1, P3, P4, and P5) was pooled from 10–20 individuals in 2-ml tubes containing 750 µl sodium phosphate buffer (120 mM; pH 8.0), homogenized them with sterile micropestles (Eppendorf, Hamburg, Germany) and purified using a bead-beating protocol as previously described (Paul *et al.*, 2012). DNA yield and purity in the extracts were assessed spectrophotometrically using the NanoDrop ND-1000 (Thermo Scientific, Schwerte, Germany).

Table 2.1 Termites used in the study of homologous gut compartments of higher termites, their feeding groups and dietary preferences. Species IDs of the termite hosts are used throughout the thesis.

ID	Host species (subfamily[a])	Feeding group[b]	Diet[c]	Origin	COII gene accession number
1	*Nasutitermes corniger* Nc150 (Nt)	W	Wood	L1	AIZ68286
2	*Microcerotermes parvus* Mp193 (Tt)	W	Wood	F1	AIZ68273
3	*Trinervitermes* sp.Tx114 (Nt)	W	Grass	F2	KT184474
4	*Cornitermes* sp. Co191 (St)	L	Litter[d]	F3	AIZ68247
5	*Embiratermes neotenicus* En289 (St)	H	Humus	F3	KY436202
6	*Palmitermes impostor* Pi290 (Tt)	H	Humus	F3	KY224567
7	*Neocapritermes taracua* Nt197, Nt323 (Tt)	H	Humus	F3	AIZ68299
8	*Termes hospes* Th196 (Tt)	H	Humus	F1	AIZ68312
9	*Promirotermes* sp. Px188 (Tt)	H	Humus	F4	KT184479
10	*Labiotermes labralis* Ll288(St)	S	Soil	F3	KY436201
11	*Cubitermes ugandensis* Cu122 (Tt)	S	Soil	F5	AIZ68260
12	*Amitermes* sp. Ax121 (At)	S	Soil	F2	KY224581
13	*Ophiotermes* sp. Ox79b (Tt)	S	Mound[e]	F6	KT184477
14	*Cubitermes umbratus* TD83 (Tt)	S	Soil	F7	AB304487
15	*Nasutitermes matangensis* Nx348 (Nt)	W	Wood	F8	KY224422

[a] Subfamilies: Tt, *Termitinae*; Nt, *Nasutitermitinae*; St, *Syntermitinae*, At, Amitermitinae

[b] Feeding groups: W, wood or grass feeders; L, litter feeders; H, humus feeders; S, 'true' soil feeders.

[c] Based on the food types given for termite genera (Jones and Eggleton, 2010).

[d] Based on observations of Gontijo and Domingos (1991).

[e] This termite is an inquiline of the soil-feeding *C. ugandensis* and presumable feeds on its mound material, which is composed of soil and feces.

[f] F, Field collections (F1, near Pointe-Noire in the Democratic Republic of the Congo [by David Sillam-Dussès]; F2, JKUAT, Kenya [by James Nonoh]; F3, Petit Saut, French Guiana [by David Sillam-Dussès]; F4, ARC-PPRI Rietondale Research Station, Pretoria, South Africa [by Michael Poulsen]; F5, Eldoret, Kenya [by D. Kamanda]; F6, Nagada, Papua New Guinea [by Mgr. Jan Šobotník]; F7, Kalunja Gl., Kakamega, Kenya [by D. Kamanda]; F8, Kalunja Gl., Kakamega, Kenya [by D. Kamanda]. L, laboratory colonies (L1, Rudolf Scheffrahn, University of Florida, Fort Lauderdale, FL, USA).

For Miseq of *nifH* genes, termites were taken from colonies maintained in the laboratory or were collected in the field (Table 2.2). Cockroaches and other insects were purchased from a commercial breeder (J. Bernhardt, Halsbrücke, Germany, www.schaben-spinnen.de), and the hindguts were dissected immediately upon arrival. In some cases, field-collected termites had to be preserved in ethanol for transport. Since the entire guts of ethanol-preserved specimens were processed within less than 1 week, detrimental effects of this treatment on community structure can be excluded (Deevong et al., 2006).

Table 2.2 List of termites and cockroaches used in studying nitrogen fixation

Family	subfamily	species	Short saying	Feeding[a]	Origin[b]	NCBI accession number[c]
Termitidae	Nasutitermitinae	*Nasutitermes takasagoensis*	Nt	Wood	F1	472
Termitidae	Nasutitermitinae	*Nasutitermes corniger*	Nc	Wood	L1	473
Termitidae	Nasutitermitinae	*Nasutitermes ephratae*	Ne	Wood	F2	474
Termitidae	Nasutitermitinae	*Nasutitermes gaigei*	Ng	Wood	F2	475
Termitidae	Nasutitermitinae	*Constrictotermes cyphergaster*	Ccy	Wood/Liquen	F3	476
Termitidae	Nasutitermitinae	*Trinervitermes sp*	Ts	Grass	F4	477
Termitidae	Syntermitinae	*Silvestritermes holmgreni*	Sh	Wood/Soil	F2	478
Termitidae	Syntermitinae	*Cornitermes cumulans*	Ccm	Soil/Grass	F5	479
Termitidae	Termitinae	*Microcerotermes indistinctus*	Mi	Wood	F2	480
Termitidae	Termitinae	*Cylindrotermes sapiranga*	Cs	Wood	F2	481
Rhinotermitidae		*Coptotermes acinaciformis*	Ca	Wood	F6	482
Rhinotermitidae		*Coptotermes niger*	Cn	Wood	L2	483
Rhinotermitidae		*Reticulitermes grassei*	Rg	Wood	F7	484
Rhinotermitidae		*Reticulitermes santonensis*	Rs	Wood	L3	485
Rhinotermitidae		*Prorhinotermes inopinatus*	Pi	Wood	F8	486
Rhinotermitidae		*Prorhinotermes canalifrons*	Pc	Wood	F9	487
Mastotermitidae		*Mastotermes darwiniensis*	Md	Wood	L2	488
Kalotermitidae		*Cryptotermes cavifrons*	Cc	Wood	F10	489
Kalotermitidae		*Cryptotermes brevis*	Cb	Wood	F10	490
Kalotermitidae		*Glyptotermes barbouri*	Gb	Wood	F11	491
Kalotermitidae		*Incisitermes snyderi*	Is	Wood	F10	492
Kalotermitidae		*Incisitermes marginipennis*	Im	Wood	L2	493
Kalotermitidae		*Incisitermes tabogae*	It	Wood	L2	494
Kalotermitidae		*Incisitermes schwarzi*	Isch	Wood	F10	495
Kalotermitidae		*Neotermes jouteli*	Nj	Wood	F10	496

Family	subfamily	species	Short saying	Feeding[a]	Origin[b]	NCBI accession number[c]
Hodotermitidae		*Hodotermes mossambicus*	Hm	Grass	F12	497
Termopsidae		*Zootermopsis nevadensis*	Zn	Wood	L3	498
Termopsidae		*Hodotermopsis sjoestedti*	Hs	Wood	L2	499
Cryptocercidae		*Cryptocercus punctulatus*	Cp	Wood	F13	500
Blaberidae	Panesthiinae	*Panesthia angustipennis*	Pa	Wood	B1	501
Blaberidae	Panesthiinae	*Salganea esakii*	Se	Wood	B1	502
Blaberidae		*Rhyparobia maderae*	Rm	Generalists	B1	503

[a] Based on the food types given for termite genera (Jones and Eggleton, 2010).

[b] F, field collections (F1, near Nishihara, Japan [by Gaku Tokuda]; F2, João Pessoa – PB, Brazil [by Ricardo Augusto Nink]; F3, São João do Cariri- PB, Brazil [by Ricardo Augusto Nink]; F4, near Nairobi, Kenya [by James Nonoh]; F5, Brasília - DF, Brazil [by Ricardo Augusto Nink]; F6, Australia [by Ghislaine Small]; F7, Pointe des Espagnols, Forêt de la Coubre, France [by Katja Meuser]; F8, Kau Wildlife Area, Madang Province, Papua-New Guinea [by Robert Hanus]; F9, Réunion Island, France [by Robert Hanus]; F10, Fort Lauderdale, FL, USA [by Rudolf Scheffrahn]; F11, Petit Saut, French Guiana [by Jan Šobotník; F12, near Pretoria, South Africa [by Jeffery Rohland]; F13, Heywood County, NC, USA [by Christine Nalepa]]); L, laboratory colonies (L1, Rudolf Scheffrahn, University of Florida, Fort Lauderdale, FL, USA; L2, Rudy Plarre, Federal Institute for Materials Research and Testing, Berlin, Germany; L3, MPI Marburg;). B, commercial breeders (B1, Jörg Bernhardt, Helbigsdorf, Germany [http://www.schaben-spinnen.de];

[c] All biosamples were submitted to the the BioSample database of NCBI. The full accession number is SAMN07985nnn – the last three digits are indicated in the table.

Microsensor

Intestinal hydrogen concentrations, pH and redox potential were measured with microsensors (Unisense, Aarhus, Denmark). Hydrogen (H_2-10, 8-12 μm tip diameter) microsensors were calibrated in Ringer's solution as described previously (Brune, Emerson and Breznak 1995) using synthetic air or a H_2/N_2 mixture (5/95, v/v). The redox microelectrode (RD-10, 8-12 μm tip diameter) was calibrated with saturated solutions of quinhydrone in pH standards of pH 4.0 and 7.0. For pH and redox microsensors, the electric potential was measured against a custom-built Ag-AgCl reference electrode.

The glass pH microelectrodes (pH-10, 8-12 μm tip diameter, 50-100 μm tip length) were calibrated with commercial standard solutions of pH 4.0, 7.0, 10.0 (HANNA Instruments, Rhode Island, USA), 11.0, and 13.0 (Buddeberg, Mannheim, Germany). The calibration standards in the range of pH 11.0 to 12.0 (intervals of 0.2 units) were freshly prepared by mixing 0.05 M Na_2HPO_4 and 0.1 M NaOH, those in the range of pH 12.0 to 13.0 by mixing 0.2 M KCl and 0.2 M NaOH using commercial stock solutions (Sigma-Aldrich, Taufkirchen, Germany) at the volumes required by the buffer equation. All standards for pH 11 and above were freshly prepared from titrated stock solutions in CO_2-free distilled water, which were kept in glass-stoppered bottles to prevent absorption of atmospheric CO_2. pH values were calculated using a non-linear regression (polynomial) because the response of the pH microelectrodes was linear between pH 4 and10, but decreased progressively at higher pH values (Figure S6.1), which is consistent with previous observations (Brune and Kühl, 1996).

For all microsensor measurements, the termites were dissected using sterile fine-tipped forceps and then the entire intact whole gut was placed in glass-faced micro-chambers and embedded with insect Ringer's solution with 0.5% agarose using the same setup as described previously (Brune and Kühl, 1996; Li et al., 2012).

Quantification of abundance of intestinal bacteria

The relative density of bacterial cells was estimated by determining the abundance of bacterial 16S rRNA genes in the respective gut compartments with quantitative real-time PCR (qPCR). The reactions were performed in a CFX Connect™ Real-Time System (BIO-RAD) in volumes of 25 μl and containing 5 μl of 100 fold diluted extracted DNA, 12.5 μl of Universal SybrGreen Supermix (BIO-RAD), and 4 μl 25mM Mg ion solution, 0.5 μM primers 341F/530R (341F, *5′*-CCTACGGGRSGCAGCAG-*3′*; 530R, *5′*-

ATTACCGCGGCKGCTG-*3′*) (Watanabe et al., 2001). The amplifications of 16S rRNA were performed with 2 minutes enzyme activation at 50°C, one initial denaturation step at 95°C for 15 min, followed by 51 amplification cycles of denaturation step at 94°C for 15 s, 60°C for 30s, an extension of 30s min at 72°C and the fluorescent signal collection step at 80°C for 30s. The specificity of the amplification products was confirmed by melting curve analysis, and the expected sizes of the amplified fragments were checked in 1%-agarose gels stained with GelRed™ (Biotium). Standard curves were obtained using serial dilutions of the pGEM®-T vector (Promega, USA) containing 16S rRNA genes of *E. coli* strain RM13516 using 10^3 to 10^8 gene copies μl^{-1}. Threshold values obtained from sample amplification were interpolated in the standard curve determining the number of 16S rRNA genes.

The abundance of nitrogen-fixing bacteria was quantified by quantitative PCR (qPCR) targeting the *nifH* gene. The reactions were performed in a CFX Connect™ Real-Time System (BIO-RAD) in volumes of 25 µl and containing 5 µl of 100 fold diluted extracted DNA, 12.5 µl of Power SYBR Green JumpStart™ *Taq* ReadMix™ (Sigma), and 3 µl 25mM Mg ion solution, 1.25 µM BSA, 0.5 µM primers YAA/IGK for *nifH* gene, or same amount of DNA and SYBR Green mixture, and 4 µl 25mM Mg ion solution, 0.5 µM primers 341F/530R (341F, *5′*-CCTACGGGRSGCAGCAG-*3′*; 530R, *5′*-ATTACCGCGGCKGCTG-*3′*) (Watanabe et al., 2001) for 16S rRNA gene. Briefly, the amplifications of *nifH* were performed with one initial denaturation step at 94°C for 5 min, followed by 40 amplification cycles of a denaturation step at 94°C for 45 s, 55°C for 1 min and an extension of 1 min at 72°C. The amplifications of 16S rRNA were performed with 2 minutes enzyme activation at 50°C, one initial denaturation step at 95°C for 15 min, followed by 51 amplification cycles of denaturation step at 94°C for 15 s, 60°C for 30s, an extension of 30s min at 72°C and the fluorescent signal collection step at 80°C for 30s. The specificity of the amplification products was confirmed by melting curve analysis, and the expected sizes of the amplified fragments were checked in a 1% agarose gel stained with GelRed™ (Biotium). Standard curves were obtained using serial dilutions of the pGEM®-T vector (Promega, USA) containing 16S rRNA genes from strain RM13516 and a cloned *nifH* gene from *Zootermopsis nevadensis*, using 10^2 to 10^7 gene copies μl^{-1}. Threshold values obtained from sample amplification were interpolated in the standard curve determining the number of 16S rRNA genes and *nifH* genes.

The concentrations of inorganic nitrogen species

Twenty-five guts sections of each termite were pooled in 1 ml of ice-cold 10 mM HCl for ammonia extraction or 2 M KCl for nitrite and nitrate extraction. Pooled guts were homogenized using a microprobe (10 W for 10 s) and incubated at 30°C with gentle shaking for 1 h. Homogenates were centrifuged (10,000 × *g* for 20 min.). Ammonia was determined by flow-injection analysis using a conductivity detector (Ji and Brune, 2006). Nitrite and nitrate were quantified with colorimetric assays of transnitration of salicylic acid and the Griess diazotization reaction (DIN EN 26777; Cataldo et al., 1975).

Library construction and sequencing

The V3–V4 region of the 16S rRNA genes in each sample was amplified using the universal bacterial primers 343Fmod and 784Rmod (Köhler et al., 2012), which were modified to include an M13-specific priming site at the 5´ end (Daigle et al., 2011). The cycle conditions for this first PCR step were as described previously (Köhler et al., 2012). The resulting amplicons were used as templates for a second PCR step using M13-specific primers tagged with sample-specific decameric barcodes (454 Roche, Branford, CT, USA), and Herculase II Fusion DNA Polymerase Kit (Agilent Technologies, Santa Clara, CA, USA). Cycle conditions and purification of the PCR products were as previously described (Mikaelyan et al., 2015a). Samples were commercially sequenced (GATC Biotech, Konstanz, Germany) using an Illumina platform (paired-end; Illumina MiSeq).

Processing of sequence data

Forward and reverse reads in the iTag libraries (generated by the Illumina MiSeq platform) were merged to form contigs using *mothur* (Schloss et al., 2009). Merged iTag contigs were processed for quality using the standard operating procedures described previously for Illumina (Kozich et al., 2013) libraries; only reads with a minimum length of 400 bp were used for further analysis.

The group file of quality-checked sequences was by their sample-specific barcodes. After removal of barcodes and primers, sequences in each sample were clustered into operational taxonomic units (OTUs) at a 97% similarity threshold in mothur. Centroid sequences were selected as representatives of each OTU (preserving the number of reads in each OTU) and

aligned with the *mothur* aligner, using the *Silva* reference alignment (SSURef release 128) as a template.

Analysis of community structure

Phylogeny-based similarities in community composition and structure were determined using the unweighted and weighted UniFrac metrics (Lozupone and Knight, 2005) implemented in *mothur*. Generalized unifrac was carried on in R using *GUniFrac* package (Chen et al., 2012). In all cases, a maximum-likelihood tree constructed using *FastTree2* (Price et al., 2010) was used as input. Unifrac distances were analyzed by non-metric multidimensional scaling (NMDS) using the *vegan* package (Oksanen, 2015) in the *R* statistical software suite (R Core Team 2016).

OTU representatives from the libraries were classified using the *RDP* classifier (Wang et al., 2007) implemented in *mothur* with the Dictyoptera taxonomic reference database (DictDb) v. 3.0 (Mikaelyan et al., 2015b), accounting for the number of reads in each OTU, and using a confidence cutoff set at 80%. The heatmap was displayed by the *pheatmap* package (Kolde, 2015) in *R* statistical software suite (R Core Team 2016). A canonical correspondence analysis (CCA) using the cca function in the *vegan* R package (Oksanen, 2015).

DNA amplification, sequencing, and data analysis of *nifH* genes

DNA was extracted from the pooled gut homogenates of 3 to 10 individuals of each species (depending on gut volume) using a bead-beating protocol with phenol-chloroform purification (Paul et al., 2012). The conserved region of the *nifH* gene was amplified in each sample using PCR primers IGK, (*5′*-ATAGGATCCAARGGNGGNATHGGNAA-*3′*); and YAA, (*5′*-GACCTGCAGATRTTRTTNGCNGCRTA-3′) (Ohkuma et al., 1996). In these sequences, N represents A, C, G, or T; R represents A or G and H represents A, C, or T. Each sample was amplified under the following conditions: 94°C for 3 min, 35 cycles of 94°C for 30 s, 48°C for 45 s, and 72°C for 2 min, then 5 min at 72°C. The purified PCR amplicons were analyzed using a Miseq Benchtop Sequencer for 2×250 bp paired-end sequencing (GATC, Germany).

The pyrotag sequences were preprocessed and aligned using the Mothur software suite (Schloss et al., 2009) under stringent conditions (reads of 200 bp, no ambiguous bases, and a maximum number of homopolymers of 10). All sequences were aligned using the core alignment in Gaby and Buckley's Database (Gaby and Buckley, 2011), and the complete

linkage clustering method was used to define operational taxonomic units (OTUs) using 99% identity as a cutoff. All samples were subsampled to the smallest number of reads per sample in the data set (579 reads). Classification-independent ordinations with UniFrac (Lozupone and Knight, 2005) displayed using principal-coordinate analysis (PCoA). PCoA analysis was made by R statistical software suite (R Core Team 2016) using *ape* and *vegan* packages (Oksanen, 2015; Paradis et al., 2004). A phylogenic tree of *nifH* genes based on deduced amino acid sequences was calculated by FastTree2 (Price et al., 2010) using WAG model (Whelan and Goldman, 2001). Heatmaps were also made by R using *pheatmap* package (Kolde, 2015). The *nifH* genes from annotated metagenomes (Rossmassler et al., 2015) were also analyzed and filtered based on the core alignment in Gaby and Buckley's Database (Gaby and Buckley, 2011).

Chapter 3

Results

3.1 Environmental factors in the gut compartments of humivorous higher termites

Summary

Higher termites have a highly compartmentalized gut and a broad range of diet along the humification gradient of substrate from wood to soil. Analysis of amplified 16S rRNA genes of gut microbiota showed that the community structure of homologous compartments in the major hindgut (P1 to P4) of different species shared similarity. Besides, P3 and P4 colonized by specific bacteria lineages associated with termite feeding groups. However, the physiochemical condition in the major hindgut compartments of humivorous species has not been studied. Here, we identified the whole hindgut (P1 to P5) of more representative termites fed on various diet and even the whole gut (Crop to P5) of soil-feeder (*Labiotermes labralis*) and humus-feeder (*Embiratermes neotenicus* and *Palmitermes impostor*), whose microenvironments were studied in a companion project. Homologous compartments drove the similarity of gut communities in humus- and soil-feeding termites but not in wood- and grass-feeding termites in which case host phylogeny and diet were decisive. In wood- and grass- feeding termites, dominating gut microbiota were from *Actinobacteria*, TG3, *Fibrobacteres* and *Spirochaetes*. On the other hand, abundant genera were from *Bacteroidetes*, *Spirochaetes* and *Firmicutes* in humus- and litter-feeding termites. The distribution of specific core bacteria like *Candidatus* Arthromitus and *Candidatus* Armantifilum in consecutive gut sections showed the adaption to microenvironments. The results underscore that the intestinal bacteria in each gut section are influenced by multiple environmental factors like pH, H_2 and dietary substrate, and enhance the digestion process of host.

Results

The continuous decreasing fiber and increasing nitrogenous contents during the humification of lignocellulose, therefore making the dietary component of humivorous termites in tropical soils far more complex. In this study, we show fundamentally distinct physicochemical gut conditions, intestinal anatomy, bacterial distributions and inorganic nitrogen concentrations in wood-, humus- and soil-feeding termites belong to evolutionary independent lineages, which shed considerable new light on the adaptations to different humivorous diets.

Gut structure in different feeding groups

In higher termites involved in this study here, the hindgut is further elongated and more compartmentalized than lower termites (Fig. 3.1). The gut segments of different feeding group termites differed in gut volume and weight (Table S6.2). P1 is generally larger in soil feeders and humus feeders than in wood-feeders. The size of P1 is characteristic feature in monophyletic genera (Noirot 2001). The anterior hindgut gut P1-to-P3 volume ratios, determined from soil-feeders *C. orthognathus*, *C. umbratus*, *L. labralis*, and humus feeders *E. neotenicus*, *P. impostor* were 1.60, 1.41, 0.64, 0.23, and 0.54, respectively, compared with 0.05 in the wood-feeder *N. matangensis* (Table S6.2). Moreover, the anterior hindgut gut P1-to-P3 weight ratios were 1.00, 0.18 for soil-feeders, and 0.20, 0.67 for humus-feeders, compared with 0.1 in the wood-feeder (Table 3.1).

Bacterial population distribution

The bacterial abundance in the gut sections differed greatly. The highest numbers were found in the P3 compartment of all feeding guilds termites (Table 3.1). The number of bacterial copies was highest in P3 of wood-feeder *N. matangensis* (15.20×10^6 copies per gram fresh weight), but decreased strongly in the humus-feeder *E. neotenicus* and *P. impostor* (1.22×10^6 and 5.04×10^6, respectively). Whereas the same decreases also exist in the soil-feeder *L. labralis* and *C. ugandensis* (0.73×10^6 and 5.04×10^6). In the anterior hindgut P1 compartment, the lowest bacterial copies in *L. labralis* and *C. ugandensis* were 0.23×10^6 copies per gram fresh weight.

Table 3.1 Bacterial abundance in different gut sections of termite species included in this study. The fresh weight of each section allows to estimate cell density and their volumes (if available) are shown in Table S6.1. The data of *Cubitermes ugandensis* and bacterial abundance of *Nasutitermes matagensis* are from other studies (Nonoh, 2013; Köhler et al., 2012).

Termite	Gut section	Fresh wt (mg)[a]	Bacterial abundance (10^6 copies gut section^{-1})[b]
Embiratermes neotenicus	Crop	0.2±0.0	0.01±0.01
	Midgut/ms	0.3±0.4	0.09±0.02
	P1	0.4±0.5	0.15±0.03
	P3	2.0±0.0	2.43±0.43
	P4	0.2±0.0	0.18±0.04
	P5	0.2±0.0	0.14±0.01
Labiotermes labralis	Crop	0.1±0.0	0.04±0.02
	Midgut/ms	0.9±0.1	0.23±0.03
	P1	1.6±0.3	0.37±0.05
	P3	2.2±0.4	1.61±0.40
	P4	2.6±0.1	0.44±0.04
	P5	0.9±0.2	0.36±0.09
Cubitermes ugandensis	Crop	0.1±0.2	0.04±0.02
	Midgut/ms	0.1±0.3	0.04±0.03
	P1	0.7±2.2	0.16±0.06
	P3	0.7±1.2	3.53±0.48
	P4	0.1±0.3	1.03±0.04
	P5	0.1±0.4	0.04±0.02
Palmitermes impostor	Crop	0.2±0.2	0.22±0.12
	Midgut/ms	0.4±0.4	0.13±0.05
	P1	0.2±0.2	0.64±0.43
	P3	0.3±0.2	4.23±1.67
	P4	0.4±0.0	3.00±1.52
	P5	1.1±0.0	0.68±0.21
Nasutitermes matangensis	Crop	0.1±0.1	0.15±0.03
	Midgut/ms	0.2±0.1	0.08±0.02
	P1	0.1±0.1	0.10±0.04
	P3	1.0±0.1	15.20±3.10
	P4	0.1±0.1	0.08±0.01
	P5	0.1±0.1	0.04±0.01
Amitermes sp.	Crop	NM[c]	0.02±0.00
	Midgut/ms	NM	0.58±0.00
	P1	NM	1.02±0.00
	P3	NM	3.80±0.38
	P4	NM	1.99±0.84
	P5	NM	1.18±0.21

[a] Values are mean ± mean deviation of three independent sample preparations except P3 of *Labiotermes labralis* based on two independent preparations. One preparation included five guts in all cases.

[b] Values are mean ± mean deviation of two independent sample preparations.

[c] Not measured.

Inorganic nitrogen

The concentrations of inorganic nitrogen species found in the gut of soil-feeding groups were higher than in the humus-feeders (Fig. 3.1). Moreover, the lowest concentrations were found in wood-feeders *N. matangensis*. For ammonia concentrations, the highest values were always found in the posterior hindgut P4 and P5, and the lowest concentrations were always found in the anterior hindgut P1 and P3, irrespective of feeding guilds. Also, nitrate and nitrite concentrations differed among gut sections of feeding guilds. The high nitrate concentrations were found in the soil-feeders *C. ugandensis* and *C. umbratus*.

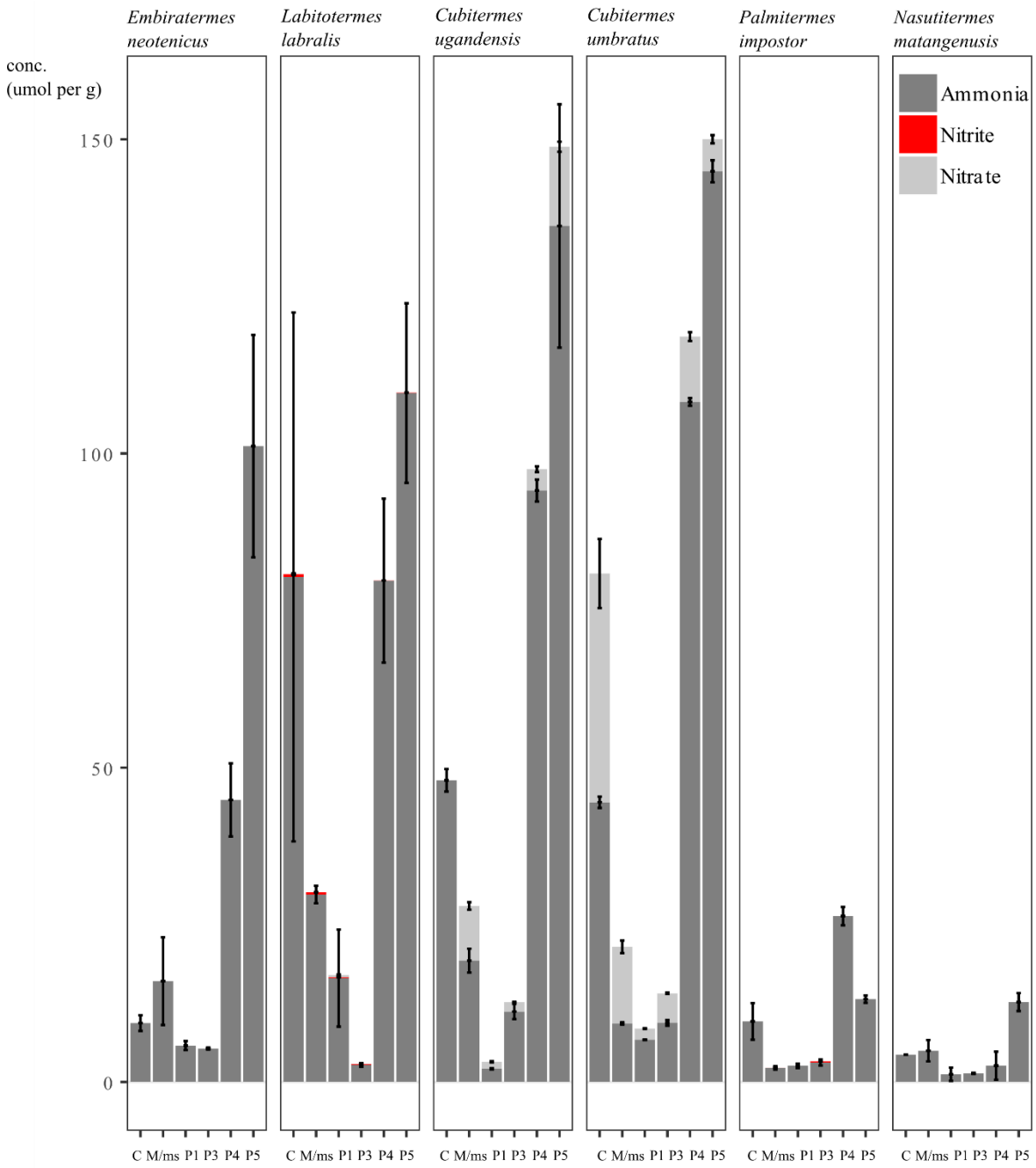

Figure 3.1 The concentrations of inorganic N species in different gut sections of the termite species investigated in this study. Each sample has three replication. The intact error bar stands for the sd value. The data of *Cubitermes* spp. are from previous study (Ngugi, Ji and Brune, 2011).

Physicochemical conditions

The pH calibration curve at high alkaline condition above pH 10 is not a linear standard curve in the previous microsensor measurement (Brune and Kühl, 1996). Therefore, we also freshly prepared pH standard solution to overcome the problem of atmospheric carbon dioxide absorption in the range of high pH value. The microelectrode showed a linear response from pH 4 to 11. With 0.2 pH unit, the calibration curve of pH microelectrode became non-linear and fitted polynomial from pH 11 to 13 (Figure S6.2).

In agreement with previous reports, the pH sharply increases in the mixed segment of all the higher termites particularly in the soil-feeders *C. ugandensis* and *L. labralis*, and in general about 3-4 units above midgut pH. The pH continued to reach maximum values in the dilated P1 gut compartment in all termites, and the most alkaline values recorded in the soil-feeders *C. ugandensis* and *L. labralis* and the humus-feeder *E. neotenicus*, with the pH values from 11.06 to 11.56 (Fig. 3.2). By contrast, the pH values found in the P1 of humus-feeder *P. impostor* and *Amitermes* sp. were less alkaline ranging from 10 to 11 (Fig. 3.2 and Fig. S6.2). The lowest pH values of all P1 compartments were found in the tubular P1 of wood-feeder *N. matangensis*, which were as less alkaline as around 10.

The redox potentials, the only negative values occurred in the P3 region of the *N. matangensis*. Whereas the negative values were found both in the P1 and P3 for the rest termites.

The accumulation of H_2 is restricted to P3 region for *N. matangensis* and *L. labralis*. While the maximal values occurred in the P1 region for *E. neotenicus*, *P. impostor*, and *Amitermes* sp. Interestingly, in the *C. ugandensis*, both mixed segment and P3 accumulated H_2. The highest hydrogen partial pressures were found in *N. matangensis*, whereas the *C. ugandensis* and *L. labralis* with relative low hydrogen pressures.

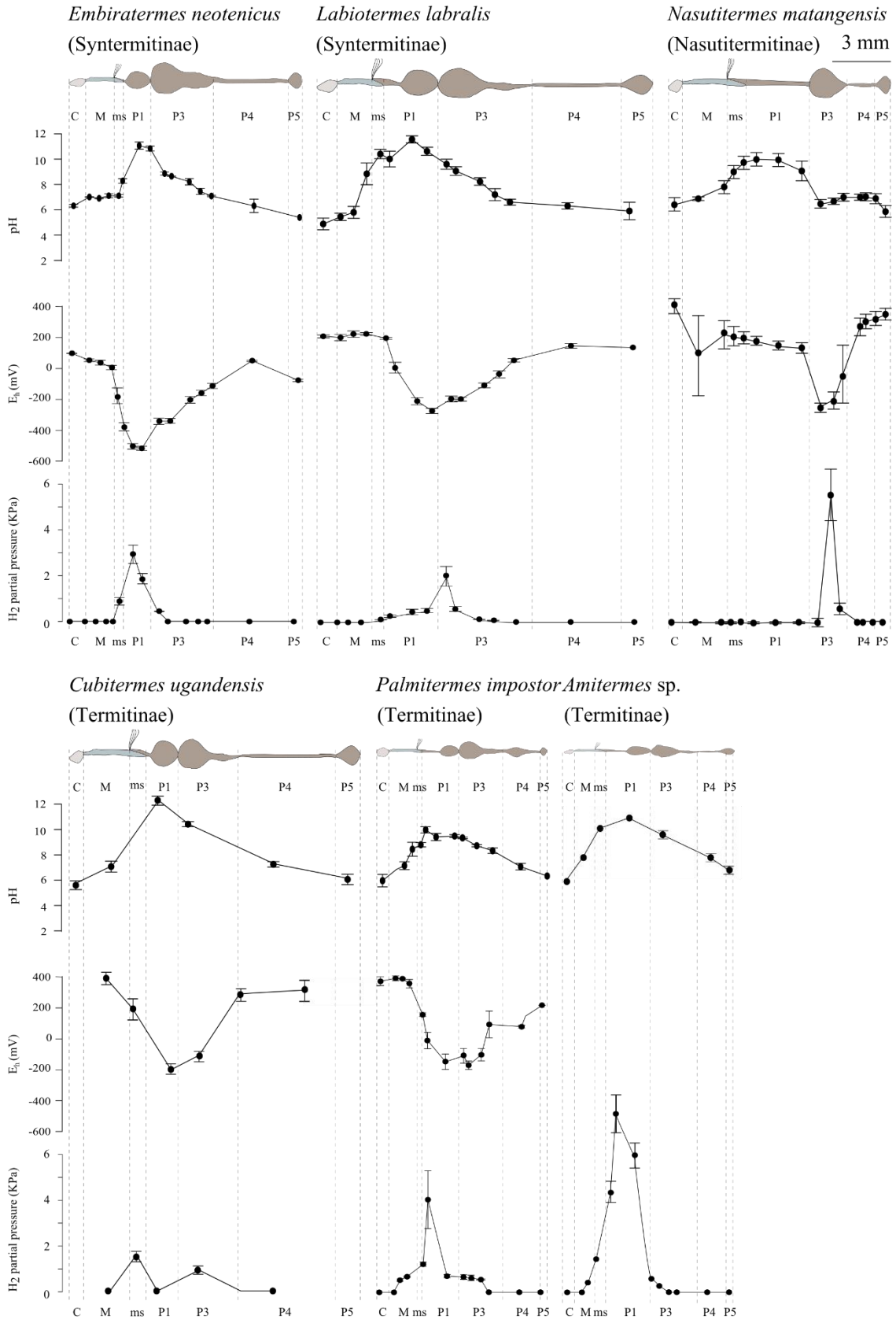

Figure 3.2 Axial profiles of pH, redox potential, and hydrogen partial pressure along the gut of the termite species investigated in this study. The data of Nasutitermes matagensis, Cubitermes ugandensis and Amitermes sp. are from other studies (Köhler et al., 2012; Kappler and Brune, 2002; Nonoh, 2013). The error bars were determined from three replications.

3.2 Effect of diet and gut environment on community structure in higher termites of different feeding groups

Summary

Higher termites have highly compartmentalized gut and a broad range of diet along the humification gradient of substrate. By amplified 16S rRNA genes of gut microbiota, it showed that the community structure of homologous compartments in the major hindgut (P1 to P4) shared similarity. Besides, P3 and P4 colonized by specific bacteria lineages associated with termite feeding groups. Here, we identified the whole hindgut (P1 to P5) of more representative termites fed on various diet and even the whole gut (Crop to P5) of soil-feeder (*Labiotermes labralis*) and humus-feeder (*Embiratermes neotenicus* and *Palmitermes impostor*), whose microenvironments were studied in a companion project. Homologous compartments drove the similarity of gut communities in humus- and soil-feeding termites but not in wood- and grass-feeding termites in which case host phylogeny and diet were decisive. In wood- and grass- feeding termites, dominating gut microbiota were from *Actinobacteria*, TG3, *Fibrobacteres* and *Spirochaetes*. On the other hand, abundant genera were from *Bacteroidetes*, *Spirochaetes* and *Firmicutes* in humus- and litter-feeding termites. The distribution of specific core bacteria like *Candidatus* Arthromitus and *Candidatus* Armantifilum in consecutive gut sections showed the adaption to microenvironments. The results underscore that the intestinal bacteria in each gut section are influenced by multiple environmental factors like pH, H_2 and dietary substrate, and enhance the digestion process of host.

Results

Quality processing of the amplicon libraries yielded (on average) about 2,500 sequence reads for each of the pyrotag libraries and 60,000 reads for each of the iTag libraries (Table S6.2). Classification results at different taxonomic levels are shown in Table S6.3. Classification with the taxonomic framework of DictDb (Mikaelyan et al., 2015b) successfully assigned taxonomic ranks to above 97% reads in the libraries at the phylum level. Classification success decreased with taxonomic depth but remained high (67% on average) down to the genus level (Table S6.2).

Community similarity driven by multiple factors

Ordination analysis based on a classification-independent weighted Unifrac revealed that the similarity of gut microbiota communities did not appear only in homologous compartments, same diet group or phylogeny of hosts (Fig. 3.3; Fig. S6.2). For example, a similarity of homologous compartments appeared in most litter and humus feeders and soil feeders with the exceptions from P1, P3 in *Promirotermes* sp. and P3 in *Amitermes* sp. In wood and grass feeders, gut bacteria community structures showed similarity in the same termite or in the same diet, such as P1 and P4 compartments in *Nasutitermes corniger*, P1 in *Microcerotermes parvus* and P4 in *Trinervitermes* sp. This indicates that the similarity of homologous compartments from different termites was driven by more than one factors.

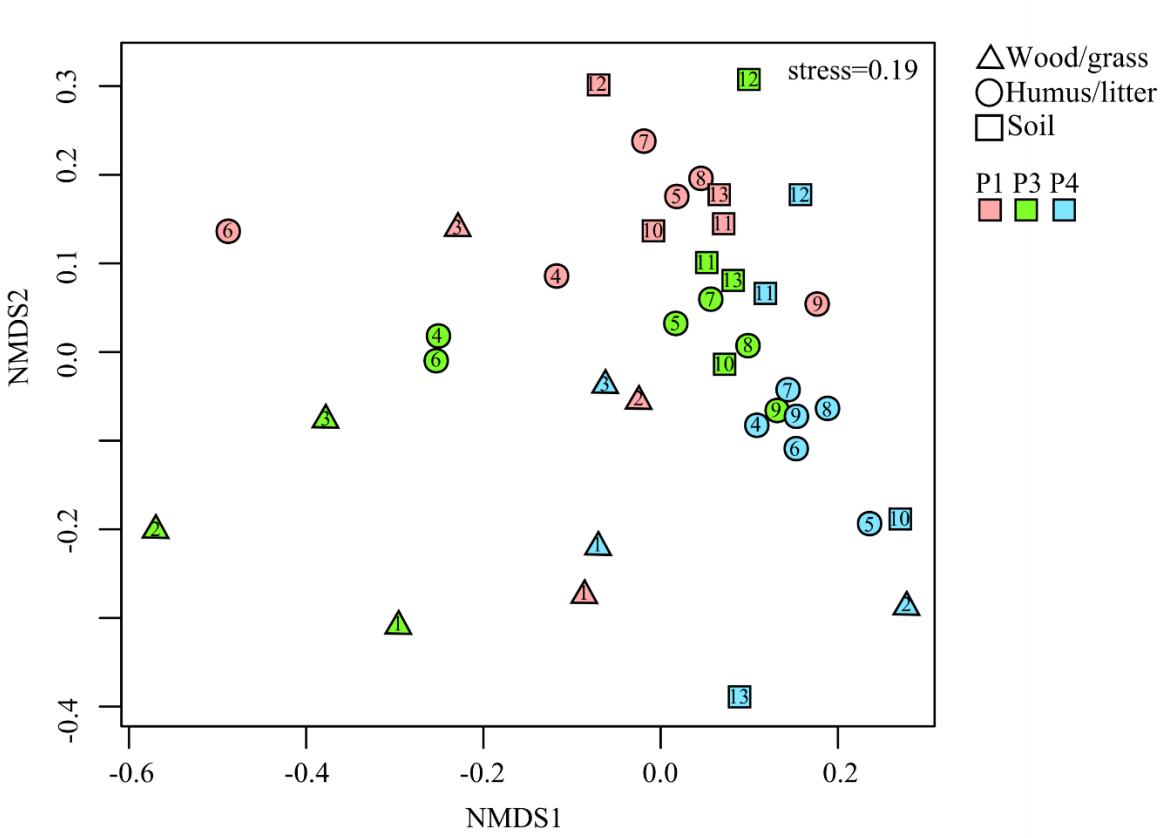

Figure 3.3 NMDS ordination of community structure based on weighted UniFrac distances of the bacterial microbiota associated with the major hindgut compartments of higher termites from different feeding groups. Gut compartments are color-coded; species IDs of the termite hosts are the same as those used in Table 2.1.

Core taxa at genus level

These multiple factors trends were substantiated by core genus-level taxa in homologous compartments, diet groups and major host groups (Fig. 3.4). In homologous gut sections, crop, midgut and mixed segment showed a high proportion (about 30% to 60%) of sequence reads appeared in the entire data set, while in rest gut sections only P3 and P5 had a small number (below 10%). Considering the core taxa based on diet or host subfamily, the sequences appearance in entire dataset did not exist in all datasets. When we regarded core status as a presence in >70% of the hosts, core taxa made up a considerable portion of the reads in the entire data set in all cases.

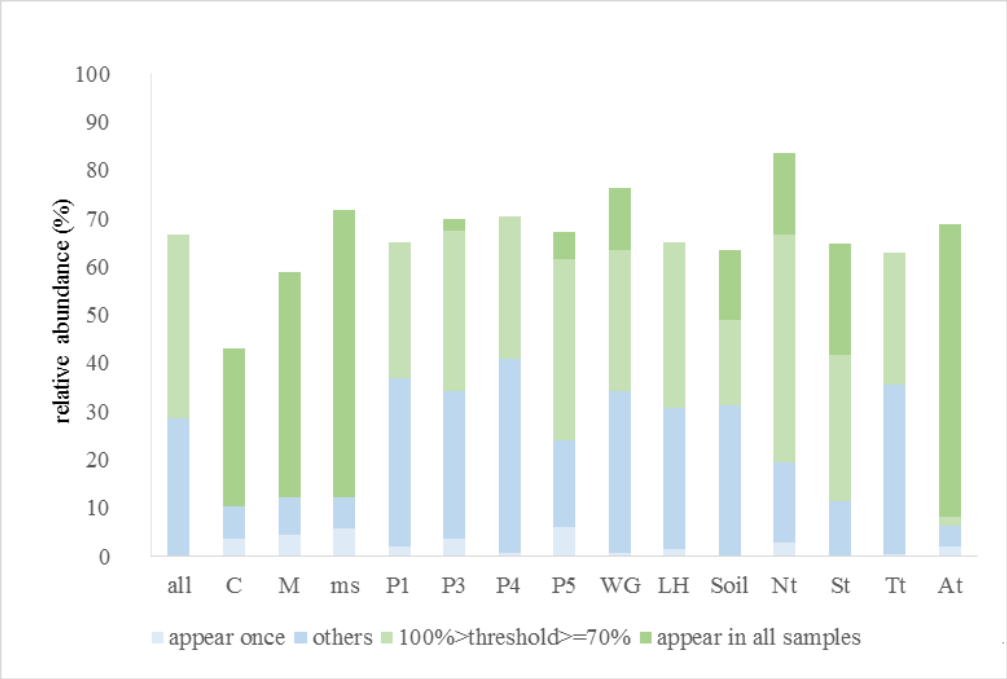

Figure 3.4 Proportion of reads from core taxa among all samples, same gut homologous compartment, same diet and same host family at the genus level. WG: Wood/grass; LH: Litter/humus. Subfamilies: Tt, *Termitinae*; Nt, *Nasutitermitinae*; St, *Syntermitinae*; At, *Amitermitinae*

Specific genus by compartment homology

Compartment-specific bacteria lineages were observed in the distribution and abundance of bacterial populations at the genus level. For example, *Candidatus* Arthromitus and *Candidatus* Armantifilum were core taxa exemplified by the core genera analysis based on homologous compartments. They showed the specific distribution in the anterior gut and posterior gut, respectively (Fig. 3.5). Mixed segment and P1 compartments had the most abundant *Ca.* Arthromitus in grass, litter, humus and soil-feeding termites. By contrast, *Ca.* Armantifilum in P4 compartment had a higher proportion than other compartments (with the exception of *Palmitermes impostor*, *Cubitermes ugandensis* and *Amitermes.* sp.).

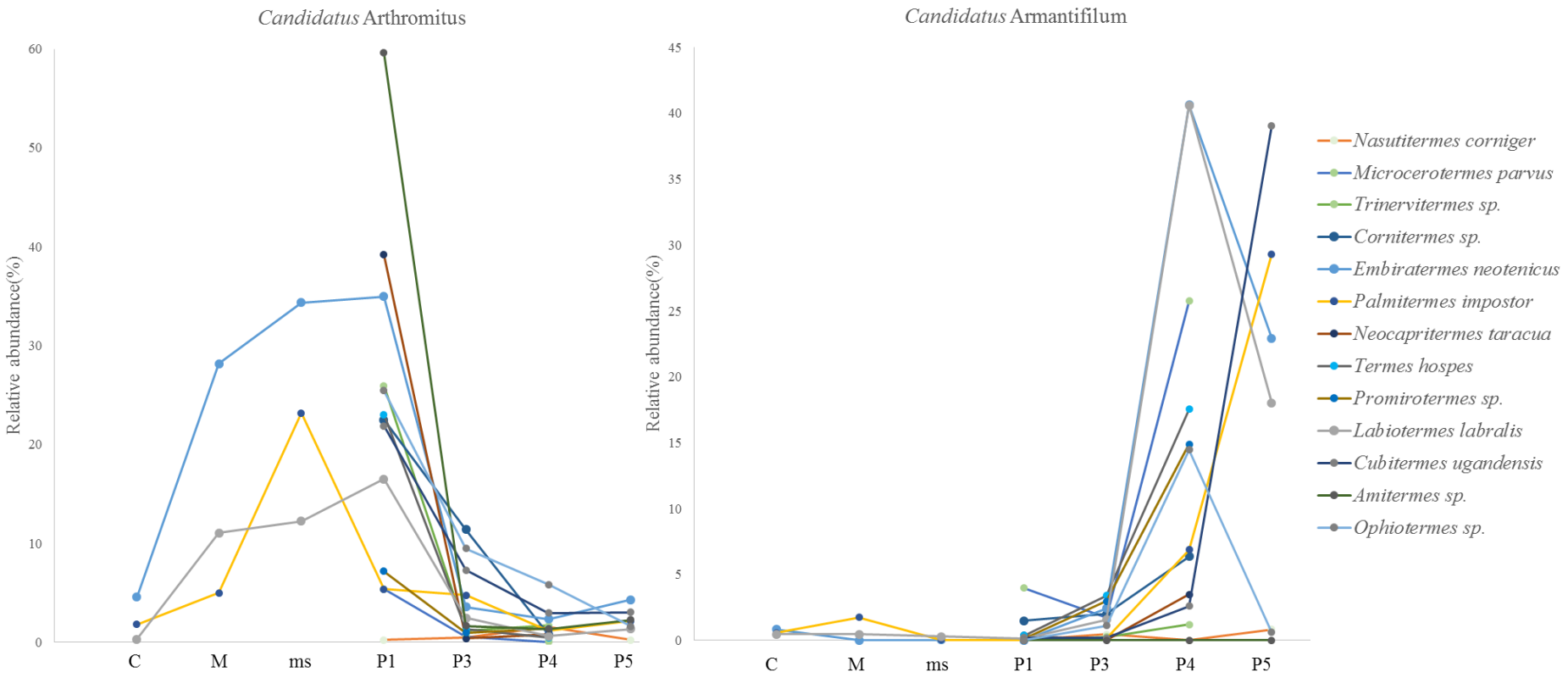

Figure 3.5 Relative abundance of the core taxa *Candidatus* Arthromitus and *Candidatus* Armantifilum in different gut sections. The dots have three color series, green, blue and grey. The green dots stands for wood and grass feeder. The blue dots stand for humus and litter feeders. The grey dots stand for soil and mound feeders.

Major patterns at the genus level

When we focused on the selected candidate genera that showed a potential response to different gut compartments, the hierarchical clustering analysis of samples based on classification showed the influences from multiple factors as well (Fig. 3.6). As in the classifications-independent analysis, wood and grass feeders showed a similarity signal in the same termite (*N. corniger*). In humus-, litter- and soil-feeding termites, all P3 compartments clustered within same diet group except *Embiratermes neotenicus*. From the perspective of gut sections, P1 and P3 clustered with homologous compartments in *Amitermes* sp., *E. neotenicus* and *Labiotermes labralis*, while this was true for P5 in *P. impostor*, *C. ugandensis*, *E. neotenicus* and *L. labralis*.

In wood- and grass- feeding termites, gut microbiota were typically dominated by several genus-level taxa from *Actinobacteria*, TG3, *Fibrobacteres* and *Spirochaetes*. Meanwhile, in humus- and litter-feeding termites, abundant lineages were from *Bacteroidetes*, *Spirochaetes* and *Firmicutes*. However, the relative abundance of *Ca.* Arthromitus and *Synergistaceae* were high in soil-feeding termites.

The most predominant bacterial taxon in the P1 compartment of both humus- and soil-feeding termites was *Ca.* Arthromitus, which formed 5–59% of the total reads among the hosts. In the P3 compartment, 2-8% of reads from humus and litter feeders can be assigned to Termite cluster 3 or *Tannerella* in *Porphyromonadaceae*. Both gut cluster 4 and uncultured 24 lineages in *Ruminococcaceae* family were abundant in humus-, litter- and soil-feeding termites. The P4 compartment of humus feeders was typically associated with a higher proportion of *Ca.* Armantifilum (*Bacteroidales* Cluster V; accounting for 3–40% of the reads) than other diet groups (with the exception of wood-feeding *M. parvus*, soil-feeding *Ophiotermes* sp. and *L. labralis*). By contrast, *Sanguibacter* was abundant in P4 of wood- and grass-feeders. About 8-12% of the reads from P5 compartments of humus feeders and soil-feeding *L. labralis* can be assigned to *Desulfovibrio*.

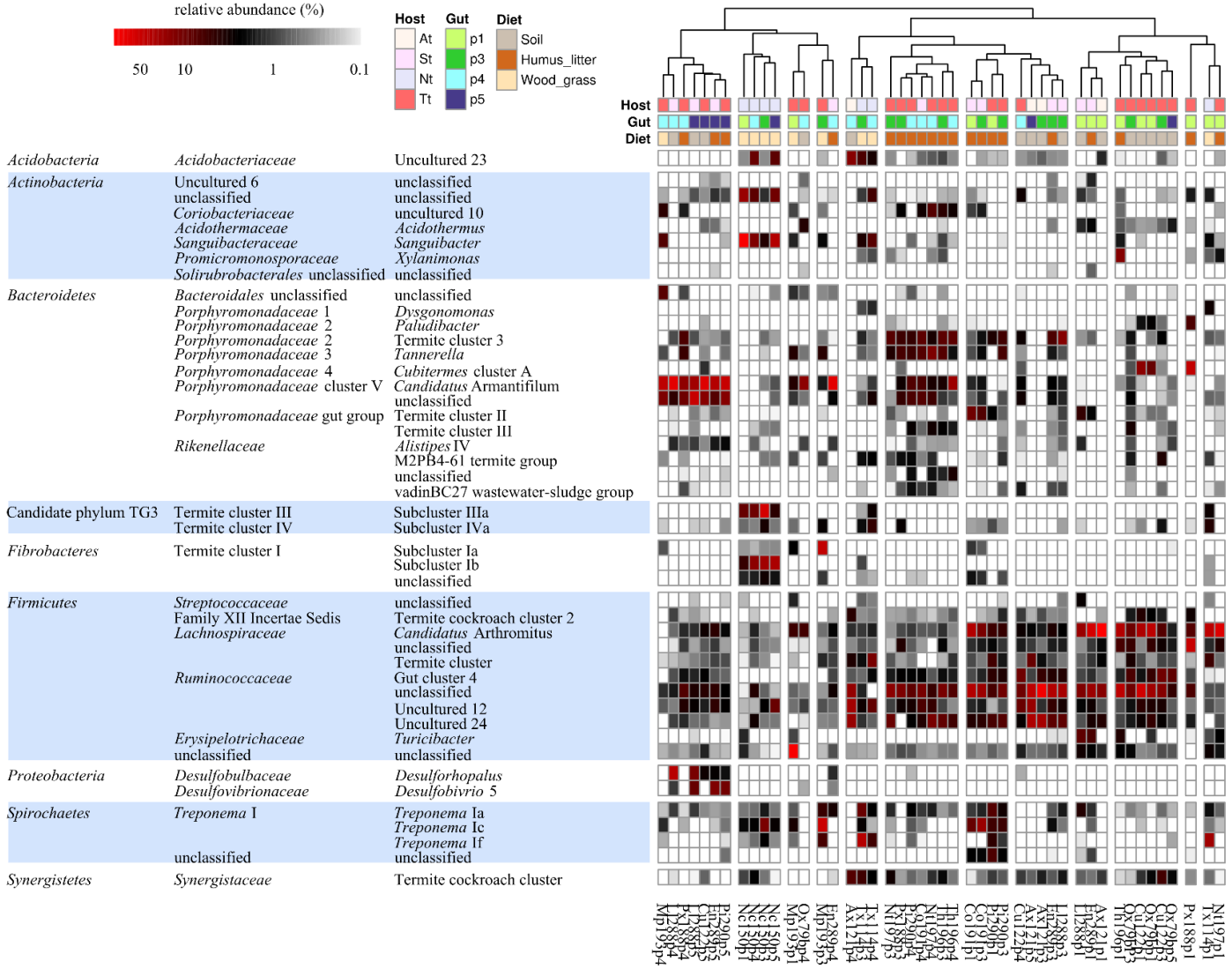

Figure 3.6 Relative abundance of selected genus-level taxa that showed a potential response to gut compartments after filter less abundant taxa (average abundance and maximal abundance are both below 1%)

Canonical correspondence analysis

From the canonical correspondence analysis (Fig. 3.7), the microenvironments of gut sections showed similarity in homologous compartments (P1, P3 and P5 in *C. ugandensis*, *E. neotenicus* and *L. labralis*) or samples from same hosts (P1 and P3 in *P. impostor*). It was consistent with the trend in community structure in NMDS analysis (Fig. 3.3). *Desulfobulbaceae*, *Desulfovibrionaceae*, *Porphyromonadaceae* Cluster V were abundant in P4 in *E. neotenicus* and *L. labralis* and all P5 compartments. These taxa were positively associated with gut ammonia concentration, redox potential, soil feeding diet and Syntermitinae. This top abundant bacteria families gave clues about environmental influences on bacteria distribution. This positive correlation also existed in core genera. The gut cluster 4 (*Ruminococcaceae*) and *Ca.* Arthromitus with hydrogen, gut diameter and pH can be interpreted as well.

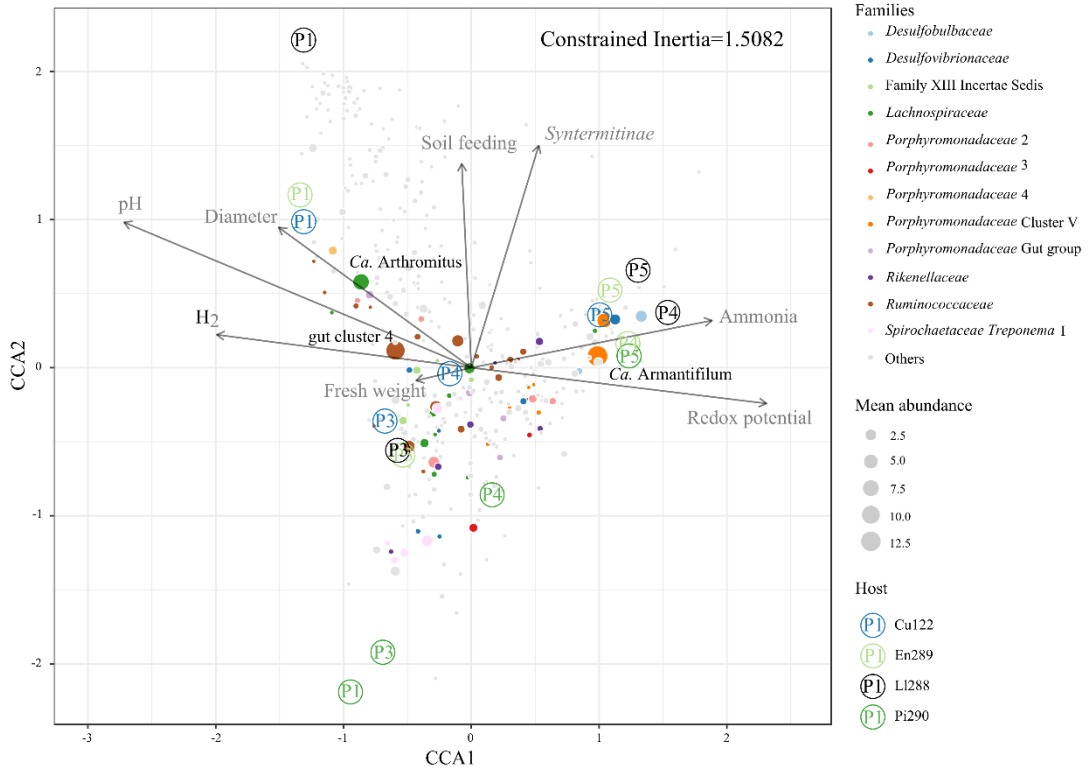

Figure 3.7 Canonical correspondence analysis (CCA) ordination plot of community structure in major gut compartments. The diagram display filled circles that represent genera, hollow circles representing gut sections, and arrows that symbolize environmental and phylogenetic factors.

3.3 Abundance and diversity of nitrogen-fixing bacteria in termite and cockroach guts

Summary

Nitrogen fixation by the gut microbiota is an important nitrogen source for wood-feeding termites, but the identity of the bacteria responsible for the activity and their numerical abundance among the gut microbiota is largely obscure. In this study, we used high-throughput sequencing of amplified *nifH* genes to characterize the potential diazotrophs in various termite families and in related cockroaches, which considerably increased taxon sampling and diversity coverage over previous studies. Ordination analyses showed a clear clustering of the diazotrophic communities among cockroaches (*Blattidae*) and higher termites (*Termitidae*). A discrete clustering was also obtained for lower termites, although they were not fully resolved at the family level. While the majority of the *nifH* sequence reads fell into the radiation of classical nitrogenases (mostly Group III), a relatively large proportion belonged to Group IV, whose function in nitrogen fixation is just emerging. In lower termites, many *nifH* phylotypes clustered with homologs of bacteria associated with termite gut flagellates, corroborating previous evidence for their importance in nitrogen fixation in certain lower termites. In higher termites, the most abundant phylotypes were assigned to homologs in fiber-associated bacteria (*Fibrobacteres* and TG3 phylum). The low ratio of *nifH* genes to 16S rRNA genes in most termites, determined by qPCR, suggests that only a surprisingly small number of gut microbes are diazotrophs. Further studies will have to address the relative expression levels of different *nifH* homologs and the basis of the highly divergent nitrogen-fixing activities in different termite species.

Results

Quantification of *nifH* gene copies by qPCR

Quantitative PCR (qPCR) assays were used to target the universal 16S rRNA genes and the four major *nifH* phylotypes. The results of the qPCR, estimating the abundance of nitrogenase genes in the different gut communities, are presented in Fig. 1. The low ratio of *nifH* genes to 16S rRNA genes in most termites suggests that only a small number of gut microbes are diazotrophs. The ratio of *nifH* to 16S rRNA was variable throughout different host species. The host species with a *nifH* to 16S rRNA ratio higher than 0.0105 all belonged to Kalotermitidae and Rhinotermitidae. Also, cockroaches (Blaberidae) only showed the low ratios of *nifH* genes to 16S rRNA, which are around 0.001. (Fig. 3.8)

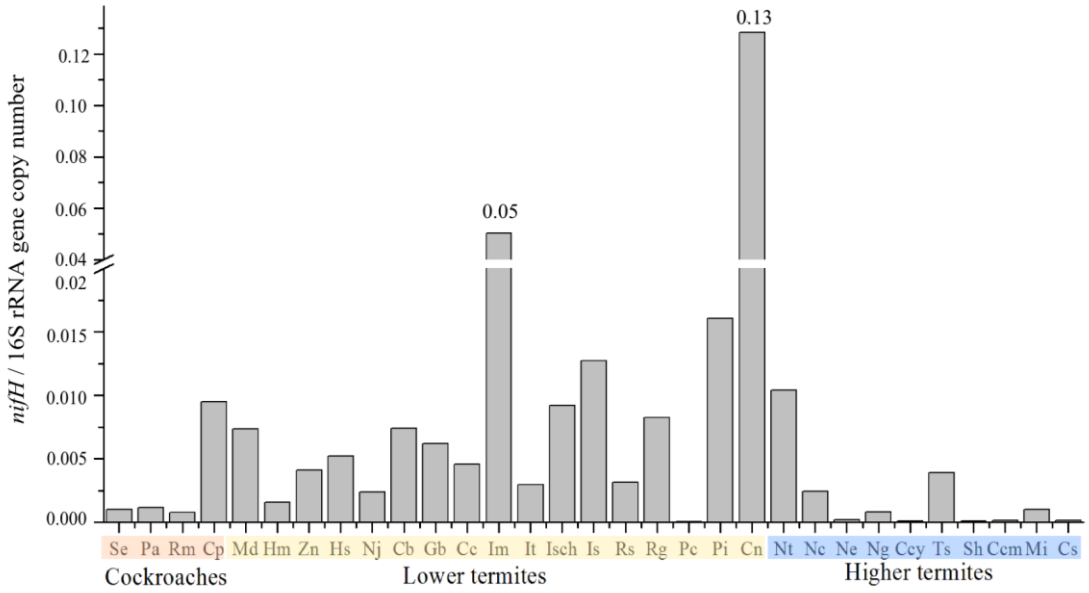

Figure 3.8: quantitative PCR results of the *nifH* gene to 16S rRNA gene copy number ratio. The host species abbreviations on the x-axis refer to Table 2.2. Colors below the graph represent cockroaches (pink), lower termites (yellow), and higher termites (blue). Broken y-axis between 0.02 and 0.04.

Cluster analysis of diazotrophic communities

The diazotrophic community composition across different hosts was assessed by multivariate analyses of the *nifH* OTU based on weighted Unifrac test (Fig. 3.9). The first two principal coordinates accounted for 38.9 % of the variation in the data. From PCoA analysis, we found that the resulting patterns reflected the phylogeny of the

respective hosts in some families. The communities of blaberid cockroaches were clearly separated from those of higher termites (Termitidae) and lower termites except grass-feeding *Hodotermes mossambicus*. The wood-feeding cockroach *Cryptocercus punctulatus* did not cluster among the other cockroaches but clustered with lower termites. The *nifH* groups detected in lower termites Termopsidae and Kalotermitidae were similar within each family, while the *nifH* groups in higher termites (Nasutitermitinae) shared similarity.

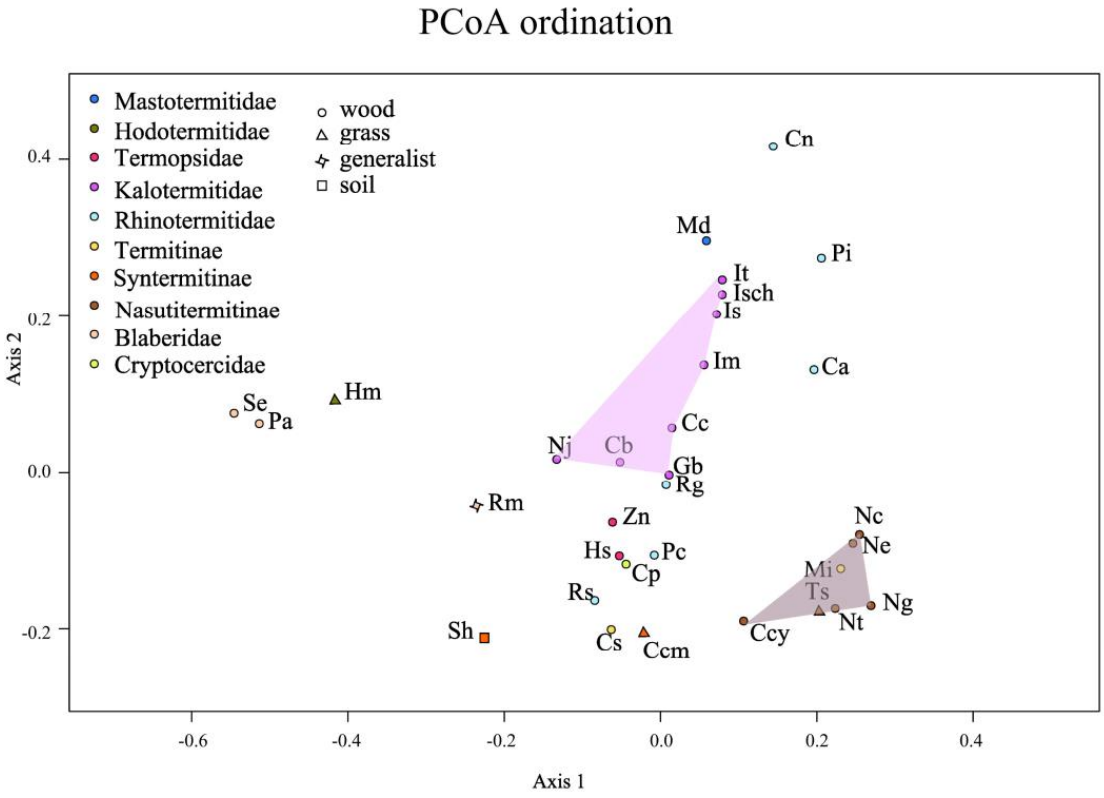

Figure 3.9: Principal coordinate analysis (PCoA) of *nifH* gene diversity in the hindgut of cockroaches and termites, based on weighted UniFrac metric. Horizontal and vertical axes correspond to the first and second principal coordinates, respectively, and percentages of variation explained by these principal coordinates were 23.5% and 15.4%, respectively.

nifH gene phylogeny

Phylogenetic diversity of *nifH* genes obtained by amplicon sequencing from various termites and cockroaches, and their relationship to phylotypes previously assigned to flagellate symbionts and other bacteria from termite guts. While the majority of the *nifH* sequence reads fell into the radiation of classical nitrogenases (mostly Group

III), a relatively large proportion belonged to Group IV nitrogenases, whose function in nitrogen fixation is just emerging.

Using the WAG model, a maximum-likelihood tree based on amino acid alignment was inferred with *nifH* genes from known strains, previous clones from termite libraries and OTUs which represented more than one percentage in corresponding *nifH* composition. There were 248, 98, 4344, 2904 OTUs (cutoff=99%) in Group I, II, III and IV respectively (Fig. 3.10 to Fig. 3.17). The OTU representatives in Group I clustered with *Arcobacter nitrofigilis* and *Azoarcus* sp. BH. In Group II, there were near *Candidatus* Adiutrix intracellularis, *Dickeya dadantii* Ech703, *Azotobacter vinelandii*, *Paludibacter propionicigenes* and termites isolate *Treponema primita* ZAS-2 (Fig. 3.10). Group III and IV had the most abundant and divergent OTUs. Group III had 41 subgroups (Fig. 3.11 to Fig. 3.13). Among them, TG3, *Clostridium*, *Fibrobacter*, *Methanosaeta*, *Treponema*, CFP2, *Opitutaceae* and *Desulfovibrio* groups were representative subgroups containing strains with whole genomes. The *nifH* genes from *Treponema azotonutricium* and *Treponema primitia* in *Zootermopsis* were in *Treponema* group (Fig. 3.12), while *nifH* genes from termite symbionts *Candidatus* Azobacteroides pseudotrichonymphae and TG3 from *Nasutitermes* clustered in CFP2 and TG3 group respectively (Fig. 3.11 and Fig. 3.12). Group IV had 57 subgroups (Fig. 3.14 to Fig. 3.17). *Endomicrobium* group contain a *nifH* gene from *Endomicrobium proavitum* (Fig. 3.16). *nifH* genes of flagellate symbiont distributed from Group II to Group IV (Fig. 3.10 to Fig. 3.13, Fig. 3.17). They clustered in Adiutrix group and *Paludibacter* group in Group II (Fig. 3.10), Flagellate group/Flagellate group 2/Flagellate *Snyderella* group/*Treponema* group/*Clostridium* group 4 in Group III (Fig. 3.11 to Fig. 3.13) and *Bacteroides* group/cockroaches lower termites group in Group IV (Fig. 3.17).

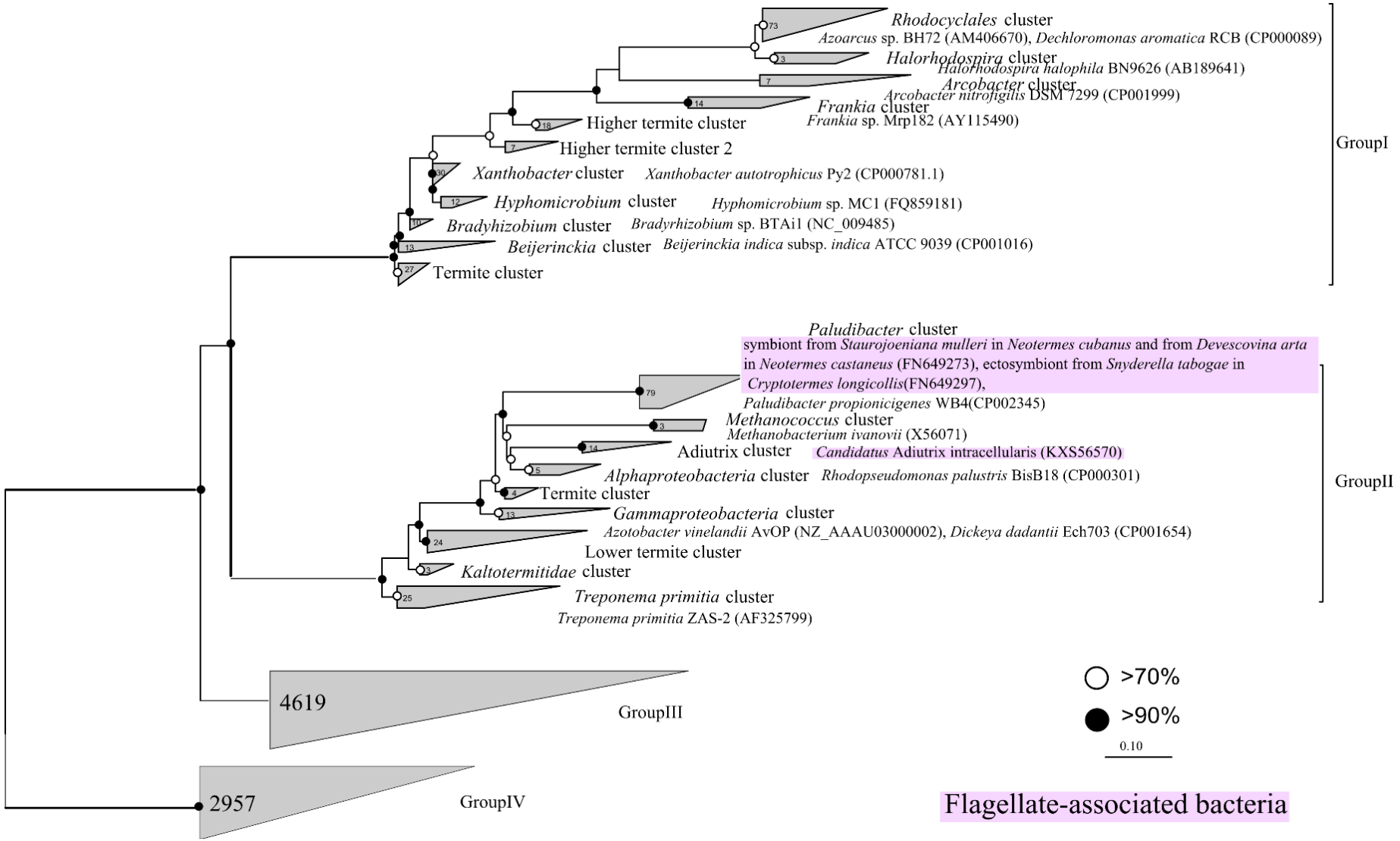
Rhodocyclales cluster
Azoarcus sp. BH72 (AM406670), Dechloromonas aromatica RCB (CP000089)
Halorhodospira cluster
Halorhodospira halophila BN9626 (AB189641)
Arcobacter cluster
Arcobacter nitrofigilis DSM 7299 (CP001999)
Frankia cluster
Frankia sp. Mrp182 (AY115490)
Higher termite cluster
Higher termite cluster 2
Xanthobacter cluster
Xanthobacter autotrophicus Py2 (CP000781.1)
Hyphomicrobium cluster
Hyphomicrobium sp. MC1 (FQ859181)
Bradyhizobium cluster
Bradyrhizobium sp. BTAi1 (NC_009485)
Beijerinckia cluster
Beijerinckia indica subsp. indica ATCC 9039 (CP001016)
Termite cluster
GroupI
Paludibacter cluster
symbiont from Staurojoeniana mulleri in Neotermes cubanus and from Devescovina arta in Neotermes castaneus (FN649273), ectosymbiont from Snyderella tabogae in Cryptotermes longicollis(FN649297),
Paludibacter propionicigenes WB4(CP002345)
Methanococcus cluster
Methanobacterium ivanovii (X56071)
Adiutrix cluster
Candidatus Adiutrix intracellularis (KXS56570)
Alphaproteobacteria cluster
Rhodopseudomonas palustris BisB18 (CP000301)
Termite cluster
Gammaproteobacteria cluster
Azotobacter vinelandii AvOP (NZ_AAAU03000002), Dickeya dadantii Ech703 (CP001654)
Lower termite cluster
Kaltotermitidae cluster
Treponema primitia cluster
Treponema primitia ZAS-2 (AF325799)
GroupII
4619
GroupIII
2957
GroupIV
>70%
>90%
0.10
Flagellate-associated bacteria

Figure 3.10: Phylogenetic relationships of the *nifH* sequences in Group I and II. Details of Group III and IV are in Figure 3.11 to 3.13 and Figure 3.14 to 3.17, respectively. The maximum-likelihood tree was calculated by deduced amino acid sequences. The local-bootstrap support values were analyzed from 1000 resampling. Nodes are labelled with black circles for local-bootstrap support values > 90% and white circles for values > 70%. Scale bar represents 0.10 substitutions per position.

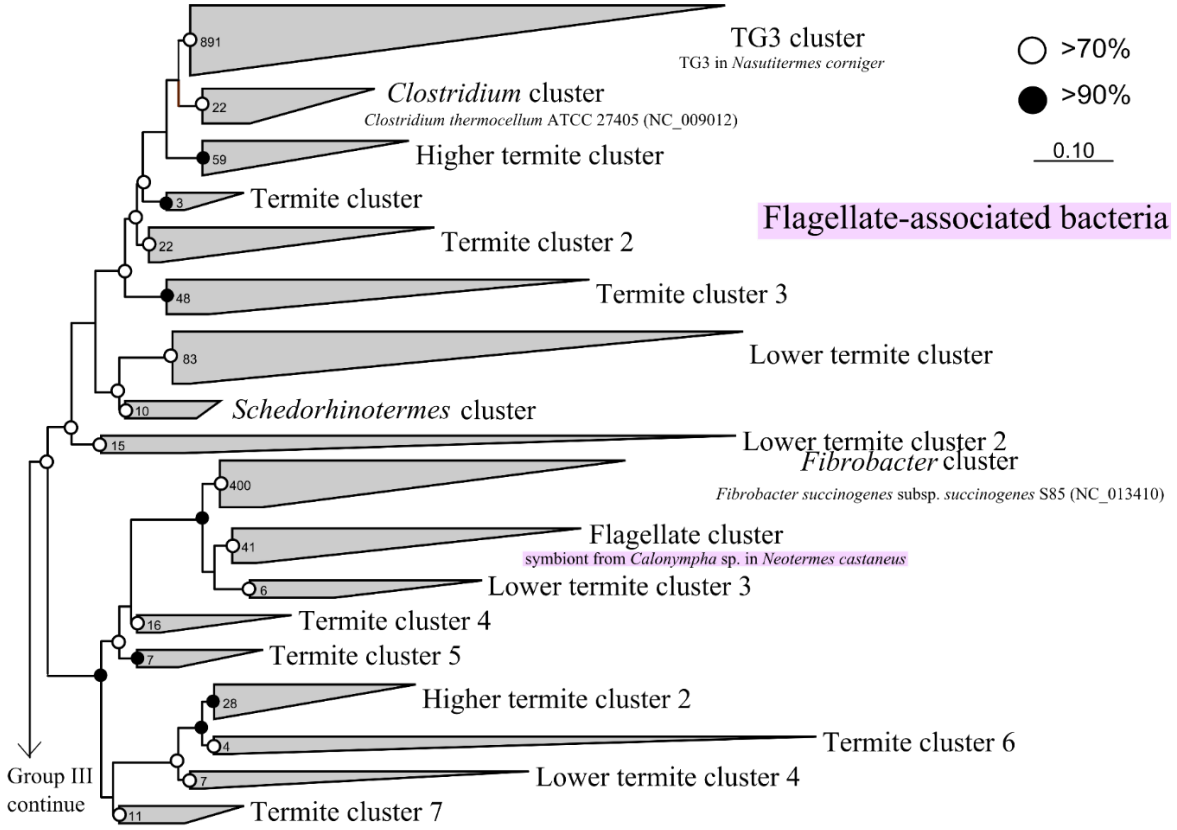

Figure 3.11: Phylogenetic relationships of the *nifH* sequences in Group III part 1. Refer to Fig. 3.10 legend for other explanations.

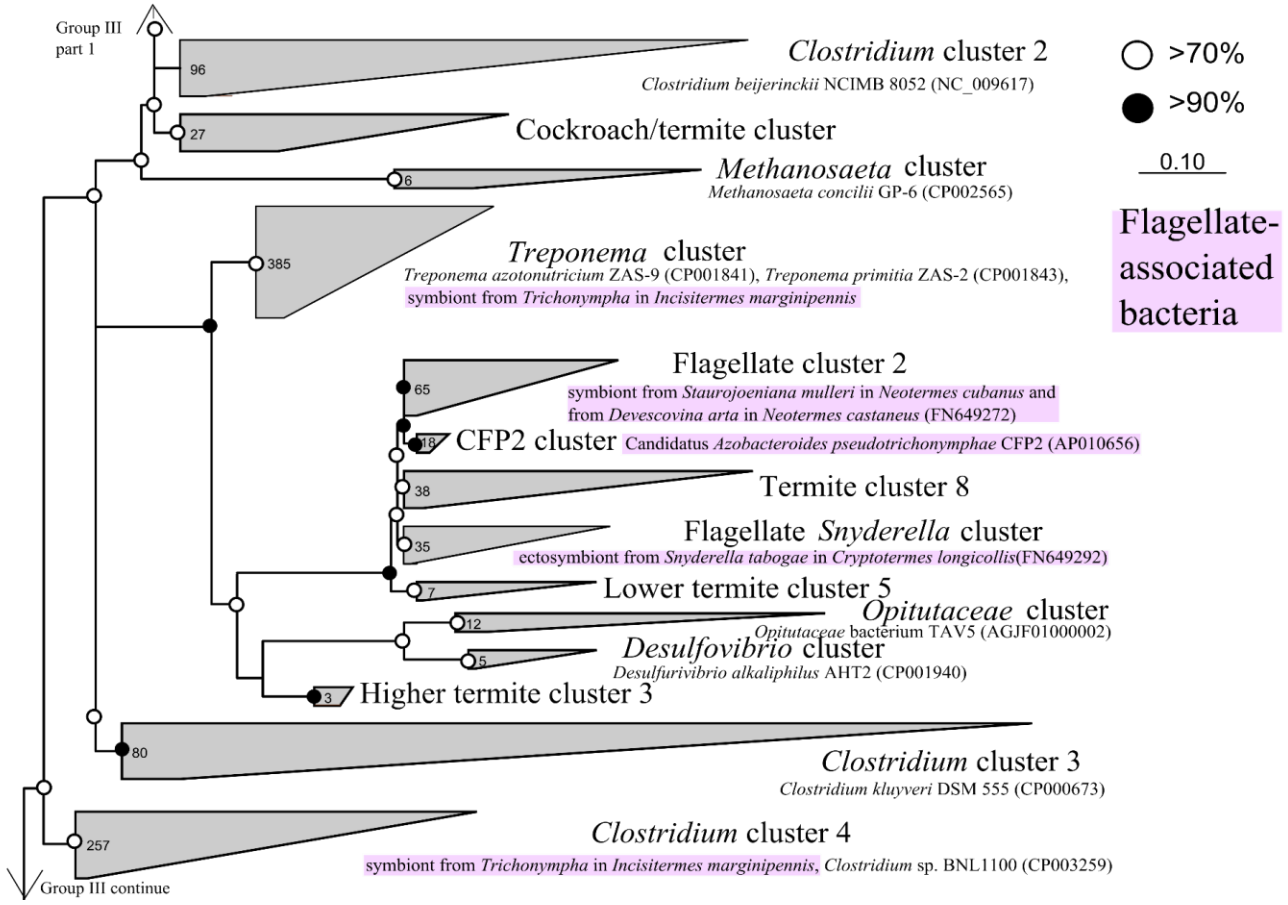

Figure 3.12: Phylogenetic relationships of the *nifH* sequences in Group III part 2. Refer to Fig. 3.10 legend for other explanations.

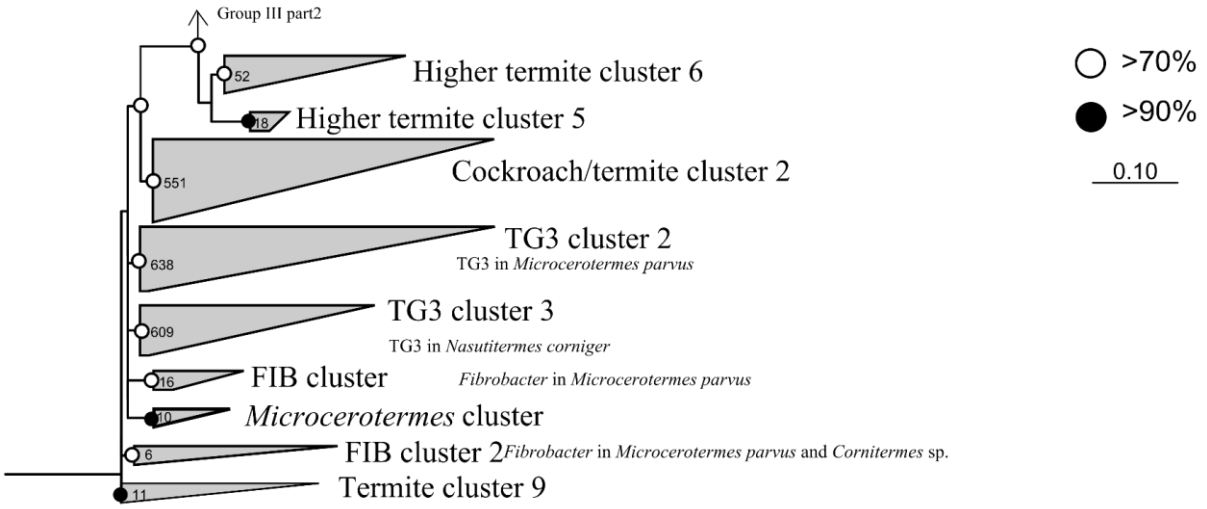

Figure 3.13: Phylogenetic relationships of the *nifH* sequences in Group III part 3. Refer to Fig. 3.10 legend for other explanations.

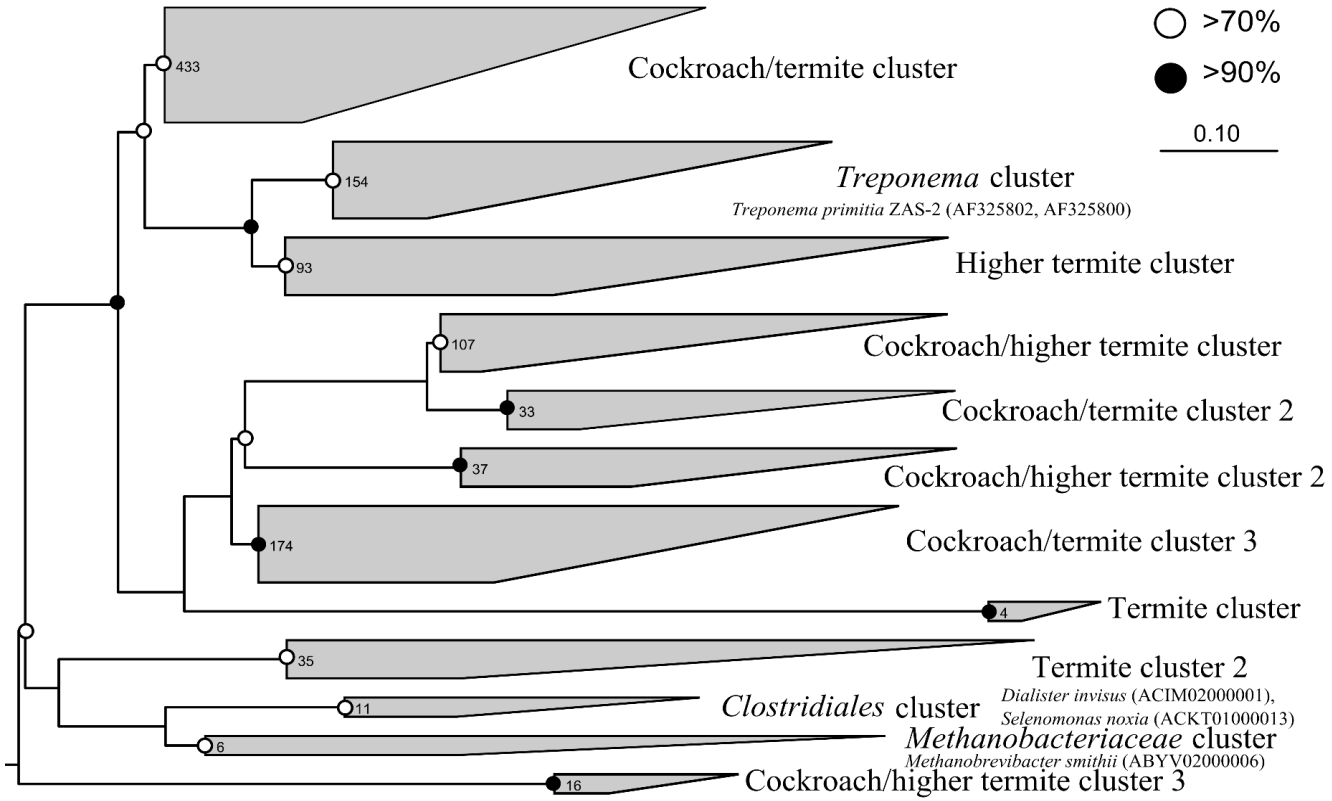

Figure 3.14: Phylogenetic relationships of the *nifH* sequences in Group IV part 1. Refer to Fig. 3.10 legend for other explanations.

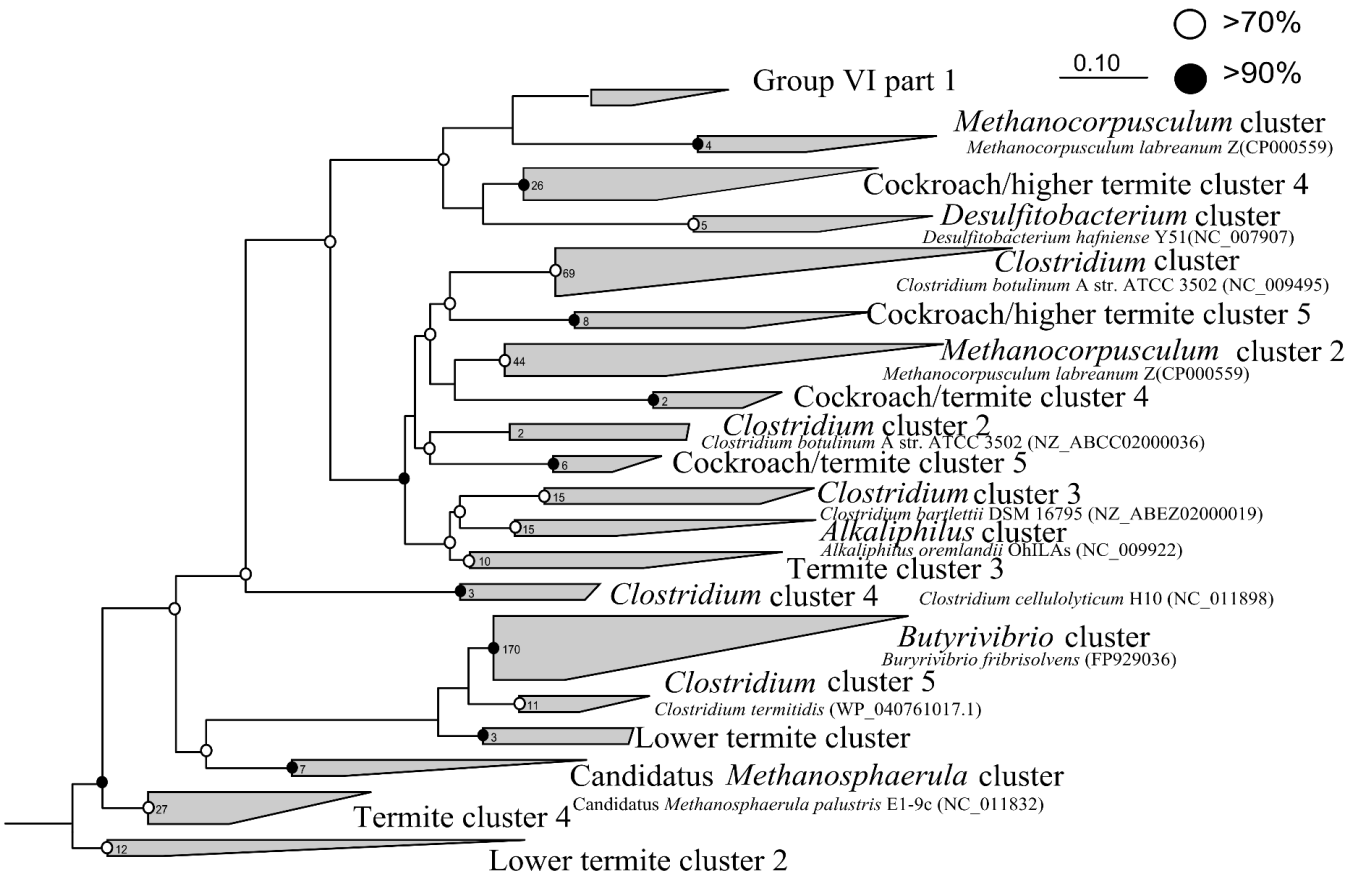

Figure 3.15: Phylogenetic relationships of the *nifH* sequences in Group IV part 2. Refer to Fig. 3.10 legend for other explanations.

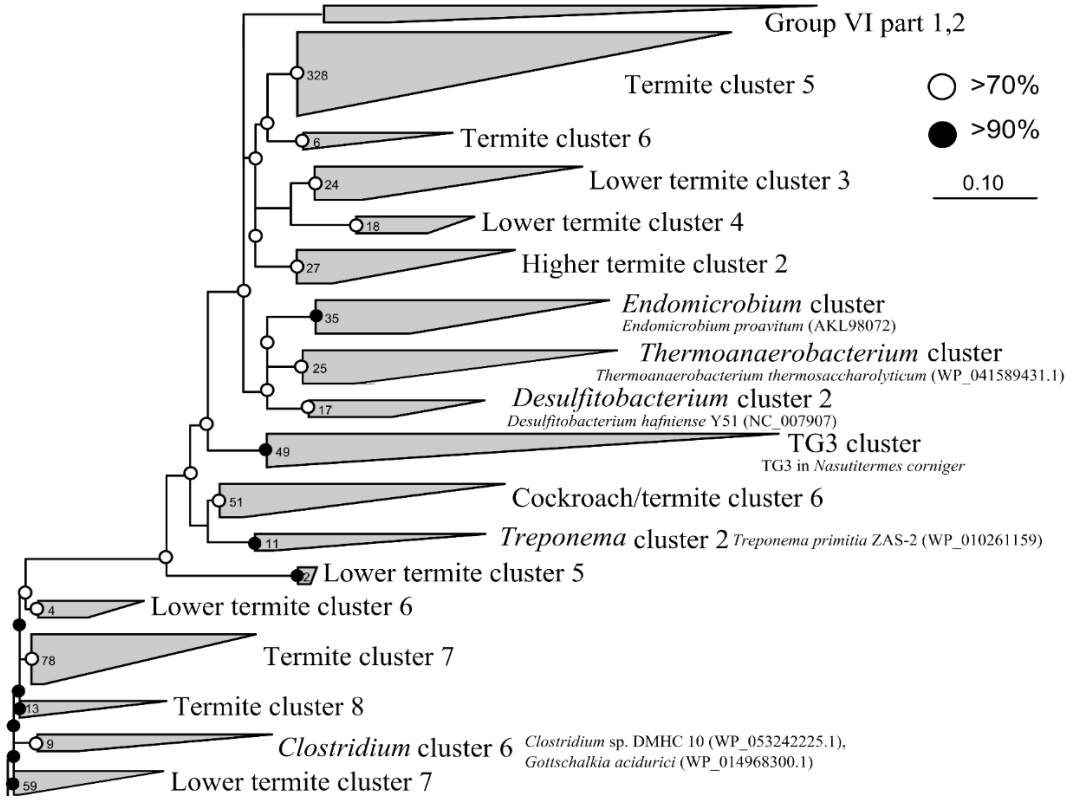

Figure 3.16: Phylogenetic relationships of the *nifH* sequences in Group IV part 3. Refer to Fig. 3.10 legend for other explanations.

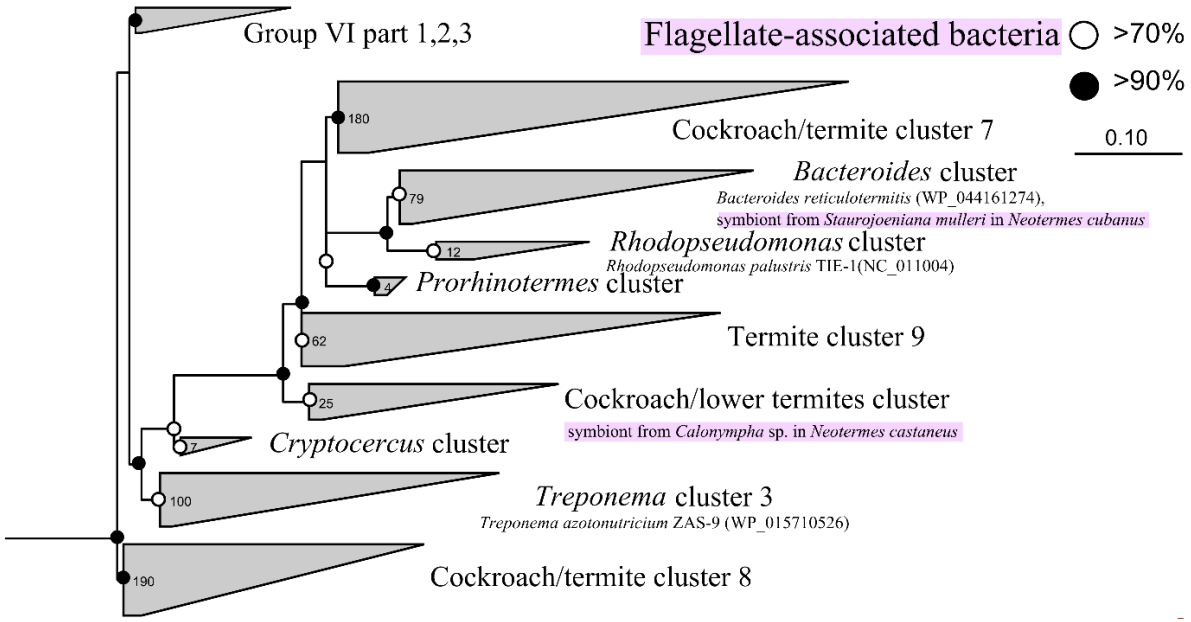

Figure 3.17: Phylogenetic relationships of the *nifH* sequences in Group IV part 4. Refer to Fig. 3.10 legend for other explanations.

Composition of the diazotrophic communities

Based on the classification of the *nifH* gene, the diazotrophic communities of the hosts clustered in Kalotermitidae, Termitidae and Rhinotermitidae except *Hodotermopsis sjoestedti* and *Coptotermes niger* (Fig. 3.18). Kalotermitidae had gut microbes with *nifH* genes distributed in Group I to IV. This is the only host family that contains significant copy numbers of all four *nifH* groups (Table S6.5). The diverse clusters in this family in Group III are flagellate clusters, *Clostridium* clusters and *Treponema* cluster, while the ones in Group IV are *Butyrivibrio* cluster, *Endomicrobium* cluster and *Treponema* cluster. In higher termites, *Microcerotermes indistinctus* and *Nasutitermes* except *Constrictotermes cyphergaster* were abundant in fiber-associated bacteria *Fibrobacteres* and TG3 clusters in Group III and *Butyrivibrio* cluster and TG3 cluster in Group IV. In clustered Rhinotermitidae, *nifH* genes falled into the cluster with *Clostridium* clusters and *Treponema* cluster in Group III. In addition, *nifH* genes from intestinal microbiota in Rhinotermitidae clustered with *Butyrivibrio* cluster and *Treponema* cluster in Group IV.

The *nifH* sequence reads from Blaberidae belonged almost exclusively to Group IV, and clustered with previously published sequences from both termites and cockroaches. For gut microbiota in *Cryptocercus punctulatus*, *nifH* genes were distributed between Group II–IV and were more similar to those from lower termites than those in Blaberidae. *nifH* gene sequences from the gut of *Hodotermes mossambicus*, the only representative of Hodotermitidae, clustered with previous clones from cockroaches and termites. The difference is that *nifH* genes in Hodotermitidae distributed in *Paludibacter* and *Treponema primitia* cluster in Group II. In *Mastotermes darwiniensis*, *nifH* genes *Treponema* cluster in Group III had the highest abundance.

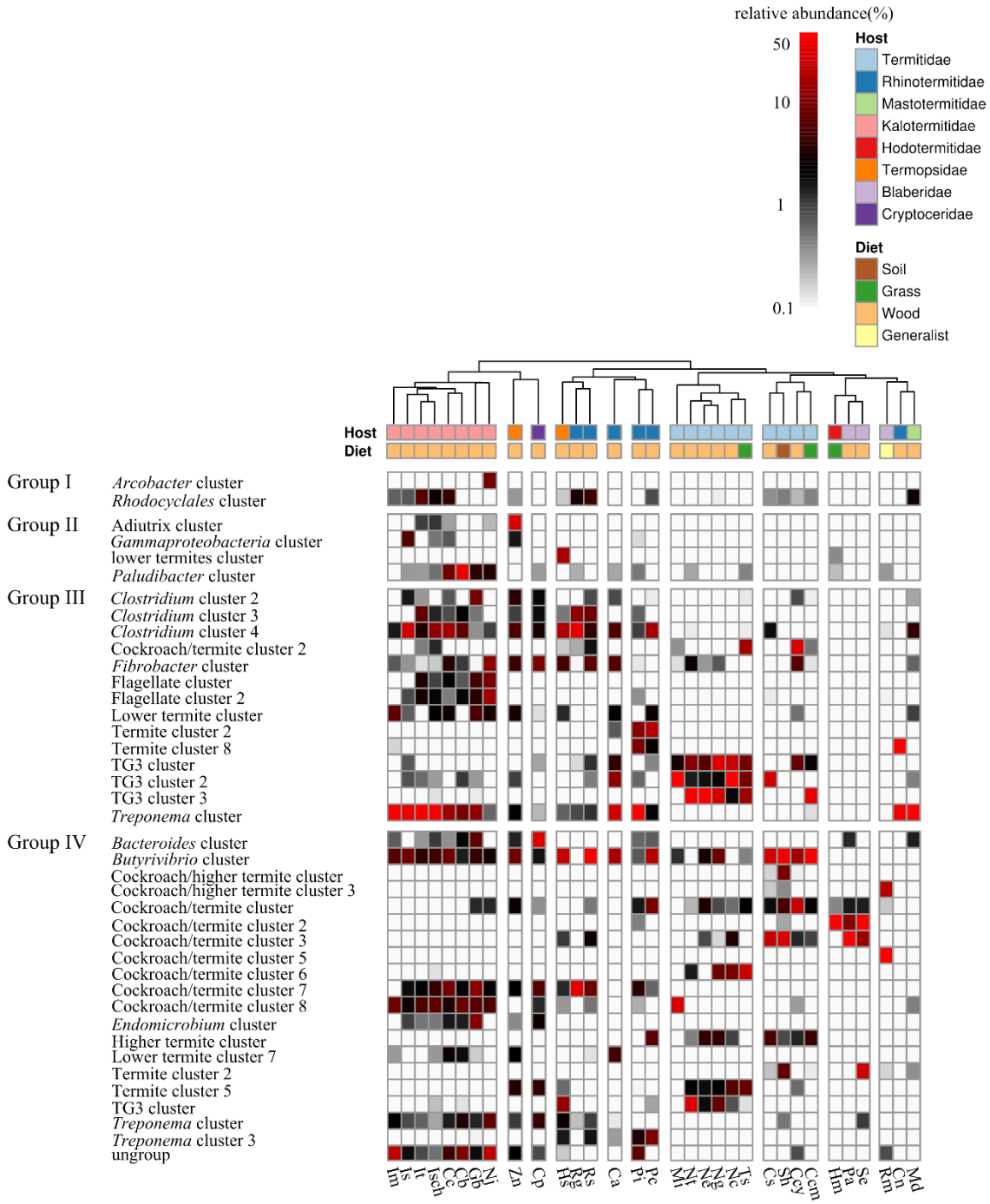

Figure 3.18: Clustering of host samples based on Euclidean distance and heatmap of the top 50 abundant taxa of *nifH* groups. Host abbreviations refer to table 2.2. The dendrogram of hosts is based on the classification of *nifH* groups whose average or lower abundance is larger than 1%.

Metagenomes showed compartment-specific communities

When we retrieved the *nifH* genes from the metagenomes of several higher termites, we found that their abundance differed markedly between feeding groups and individual gut compartments. None of the recovered *nifH* genes clustered with *Endomicrobium* cluster in Group IV. Although there were a large amount of *nifH* genes in some gut sections in interface and soil feeders, the majority of *nifH* genes were in Group IV (Fig. 3.19). While soil feeders had only relatively small populations of potential diazotrophs, termites feeding on wood and grass litter were abundantly associated with phylotypes that clustered with the *nifH* genes of fiber-associated bacteria (Fig. 3.20).

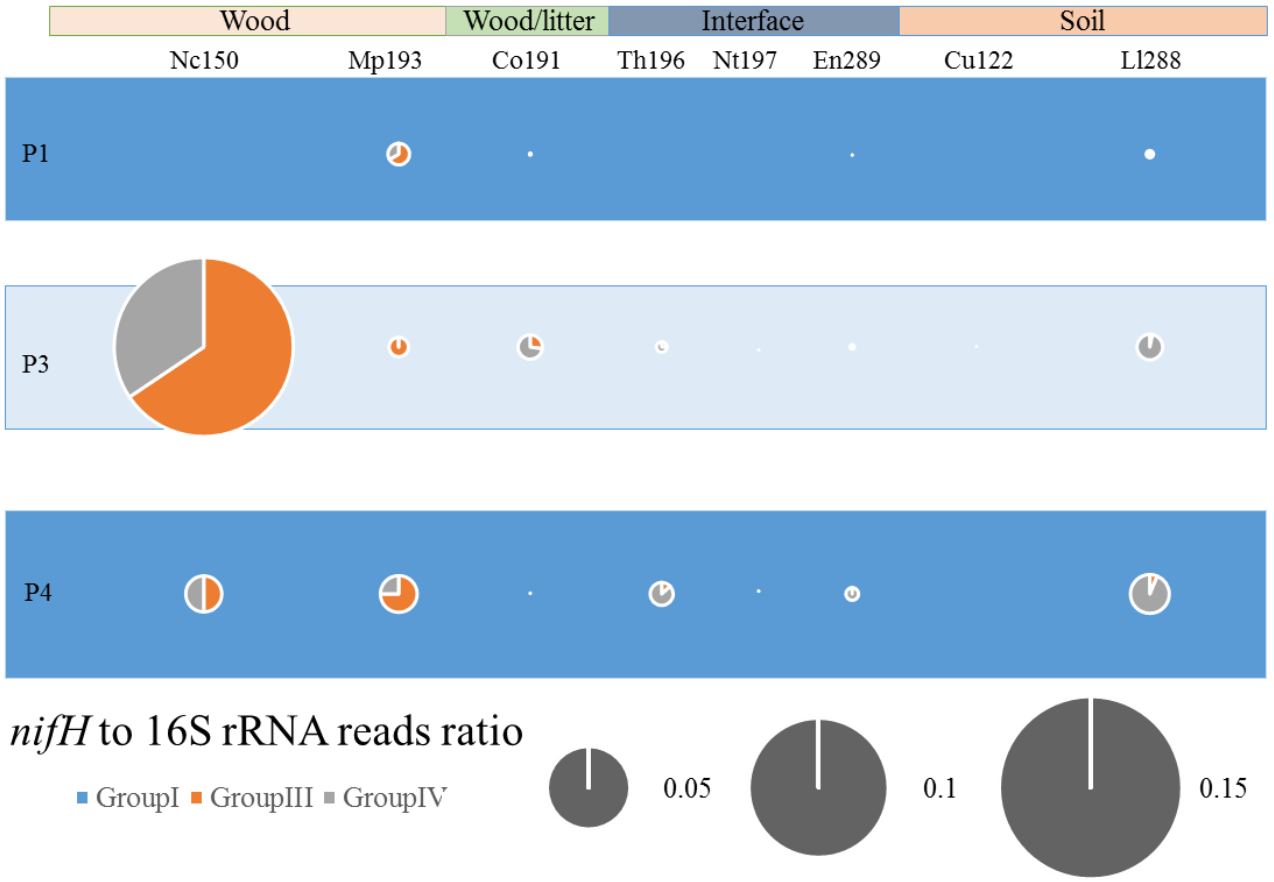

Figure 3.19: *nifH* abundances from various *nifH* groups (on group level) in different gut compartments, based on metagenomic analysis of termites from various feeding groups (data from Rossmassler et al., 2015). Nc150: *Nasutitermes corniger*; Mp193: *Microcerotermes parvus*; Co191: *Cornitermes* sp.; Th196: *Termes hospes*; Nt197: *Neocapritermes taracua*; En289: *Embiratermes neotenicus*; Cu122: *Cubitermes* sp.; Ll288: *Labiotermes labralis.* The area of the circles reflects the number of reads in the respective gut sections normalized by 16S rRNA gene reads.

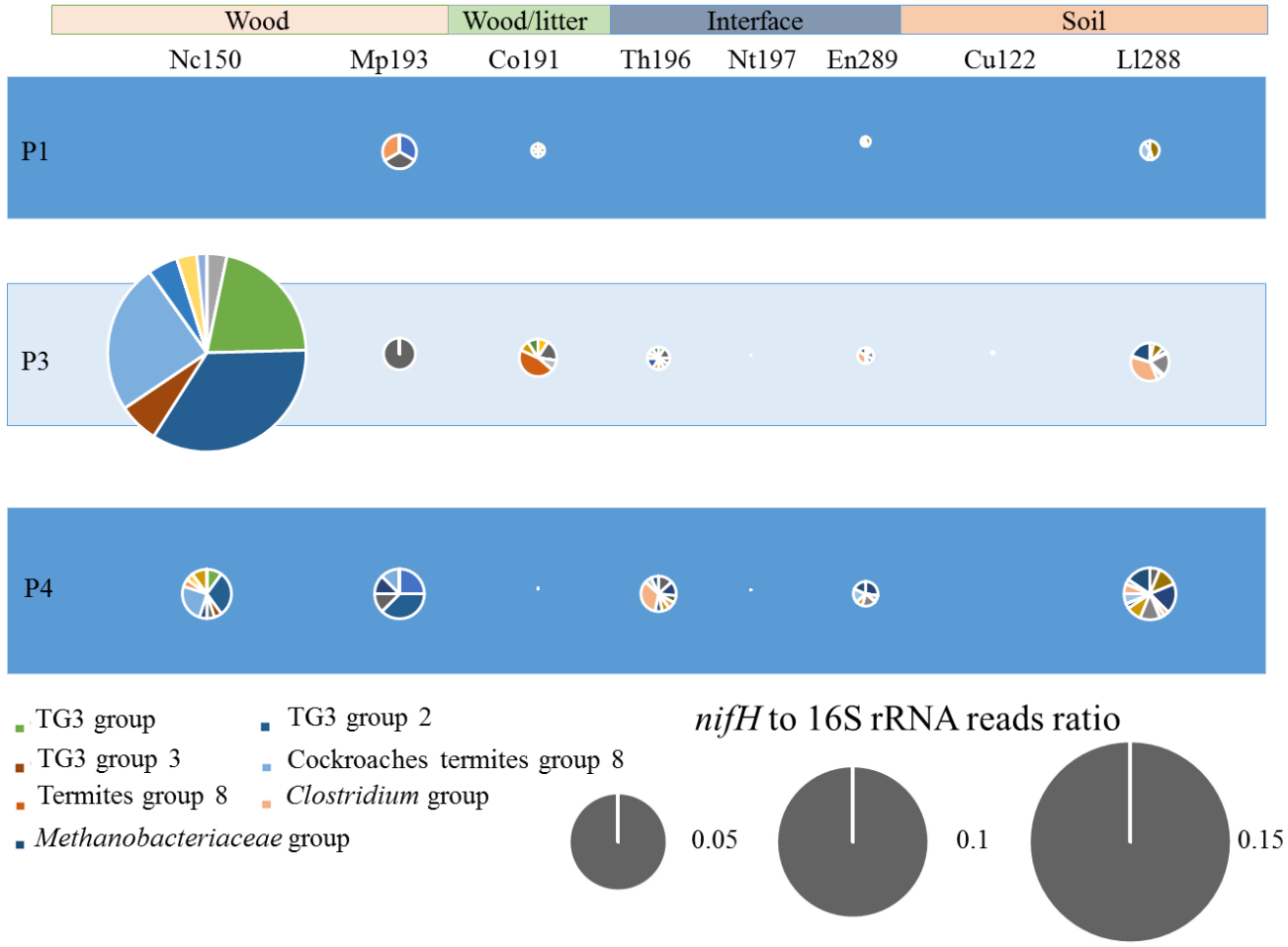

Figure 3.20: *nifH* abundances from various *nifH* groups (on subgroup level) in different gut compartments, based on metagenomic analysis of termites from various feeding groups (data from Rossmassler et al., 2015). Refer to Fig. 3.19 legend for other explanations.

Chapter 4

Discussion

The present thesis investigates the bacterial communities colonizing the highly compartmented intestinal tract of higher termites. The preceding chapters have provided a detailed characterization of the microenvironments in individual gut compartments of hitherto unstudied soil- and humus-feeding species, presented the factors driving community structure in the respective habitats, and highlighted the difference in the composition of the diazotrophic communities in termites and cockroaches. In this chapter, I present discussion of the interactions between microenvironment and gut microbiota in higher termites, the differences in nitrogen supply between termites feeding on sound wood or lignocellulose in advanced stages of humification, and influence of legacy effect and gut habitat effect on microbial community structure.

4.1 Environmental factors in the gut compartments of humivorous higher termites

Gut structure in different feeding groups

The P1 compartment of wood-feeder was generally tubular, but became more pronounced and dilated in the humus-feeders, and even further enlarged in the soil-feeders. Compared with P1, the P3 morphology are more correlated to the diet. It forms a unique compartment without separation of P3a and P3b in the wood- and grass-feeders, while it is more complicated, elongated and compartmentalized in most of the soil-feeders (Noirot, 2001).

The guts of fungus-cultivating higher Macrotermitinae termites are relatively short and resemble those of the lower termites in both morphology and physicochemical conditions (Li et al., 2012, 2016). The intestinal anatomy data here, especially the continuous enlarged P1 along with the dietary humification, suggesting that critical roles of the P1 compartment in the digestion of humus components. In general, the hindguts in soil feeders are larger than the ones in humus feeders, although they may from the same phylogenetic subfamilies (Syntermitinae and Termitinae).

Bacterial population distribution

In soil feeders, *L. labralis* and *C. ugandensis*, the incresase in gut pH from midgut to the first hindgut segement results in the drop of cell density from 0.26×10^6 to 0.23×10^6 and 0.4×10^6 to 0.23×10^6 copies per gram fresh weight respectively. This evidence confirm the previous hypothesis that the midgut-hindgut junction serves as a barrier to inhibit bacteria passing through termite guts (Schmitt-Wagner et al., 2003). Along the gut axial, the cell density dropped from P3 to P4 compartments in humus and soil feeders, *E. neotenicus*, *L. labralis* and *P. impostor*. This is consist with previous study in *N. matangensis* (Köhler et al., 2012) suggests that the digestion of microbial biomass with the flow of the digesta.

Inorganic nitrogen

Our results are in general agreement with previous reports on the intestinal ammonia pools in other soil-feeding termites *Cubitermes*, *Procubitermes*, *Ophiotermes* spp. (Ji and Brune, 2006; Ngugi et al., 2011; Ngugi and Brune, 2012), which corroborated the capacity of humus-, soil-feeding termites to mineralize organic nitrogenous components of humus matter to ammonia. Regarding ammonia oxidation, despite the high concentrations of nitrate and nitrite were found in the P4 compartment of two soil-feeding Termitinae *Cubitermes* and one *Ophiotermes* species indicate the anaerobic ammonia oxidation occurrence in the intestinal tract (Ngugi and Brune, 2012), the oxidation of ammonia to nitrite was not as significant as soil-feeding Syntermitinae *L. labralis*. Also, in the humus feeders, including *E. neotenicus* and *P. impostor*, the ammonia oxidation was not significant. In fact, the isotopic concentration of nitrite in *E. neotenicus* was only one-tenth of the one in *Cubitermes* species under same experiment setup (data not published). Moreover, this anaerobic ammonia oxidation in this termite did not enhance by adding ferric iron, which supposed to be an electron acceptor in *Cubitermes* species (Kappler and Brune, 2002). Together, our results indicate the possible nitrogen metabolism difference in humus- and soil-feeders, as well as in Termitinae and Syntermitinae.

Physicochemical conditions

In the higher termite, a large number of termite species have been surveyed on the pH of major gut regions, which show important insights into the distribution of elevated pH among the Termitidae. However, most of these were using pH indicator paper to measured pooled, disrupted gut homogenates (Bignell and Anderson, 1980; Bignell and Eggleton, 1995). Our microsensor measurements of *Cubitermes ugandensis* P1 compartment is two pH units above the values from pH-sensitive paper, which indicate pH paper measurement might not well represent the *in situ* intestinal conditions.

The strong differences between the intestinal pH of consecutive gut compartments and the anterior hindgut P1 compartments appear to be more alkaline in all termite species investigated (Figure 3.2). The intestinal pH of P1 compartments were extreme higher in soil-feeding termites but less alkaline in humus-feeding groups, and even reduced in the wood-feeder, irrespective of subfamilies, which indicates that the pH of anterior hindgut is the most characteristic element of the dietary guilds in higher termites. Given highly alkaline condition in anterior hindgut has long been assuming enables release peptidic or other nitrogenous residues from humic substances of soil organic matter (Brune, 1998; Kappler and Brune, 1999; Swift and Posner, 1972;(Bignell and Eggleton, 1995; Brune and Kühl, 1996; Ji et al., 2000), our results of diet-dependent pH condition reinforce the hypothesis that the highly alkaline pH in the P1 compartments play an important role in digestion of stabilized humus components in intake soil.

Despite the similarity of the intestinal pH among homologous gut compartments of the same feeding groups, there are clear differences that distinct representatives of the subfamily. The anterior P3 significantly more alkaline in Termitinae, and slightly reducing in Syntermitinae, but in *Nasutitermes matangensi* (Nasuitermitinae) the P3 reached to neutrality (Figure 3.2). The differences in alkalinity between subfamilies can explain the community structure in the P3 compartments of soil-feeding *L. labralis* was more similar to the homologous compartments of humus-feeding termites *Amitermes* sp. (Syntermitinae), than to members of its own feeding groups.

As a major fermentation product of carbohydrates (Pester and Brune, 2007), our results of completely different hydrogen accumulated profiles across different feeding

groups indicate the distributions of bacterial populations responsible for hydrogen production and consumption are fundamentally distinct. Our high-resolution analysis of bacterial community structures showed the potential hydrogen-producing lineages spirochetes were abundant in hydrogen accumulating gut sections, whereas hydrogen-consumers *Ruminococcus* species were rare, which likely explain the hydrogen accumulated distributions.

Our microsensor study provides comprehensive intestinal physicochemical parameters in diet-diverse higher termites. Clearly, phylogeny is not the only factor of high alkaline gut. The distinct diet-specific patterns observed in the intestinal physicochemical conditions of higher termites, which add to the growing body of evidence that dietary specialization of termites involved concerted adaptions in intestinal anatomy and physicochemical gut conditions.

4.2 Effect of diet and gut environment on community structure in higher termites of different feeding groups

In our highly resolved analysis of the gut microbiota in homologous compartments, the presence of distinct communities in each of the major hindgut compartments showed similarity not simply in one factor, like homologous gut compartments of termites from different lineages, the major feeding groups or same host. Rather, ordination analysis emerges that a high similarity of bacterial community structure among homologous gut compartments in litter-, humus- and soil-feeding termites. Meanwhile, gut bacteria community structures showed similarity in the same termite or from same diet group in wood and grass feeders. In the following, we will discuss the influence of environmental factors and different diets on the distribution of particular microbial lineages in the highly compartmentalized guts of higher termites.

Community structure in homologous compartments

Across different termites we studied, there are remarkable differences in consecutive gut compartments and similarities in some homologous gut sections from various termite subfamilies. The microenvironment of consecutive gut compartments in each species much differed than that of homologous compartments. This indicates gut environmental conditions drive the formation of communities. The intestinal pH is one essential environmental factor in higher termites. Unlike the neutral hindgut of lower termites (Bignell and Anderson, 1980; Brune et al., 1995; Eutick et al., 1976; Veivers et al., 1980), the anterior hindgut of most higher termites (except Macrotermitinae) is highly alkaline (Anklin-Mühlemann et al., 1995; Bignell, 1994; Bignell and Anderson, 1980; Bignell and Eggleton, 1995; Kovoor, 1967). The most alkaline gut sections are in soil-feeding Termitinae with pH 11 to 12.5. The sharp increase of pH value coincides with the mixed segment, morphologically unique compartment of higher termites except Macrotermitinae (Noirot, 2001). The highly alkaline pH values in the anterior hindgut compartments play an important role in the digestion of stabilized humus components in intake soil (Bignell and Eggleton, 1995; Brune and Kühl, 1996). Alkaline condition in anterior gut enables the release of amino acids from humic acids and extraction of organic matter from the soil (Kappler and Brune, 1999; Swift and Posner, 1972).

Certain *Clostridiales* lineages (*Ruminococcaceae* and *Lachnospiraceae*) associated with alkaline gut compartments (this study; Mikaelyan et al., 2016; Schmitt-Wagner et al., 2003;

Thongaram et al., 2005). For *Ca.* Arthromitus, the distribution is positively related to pH value (Figure S6.3). This also consists with the previous assumption that different members of this lineage (Thompson et al., 2012) are adapted to various gut physicochemical conditions (Mikaelyan et al., 2016) based on the phylogenetic analysis of OTUs in this genus. At present, *Ca.* Armantifilum is uncultured and there is no pH preference information in this lineage. In our study, the whole genus showed accumulation in posterior hindgut in humus- and soil-feeders (pH 6.1 to 8). *Ca.* Armantifilum has a representative, *Ca.* Armantifilum devescovinae, which cospeciated with devescovinid flagellates in dry-wood termites (Desai et al., 2010). Flagellate occupied in the hindgut of lower termites. Although there is no flagellate in higher termites, the relative abundance of genus *Ca.* Armantifilum is higher in hindgut as well. Moreover, the relative abundance of genus *Turicibacter* (*Firmicutes*, *Erysipelotrichaceae*) in all compartments show a similar trend with pH value in this study (Figure S6.3). Representatives of this cluster have also been detected in the alkaline P1 regions of the soil-feeding *Pericapritermes latignathus* and a grass-feeding *Speculitermes* sp. (Thongaram et al., 2005) and a wood-feeding *Nasutitermes corniger* and in the alkaline midgut of the humivorous larva of the scarab beetle *Pachnoda ephippiata* (Egert et al., 2003), which indicates an adaptation to high pH. The occurrence of *Turicibacter* spp. in the gut of a *Microcerotermes* sp. (Hongoh et al., 2005), which also comprises an alkaline P1 (Brune et al., 1995), is in agreement with this assumption.

The distribution and activity of microbiota are not only reflected and influenced by pH but also by hydrogen gradient.Through phylogenetic analyses of conserved single-copy protein-coding genes, Warnecke et al. (Warnecke et al., 2007) could link the iron-only hydrogenases in the metagenome of a *Nasutitermes* sp. to members of the *Spirochaetes*. Molecular hydrogen is a major fermentation product of carbohydrates in many species of Spirochaeta (Canale-Parola, 1992) and also in *Treponema azotonutricium*, an isolate from the lower termite *Zootermopsis angusticollis* (Graber et al., 2004). All isolates of termite gut treponemes possess several [FeFe] hydrogenases (Ballor et al., 2012), and related hydrogenase genes are also present in other lower termites (Ballor and Leadbetter, 2012). It is spirochetal lineages and many *Ruminococcus* species have the potential to be involved in reductive acetogenesis (Leaphart and Lovell, 2001; Rieu-Lesme et al., 1996; Warnecke et al., 2007).

Crop and P5 shared common genus in *Bacteroidetes*, *Spirochaetes* and *Actinobacteria*. The similarity of crop and P5 is accomplished by digesting microbial biomass derived from the

hindgut contents—either by coprophagy or by proctodeal trophallaxis. The community of the midgut and mixed segment are dominated by *Firmicutes*, particular members of *Lachnospiraceae* (Table S6.3), which including *Candidatus* Arthromitus. Nothing is known about the uncultured lineages in termite guts, but the family *Lachnospiraceae* comprises many species with high proteolytic, xylanolytic, and also cellulolytic activities from the gastrointestinal tract of mammals (e.g., *Butyrivibrio* spp.) (Cotta and Forster, 2006).

Community similarity in feeding groups

In humus- and soil-feeding termites show an increased abundance of *Firmicutes* in the anterior gut with highest abundant in P1, primarily members of *Ruminococcaceae* and *Lachnospiraceae* (Mikaelyan et al., 2015a). These two bacterial families have high number glycoside hydrolase genes and specific metabolic pathways to cleave the cellulose and hemicellulose components of complex plant material and degrade a wide variety of polysaccharides (Barelli et al., 2015). In our analysis, members of these families were identified as core genera which appeared in all samples of litter-, humus- and soil feeders (Table S6.3).

One significant difference of feeding groups is the nitrogen supply from the diet. Wood-feeding termites thrive on a diet with an extremely low nitrogen content, and N_2 fixation by their intestinal microbiota is an important factor in the nitrogen economy of the host. In soil-feeding termites, they get sufficient nitrogen supply from food and excrete a large amount of ammonia as waste (Ji and Brune, 2006; Ngugi and Brune, 2012). In all gut compartments, ammonification is the major process for nitrate ingestion in *Cubitermes* and *Ophiotermes* species (Ngugi et al., 2011; Ngugi and Brune, 2012). In the posterior hindgut, the microbiota also catalyze denitrification and the anaerobic oxidation of ammonia, possibly with ferric iron as electron acceptor (Ngugi et al., 2011; Ngugi and Brune, 2012). The prokaryotes that carry out these process are not clear, but they may contribute to the niches among feeding group and in the posterior hindgut of humus and soil feeders.

4.3 Environmental factors in higher termites interact with gut microbiota

This is the first comprehensive analysis that directly links environmental factors and gut microbiota in individual gut sections in the digestive tracts of humus-feeding and soil-feeding higher termites, combining a detailed assessment of bacterial community structure with a highly resolved analysis of the physicochemical gut conditions.

A study about hydrogen accumulation in higher termites including *Cubitermes* spp. showed that the mixed segment and P3 accumulate substantial amounts of hydrogen (Schmitt-Wagner and Brune, 1999). Interestingly, in soil-feeding *Labiotermes labralis* hydrogen accumulated in the P3 compartment, whereas in the wood-feeding termite *N. corniger* the P3 compartment was the only region with observed hydrogen accumulation as well (Köhler et al., 2012). However, the location of hydrogen accumulation in humus-feeding *Embiratermes neotenicus* and *Palmitermes impostor* was in the anterior part of the hindgut. Through phylogenetic analyses of conserved single-copy protein-coding genes, Warnecke et al. (2007) could link many iron-only hydrogenases in the metagenome of a *Nasutitermes* sp. to members of the *Spirochaetes* in the genus *Treponema* . Molecular hydrogen is a major fermentation product of carbohydrates in many *Spirochaeta* spp. (Leschine et al., 2006) and also in *Treponema azotonutricium*, an isolate from the lower termite *Zootermopsis angusticollis* (Graber et al., 2004). All isolates of termite gut treponemes possess several [FeFe] hydrogenases (Ballor et al., 2012), and related hydrogenase genes are also present in other lower termites (Ballor and Leadbetter, 2012). Spirochetal lineages and *Ruminococcus* lineages are potentially involved in reductive acetogenesis (Leaphart and Lovell, 2001; Rieu-Lesme et al., 1996; Warnecke et al., 2007). In the corresponding hydrogen accumulating gut section, the abundance of *Spirochaetes* is in the range of 2.4 to 7.0%. Another potential candidate for hydrogen production is *Clostridia*. Some *Clostridia* have been successfully isolated from termite guts, like *Clostridium beijerinckii* (Taguchi et al., 1993), *Clostridium* sp. strain X53 (Taguchi et al., 1996). The study of the gut community structure in different gut compartments showed that a number of 16S rRNA genes from *Cubitermes orthognathus* guts are related to *Clostridium termitidis* isolated from *Nasutitermes luja* (Hethener et al., 1992).

The intestinal pH of soil- and humus-feeding termites is also characterized along axial distribution (Bignell and Eggleton, 1995; Brune and Kühl, 1996). In humus- and soil-feeding termites the anterior hindgut region with alkaline situation (the highest pH ever reported was in P1 of *Cubitermes* species) correlates with the high abundance of several lineages of *Ruminococcaceae* and *Lachnospiraceae*, like *Candidatus* Arthromitus. There are other

specific bacterial genera associated with pH value in termite guts. The presence of *Desulfitibacter* is consistent with high pH value in humus- and soil-feeding termites. Members of the genus *Desulfitibacter* are alkalitolerant and grow either by fermentation or by the reduction of sulfite or thiosulfate (Nielsen et al., 2006). The genus *Paludibacter* accumulated mostly in alkaline gut compartments (pH 10.3 to 12.2) of soil-feeding termites, although members of this genus are found also in the slightly acidic P5 of *Cubitermes ugandensis* (pH 6.1). It has a fermentative metabolism and was enriched in sludge fermentation systems at pH 10 (Liu et al., 2016; Ueki et al., 2006).

4.4 Abundance and diversity of nitrogen-fixing bacteria in termite and cockroach guts

A few previous studies have addressed the composition of the termite gut diazotrophs, but further exploring and understanding are still need in terms of its functional groups (Du et al., 2012; Ohkuma et al., 1999; Yamada et al., 2007). In our study, the qPCR data revealed the high ratio of *nifH* to 16S rRNA gene copy numbers presented in Kalotermitidae and Rhinotermitidae. The low ratio of *nifH* genes to 16S rRNA genes in most termites, determined by qPCR, suggests that only a surprisingly small proportion of gut microbes is diazotrophs. However, this limited number of diazotrophs is sufficient to carry out the nitrogen-fixing activity determined by acetylene reduction assays. For example in *Zootomopsis* sp., 0.272 nmol C_2H_4 per h per g termites is formed (Breznak and Canale-Parola, 1973). The *Treponema* strain ZAS-9 isolated from *Zootermopsis angusticollis* can fix 1.2 μg N_2 (hour × mg protein)$^{-1}$ (Lilburn et al., 2001) which matches the nitrogen fixation ability of whole termites normalized by *nifH* gene copy numbers. For those species with a high ratio of *nifH* to 16S rRNA gene, nitrogen fixation ability has been documented previously (Breznak et al., 1973; French et al., 1976; Tayasu et al., 1994). However, not all species with high nitrogen fixation rates had a high *nifH* to 16S rRNA gene ratio. These involved the regulation of nitrogenase production on transcription and translation levels (Noda et al., 2002; Noda et al., 1999). *nifH* genes from termites guts that are closely related to *nifH* genes of *Spirochaetes* (Lilburn et al., 2001) were shown to be transcribed using RT-PCR (Noda et al., 1999; Ohkuma et al., 1999). The abundance of *nifH* genes in this cluster in *Kalotermitidae* and *Rhinotermitidae* showed the potential role of *Spirochaetes* in nitrogen fixation in the gut of termites.

Our study supports the hypothesis that flagellate symbionts contribute to nitrogen fixation in termite guts. It is known that there are specific flagellates at each host species or family level in Cryptocercidae and termites. The flagellates of *Incisitermes tabogae* and *Incisitermes marginipennis*, *Pyrsonympha* and *Oxymonas*, have spirochetes as ectosymbionts (Tamschick and Radek, 2013). The existence of these flagellate symbionts together with the abundance of *nifH* genes in Group III *Treponema* cluster in the heatmap from these two hosts shows the link between flagellate symbionts and nitrogen fixation. These kinds of phenomena appeared in different *nifH* groups as well. For example, the *Paludibacter* cluster in Group II and flagellate cluster 2 in Group III contained *nifH* genes from *Snyderella tabogae* and *Devescovina* sp., in the gut of *Cryptotermes longicollis* and *Neotermes castaneus*, respectively. These two clusters showed high relative abundance in *Neotermes jouteli* and

Cryptotermes cavifrons. In previous studies about symbiotic *Bacteroidales* bacteria of various protists (Noda et al., 2009), *C. cavifrons* have *Bacteroidales* in *Caduceia versatili*, *Stephanonympha* sp. and *Snyderella* sp. It was consistent with the *nifH* genes abundance in Group II *Paludibacter* cluster. The trichomonad family Devescovinidae is restricted to Kalotermitidae, with a few exceptions (in Hodotermitidae, *Anacanthotermes*). With one exception, *Metadevescovina extranea* Kirby found in *Mastotermes*, the flagellate genera *Caduceia*, *Macrotrichomonas*, *Metadevescovina* and *Hyperdevescovina* are exclusively found in Kalotermitidae (Honigberg, 1970). This explains the high abundance of *nifH* genes from gut communities in Kalotermitidae in Group III flagellate cluster 2. The *nifH* sequences in the *Treponema* cluster in *Hodotermopsis sjoestedti* are consistent with those from a previous study (Ohkuma et al., 2015) of the associated *Candidatus* Treponema intracellularis of *Eucomonympha* in the same species. Nitrogen fixed by the endosymbiont can be converted to more valuable nitrogenous compounds such as amino acids and supplied directly for protein synthesis of the protist. This asset allows the protist to grow stably and independently, and ensures that the host termite maintains the essential cellulolytic protists.

The comparison of *nifH* phylogenetic divergence among hosts revealed that *nifH* phylogenetic diversity to some degree reflected host phylogeny, like Kalotermitidae, Termopsidae and Nasutitermitinae (Fig. 3.9). The classification of the *nifH* group is also concordant with the close relationship between the *nifH* communities within Kalotermitidae, Rhinotermitidae and Termitidae. In wood-feeding higher termites, the representative abundant taxa are fiber-associated bacteria related which occupy the ecological niche after the loss of flagellates (Mikaelyan et al., 2014). In lower termites, this within-family similarity may result from the symbiosis of flagellates as discussed above and other family-specific habits like proctodeal trophallaxis. A salient finding of this study is the abundance of the Group VI *Endomicrobium* cluster in Kalotermitidae, Termopsidae and Cryptocercidae. *Endomicrobium proavitum* fixes nitrogen with a Group IV nitrogenase (Zheng et al., 2016), suggesting that the *Endomicrobium* cluster in Group VI comprises functional diazotrophs that use a Group IV nitrogenase.

4.5 Nitrogen nutrition supply strategies in termites

In xylophagous termites, the limiting nitrogen supply from food drives them to rely on nitrogen from other sources like atmospheric nitrogen. Although diazotrophs have been isolated from termite guts, the majority of the nitrogen-fixing gut microbiota is uncultivated. In lower termites, flagellates play an important role in the supply of nutrients, especially via the digestion of cellulose. Recently, a culture-independent study also offered evidence for nitrogen fixation of flagellate symbionts (Desai and Brune, 2011). Capillary-picked flagellate suspensions of *Staurojoeniana mulleri* from the termite *Neotermes cubanus* clustered with clones from flagellate (*Devescovina* spp.) suspensions from *Neotermes castaneus* (Desai and Brune, 2011) and *Paludibacter propionicigenes* in Group II of the *nifH* phylogenetic tree. In the gut of the wood-feeding beetle, *Odontotaenius disjunctus*, the most abundant transcript of the diazotrophic marker gene is related to the Ni–Fe nitrogenase of *Paludibacter propionicigenes* belonging to the phylum of *Bacteroidetes* (Ceja-Navarro et al., 2014). In Group III of *nifH*, symbionts from same flagellate cluster formed a subcluster with diazotrophic *Treponema azotonutricium* and *Treponema primitia* isolates from *Zootermopsis angusticollis* (Graber et al., 2004).

Despite the flagellate symbionts, another interesting bacteria group is the diazotrophs with previous unfunctional *nifH*. *Endomicrobium proavitum*, isolated from *Reticulitermes santonensis*, has only a *nifH* gene in the Group IV of *nifH*. It shows nitrogen fixation activity in acetylene reduction assay and ^{15}N-labeled nitrogen isotopic tracer analysis (Zheng et al., 2016). In the previous culture-independent study of *nifH* gene in gut microbiota of termites (Yamada et al., 2007; Ohkuma et al., 1999), researchers merely focused on the *nifH* genes from Group I to Group III. They excluded the Group IV of *nifH* because they thought that they were unfunctional in nitrogen fixation. In my study, *nifH* genes from the subgroup *Endomicrobium* cluster were abundant in Kalotermitidae, Termopsidae and Cryptoceridae. There may present potential diazotrophs with functional *nifH* genes in Group IV.

The nitrogen content of lignocellulose is generally low, but increases considerably during humification. The ammonia concentration in the gut of soil- and humus-feeding termites shows that mineralization starts from the anterior gut at a low level while peptide mineralization has a high rate in the hindgut section (Ngugi and Brune, 2012). The high ammonia concentrations in soil- and humus- feeders agree with the previous finding in *Cubitermes* species (Ji and Brune, 2006). In addition, the high δ15N-ammonia throughout the

gut and in the nest material of soil feeders suggest that peptidic compounds are essential substrates (Ji and Brune, 2006). Soil- and humus-feeding termites have a remarkable intestinal ammonia pool and showed the highest ammonia values (145 umol per gram fresh weight) ever reported among mammals and insects feeding on a protein-rich diet (Prusch, 1972; Wright, 1995). Ammonia inhibits nitrogenase activity (Breznak, 2000) and bacteria avoid wasting ATPs when nitrogen nutrition is sufficient. The accumulation of ammonia in *Labiotermes* sp. and *Cubitermes* sp. is in agreement with the low acetylene reduction activity in these species (Rohrmann and Rossman, 1980; Sylvester-Bradley et al., 1978).

4.6 How intestinal microbial communities are formed: Legacy effect or habitat effect?

The intestinal symbionts and termites form an important symbiosis. The origins of symbionts are from two possible routes, vertical transfer of microbiota and environmental uptake. As social insects, termites can transmit specialized gut bacteria via the fecal route or procodeal trophallaxis. The closest relative of termites, cockroaches, feed on faeces (Nalepa and Bandi, 2000). This behavior is known as coprophagy and it provides microbes for its hosts (Anderson and Bignell, 1980). After entering the gut, microbes interact with the microenvironment and establish a relatively stable mutualism. This symbiosis is passed by proctodeal trophallaxis from generation to generation in *Cryptocercus*-like species and all termites with flagellates (Nalepa and Bandi, 2000; Nalepa et al., 2001). In higher termites, they lost flagellates and carry out stomatodeal trophallaxis, mouth to mouth feeding. The horizontal transmission of symbionts is not clear (Aanen et al., 2002).

In culture-independent study of termite gut communities, most sequences of 16S rRNA gene are termite gut specialists and highly compartmentalized gut sections have different bacteria communities (Hongoh et al., 2006; Ohkuma and Brune, 2010; Köhler, 2011). Insect gut microorganisms that are transmitted directly between hosts adapt to specific host gut niches whereas bacteria from external environments in each host generation specifically respond to the host-associated niche (Ruby et al., 2004; Wier et al., 2010).

Studies about the gut microbiota of zebrafish and mice showed that legacy effect which inherited from the mother (Ley et al., 2005) possibly rise the differences and combines with gut habitat effects to shape the community structure (Rawls et al., 2006). Phylogenetic patterns in the community structure of gut bacteria showed high similarity within lower termites, cockroaches and individual higher termite subfamilies (Dietrich et al., 2014). This legacy effect may be a result from specific termite behaviors like proctodeal trophallaxis or coprophagy (Nalepa et al., 2001). The legacy effect also interacts with functional bacteria lineages like diazotrophs when a limited number of hosts were examined (Ohkuma et al., 1999; Yamada et al., 2007). From previous studies, we already see that the influence of the legacy effect on diazotroph or whole gut community decreases with the increase of representative host species or resolution of the gut compartment (Mikaelyan et al., 2016; Yamada et al., 2007).

Since the hosts involved in the study were limited (Ohkuma et al., 1999; Yamada et al., 2007), we studied diazotrophs in lower termites, higher termite and cockroach representatives from more subfamilies.In addition, the gut microbes from the compartmentalized gut of higher termite were also examined to clarify the contribution of each effect.

Lower termites mainly live on a nitrogen-poor diet with a high C: N ratio. Intestinal nitrogen-fixing bacteria in the same family of lower termites formed similar communities, like Kalotermitidae. In higher termites, the *nifH* groups from the gut of wood-feeding Nasutitermitinae clustered together in an ordination test. This showed the influence of legacy effect. When we considered the bacterial community structure in each gut section, the major hindgut compartments (P1, P3 and P4) of *Nasutitermes corniger* were primarily influenced by the host phylogeny.

Regardless whether we consider diazotrophs or the entire bacterial microbiota of termites and cockroaches, it is not possible to identify a single effect that explains the community structure in all cases. Since nitrogen fixation is a high-ATP-consuming process, the hosts prefer not to undergo nitrogen fixation in nitrogen-rich situations. Grass-feeding termites were more similar to cockroaches than other wood-feeding lower termites. Diet also plays a role in gut community structure of higher termites. Grass- and wood-feeding higher termites shared abundant core families in *Actinobacteria*, *Fibrobacteres* and TG3. *Bacteroidetes* and *Firmicutes* contained major core families in litter-, humus- and soil-feeders. Despite differing diets, other environmental factors also influence the formation of intestinal microbial community. In the homologous gut compartment, the microenvironments are more similar with homologs than consecutive sections. For example, the core families in the crop are mainly in *Lactobacillales* and *Xanthomonadales*. The tolerance of acidity of *Lactobacillales* and the capability of survival and growth in an oxygenated environment of *Xanthomonadales* fit the crop environment. The core families were *Treponema* I and *Lachnospiraceae* in the midgut, while *Ruminococcaceae* and *Porphyromonadaceae* Cluster V were typical in the hindgut.

In lower termite, the majority feed on a nitrogen-limiting diet and their symbionts, like bacteria and flagellates, help them to fix nitrogen and balance the C:N ratio. The diazotrophs community structure is determined by flagellates, host phylogenies and host diets. In the termite evolution process, the higher termites feed on a diverse diet and have highly compartmentalized guts. From this thesis, we saw how multi-factors influence the intestinal microbiota in each gut section in higher termites. The microenvironment conditions, like pH,

redox potential, hydrogen partial pressure and N pool size shape the gut community structure. The diet differences between wood/grass feeders and litter/humus/soil feeders reflect on several abundant representative genera in particular diet groups, while the majority of homologous gut compartments showed similarity even when the hosts are from different subfamilies.

Chapter 5

References

Aanen, D. K., Eggleton, P., Rouland-Lefevre, C., Guldberg-Froslev, T., Rosendahl, S., and Boomsma, J. J. (2002). The evolution of fungus-growing termites and their mutualistic fungal symbionts. *Proc. Natl. Acad. Sci. U. S. A.* 99, 14887–14892. doi:10.1073/pnas.222313099.

Anderson, J. M., and Bignell, D. E. (1980). Bacteria in the food, gut contents and faeces of the litter-feeding millipede *glomeris marginata* (villers). *Soil Biol. Biochem.* 12, 251–254. doi:10.1016/0038-0717(80)90070-X.

Anklin-Mühlemann, R., Bignell, D. E., Veivers, P. C., Leuthold, R. H., and Slaytor, M. (1995). Morphological, microbiological and biochemical studies of the gut flora in the fungus-growing termite *Macrotermes subhyalinus*. *J. Insect Physiol.* 41, 929–940. doi:10.1016/0022-1910(95)00062-Y.

Austin, J. W., Szalanski, A. L., and Cabrera, B. J. (2004). Phylogenetic analysis of the subterranean termite family Rhinotermitidae (Isoptera) by using the mitochondrial cytochrome oxidase II Gene. *Ann. Entomol. Soc. Am.* 97, 548–555. doi:10.1603/0013-8746(2004)097[0548:PAOTST]2.0.CO;2.

Brune, A. (1998). Termite guts: the world's smallest bioreactors. *Trends Biotechnol.* 16, 16–21. doi:10.1016/S0167-7799(97)01151-7.

Brune, A. (2013). "Symbiotic associations between termites and prokaryotes," in *The Prokaryotes: Prokaryotic Biology and Symbiotic Associations*, eds. M. Dworkin, S. Falkow, E. Rosenberg, K. Schleifer, E. Stackebrandt (Dordrecht: Springer Netherlands) doi:10.1007/978-3-642-30194-0_20.

Brune, A. (2014). Symbiotic digestion of lignocellulose in termite guts. *Nat. Rev. Microbiol.* 12, 168–180. doi:10.1038/nrmicro3182.

Brune, A., and Kühl, M. (1996). pH profiles of the extremely alkaline hindguts of soil-feeding termites (Isoptera: Termitidae) determined with microelectrodes. *J. Insect Physiol.* 42, 1121–1127. doi:10.1016/S0022-1910(96)00036-4.

Ballor, N. R., and Leadbetter, J. R. (2012). Analysis of extensive [FeFe] hydrogenase gene diversity within the gut microbiota of insects representing five families of Dictyoptera. *Microb. Ecol.* 63, 586–595. doi:10.1007/s00248-011-9941-5.

Ballor, N. R., Paulsen, I., and Leadbetter, J. R. (2012). Genomic analysis reveals

multiple [FeFe] hydrogenases and hydrogen sensors encoded by Treponemes from the H_2-rich termite gut. *Microb. Ecol.* 63, 282–294. doi:10.1007/s00248-011-9922-8.

Barelli, C., Albanese, D., Donati, C., Pindo, M., Dallago, C., Rovero, F., Cavalieri, D., Tuohy, K. M., Hauffe, H. C., and Filippo, C. D. (2015). Habitat fragmentation is associated to gut microbiota diversity of an endangered primate: implications for conservation. *Sci. Rep.* 5, 14862. doi:10.1038/srep14862.

Benemann, J. R. (1973). Nitrogen fixation in termites. *Science* 181, 164–165. doi:10.1126/science.181.4095.164

Bentley, B. L. (1984). Nitrogen fixation in termites: Fate of newly fixed nitrogen. *J. Insect Physiol.* 30, 653-655. doi:10.1016/0022-1910(84)90050-7.

Bignell, D. (1994). "Soil-feeding and gut morphology in higher termites," in *Nourishment Evol. insect Soc.*, eds. J. H. Hunt and C. A. alepa (Boulder, Westview Press), 131-158.

Bignell, D. E., and Eggleton, P. (1995). On the elevated intestinal pH of higher termites (Isoptera: Termitidae). *Insectes Soc.* 42, 57–69. doi:10.1007/BF01245699.

Bignell, D. E., and Eggleton, P. (2000). "Termites in ecosystems," in *Termites: Evolution, Sociality, Symbioses, Ecology*, eds. Y. Abe, D. E. Bignell, T. Higashi (Dordrecht: Springer Netherlands), 363–387. doi:10.1007/978-94-017-3223-9_17.

Bignell, D. E. (2006). "Termites as soil engineers and soil processors," in *Intestinal Microorgansims of Termites and Other Invertebrates*, eds. König, H., and Varma, A (Berlin/Heidelberg: Springer-Verlag), 183-220.

Bignell, D. E. (2010). "Morphology, physiology, biochemistry and functional design of the termite gut: an evolutionary wonderland," in *Biology of Termites: a Modern Synthesis*, eds. D. E. Bignell, Y. Roisin, and N. Lo (Dordrecht: Springer Netherlands), 375–412. doi:10.1007/978-90-481-3977-4_14.

Bignell, D. E. (2016). "The role of symbionts in the evolution of termites and their rise to ecological dominance in the tropics," in *The Mechanistic Benefits of Microbial Symbionts*, ed. C. J. Hurst (Cham, Springer), 121–172.

doi:10.1007/978-3-319-28068-4_6.

Bignell, D. E., and Anderson, J. M. (1980). Determination of pH and oxygen status in the guts of lower and higher termites. *J. Insect Physiol.* 26, 183–188. doi:10.1016/0022-1910(80)90079-7.

Bignell, D. E., and Eggleton, P. (1995). On the elevated intestinal pH of higher termites (Isoptera: Termitidae). *Insectes Soc.* 42, 57–69. doi:10.1007/BF01245699.

Bignell, D. E., and Roisin Y, Lo N. (2011). "Global biogeography of termites," in *Biology of Termites: A Modern Synthesis*, eds. D. E. Bignell, Y. Roisin, and N. Lo (Dordrecht: Springer Netherlands), 477–498.

Bourguignon, T., Šobotník, J., Lepoint, Gi., Martin, J.-M., Hardyt, O. J., Dejean, A., Roisin, Y. (2011). Feeding ecology and phylogenetic structure of a complex neotropical termite assemblage, revealed by nitrogen stable isotope ratios. *Ecol. Entomol.* 36, 261–269. doi:10.1111/j.1365-2311.2011.01265.x.

Brauman, A., Bignell, D. E., and Tayasu, I. (2000). "Soil-feeding termites: biology, microbial associations and digestive mechanisms," in *Termites: Evolution, Sociality, Symbioses, Ecology*, eds. Y. Abe, D. E. Bignell, T. Higashi (Dordrecht: Springer Netherlands), 233–259. doi:10.1007/978-94-017-3223-9_11.

Breznak, John A., Winston J. Brill, James W. Mertins, and H. C. C. (1973). Nitrogen fixation in termites. *Nature* 244, 577–580. doi:10.1038/244577a0.

Breznak, J. A., and Canale-Parola, E. (1973). Biology of nonpathogenic, host-associated Spirochetes. *CRC Critical Reviews in Microbiology* 2, 457–489.

Breznak, J. A., and Pankratz, H. S. (1977). In situ morphology of the gut microbiota of wood-eating termites [*Reticulitermes flavipes* (Kollar) and *Coptotermes formosanus* Shiraki]. *Appl. Environ. Microbiol.* 33, 406–26.

Breznak, J. A. (2000). "Ecology of prokaryotic microbes in the guts of wood- and litter-feeding termites," in *Termites: Evolution, Sociality, Symbioses, Ecology* eds T. Abe, D. E. Bignell and M. Higashi (Dordrecht: Springer Netherlands), 209-231.

Breznak, J. A., and Canale-Parola, E. (1973). Biology of nonpathogenic, host-associated Spirochetes. *CRC Critical Reviews in Microbiology* 2, 457–489.

Brune, A. (1998). Termite guts: the world's smallest bioreactors. *Trends Biotechnol.* 16, 16–21. doi:10.1016/S0167-7799(97)01151-7.

Brune, A. (2012). "Microbial symbioses in the digestive tract of lower termites," in *Beneficial Microorganisms in Multicellular Life Forms*, eds. E. Rosenberg, U. Gophna (Berlin, Heidelberg: Springer Berlin Heidelberg), 3–25. doi:10.1007/978-3-642-21680-0_1.

Brune, A. (2013). "Symbiotic associations between termites and prokaryotes," in *The Prokaryotes: Prokaryotic Biology and Symbiotic Associations*, eds. M. Dworkin, S. Falkow, E. Rosenberg, K. Schleifer, E. Stackebrandt (Dordrecht: Springer Netherlands), 1, 439-474. doi:10.1007/978-3-642-30194-0_20.

Brune, A. (2014). Symbiotic digestion of lignocellulose in termite guts. *Nat. Rev. Microbiol.* 12, 168–180. doi:10.1038/nrmicro3182.

Brune, A., and Dietrich, C. (2015). The gut microbiota of termites: digesting the diversity in the light of ecology and evolution. *Annu. Rev. Microbiol* 69, 145–66. doi:10.1146/annurev-micro-092412-155715.

Brune, A., Emerson, D., and Breznak, J. A. (1995). The termite gut microflora as an oxygen sink: microelectrode determination of oxygen and pH gradients in guts of lower and higher Termites. *Appl. Environ. Microbiol.* 61, 2681–7.

Brune, A., and Kühl, M. (1996). pH profiles of the extremely alkaline hindguts of soil-feeding termites (Isoptera: Termitidae) determined with microelectrodes. *J. Insect Physiol.* 42, 1121–1127. doi:10.1016/S0022-1910(96)00036-4.

Brune, A., and Ohkuma, M. (2011). "Role of the termite gut microbiota in symbiotic digestion," in *Biology of Termites: A Modern Synthesis* doi:10.1007/978-90-481-3977-4_16.

Canale-Parola, E. (1992). "Free-living saccharolytic Spirochetes: the genus *Spirochaeta*," in *The Prokaryotes* (New York, NY: Springer New York), 3524–3536. doi:10.1007/978-1-4757-2191-1_29.

Cataldo, D. A., Maroon, M., Schrader, L. E., and Youngs, V. L. (1975). Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun. Soil Sci. Plant Anal.* 6, 71–80. doi:10.1080/00103627509366547.

Ceja-Navarro, J. A., Nguyen, N. H., Karaoz, U., Gross, S. R., Herman, D. J.,

Andersen, G. L., Bruns, T. D., Pett-Ridge, J., Blackwell, M., and Brodie, E. L. (2014). Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of *Odontotaenius disjunctus*. *ISME J.* 8, 6–18. doi:10.1038/ismej.2013.134.

Chen, J., Bittinger, K., Charlson, E. S., Hoffmann, C., Lewis, J., Wu, G. D, Collman, R. G., Bushman, F. D., and Li, H. (2012). Associating microbiome composition with environmental covariates using generalized UniFrac distances. *Bioinformatics*, 28, 2106-2113.

Cotta, M., and Forster, R. (2006). "The Family *Lachnospiraceae*, including the genera *Butyrivibrio*, *Lachnospira* and *Roseburia*," in *The Prokaryotes* (New York, NY: Springer US), 1002–1021. doi:10.1007/0-387-30744-3_35.

d'Ettorre, P. (2017). "Distributed agency in ants," in *Distributed Agency*, ed. N. J. Enfield and Paul Kockelman (Oxford Scholarship Online), 131–137. doi:10.1093/acprof:oso/9780190457204.001.0001.

Daigle, D., Simen, B. B., Pochart, P., Daigle, D., Simen, B. B., and Pochart, P. (2011). "High-throughput sequencing of PCR products tagged with universal primers using 454 Life Sciences systems," in *Current Protocols in Molecular Biology* (Hoboken, NJ, USA: John Wiley & Sons, Inc.), 7.5.1-7.5.14. doi:10.1002/0471142727.mb0705s96.

De, B. L., and Conacher, A. (1990). The role of termites and ants in soil modification - a review. *Aust. J. Soil Res.* 28, 55-93. doi:10.1071/SR9900055.

Deevong, P., Hongoh, Y., Inoue, T., Trakulnaleamsai, S., Kudo, T., Noparatnaraporn, N., Ohkuma, M. (2006). Effect of temporal sample preservation on the molecular study of a complex microbial community in the gut of the termite *Microcerotermes* sp. *Microbes Environ.* 21, 78–85. doi:10.1264/jsme2.21.78.

Desai, M. S., and Brune, A. (2011). Bacteroidales ectosymbionts of gut flagellates shape the nitrogen-fixing community in dry-wood termites. *ISME J. Adv. online Publ.* 6, 1302-1313. doi:10.1038/ismej.2011.194.

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

Environ. Microbiol. 12, 2120-2132. doi:10.1111/j.1462-2920.2009.02080.x.

Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung (DIN EN 26777))

Dietrich, C., Kohler, T., and Brune, A. (2014). The cockroach origin of the termite gut microbiota: patterns in bacterial community structure reflect major evolutionary events. *Appl. Environ. Microbiol.* 80, 2261–2269. doi:10.1128/AEM.04206-13.

Donovan, S. E., Eggleton, P., and Bignell, D. E. (2001). Gut content analysis and a new feeding group classification of termites. *Ecol. Entomol.* 26, 356–366. doi:10.1046/j.1365-2311.2001.00342.x.

Doolittle, M., Raina, A., Lax, A., and Boopathy, R. (2008). Presence of nitrogen fixing *Klebsiella pneumoniae* in the gut of the Formosan subterranean termite (*Coptotermes formosanus*). *Bioresour. Technol.* doi:10.1016/j.biortech.2007.07.013.

Du, X., Li, X., Wang, Y., Peng, J., Hong, H., and Yang, H. (2012). Phylogenetic diversity of nitrogen fixation genes in the intestinal tract of *Reticulitermes chinensis* snyder. *Curr. Microbiol.* 65, 547-551. doi:10.1007/s00284-012-0185-5.

Ebert, A., and Brune, A. (1997). Hydrogen concentration profiles at the oxic-anoxic interface: a microsensor study of the hindgut of the wood-feeding lower termite *Reticulitermes flavipes* (Kollar). *Appl. Environ. Microbiol.* 63, 4039–4046.

Egert, M., Wagner, B., Lemke, T., Brune, A., and Friedrich, M. W. (2003). Microbial community structure in midgut and hindgut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). *Appl. Environ. Microbiol.* 69, 6659–6668. doi:10.1128/AEM.69.11.6659-6668.2003.

Eggleton, P. (2011). "An introduction to termites: Biology, taxonomy and functional morphology," in *Biology of Termites: A Modern Synthesis* doi:10.1007/978-90-481-3977-4_1.

Eggleton, P., and Tayasu, I. (2001). Feeding groups, lifetypes and the global ecology of termites. *Ecol. Res.* 16, 941–960. doi:10.1046/j.1440-1703.2001.00444.x.

Eutick, M. L., O'Brien, R. W., and Slaytor, M. (1976). Aerobic state of gut of

Nasutitermes exitiosus and *Coptotermes lacteus*, high and low caste termites. *J. Insect Physiol.* 22, 1377–1380. doi:10.1016/0022-1910(76)90161-X.

Fall, S., Hamelin, J., Ndiaye, F., Assigbetse, K., Aragno, M., Chotte, J. L., and Brauman, A. (2007). Differences between bacterial communities in the gut of a soil-feeding termite (*Cubitermes niokoloensis*) and its mounds. *Appl. Environ. Microbiol.* 73:5199-5208 doi:10.1128/AEM.02616-06.

French, J. R., Turner, G. L., and Bradbury, J. F. (1976). Nitrogen fixation by bacteria from the hindgut of termites. *J. Gen. Microbiol.* 96, 202–206. doi:10.1099/00221287-95-2-202.

Fröhlich, J., Koustiane, C., Kämpfer, P., Rosselló-Mora, R., Valens, M., Berchtold, M., Kuhnigk, T., Hertel, H., Maheshwari, D. K., and König, H. (2007). Occurrence of rhizobia in the gut of the higher termite *Nasutitermes nigriceps*. *Syst. Appl. Microbiol.* 30, 68–74. doi:10.1016/j.syapm.2006.03.001.

Gaby, J. C., and Buckley, D. H. (2011). A global census of nitrogenase diversity. *Environ. Microbiol.* 13, 1790–1799. doi:10.1111/j.1462-2920.2011.02488.x.

Garnier-Sillam, E., and Harry, M. (1995). Distribution of humic compounds in mounds of some soil-feeding termite species of tropical rainforests: its influence on soil structure stability. *Insectes Soc.* 42, 167–185. doi:10.1007/BF01242453.

Gontijo, T. A., and Domingos, D. J. (1991). Guild distribution of some termites from Cerrado Vegetation in south-east Brazil. *J. Trop. Ecol.* 7, 523–529. doi:10.2307/2559217.

Graber, J. R., Leadbetter, J. R., and Breznak, J. A. (2004). the first Spirochetes isolated from termite guts. *Appl. Environ. Microbiol.* 70, 1315–1320. doi:10.1128/AEM.70.3.1315–1320.2004.

Hethener, P., Brauman, A., and Garcia, J. L. (1992). *Clostridium termitidis* sp. nov., a cellulolytic bacterium from the gut of the wood-feeding termite, *Nasutitermes lujae*. *Syst. Appl. Microbiol.* 15, 52–58. doi:10.1016/S0723-2020(11)80138-4.

Honigberg, B. M. (1970). "Protozoa associated with termites and their role in digestion," in *Biology of termites*, ed. K. Krishna and F. M. Weesner (New York: Academic Press), 2, 1-36.

Hongoh, Y. (2011). Toward the functional analysis of uncultivable, symbiotic

microorganisms in the termite gut. *Cell. Mol. Life Sci.* 68, 1311–1325. doi:10.1007/s00018-011-0648-z.

Hongoh, Y., Deevong, P., Inoue, T., Moriya, S., Trakulnaleamsai, S., Ohkuma, M., Vongkaluang, C., Noparatnaraporn, N., and Kudo, T. (2005). Intra- and interspecific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host. *Appl. Environ. Microbiol.* 71, 6590–6599. doi:10.1128/AEM.71.11.6590-6599.2005.

Hongoh, Y., Deevong, P., Hattori, S., Inoue, T., Noda, S., Noparatnaraporn, N., Kudo, T., and Ohkuma M. (2006). Phylogenetic diversity, localization, and cell morphologies of members of the candidate phylum TG3 and a subphylum in the phylum *Fibrobacteres*, recently discovered bacterial groups dominant in termite guts. *Appl. Environ. Microbiol.* 72, 6780–8. doi:10.1128/AEM.00891-06.

Hongoh, Y., Ekpornprasit, L., Inoue, T., Moriya, S., Trakulnaleamsai, S., Ohkuma, M., Noparatnaraporn, N., and Kudo, T. (2005). Intracolony variation of bacterial gut microbiota among castes and ages in the fungus-growing termite *Macrotermes gilvus*. *Mol. Ecol.* 15, 505–516. doi:10.1111/j.1365-294X.2005.02795.x.

Hongoh, Y., Sharma, V. K., Prakash, T., Noda, S., Toh, H., Taylor, T. D., Kudo, T., akaki, Y., Toyoda, A., Hattori, M., and Ohkuma, M. (2008). Genome of an endosymbiont coupling N_2 fixation to cellulolysis within protist cells in termite gut. *Science* 322, 1108–1109. doi:10.1126/science.1165578.

Ikeda-Ohtsubo, W., and Brune, A. (2009). Cospeciation of termite gut flagellates and their bacterial endosymbionts: *Trichonympha* species and "*Candidatus* Endomicrobium trichonymphae". *Mol. Ecol.* 18, 332–42. doi:10.1111/j.1365-294X.2008.04029.x.

Inoue, T., Kitade, O., Yoshimura, T., and Yamaoka, I. (2000). "Symbiotic associations with protists," in *Termites: Evolution, Sociality, Symbioses, Ecology* (Dordrecht: Springer Netherlands), 275–288. doi:10.1007/978-94-017-3223-9_13.

Inward, D. J. G., Vogler, A. P., and Eggleton, P. (2007). A comprehensive phylogenetic analysis of termites (Isoptera) illuminates key aspects of their evolutionary biology. *Mol. Phylogenet. Evol.* 44, 953-967.

doi:10.1016/j.ympev.2007.05.014.

Ji, R. (2000). Transformation and mineralization of organic matter by soil-feeding termites. [dissertation]. [Konstanz]: University of Chicago

Ji, R., and Brune, A. (2001). Transformation and mineralization of 14 C-labeled cellulose, peptidoglycan, and protein by the soil-feeding termite *Cubitermes orthognathus*. *Biol. Fertil. Soils* 33, 166–174. doi:10.1007/s003740000310.

Ji, R., and Brune, A. (2005). Digestion of peptidic residues in humic substances by an alkali-stable and humic-acid-tolerant proteolytic activity in the gut of soil-feeding termites. *Soil Biol. Biochem.* 37, 1648–1655. doi:10.1016/j.soilbio.2005.01.026.

Ji, R., and Brune, A. (2006). Nitrogen mineralization, ammonia accumulation, and emission of gaseous NH_3 by soil-feeding termites. *Biogeochemistry* 78, 267–283. doi:10.1007/s10533-005-4279-z.

Ji, R., Kappler, A., and Brune, A. (2000). Transformation and mineralization of synthetic 14C-labeled humic model compounds by soil-feeding termites. *Soil Biol. Biochem.* 32, 1281–1291. doi:10.1016/S0038-0717(00)00046-8.

John A. HoltA, M. L. (2000). "Termites and soil properties," in *Termites: Evolution, Sociality, Symbioses, Ecology*, eds. Y. Abe, D. E. Bignell, T. Higashi (Dordrecht: Springer Netherlands), 389-407.

Jones, D. T., and Eggleton, P. (2010). "Global biogeography of termites: a compilation of sources," in *Biology of Termites: a Modern Synthesis* (Dordrecht: Springer Netherlands), 477–498. doi:10.1007/978-90-481-3977-4_17.

Kappler, A., and Brune, A. (1999). Influence of gut alkalinity and oxygen status on mobilization and size-class distribution of humic acids in the hindgut of soil-feeding termites. *Appl. Soil Ecol.* 13, 219–229. doi:10.1016/S0929-1393(99)00035-9.

Kappler, A., and Brune, A. (2002). Dynamics of redox potential and changes in redox state of iron and humic acids during gut passage in soil-feeding termites (*Cubitermes* spp.). *Soil Biol. Biochem.* 34, 221–227. doi:10.1016/S0038-0717(01)00176-6.

Knicker, H., Schmidt, M. W. I., and Kögel-Knabner, I. (2000). Nature of organic

nitrogen in fine particle size separates of sandy soils of highly industrialized areas as revealed by NMR spectroscopy. *Soil Biol. Biochem.* 32, 241–252. doi:10.1016/S0038-0717(99)00154-6.

Köhler, T. (2011) Physicochemical gradients and deep sequencing of the bacterial microbiota indicate functional compartmentation in the gut of the higher termite *Nasutitermes corniger*. Diversity and evolutionary patterns in the bacterial gut microbiota of termites and cockroaches. PhD Thesis, Philipps-Universität Marburg, Marburg, Germany, 11–42.

Köhler, T., Dietrich, C., Scheffrahn, R. H., and Brune, A. (2012). High-resolution analysis of gut environment and bacterial microbiota reveals functional compartmentation of the gut in wood-feeding higher termites (*Nasutitermes* spp.). *Appl. Environ. Microbiol.* 78, 4691–701. doi:10.1128/AEM.00683-12.

König, H., and Varma, A. (Ajit) (2006). *Intestinal microorganisms of termites and other invertebrates*. Springer.

Kolde, R. (2015). pheatmap: Pretty Heatmaps. R package version 1.0.8. http://CRAN.R-project.org/package=pheatmap

Kovoor, J. (1967). Le pH intestinal d'un Termite supérieur (*Microcerotermes edentatus*, Was., amitermitinæ). *Insectes Soc.* 14, 157–160. doi:10.1007/BF02223265.

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., and Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl. Environ. Microbiol.* 79, 5112–5120. doi:10.1128/AEM.01043-13.

Krishna, K., Grimaldi, D. A., Krishna, V., and Engel, M. S. (2013). Treatise on the Isoptera of the World. *Bull. Am. Museum Nat. Hist.* 377, 1–202. doi:10.1206/377.6.

Kudo, T., Ohkuma, M., Moriya, S., Noda, S., and Ohtoko, K. (1998). Molecular phylogenetic identification of the intestinal anaerobic microbial community in the hindgut of the termite, *Reticulitermes speratus*, without cultivation. *Extremophiles* 2, 155–161.

Kuhnigk, Thomas, Jürgen Branke, Daniel Krekeler, Heribert Cypionka, and H. K. (1996). A feasible role of sulfate-reducing bacteria in the termite gut. *Syst. Appl. Microbiol.* 19, 139–149.

Leaphart, A. B., and Lovell, C. R. (2001). Recovery and analysis of formyltetrahydrofolate synthetase gene sequences from natural populations of acetogenic bacteria. *Appl. Environ. Microbiol.* 67, 1392–1395. doi:10.1128/AEM.67.3.1392-1395.2001.

Leschine, S., Paster, B. J., and Canale-Parola, E. (2006). "Free-living saccharolytic spirochetes: The genus *Spirochaeta*," in *The Prokaryotes* (New York, NY: Springer New York), 195–210. doi:10.1007/0-387-30747-8_7.

Ley, R. E., Bäckhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., and Gordon, J. I. (2005). Obesity alters gut microbial ecology. *Proc. Natl. Acad. Sci. U. S. A.* 102, 11070–5. doi:10.1073/pnas.0504978102.

Lilburn, T. G., Kim, K. S., Ostrom, N. E., Byzek, K. R., Leadbetter, J. R., & Breznak, J. A. (2001). Nitrogen fixation by symbiotic and free-living Spirochetes. *Science* 292, 2495–2498.

Liu, H., and Beckenbach, A. T. (1992). Evolution of the mitochondrial cytochrome oxidase II gene among 10 orders of insects. *Mol. Phylogenet. Evol.* 1, 41–52. doi:10.1016/1055-7903(92)90034-E.

Liu, Y., Yang, H., Zhang, X., Xiao, Y., Guo, X., and Liu, X. (2016). Genomic analysis unravels reduced inorganic sulfur compound oxidation of heterotrophic acidophilic *Acidicaldus* sp. strain DX-1. *Biomed Res. Int.* doi:10.1155/2016/8137012.

Li, H., Dietrich, C., Zhu, N., Mikaelyan, A., Ma, B., Pi, R., Liu, Y., Yang, M., Brune, A. (2016). Age polyethism drives community structure of the bacterial gut microbiota in the fungus-cultivating termite *Odontotermes formosanus*. *Environ. Microbiol.* 18, 1440–1451. doi:10.1111/1462-2920.13046.

Li, H., Sun, J., Zhao, J., Deng, T., Lu, J., Dong, Y., Deng, W., Mo, J. (2012). Physicochemical conditions and metal ion profiles in the gut of the fungus-growing termite *Odontotermes formosanus*. *J. Insect Physiol.* 58, 1368–1375. doi:10.1016/J.JINSPHYS.2012.07.012.

Lo, N., and Eggleton, P. (2011). "Termite phylogenetics and co-cladogenesis with symbionts," in *Biology of Termites: A Modern Synthesis*, eds. D. Bignell, Y. Roisin, N. Lo (Dordrecht: Springer Netherlands), 27–50. doi:10.1007/978-90-481-3977-4_2.

Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi:10.1128/AEM.71.12.8228-8235.2005.

Makonde, H. M., Boga, H. I., Osiemo, Z., Mwirichia, R., Mackenzie, L. M., Göker, M., and Klenk, H. P. (2013). 16S-rRNA-based analysis of bacterial diversity in the gut of fungus-cultivating termites (*Microtermes* and *Odontotermes* species). *Antonie Van Leeuwenhoek* 104, 869–883. doi:10.1007/s10482-013-0001-7.

Matsumoto, T. (1976). The role of termites in an equatorial rain forest ecosystem of west Malaysia I. population density, biomass, carbon, nitrogen and calorific content and respiration rate. *Oecologia (Berl.)* 22, 153–178.

Mikaelyan, A., Strassert, J. F. H., Tokuda, G., and Brune, A. (2014). The fibre-associated cellulolytic bacterial community in the hindgut of wood-feeding higher termites (*N asutitermes* spp.). *Environ. Microbiol.* 16, 2711–2722. doi:10.1111/1462-2920.12425.

Mikaelyan, A., Dietrich, C., Köhler, T., Poulsen, M., Sillam-Dussès, D., and Brune, A. (2015a). Diet is the primary determinant of bacterial community structure in the guts of higher termites. *Mol. Ecol.* 24, 5284–5295. doi:10.1111/mec.13376.

Mikaelyan, A., Köhler, T., Lampert, N., Rohland, J., Boga, H., Meuser, K., and Brune, A. (2015b). Classifying the bacterial gut microbiota of termites and cockroaches: A curated phylogenetic reference database (DictDb). *Syst. Appl. Microbiol.* 38, 472–482. doi:10.1016/j.syapm.2015.07.004.

Mikaelyan, A., Meuser, K., and Brune, A. (2016). Microenvironmental heterogeneity of gut compartments drives bacterial community structure in wood-and humus-feeding higher termites. *FEMS Microbiology Ecology*, 93. doi:10.1093/femsec/fiw210.

Miltner, A., Bombach, P., Schmidt-Brücken, B., and Kästner, M. (2012). SOM genesis: microbial biomass as a significant source. *Biogeochemistry* 111, 41–55.

doi:10.1007/s10533-011-9658-z.

Nalepa, C. A., and Bandi, C. (2000). “Characterizing the ancestors: paedomorphosis and termite evolution,” in *Termites: Evolution, Sociality, Symbioses, Ecology* (Dordrecht: Springer Netherlands), 53–75. doi:10.1007/978-94-017-3223-9_3.

Nalepa, C. A., Bignell, D. E., and Bandi, C. (2001). Detritivory, coprophagy, and the evolution of digestive mutualisms in Dictyoptera. *Insectes Soc.* 48, 194–201. doi:10.1007/PL00001767.

Ngugi, D. K., and Brune, A. (2012). Nitrate reduction, nitrous oxide formation, and anaerobic ammonia oxidation to nitrite in the gut of soil-feeding termites (*Cubitermes* and *Ophiotermes* spp.). *Environ. Microbiol.* 14, 860–871. doi:10.1111/j.1462-2920.2011.02648.x.

Ngugi, D. K., Ji, R., and Brune, A. (2011). Nitrogen mineralization, denitrification, and nitrate ammonification by soil-feeding termites: A 15N-based approach. *Biogeochemistry* 103, 355–369. doi:10.1007/s10533-010-9478-6.

Nielsen, M. B., Kjeldsen, K. U., and Ingvorsen, K. (2006). *Desulfitibacter alkalitolerans* gen. nov., sp. nov., an anaerobic, alkalitolerant, sulfite-reducing bacterium isolated from a district heating plant. *Int. J. Syst. Evol. Microbiol.* doi:10.1099/ijs.0.64356-0.

Noirot, C. (1992). "From wood- to humus-feeding: an important trend in termite evolution," in *Biology and evolution of social insects*, ed J. Billen (Belgium, Leuven University Press), 107–119.

Noirot, C. (1995). The gut of termites (isoptera): Comparative anatomy, systematics, phylogeny. I: Lower termites. *Ann. la Société Entomol. Fr.* 31, 197–226.

Noirot, C. (2001). The gut of termites (Isoptera) comparative anatomy systematics, phylogeny,. II. -higher termites (Termitidae). *Ann. Soc. Entomol. Fr* 37, 431–471.

Noda, S., Hongoh, Y., Sato, T., and Ohkuma, M. (2009). BMC Evolutionary biology complex coevolutionary history of symbiotic Bacteroidales bacteria of various protists in the gut of termites. *BMC Evol. Biol.* 9. doi:10.1186/1471-2148-9-158.

Noda, S., Mantini, C., Meloni, D., Inoue, J. I., Kitade, O., Viscogliosi, E., and Ohkuma, M. (2012). Molecular phylogeny and evolution of parabasalia with

improved taxon sampling and new protein markers of actin and elongation factor-1α. *PLoS One*. doi:10.1371/journal.pone.0029938.

Noda, S., Ohkuma, M., and Kudo, T. (2002). Nitrogen fixation genes expressed in the symbiotic microbial community in the gut of the termite *Coptotermes formosanus*. *Microbes and Environments.* 17, 139–143.

Noda, S., Ohkuma, M., Usami, R., Horikoshi, K., and Kudo, T. (1999). Culture-independent characterization of a gene responsible for nitrogen fixation in the symbiotic microbial community in the gut of the termite *Neotermes koshunensis*. *Appl. Environ. Microbiol.* 65, 4935-4942.

Nonoh, J. O. (2013). Archaeal diversity and community structure in the compartmented gut of higher termites. [dissertation] Philipps-Universität Marburg.

Ohkuma, M., and Brune, A. (2010). "Diversity, structure, and evolution of the termite gut microbial community," in *Biology of Termites: a Modern Synthesis* (Dordrecht: Springer Netherlands), 413–438. doi:10.1007/978-90-481-3977-4_15.

Ohkuma, M., Noda, S., Hattori, S., Iida, T., Yuki, M., Starns, D., Inoue, J., Darby, A., C., and Hongoh, Y. (2015). Acetogenesis from H_2 plus CO_2 and nitrogen fixation by an endosymbiotic spirochete of a termite-gut cellulolytic protist. *PNAS* 112, 10224–10230. doi:10.1073/pnas.1423979112.

Ohkuma, M., Noda, S., and Kudo, T. (1999). Phylogenetic diversity of nitrogen fixation genes in the symbiotic microbial community in the gut of diverse termites. *Appl. Environ. Microbiol.* 65, 4926–4934.

Ohkuma, M., Noda, S., Usami, R., Horikoshi, K., and Kudo, T. (1996). Diversity of nitrogen fixation genes in the symbiotic intestinal microflora of the termite *Reticulitermes speratus*. *Appl. Environ. Microbiol.* 62, 2747–2752.

Oksanen, J. (2015). Multivariate analysis of ecological communities in R: vegan tutorial. *University Oulu, Finland*

Okwakol, M. J. N. (1980). Estimation of soil and organic matter consumption by termites of the genus *Cubitermes*. *Afr. J. Ecol.* 18, 127–131. doi:10.1111/j.1365-2028.1980.tb00276.x.

Otani, S., Mikaelyan, A., Nobre, T., Hansen, L. H., Koné, N. A., Sørensen, S. J., Aanen, D. K., Boomsma, J. J., Brune, A., and Poulsen, M. (2014). Identifying the core microbial community in the gut of fungus-growing termites. *Mol. Ecol.* 23. doi:10.1111/mec.12874.

Paradis, E., Claude, J., and Strimmer, K. (2004). APE: Analyses of phylogenetics and evolution in R language. *Bioinformatics* 20, 289–290. doi:10.1093/bioinformatics/btg412.

Paster, B. J., Dewhirst, F. E., Cooke, S. M., Fussing, V., Poulsen, L. K., and Breznak, J. A. (1996). Phylogeny of not-yet-cultured spirochetes from termite guts. *Appl. Environ. Microbiol.* 62, 347–352.

Paul, K., Nonoh, J. O., Mikulski, L., and Brune, A. (2012). Methanoplasmatales, Thermoplasmatales-related archaea in termite guts and other environments, are the seventh order of methanogens. *Appl. Environ. Microbiol.* 78, 8245–8253. doi:10.1128/AEM.02193-12.

Pester, M., and Brune, A. (2006). Expression profiles of fhs (FTHFS) genes support the hypothesis that spirochaetes dominate reductive acetogenesis in the hindgut of lower termites. *Environ. Microbiol.* 8, 1261–1270. doi:10.1111/j.1462-2920.2006.01020.x.

Piccolo, A. (1996). "Humus and soil conservation," In *Humic substances in terrestrial ecosystems*. ed. A. Piccolo (Amsterdam, Elsevier), 225–264.

Potrikus, C. J., and Breznak, J. A. (1977). Nitrogen-fixing *Enterobacter agglomerans* isolated from guts of wood-eating termites. *Appl. Environ. Microbiol.* 33, 392–399.

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2 – Approximately maximum-likelihood trees for large alignments. *PLoS One* 5, e9490. doi:10.1371/journal.pone.0009490.

Prusch, R. D. (1972). Secretion of NH_4Cl by the hindgut of *Sarcophaga bullata* larva. *Comp. Biochem. Physiol. Part A Physiol.* 41, 215–223. doi:10.1016/0300-9629(72)90049-7.

R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-

project.rg/.

Rawls, J. F., Mahowald, M. A., Ley, R. E., and Gordon, J. I. (2006). Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. *Cell* 127, 423–33. doi:10.1016/j.cell.2006.08.043.

Raymond, J., Siefert, J. L., Staples, C. R., and Blankenship, R. E. (2004). The natural history of nitrogen fixation. *Mol. Biol. Evol.* 21, 541–54. doi:10.1093/molbev/msh047.

Rieu-Lesme, F., Morvan, B., Collins, M. D., Fonty, G., and Willems, A. (1996). A new H_2/CO_2-using acetogenic bacterium from the rumen: description of *Ruminococcus schinkii* sp. nov. *FEMS Microbiol. Lett.* 140, 281–6.

Rohrmann, G. F., and Rossman, A. Y. (1980). Nutrient strategies of *Macrotermes ukuzii* (Isoptera: Termitidae). *Pedobiologia* 20, 61–73.

Rossmassler, K., Dietrich, C., Thompson, C., Mikaelyan, A., Nonoh, J. O., Scheffrahn, R. H., Sillam-Dussès, D., and Brune, A. (2015). Metagenomic analysis of the microbiota in the highly compartmented hindguts of six wood- or soil-feeding higher termites. *Microbiome* 3, 56. doi:10.1186/s40168-015-0118-1.

Rubio, L. M., and Ludden, P. W. (2002). "The Gene Products of the nif Regulon," in *Nitrogen Fixation at the Millennium* ed. G.J. Leigh (Elsevier), 101–136. doi:10.1016/B978-044450965-9/50004-5.

Ruby, E., Henderson, B., and McFall-Ngai, M. (2004). Microbiology. We get by with a little help from our (little) friends. *Science* 303, 1305–1307. doi:10.1126/science.1094662.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Horn, D. J. V., and Weber, C. F. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi:10.1128/AEM.01541-09.

Schmitt-Wagner, D., and Brune, A. (1999). Hydrogen profiles and localization of methanogenic activities in the highly compartmentalized hindgut of soil-feeding higher termites (*Cubitermes* spp.). *Appl. Environ. Microbiol.* 65, 4490–4496.

Schmitt-Wagner, D., Friedrich, M. W., Wagner, B., and Brune, A. (2003). Phylogenetic diversity, abundance, and axial distribution of bacteria in the intestinal tract of two soil-feeding termites (*Cubitermes* spp.). *Appl. Environ. Microbiol.* 69, 6007–6017. doi:10.1128/AEM.69.10.6007-6017.2003.

Schulten, H.-R. (1995). The three-dimensional structure of humic substances and soil organic matter studied by computational analytical chemistry. *Fresenius. J. Anal. Chem.* 351, 62–73. doi:10.1007/BF00324293.

Schulz, M., Slaytor, M., Hogan, M., and O'brien, R. (1986). Components of cellulase from the higher termite, *Nasutitermes walkeri*. *Insect Biochem.* 16, 929-932.

Slaytor, M., Veivers, P., and Lo, N. (1997). Aerobic and anaerobic metabolism in the higher termite *Nasutitermes walkeri* (Hill). *Insect Biochem. Mol. Biol.* 27, 291-303.

Sleaford, F., Bignell, D. E., and Eggleton, P. (1996). A pilot analysis of gut contents in termites from the Mbalmayo Forest Reserve, Cameroon. *Ecol. Entomol.* 21, 279–288. doi:10.1111/j.1365-2311.1996.tb01245.x.

Stevenson, F. J. (1994). *Humus chemistry : genesis, composition, reactions*. 2nd ed. (New York, John Wiley & Sons).

Swift, R. S., and Posner, A. M. (1972). Autoxidation of humic acid under alkaline conditions. *J. Soil Sci.* 23, 381–393. doi:10.1111/j.1365-2389.1972.tb01669.x.

Sylvester-Bradley, R., Bandeira, A. G., Oliveira, L. A. de, Sylvester-Bradley, R., Bandeira, A. G., and Oliveira, L. A. de (1978). Fixação de nitrogênio (redução de acetileno) em cupins (Insecta: Isoptera) da Amazônia Central. *Acta Amaz.* 8, 621–627. doi:10.1590/1809-43921978084621.

Taguchi, F., Dan Chang, J., Mizukami, N., Saito-taki, T., Hasegawa, K., and Morimoto, M. (1993). Isolation of a hydrogen-producing bacterium, *Clostridium beijerinckii* strain AM21B, from termites. *Canadian Journal of Microbiology* 39, 726-730.

Taguchi, F., Hasegawa, K., Saito-Taki, T., and Hara, K. (1996). Simultaneous production of xylanase and hydrogen using xylan in batch culture of *Clostridium* sp. strain X53. *J. Ferment. Bioeng.* 81, 178-180. doi:10.1016/0922-338X(96)87600-8.

Tai, V., Carpenter, K. J., Weber, P. K., Nalepa, C. A., Perlman, S. J., and Keeling, P. J. (2016). Genome evolution and nitrogen-fixation in bacterial ectosymbionts of a protist inhabiting wood-feeding cockroaches. *Appl. Environ. Microbiol.* 82, 4682-4695., AEM.00611-16-. doi:10.1128/AEM.00611-16.

Tamschick, S., and Radek, R. (2013). Colonization of termite hindgut walls by oxymonad flagellates and prokaryotes in *Incisitermes tabogae, I. marginipennis* and *Reticulitermes flavipes*. *Eur. J. Protistol.* 49, 1-14.doi:10.1016/j.ejop.2012.06.002.

Täyasu, I., A. Sugimoto, E. Wada, and T. A. (1994). Xylophagous termites depending on atmospheric nitrogen. *Naturwissenschaften* 81, 229–231.

Tholen, A., and Brune, A. (1999). Localization and in situ activities of homoacetogenic bacteria in the highly compartmentalized hindgut of soil-feeding higher termites (*Cubitermes* spp.). *Appl. Environ. Microbiol.* 65, 4497–505.

Thompson, C. L., Vier, R., Mikaelyan, A., Wienemann, T., and Brune, A. (2012). "*Candidatus* Arthromitus" revised: segmented filamentous bacteria in arthropod guts are members of *Lachnospiraceae*. *Environ. Microbiol.* 14, 1454–1465. doi:10.1111/j.1462-2920.2012.02731.x.

Thongaram, T., Hongoh, Y., Kosono, S., Ohkuma, M., Trakulnaleamsai, S., Noparatnaraporn, N., and Kudo, T. (2005). Comparison of bacterial communities in the alkaline gut segment among various species of higher termites. *Extremophiles* 9, 229–238. doi:10.1007/s00792-005-0440-9.

Ueki, A., Akasaka, H., Suzuki, D., and Ueki, K. (2006). *Paludibacter propionicigenes* gen. nov., sp. nov., a novel strictly anaerobic, gram-negative, propionate-producing bacterium isolated from plant residue in irrigated rice-field soil in Japan. *Int. J. Syst. Evol. Microbiol.* doi:10.1099/ijs.0.63896-0.

Veivers, P. C., O'Brien, R. W., and Slaytor, M. (1980). The redox state of the gut of termites. *J. Insect Physiol.* 26, 75–77. doi:10.1016/0022-1910(80)90112-2.

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–7. doi:10.1128/AEM.00062-07.

Warnecke, F., Luginbühl, P., Ivanova, N., Ghassemian, M., Richardson, T. H., Stege,

J. T., Cayouette, M., McHardy, A. C., Djordjevic, G., Aboushadi, N., Sorek, R., Tringe, S. G., Podar, M., Martin, H. G., Kunin, V., Dalevi, D., Madejska, J., Kirton, E., Platt, D., Szeto, E., Salamov, A., Barry, K., Mikhailova, N., Kyrpides, N. C., Matson, E. G., Ottesen, E. A., Zhang, X., Hernández, M., Murillo, C., Acosta, L. G., Rigoutsos, I., Tamayo, G., Green, B. D., Chang, C., Rubin, E. M., Mathur, E. J., Robertson, D. E., Hugenholtz, P., and Leadbetter, J. R. (2007). Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature* 450, 560–565. doi:10.1038/nature06269.

Watanabe, K., Kodama, Y., and Harayama, S. (2001). Design and evaluation of PCR primers to amplify bacterial 16S ribosomal DNA fragments used for community fingerprinting. *J. Microbiol. Methods* 44, 253–262. doi:10.1016/S0167-7012(01)00220-2.

Wertz, J. T., Kim, E., Breznak, J. A., Schmidt, T. M., and Rodrigues, J. L. M. (2012). Genomic and physiological characterization of the *Verrucomicrobia* isolate *Diplosphaera colitermitum* gen. nov., sp. nov., reveals microaerophily and nitrogen fixation genes. *Appl. Environ. Microbiol.* 78, 1544-1555. doi:10.1128/AEM.06466-11.

Whelan, S., and Goldman, N. (2001). A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Mol. Biol. Evol.* 18, 691–699.

Wier, A. M., Nyholm, S. V, Mandel, M. J., Massengo-Tiassé, R. P., Schaefer, A. L., Koroleva, I., Splinter-Bondurant, S., Brown, B., Manzella, L., Snir, E., Almabrazi, H., Scheetz, T. E., Bonaldo, Mde. F., Casavant, T. L., Soares, M. B., Cronan, J. E., Reed, J. L., Ruby, E. G., and McFall-Ngai, M. J. (2010). Transcriptional patterns in both host and bacterium underlie a daily rhythm of anatomical and metabolic change in a beneficial symbiosis. *Proc. Natl. Acad. Sci. U. S. A.* 107, 2259–2264. doi:10.1073/pnas.0909712107.

Wood, T. G. (1988). Termites and the soil environment. *Biol. Fertil. Soils* 6, 228–236. doi:10.1007/BF00260819.

Wright, P. A. (1995). Nitrogen excretion: three end products, many physiological roles. *J. Exp. Biol.* 198, 272-81.

Yamada, A., Inoue, T., Noda, S., Hongoh, Y., and Ohkuma, M. (2007). Evolutionary

trend of phylogenetic diversity of nitrogen fixation genes in the gut community of wood-feeding termites. *Mol. Ecol.* 16, 3768-77. doi:10.1111/j.1365-294X.2007.03326.x.

Yoneda, T., Yoda, K., and Kira, T. (1977). Accumulation and decomposition of big wood litter in Pasoh Forest, West Malaysia. *Jap. J. Ecol.* 27, 53–60.

Young, J. P. W. (2005). "The phylogeny and evolution of nitrogenases," in *Genomes and Genomics of Nitrogen-fixing Organisms* (Berlin/Heidelberg: Springer-Verlag), 221–241. doi:10.1007/1-4020-3054-1_14.

Zheng, H., Dietrich, C., Radek, R., and Brune, A. (2016). *Endomicrobium proavitum*, the first isolate of *Endomicrobia* class. nov. (phylum *Elusimicrobia*) – an ultramicrobacterium with an unusual cell cycle that fixes nitrogen with a Group IV nitrogenase. *Environ. Microbiol.* 18, 191–204. doi:10.1111/1462-2920.12960.

Chapter 6

Supplementary materials

Figure S6.1 Calibration curves for a glass pH microelectrode over the whole pH range in this study. The response of the microelectrodes became increasingly non-linear at alkaline pH (pH > 11).

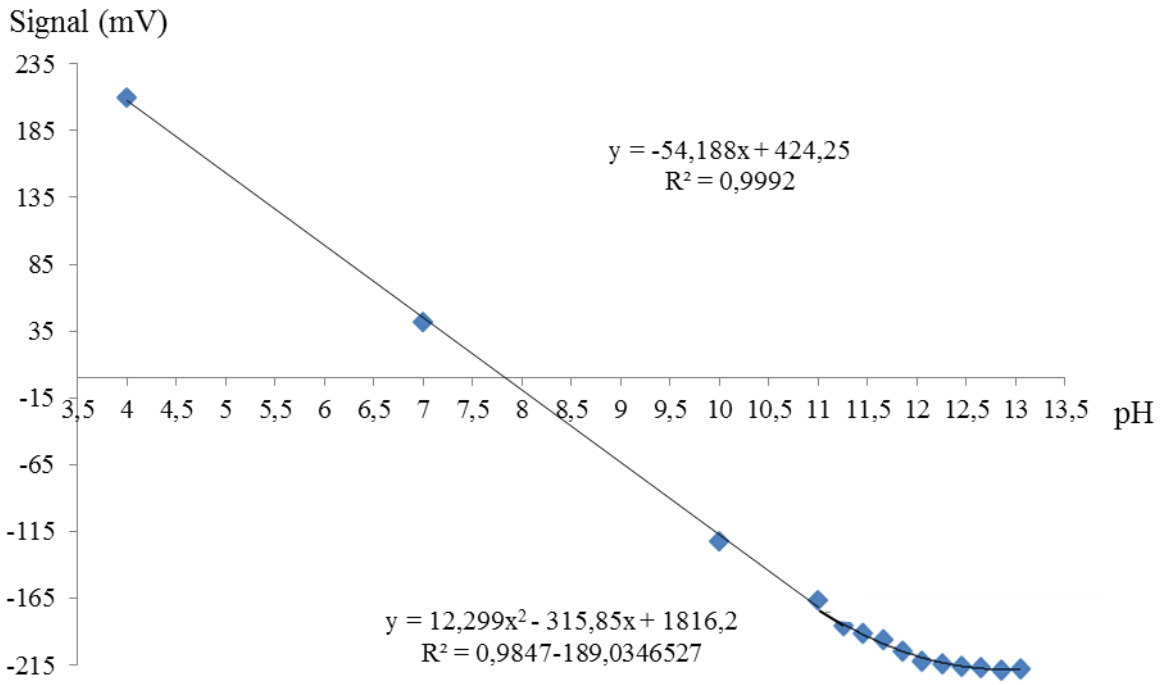

Table S6.1 Gut volumes for different gut sections of termite species used in this study (not recorded for all species). The measurements were based on four independent groups. The data of *Cubitermes* spp. are from Dr. Anne Thole.

	C	M/ms	P1	P3	P4	P5	Sum	P1/P3 ratio
Embiratermes neotenicus	0.05±0.05	0.08±0.04	0.39±0.05	1.67±0.45	0.07±0.02	0.04±0.03	2.31±0.57	0.23
Labiotermes labralis	0.07±0.05	0.37±0.06	1.72±0.17	2.68±0.78	0.33±0.25	0.13±0.22	5.30±0.85	0.64
Palmitermes impostor	0.06±0.06	0.19±0.13	0.24±0.09	0.44±0.39	0.10±0.09	0.07±0.04	1.10±0.72	0.54
Nasutitermes matangensis	0.03±0.01	0.10±0.03	0.03±0.01	0.66±0.14	0.02±0.01	0.11±0.01	0.96±0.18	0.05
Cubitermes umbratus		0.37	1.55	0.97	0.32	0.11		1.60
Cubitermes orthognathus			0.76	0.54	0.12	0.17		1.41

Table S6.2 The total number of reads after quality filtration of the amplicon libraries of bacterial 16S rRNA genes from the major gut compartments of various higher termites, and the classification success for each sample at different taxonomic levels. Species IDs of the termite hosts are the same as those used in the main publication.

Table S6.3 Relative abundance (in percent) of bacterial taxa in the amplicon libraries of 16S rRNA genes from the major hindgut compartments of various higher termites. The table is interactive: Classification results can be displayed for different taxonomic levels by clicking the corresponding boxes (1, phylum; 2, class; 3, order; 4, family; 5, genus). The samples are ordered by feeding group (see Table 2.1).

https://www.dropbox.com/s/lu0fgr1sn9ptqqo/Chapter3_Supplementary_table.xlsx?dl=0

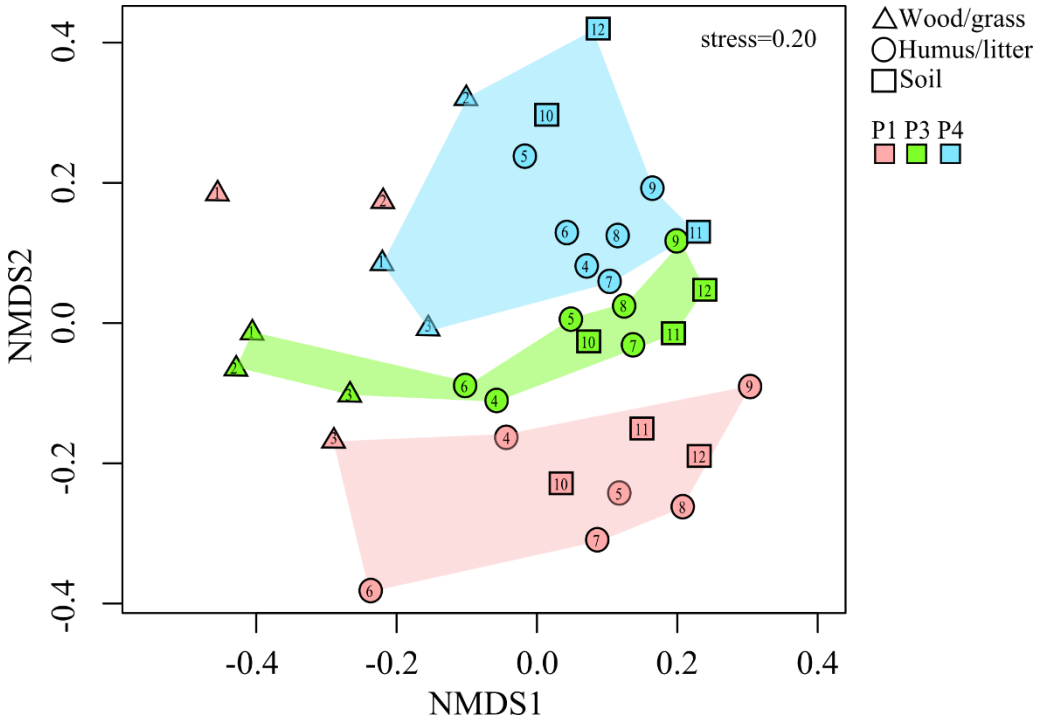

Figure S6.2 Non-metric multidimensional scaling (NMDS) representation of prokaryotic (16S rRNA gene) communities, based on generalized UniFrac distances. Gut compartments are color-coded; species IDs of the termite hosts are the same as those used in Table 2.1.

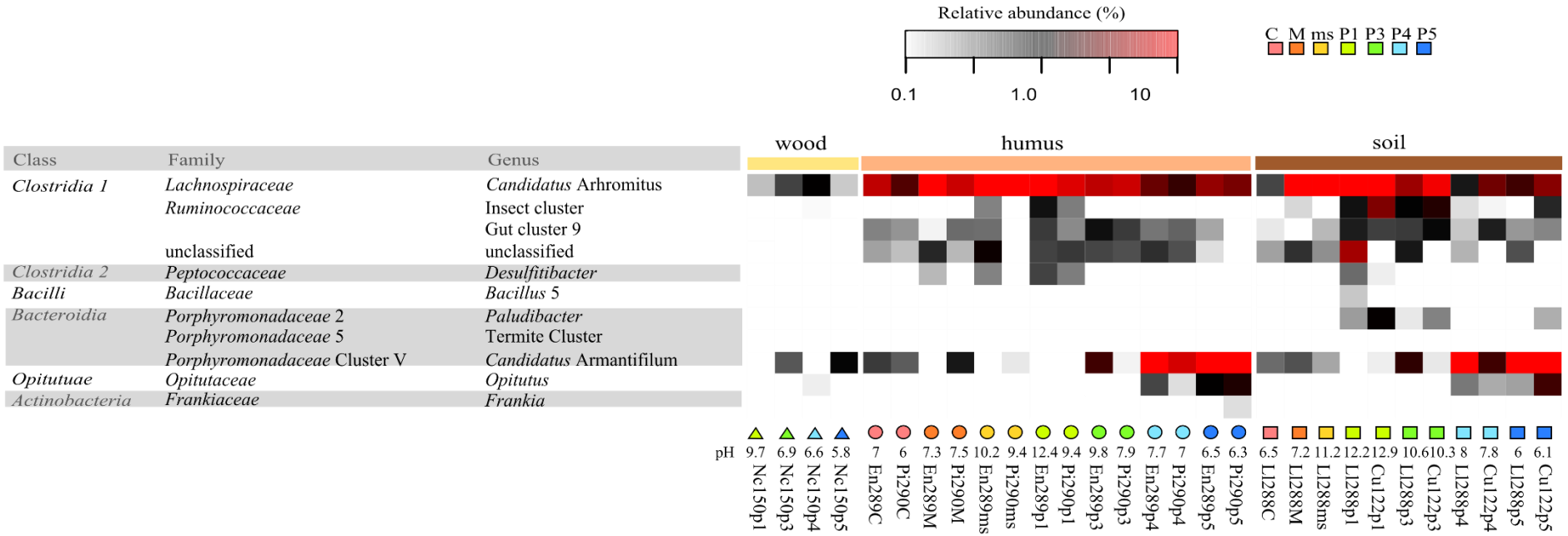

Figure S6.3 Relative abundance of top correlating lineages with pH based on the highest and lowest *adonis* correlation coefficients and correlation verified by p value

Table S6.4 The total number of reads after quality filtration of the amplicon libraries of *nifH* genes from the gut of various cockroaches and termites, and the classification success for each sample at different taxonomic levels.
Table S6.5 Relative abundance (in percent) of bacterial taxa in the amplicon libraries of 16S rRNA genes from the major hindgut compartments of various higher termites. The table is interactive: Classification results can be displayed for different taxonomic levels by clicking the corresponding boxes (1, phylum; 2, class; 3, order; 4, family; 5, genus). The samples are ordered by feeding group (see Table 2.2).

https://www.dropbox.com/s/5j5gqki6q0k63m8/Chapter4_Supplementary_table.xlsx?dl=0

List of Abbreviations

16S rRNA	Small subunit bacterial rRNA
C	Crop
CCA	Canonical correspondence analysis
ESR	Erythrocyte sedimentation rate
FIA	Flow-injection assay
FTIR	Fourier-transform infrared spectroscopy
M	Midgut
ms	Mixed segment
NMDS	Non-metric Multidimensional Scaling
NMR	Nuclear magnetic resonance
OTU	Operational Taxonomic Unit
P1	First proctodeal hindgut compartment
P2	Second proctodeal hindgut compartment
P3	Third proctodeal hindgut compartment
P4	Forth proctodeal hindgut compartment
P5	Fifth proctodeal hindgut compartment
PCoA	Principal coordinate analysis
PCR	Polymerase Chain Reaction
pyrolysis-GC-MS	Pyrolysis–gas chromatography–mass spectrometry

qPCR	Quantitative polymerase chain reaction
RDP	Ribosomal Database Project
RT-PCR	Reverse transcription polymerase chain reaction
SOM	Soil organic matter
sp.	Species (singular)
spp.	Species (plural)
TG3	Candidate phylum Termite Group 3
T-RFLP	Terminal restriction fragment length polymorphism
WAG	Whelan & Goldman

Contributions

Environmental factors in the gut compartments of humivorous higher termites

Contributions: Hongjie Li dissected termites, measured fresh weight, calculated gut volume, carried on microsensor measurements and wrote the manuscript. Wanyang Wang dissected termites, measured fresh weight, carried on microsensor measurements, FIA (Flow-injection analysis), colorimetric assays and corrected the manuscript. James Oluoch Nonoh did the experiments related to *Amitermes* sp. David Sillam-Dussès collected termites from the field. Andreas Brune conceived the study, discussed results and secured funding

Effect of diet and gut environment on community structure in higher termites of different feeding groups

Contributions: Wanyang Wang planned experiments, analyzed data, discussed results, and wrote the manuscript. Hongjie Li dissected termites and extracted DNA. Katja Meuser prepared samples for amplicon sequencing. David Sillam-Dussès collected termites from the field. Andreas Brune conceived the study, discussed results and secured funding.

Abundance and diversity of nitrogen-fixing bacteria in termite and cockroach guts

Contributions: Wanyang Wang planned and carried out experiments (extracted DNA from some samples, prepared samples for sequencing, qPCR), analyzed data (made a part of OTUs (Operational taxonomic unit), did ordination analysis, built the *nifH* tree, classified the *nifH* genes and got the *nifH* genes from annotated metagenome results), discussed results, and wrote the manuscript. Carsten Dietrich planned experiments and analyzed data (made a part of OTUs). Katja Meuser carried out experiments (prepared samples for amplicon sequencing and sequenced COII genes). Niclas Lampert analyzed data (made a part of OTUs). Aram Mikaelyan discussed tree building methods. Andreas Brune conceived the study, discussed results and secured funding

Acknowledgements

My grateful thanks go to my supervisor Professor Andreas Brune for giving me an opportunity to work in the termite world, and for his invaluable suggestions, support, patience and inspiration throughout my Ph.D. studies.

I would like to thank Prof. Dr. Wolfgang Buckel for reviewing my thesis.

Meanwhile, I am very grateful that Prof. Dr. Roland Brandl and Prof. Dr. Alfred Batschauer agree to examine this thesis, and offer their comments and criticisms.

This is also a great opportunity for me to thank Prof. Ralf Conrad, PD Dr. Werner Liesack and Dr. Tobias Erb for being in my thesis advisory committee, perceptive suggestions and great inspiration.

I am very thankful to all the present and former members of AG Brune Group for their contribution of my study and the friendly atmosphere in the laboratory. I have a good time with you not only in the lab but also after work. Especially, I want to thank Dr. Dorothee Tegtmeier, Niclas Lampert and Christopher Feldewert for their feedback on this thesis.

I thank Niclas Lampert for discussing core genus analysis and CCA analysis. I thank Christine Nalepa (North Carolina State University), Ricardo Augusto Nink (Federal Institute of Education, Science and Technology of Bahia, Bahia, Brazil), Rudy Plarre (Federal Institute for Materials Research and Testing, Berlin, Germany), Rudolf Scheffrahn (University of Florida), Gaku Tokuda (University of the Ryukyus), Robert Hanus (Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences), Jan Šobotník (The Biology Centre of the Czech Academy of Sciences), Ghislaine Small (The University of Western Australia) and our coworkers Katja Meuser and James Nonoh (MPI Marburg) for providing termites.

I am indebted to the International Max Planck Research School for the financial support. I am also thankful to the former IMPRS coordinator Dr. Marcela Hernandez and Dr. Wiebke Wlotzka, the present IMPRS coordinator Dr. Zrinka Gattin and the former IMPRS secretary Mrs. Simone Hain for the timely help, especially when I started my PhD.

I thank all my friends for their companion and understanding of my study. I am sorry for my absence during the time I wrote this thesis.

Last but not least, I would like to thank my family. Thank you for encouraging me to explore the world, continuous love and your support for all these years.

Pledge

I certify that the present thesis entitled:

"**The bacterial gut microbiota of wood- and humus-feeding termites: Diazotrophic populations and compartment-specific response of bacterial communities to environmental factors**"

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.

Wanyang Wang

Marburg, December 2017