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Identifying the core microbial community in the gut of fungus-growing termites

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Abstract

Gut microbes play a crucial role in decomposing lignocellulose to fuel termite societies, with protists in the lower termites and prokaryotes in the higher termites providing these services. However, a single basal subfamily of the higher termites, the Macrotermitinae, also domesticated a plant biomass-degrading fungus (*Termitomyces*), and how this symbiont acquisition has affected the fungus-growing termite gut microbiota has remained unclear. The objective of our study was to compare the intestinal bacterial communities of five genera (nine species) of fungus-growing termites to establish whether or not an ancestral core microbiota has been maintained and characterizes extant lineages. Using 454-pyrosequencing of the 16S rRNA gene, we show that gut communities have representatives of 26 bacterial phyla and are dominated by Firmicutes, Bacteroidetes, Spirochaetes, Proteobacteria and Synergistetes. A set of 42 genus-level taxa was present in all termite species and accounted for 56–68% of the species-specific reads. Gut communities of termites from the same genus were more similar than distantly related species, suggesting that phylogenetic ancestry matters, possibly in connection with specific termite genus-level ecological niches. Finally, we show that gut communities of fungus-growing termites are similar to cockroaches, both at the bacterial phylum level and in a comparison of the core Macrotermitinae taxa abundances with representative cockroach, lower termite and higher nonfungus-growing termites. These results suggest that the obligate association with *Termitomyces* has forced the bacterial gut communities of the fungus-growing termites towards a relatively uniform composition with higher similarity to their omnivorous relatives than to more closely related termites.

Keywords: 16S rRNA pyrosequencing, bacterial community, gut microbiota, Macrotermitinae, symbiosis, *Termitomyces*

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Introduction

The rapid growth in microbiome research, particularly during the last decade, has shown that microbial communities make crucial contributions to digestion, immunity, reproduction and other physiological functions of insect hosts (Warnecke *et al.* 2007; Werren *et al.* 2008; Lehman *et al.* 2009; Fraune & Bosch 2010; Lee &

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Mazmanian 2010; Sharon *et al.* 2010). In spite of substantial fluctuations due to different host conditions (Hongoh *et al.* 2005, 2006; Moran *et al.* 2008; Andert *et al.* 2010; Huang *et al.* 2013), hosts often associate with a specific set of microbes: their core microbiota (Turnbaugh *et al.* 2007; Hamady & Knight 2009; Huse *et al.* 2012). When that is so, core microbiota can be considered as a host species-, genus- or family-specific trait that may play key roles in determining host fitness and evolutionary potential (Hongoh *et al.* 2005; Andert *et al.* 2010; Hongoh 2010; Brucker & Bordenstein 2012). However, relative to vertebrates, explicit studies of core communities associated with insects are still few, but include the common bed bug *Cimex lactularius* (Meriweather *et al.* 2013), the honeybee *Apis mellifera* (Sabree *et al.* 2012), *Drosophila melanogaster* (Wong *et al.* 2011) and *Cephalotes varians* (Hu *et al.* 2014).

As terrestrial eusocial invertebrates, the termites (Insecta, Isoptera) occupy most available habitats in (sub) tropical regions (Donovan *et al.* 2001). They play essential roles in general plant decomposition (Jones 1990) by ingesting dead plant matter, degrading lignocellulose and other components and recycling nutrients (Brauman *et al.* 2001). This is accomplished by the combined activities of the host and its gut microbes (Watanabe & Tokuda 2010; Ni & Tokuda 2013). The composition of the intestinal microbial communities varies markedly across termite lineages (Hongoh 2011; Brune 2014). In the so-called lower termites, the gut is occupied by a dense community of protist symbionts working in concert with gut bacteria (Cleveland 1923; Brugerolle & Radek 2006; Hongoh 2010), whereas the higher termites (family Termitidae) lost these eukaryote symbionts and rely primarily on their gut bacteria to assist in decomposition (Brune & Ohkuma 2011; Brune 2014). Members of the basal higher termite subfamily Macrotermitinae have added another party to their pool of symbionts by cultivating a *Termitomyces* fungus (Tricholomataceae, Basidiomycotina), which also assists in degrading plant material (Sands 1969; Bignell *et al.* 1994; Hyodo *et al.* 2003). Via a complex process of dual gut passage, these termites first ingest a mixture of plant substrate and asexual *Termitomyces* spores and defecate this to build layers of a sponge-like fungus comb structure, which produces new nodules with asexual spores and finally is consumed in its entirety after considerable mycelial growth (Sands 1960; Nobre *et al.* 2011b; Nobre & Aanen 2012).

Fungiculture in the Macrotermitinae evolved only once ca. 35 Ma and the subfamily radiated into ca. 330 known extant species (Aanen *et al.* 2002), of which none secondarily abandoned fungus farming. Although it is generally appreciated that gut bacteria remained important after fungus farming evolved (Hongoh 2011), it has recently been shown that the presence of *Termitomyces* has induced functional division of labour with different complementary decomposition roles for *Termitomyces* and the gut bacterial community (Liu *et al.* 2013). The overall gut community structure of representatives of the Macrotermitinae investigated so far has shown considerably divergence from gut communities of other higher termite subfamilies (Hongoh *et al.* 2006; Dietrich *et al.* 2014). This does not imply that communities are mainly made up of novel bacteria lineages, as many of the bacterial clones obtained from guts of *Odontotermes* spp. (Shinzato *et al.* 2007; Makonde *et al.* 2013), *Macrotermes gilvus* (Hongoh *et al.* 2006) and *Microtermes* sp. (Makonde *et al.* 2013) are related to bacteria present in other termites, but rather that relative abundances of bacteria are shifted across members of the subfamily. It has remained unclear how much of the change in the macrotermitine gut microbiota can be explained by their common ancestry as fungus farmers. This is in part because termite species coverage has so far been limited and methods have rarely gone beyond clone libraries (Hongoh *et al.* 2006; Mackenzie *et al.* 2007; Shinzato *et al.* 2007; Zhu *et al.* 2012; Makonde *et al.* 2013), precluding comparative analyses across the Macrotermitinae. Using 454-pyrosequencing of the 16S rRNA gene, we analyse and compare the bacterial community structure of nine fungus-growing termite species, spanning five genera of the Macrotermitinae. We characterize gut microbiota compositions, identify the core community of fungus-growing termites and compare their compositions to cockroach, lower termite and other higher termite guts.

Materials and methods

The experimental material and DNA extraction

Termite workers from one colony from each of nine fungus-growing termite species were sampled in the Lamto reserve, in central Ivory Coast (6°13″ N and 5°02″ W) in November 2011: *Macrotermes subhyalinus*, *Microtermes toumodiensis*, *Ancistrotermes cavithorax*, *Ancistrotermes guineensis*, *Pseudacanthotermes militaris*, *Pseudacanthotermes minor*, *Odontotermes* sp. 1, *Odontotermes* sp. 2 and *Odontotermes* sp. 3 (cf. Aanen *et al.* 2002; Nobre *et al.* 2011a). Whole guts, excluding Malpighian tubules, were dissected from 8 to 10 foraging workers per colony and pooled, and genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer instructions. DNA was purified using phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma-Aldrich, USA) following a modified protocol from the manufacturer's instructions. Briefly, the DNA was mixed with an equal volume of

phenol/chloroform/isoamyl alcohol and kept on a rotator for 15 min, this was followed by centrifugation at 12 000 *g* for 20 min, after which the aqueous phase was transferred to a new tube and this procedure was repeated. Subsequently, 1 volume of chloroform was added, the tubes were slowly rotated for 15 min, centrifuged for 15 min at 12 000 *g*, and the resulting aqueous phase was transferred to a new tube and mixed with an equal volume of ice-cold isopropanol. The tubes were briefly mixed, and the DNA was precipitated by centrifugation for 20 min at 12 000 *g*, after which the isopropanol was removed and the samples were allowed to dry. Purified DNA was resuspended in 100 μl AE elution buffer (Qiagen) and quantified photometrically using NanoDrop ND-1000 (Thermo Scientific, Germany). Prior to 454 amplifications, 16S rRNA gene quality was assessed by positive PCR using general bacteria primers (341F - 809R, 10 μM, Hansen *et al.* 2012).

PCR amplification and pyrotag sequencing

The 16S rRNA gene (including Archaea) was amplified using the primers 341F (5′- CCTAYGGGRBGCASCAG -3′) and 806R (5′- GGACTACNNGGGTATCTAAT -3′) flanking the hypervariable V3 – V4 regions (Hansen *et al.* 2012). The primers were modified by adding sample-specific multiplex identifier barcodes (MID) (5′-Adaptor A) to the forward primer and a universal sequence (5′-Adaptor B) to the reverse primer. The amplification reaction was prepared in 20 μl final volume containing: 12.4 μl sterile distilled water, 0.4 μl dNTPs (10 μM), 4 μl 5 × HF buffer, 1 μl of each primer (10 μM), 1 μl template and 0.2 μl Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Germany). The conditions for PCR comprised 98 °C for 30 s followed by 15 cycles of 98 °C for 5 s, 56 °C for 20 s and 72 °C for 20 s with final extension step at 72 °C for 5 min. Target PCR products were visualized by agarose gel electrophoresis and then extracted and purified from the gel using Montage DNA Gel Extraction Kit (Millipore Corporation, USA). DNA concentrations were quantified using Quant-iT dsDNA High-Sensitivity Assay Kit and Qubit fluorometer (Invitrogen). The samples were subjected to sequencing on a GS FLX Titanium PicoTiterPlate using a GS FLX Titanium Sequencing Kit according to the manufacturer's instructions (Roche).

Sequence filtering and taxon classification

The raw flowgrams were fed into the QIIME pipeline (version 1.5.0 Caporaso *et al.* 2010), where multiplexed reads were assigned to samples based on unique barcodes, and erroneous reads were removed by denoizing. The resulting FASTA file was subjected to several filtering steps to remove poor-quality sequence reads using MOTHUR (version 1.27.0, Schloss *et al.* 2009). Sequences containing ambiguous bases (N), having mismatches with the 16S rRNA gene primers, with homopolymer stretch longer than 10 bases or those that were shorter than 200 bp were excluded from the subsequent analyses. Clean sequences have been deposited in MG-RAST (Meyer *et al.* 2008) under Accession nos 4536054.3-4536062.3. The resulting set of high-quality sequences was aligned against the SILVA 102 nonredundant database using MOTHUR. Aligned sequences were assigned to taxonomic groups using the *naïve Bayesian* classifier with a confidence threshold of 60% and a manually curated reference database DICTDB *v. 2.3* (Köhler *et al.* 2012). This database was generated from the SILVA database and improved by including 16S rRNA gene sequences obtained from the guts of termites and cockroaches and by renaming uninformative names to those consistent with the literature; it is available upon request. This reference-based classification clusters shorter reads in a defined phylogenetic context of longer high-quality reference sequences instead of adhering to an arbitrary cut-off value on sequence similarity. The database further divides the SILVA-defined genera into higher-resolution monophyletic subgroups (e.g. *Alistipes* 1 and *Alistipes* 2, see Results) based on detailed phylogenetic analyses (cf. Dietrich *et al.* 2014). Rarefaction curves were generated using R (package vegan, R Core Team 2013) to determine whether sequencing depth was sufficient to cover the expected number of operational taxonomic units (OTUs) at the level of 97% sequence similarity.

Analyses of community diversity and identification of the core microbiota

The representative clusters were sorted according to genus-level classification and taxa abundances were calculated as the number of reads per taxon. Unclassified reads (18.4–34.1%) were excluded from subsequent analyses. Prior to do doing so, we confirmed that the exclusion of unclassified reads did not affect patterns of community clustering, by performing OTU cluster analyses on all quality-filtered and classified reads using QIIME (version 1.8.0), followed by PCoA and NMDS community similarity analyses of Bray–Curtis distances using R (Fig. S4, Supporting information). We then estimated community richness (Chao1) and diversity indices (Shannon–Wiener, Simpson, Inverse Simpson and Evenness). Community similarity was calculated using weighted and unweighted UniFrac distances and visualized using principal coordinates analysis (PCoA). For the classification-dependent approach, principal component analysis (PCA) (R

package stats, R Core Team 2013) was used to visualize the overall dissimilarity in community structure among the samples. The contribution of genus-level taxa to the principal components (as conveyed by their loadings; Abdi & Williams 2010) was determined to identify bacterial lineages that explain most of the observed dissimilarity among gut communities.

In addition to characterizing the taxa that were most abundant and mostly affected differences in community structure between termite species, we determined the composition of the fungus-growing termite core microbiota, that is, shared taxa across all termite species. For this, we selected three core thresholds: i) taxa present in all nine samples (100% core), ii) taxa present in at least eight of nine samples (88.9% core) and iii) taxa present in at least seven of nine samples (77.9% core). The proportion of taxa represented by the core was calculated for each termite species by dividing the number of taxa in the core by the total number identified in that species. To determine the quantitative contributions of the core to the entire community, we calculated the proportion of reads assigned to the core relative to the total number of quality-filtered and classified reads for each termite species.

Abundance comparisons with other termite and cockroach gut microbiotas

Using two-tailed t-test analyses, we compared the relative abundances of the seven dominant phyla in fungus-growing termites with those observed in eight higher nonfungus-growing termite species, eight lower termite species and 15 cockroach species (Dietrich *et al.* 2014). This study used 454 pyrosequencing of the same region of the 16S rRNA gene, the same analysis pipeline (MOTHUR), and the DICTDB *v. 2.3* database for taxon assignments. The only difference to our study thus is that they used a modified primer set, which we cannot rule out might slightly affect community compositions. *P*-values were Bonferroni corrected (Bonferroni 1935) to account for multiple testing. Fisher's tests (Fisher 1932) with Bonferroni corrected *P*-values were performed to test for overall significant differences between fungus-growing termites and higher termites, lower termites and cockroaches, respectively. Furthermore, using a weighted Euclidean distance analysis in R (package vegetarian, R Core Team 2013), we performed clustering analyses comparing the relative abundance of the fungus-growing termite core members to their abundances in other termites and in cockroaches.

Results

Pyrosequencing data and taxonomic classification of sequence reads

454-pyrosequencing yielded between 9124 and 24 493 reads for each of the nine termite gut samples (Table 1), and the rarefaction analysis indicated that sufficient sampling depth was achieved for all samples (Fig. S1, Supporting information). Our diversity measures indicated that *M. subhyalinus* contained the most

Table 1 The number of sequences after denoizing and filtering of raw reads, the number of identified taxa, the percentage of reads successfully assigned to the phylum, family and genus levels (based on relative abundances) as well as the estimated richness and diversity indices for the bacterial communities (at 3% dissimilarity threshold)

Termite species	Number of sequences	Number of phylotypes	Classification success			Richness and diversity indices				
			Phylum	Family	Genus	Chao1	Shannon	Simpson	Invsimpson	Evenness
Ancistrotermes cavithorax	9124	111	97.4	89.2	73.9	342.4	3.8	1.0	23.1	0.6
Ancistrotermes guineensis	9551	102	97.0	87.2	67.9	344.8	3.8	1.0	29.7	0.6
Macrotermes subhyalinus	24 281	162	98.6	90.8	75.8	366.2	3.5	0.9	18.1	0.5
Microtermes toumodiensis	22 581	117	97.9	87.4	69.6	349.9	3.7	0.9	19.2	0.5
Odontotermes sp. 1	11 765	135	98.4	92.5	81.5	478.7	3.8	1.0	27.0	0.6
Odontotermes sp. 2	16 040	138	97.9	88.4	78.8	367.5	3.3	0.9	9.7	0.5
Odontotermes sp. 3	18 556	148	97.2	84.5	70.3	374.8	3.5	0.9	15.7	0.5
Pseudacanthotermes militaris	24 493	148	97.4	84.8	65.9	333.7	3.9	1.0	27.3	0.5
Pseudacanthotermes minor	9772	119	96.9	87.3	70.5	348.0	3.4	0.9	13.5	0.5

genus-level taxa (162, Table 1) and *A. guineensis* the least (102, Table 1), that *Odontotermes* sp. 1 was richest (Chao 1 index), while *P. militaris* was poorest, and that *A. guineensis* harboured the most diverse gut microbiota, while *Odontotermes* sp. 2 was least diverse (Inverse Simpson index, Table 1). Despite variation in community composition, Shannon–Wiener and Simpson indices were similar across communities, ranging from 3.3 to 3.9 (mean 3.6) and 0.9–1.0 (mean 0.94), respectively, and this pattern was also apparent for community evenness (range: 0.5–0.6; mean 0.53) (Table 1).

Classification using the manually curated reference database allowed for a large proportion of the reads to be assigned to taxa. Across termite species, classification success was improved compared to the RDP classifier (data not shown) at all taxonomic ranks, enabling high-resolution downstream analyses of the quality-filtered and classified reads (Table 1). In total, gut communities harboured 26 phyla, of which 11 were detected in all termite species, and the five most abundant phyla (Firmicutes, Bacteroidetes, Spirochaetes, Proteobacteria and Synergistetes) accounted for 90.5% of all sequence reads (Fig. 1). Representatives of Firmicutes and Bacteroidetes (on average 34 and 32% of all reads, respectively) were most prevalent and dominated in all fungus-growing termite host species (Fig. 1). Spirochaetes (on average 9% of all reads) were most abundant in *M. toumodiensis*, less frequent in the *Ancistrotermes* and *Odontotermes* species, and relatively rare in *Pseudacanthotermes* species and in *M. subhyalinus*, whereas Proteobacteria and Synergistetes (on average 9 and 7% of all reads, respectively) were more evenly distributed (Fig. 1).

Detailed classification results for the different phylogenetic levels are presented in Table S1 (Supporting information). At the genus level, the 10 most abundant genus-level taxa of the 321 identified were: *Alistipes* 1 (10.9% average abundance across termite species), *Alistipes* 2 (7.4%), *Treponema* 1a (5.6%), two taxa in the family Ruminococcaceae (Gut Cluster 1: 4.3% and Insect guts a: 4.1%), '*Candidatus* Tammella' (3.9%), '*Candidatus* Arthromitus' (3.4%), *Desulfovibrio* 3 (3.3%), *Paludibacter* (2.2%) and a member of the Synergistaceae (uncultured 6: 2.1%). Visualization of community similarities using UniFrac distance analyses in PCoA plots indicated that congeneric termite species were more similar to each other than to other Macrotermitinae species, irrespective of whether UniFrac analyses were unweighted (the presence/absence of taxa only) or weighted (relative

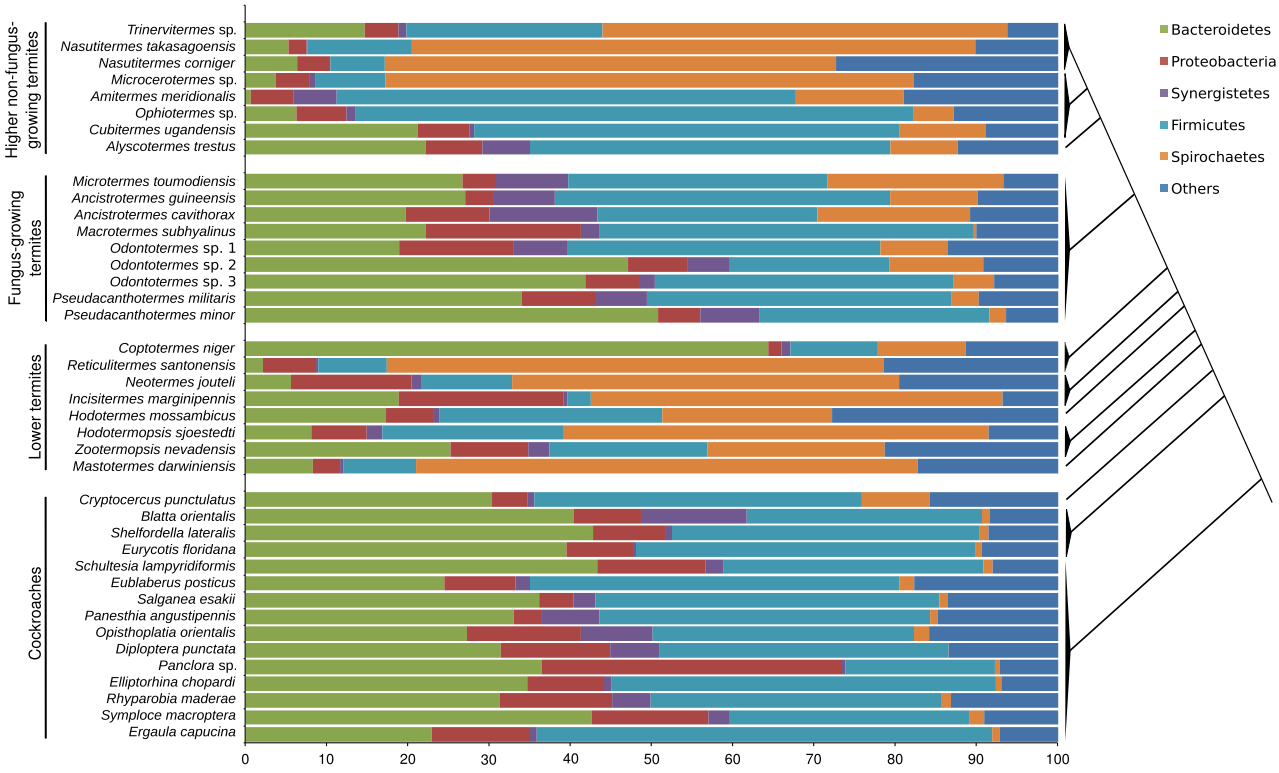

Fig. 1 Relative abundance of the five major bacteria phyla in the guts of fungus-growing termites (this study) compared to the relative abundances in the guts of 15 cockroach species, eight lower termite species and eight higher nonfungus-growing termite species (data from Dietrich *et al.* 2014), placed in a schematic phylogenetic tree of the major host groups based on Inward *et al.* (2007).

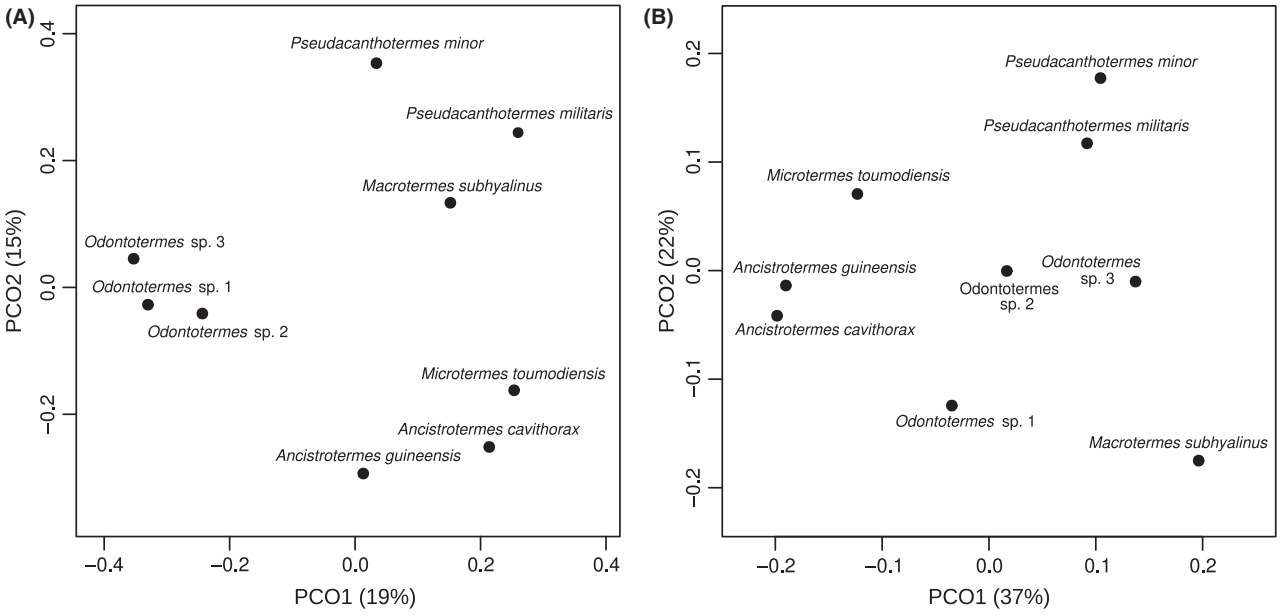

Fig. 2 Principal coordinates analysis (PCoA) visualizing bacterial community similarities across termite species, which were analysed using either unweighted (A) or weighted (B) UniFrac distances in R. Each dot represents one gut community. PCO1 and PCO2 are shown with the percentage variation explained for each axis.

abundances included) (Fig. 2A,B; Fig. S2, Supporting information).

Taxa contributing most to community structure

By calculating the loading values of each sample across the PCA axes, we determined which taxa contributed the most to community differences. The full results are given in Table S2 (Supporting information) and a heatmap of the relative abundances of the 50 most contributing taxa is given in Fig. 3A (92.5–95.3% of the total number of classified reads). Among these 50 taxa, 29 were shared by all termite species. The 10 genera with the strongest effects on the pattern observed were *Alistipes* 2 (Bacteroidetes), *Treponema* 1a (Spirochaetes), *Alistipes* 1 (Bacteroidetes), Gut Cluster 1 (Firmicutes), '*Ca*. Tammella' (Synergistetes), *Desulfovibrio* 3 (Proteobacteria), '*Ca*. Arthromitus' (Firmicutes), *Arcobacter* (Proteobacteria), *Paludibacter* (Bacteroidetes) and *Mucispirillum* (Deferribacteres). These taxa were also among the most abundant genera, and all except *Arcobacter* were present in all nine termite species (Fig. 3). However, there was variation in their relative abundances between termite species/genera; for example, although *Alistipes* 1 and 2 (Bacteroidetes) were relatively abundant in all species, they ranged from 1.7 to 28.6% in abundance across termite species. Similarly, *Treponema* 1a was relatively abundant in most genera but was present in very low abundance in *M. subhyalinus* (Fig. 3). In contrast, Gut Cluster 1 (Ruminococcaceae) was generally abundant, but particularly so in *M. subhyalinus*. '*Ca*. Tammella' (Synergistetes) was present in all the termites, ranging from 1.4% in *A. cavithorax* to 9.8% in *P. militaris*, and *Desulfovibrio* 3 and the Cockroach *Macrotermes* cluster (Proteobacteria) were more abundant in *M. subhyalinus* (9.4% and 3.6%, respectively) than in other species (1.1–3.7% and 0–0.6%, respectively) (Fig. 3). '*Ca*. Arthromitus' (Firmicutes) was least abundant in *M. subhyalinus* (0.9%) and most abundant in *A. guineensis* (6.8%). The genera *Mucispirillum* (Deferribacteres) and *Arcobacter* (Proteobacteria) were abundant in all *Odontotermes* species (particularly in *Odontotermes* sp. 1: 7.1% *Mucispirillum* and 7.7% *Arcobacter*), in contrast to the remaining termite genera, where they were virtually absent (0–0.8%) (Fig. 3, Table S1, Supporting information). Finally, the genus *Paludibacter* (Bacteroidetes) was more abundant in *Ancistrotermes* (5.8 and 7.0%) and *Pseudacanthotermes* (1.5% in both species) compared to the other termite species (0.4–1.1%).

The core microbiota of fungus-growing termites

Forty-two of the 321 genus-level taxa identified were present in all nine termite species at the 100% core threshold, 29 of which were among the 50 taxa contributing the most to community differences observed in Fig. 2, and colour-scaled abundances of the remaining 13 core taxa is given in Fig. 3B. The 42 core taxa were distributed among eight phyla, and 78.6% of these taxa were in the Bacteroidetes and Firmicutes, with the

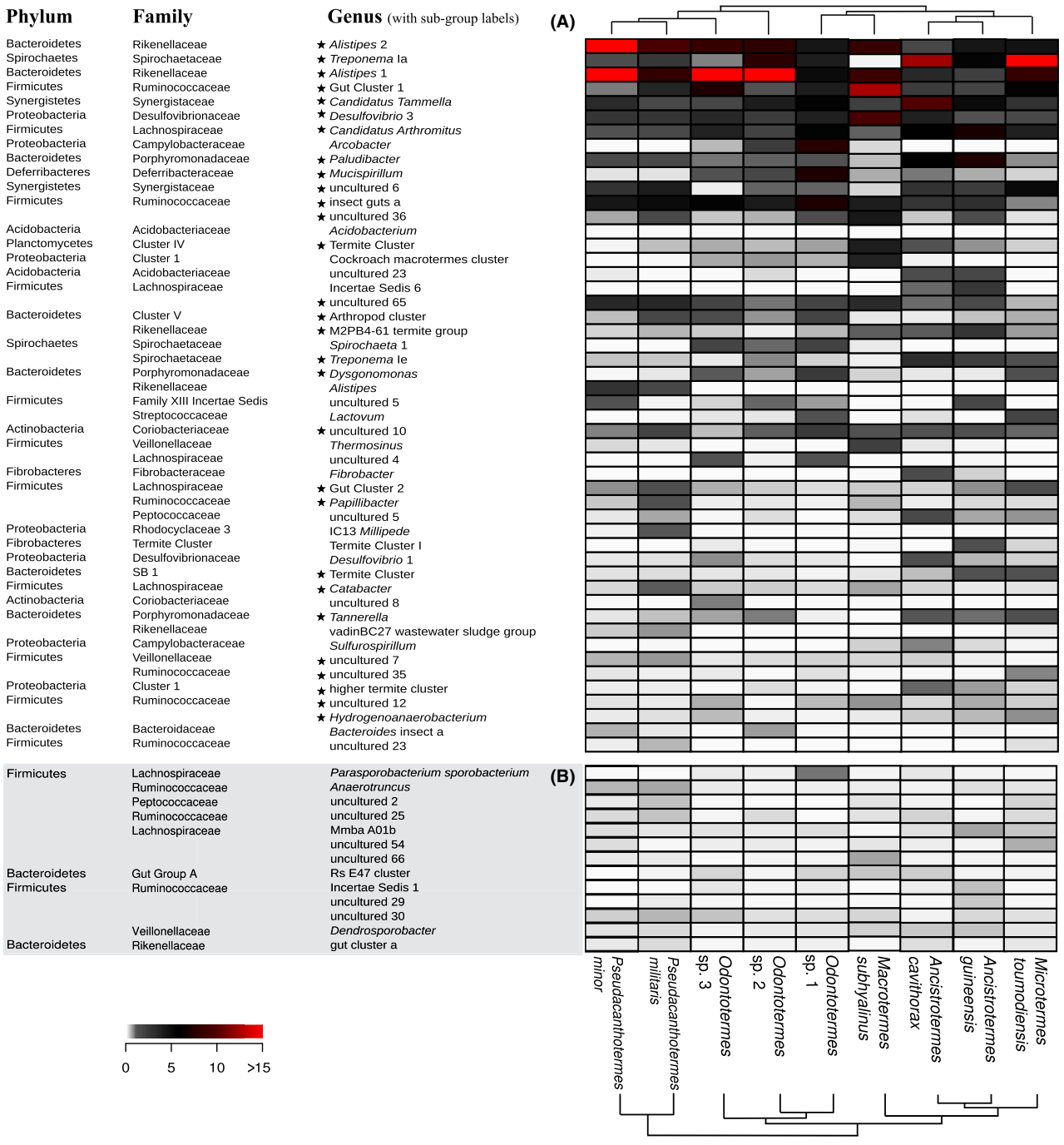

Fig. 3 Relative abundance of the 50 taxa contributing most to the principal components (as conveyed by their loadings; Abdi & Williams 2010) (A), and hence the separation of termite gut samples in Fig. 2, representing 92.5–95.3% of the quality-filtered and classified reads. Classification is presented at the phylum, family and genus levels. Twenty-nine of these were present in the 100% core microbiota (indicated with asterisks). The bottom portion of the figure (B, shaded text) shows the phylogenetic placement of the remaining 13 core taxa, which were generally in low abundance. The heatmap scale is the percentage of reads assigned to a given taxon out of the total number of the quality-filtered and classified reads for the termite species. The dendrogram at the top shows the weighted Euclidean distance analysis of community similarity generated using R (equivalent to Fig. 2B) and the dendrogram at the bottom shows a schematic termite host phylogeny (adapted from Aanen *et al.* 2002).

remaining being in Proteobacteria, Spirochaetes, Synergistetes, Planctomycetes, Deferribacteres and Actinobacteria (Fig. 3; Table S2, Supporting information). The proportion present in the 100% core out of the total number of taxa for individual termite species ranged from 25.9% in *M. subhyalinus* to 41.2% in *A. guineensis* (Fig. 4). In addition to occupying a substantial portion of taxa identified, the core community comprised 55.8–68.4% of the total number of quality-filtered and classified reads (Fig. 4). Using a slightly less strict core criterion (presence in at least eight of nine termite species, 88.9% core threshold), 55 taxa were identified, corresponding to 34-53.9% of the total number of taxa and 57.6% and 69.3% of the sequenced reads (Fig. 4). Using the even less strict criterion (present in at least seven samples; 77.9% core threshold), 71 taxa were part of the core, increasing the qualitative proportions to 43.8–69.6%, while increasing the number of reads assigned to the core only slightly (58.7–71.6%) (Figure 4).

Abundance comparisons to other termite and cockroach gut communities

Even at the phylum level, significant differences in fungus-growing termite gut microbiota compositions were apparent in the comparison to guts of cockroaches and other termites from an analysis performed by Dietrich *et al.* (2014) (Fig. 1; Table 2). Spirochaetes were significantly higher in relative abundance in lower termites compared to fungus-growing termites (Fig. 1; Table 2). The phylum was even less abundant in the cockroaches, with significantly lower abundances than in fungus-growing termites (Fig. 1; Table 2). The termite group TG3 phylum had very low relative abundance among the fungus-growing termites and cockroaches, but this phylum was overall not significantly different in abundance among the four groups (Table 2). The termite group phylum Elusimicrobia, previously known as TG1, was similar in abundance in fungus-growing and other higher termites, but significantly more abundant in lower termites and the cockroaches (Table 2). In contrast, the phylum Synergistetes was significantly more abundant in fungus-growing termites compared to other termites, but not significantly different from in cockroaches. The two most abundant phyla in fungus-growing termites, Firmicutes and Bacteroidetes, showed different relative abundance patterns in nonfungus-growing termites; Firmicutes were significantly less abundant in the lower termites (Table 2), while Bacteroidetes were significantly less abundant in other higher termites. These dominant fungus-growing termite gut phyla were not significantly different in abundance from cockroach gut microbiotas (Fig. 1; Table 2). Overall, Fisher's tests showed that fungus-growing termites were significantly different to both lower and higher nonfungus-growing termites, but not significantly different from cockroaches (Fig. 1; Table 2).

The weighted Euclidean distance analysis comparing the relative abundances of the fungus-growing termite

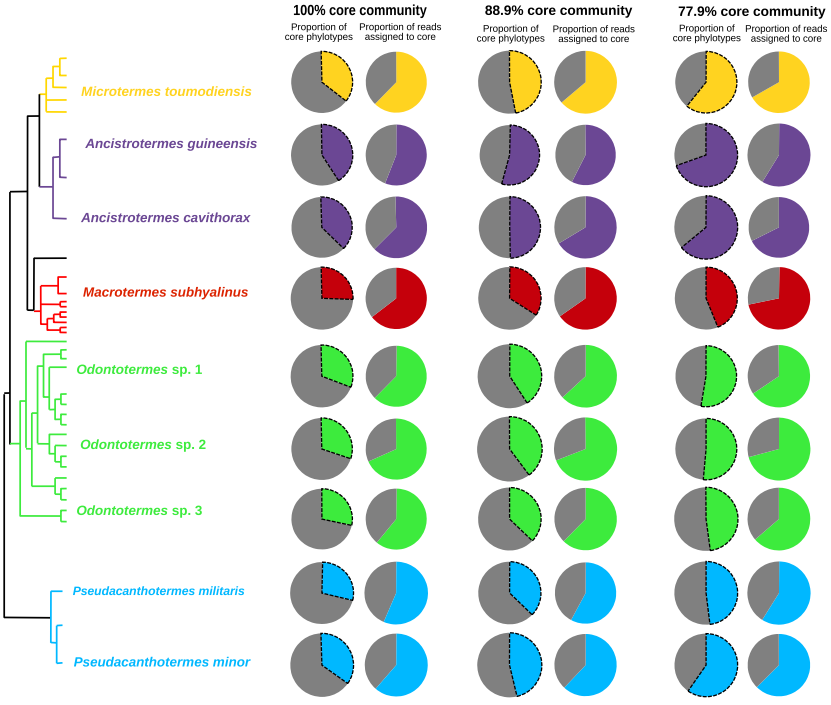

Fig. 4 The proportion of taxa making up the fungus-growing termite core microbiota at three different core thresholds: presence in nine out of nine termite species (100%, 42 taxa, left), presence in at least eight of nine termite species (88.9%, 55 taxa, middle) and presence in at least seven of nine termite species (77.9%, 71 taxa, right). For each core threshold, left pies show the proportion of total number of taxa (genus or subgenus levels) assigned to the core and right pies give the proportion of reads assigned to core taxa for each termite species. Samples are organized by the phylogenetic placement of the host termite adapted from Aanen *et al.* (2002).

Table 2 Two-sample t-test analyses of the relative abundances of seven bacterial phyla in fungus-growing termites (this study), cockroaches, lower and higher nonfungus-growing termites (Dietrich *et al.* 2014)

	FGT-HT			FGT-LT			FGT-cockroaches		
Bacteria phyla	t value	df	Adjusted *P*-value	t value	df	Adjusted *P*-value	t value	df	Adjusted *P*-value
Spirochaetes	2.52	8	0.108	**4.27**	**9**	**0.006**	**2.99**	**9**	**0.045**
TG3	2.44	7	0.135	0.82	8	1.000	0.11	11	1.000
Elusimicrobia	1.27	12	0.690	**3.3**	**7**	**0.039**	**3.53**	**19**	**0.006**
Firmicutes	0.02	8	1.000	**5.09**	**15**	**0.004**	0.98	18	1.000
Bacteroidetes	**4.45**	**14**	**0.003**	1.64	11	0.390	0.56	11	1.000
Synergistetes	**3.33**	**14**	**0.015**	**4.63**	**9**	**0.003**	2.02	18	0.174
Proteobacteria	2.2	10	0.159	0.08	14	1.000	1.03	22	0.930
Combined *P*-values			**0.006**			**0.00006**			0.160

Fisher's tests of combined *P*-values in each combination in the bottom. FGT, fungus-growing termites; HT, higher nonfungus-growing termites; LT, lower termites; significant *P*-values after Bonferroni correction in bold. The test results include all reads assigned to a phylum, but similar results were obtained if performed only with reads classified to the genus- or subgenus levels (Table S4, Supporting information).

core members to their abundances in other Dictyoptera (superorder containing the cockroaches and termites) confirmed that fungus-growing termite guts are more similar to cockroach guts than to other termites also at this finer level of classification (Fig. 5). This pattern appears to be driven mainly by the comparably high abundances of *Alistipes* 1 and 2, *Desulfovibrio* 3, *Paludibacter* and Insect guts a and Gut cluster 1 in the Ruminococcaceae, in addition to an on-average reduced abundance of the spirochete *Treponema* 1a in cockroach and fungus-growing termite guts (Fig. 5).

Discussion

Previous surveys of the gut microbiota of Macrotermitinae have been limited to clone libraries of either one or a few termite species (Hongoh *et al.* 2006; Mackenzie *et al.* 2007; Shinzato *et al.* 2007; Zhu *et al.* 2012; Makonde *et al.* 2013). In contrast, our pyrosequencing analysis included all common genera of the Macrotermitinae, and thereby provided more insight into bacterial community structure within the subfamily. Analyses from five of 11 genera of the Macrotermitinae showed that community composition to some extent resembles host phylogeny, that their gut microbiotas are distinct from those of other termites and that a core community of 42 genus-level taxa are shared across the Macrotermitinae. Although we cannot rule out that variation within and between termite species is present but not reflected given our sampling, the high resemblance of communities across diverse fungus-growing termites suggests consistency in communities. The adoption of *Termitomyces* as a central plant-biomass-degrading symbiont thus appears to have shifted the gut microbiota composition at the origin of fungus farming in termites, with implications for the role of the gut microbes in the tripartite mutualistic association.

Despite the presence of a large proportion of shared gut microbes across the fungus-farming termites, PCoA ordination of unweighted UniFrac distances (confirmed with *Bray–Curtis* PCoA and NMDS plots; Fig. S3, Supporting information) indicated that communities cluster largely according to the phylogeny of their host termite species. The slight dilution of this phylogenetic signal in the weighted UniFrac analysis is due to the inclusion of abundances of bacterial taxa in this test. For example, the three *Odontotermes* species are very similar in the unweighted analyses (Fig. 2A), as they share a high portion of taxa; however, when including relative abundances of taxa (weighted; Fig. 2B), *Odontotermes* sp. 1 is distanced from the other two species due to, among others, the relatively low abundance of *Alistipes* 1 and relatively high abundances of *Arcobacter* and *Mucispirillum* (Fig. 3). Both the phylogeny and classification-based approaches show that even at the high sampling depth obtained with pyrosequencing, congeneric fungus-growing species share a greater proportion of bacterial groups than more distantly related species: the proportion of the pairwise sharing between genera was substantially lower (mean = 61.6%) than the proportion shared between pairs of congeneric termite species (mean = 70.7%) (Table S3, Supporting information). These patterns of association suggest that community differences among members of the Macrotermitinae may be shaped by codiversification with their termite host. However, this pattern could also arise as a product of termite species with similar ecologies acquiring similar gut microbes (cf. Sanders *et al.* 2014).

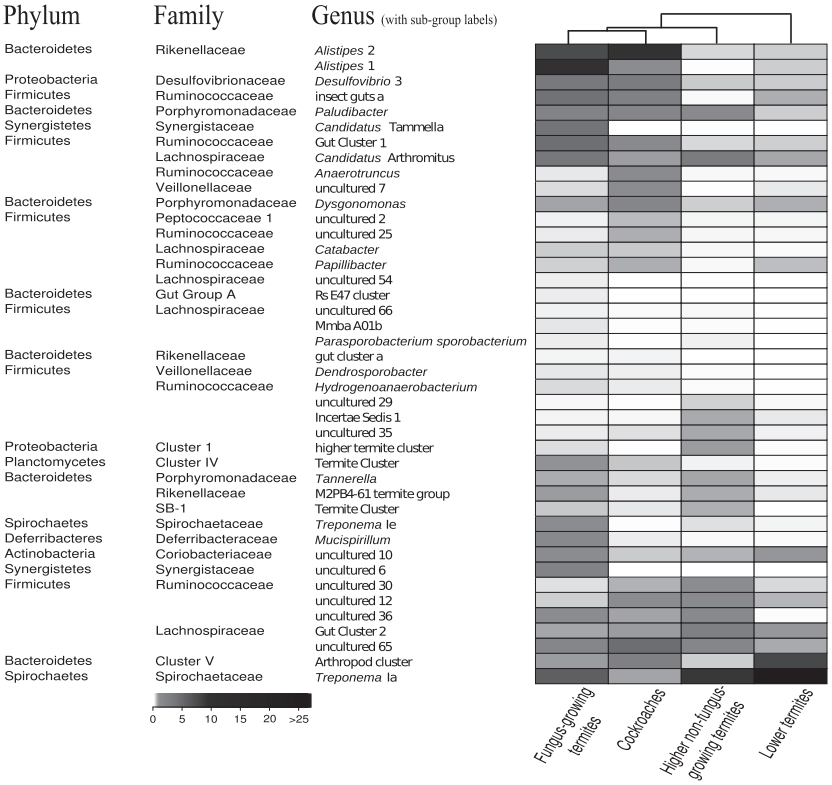

Fig. 5 Relative abundances of the 42 core taxa in fungus-growing termites in the microbiotas of cockroaches, lower termites and higher nonfungus-growing termites (data from Dietrich *et al.* 2014). Each row in the heatmap represents one bacterial taxon present in the fungus-growing termite core, and cell shades indicate relative abundances in the respective dictyopteran group. The scale is the percentage of reads assigned to a given taxon out of the total number of quality-filtered and classified reads for each group. The dendrogram at the top shows the weighted Euclidean distance analysis of community similarity between the four groups of isopterans generated using R, indicating that the relative abundances of fungus-growing termite core bacteria taxa are more similar to cockroach communities than to communities associated with lower and nonfungus-growing higher termites.

Consequently, more sampling within the Macrotermitinae will be needed to establish within-termite species variation in community composition, as well as to examine additional host species that can allow for statistical testing of whether cophylogenetic patterns are driven mainly by codiversification or acquisitions.

Firmicutes and Bacteroidetes together account for most of the bacterial communities across the species analysed. This finding confirms the results of previous studies (Hongoh *et al.* 2006; Liu *et al.* 2013; Makonde *et al.* 2013; Dietrich *et al.* 2014), indicating that the predominance of these groups is a general trend in the Macrotermitinae. Their underrepresentation, particularly in wood-feeding lineages of higher termites (Nasutitermitinae and Termitinae), contributes to the separation of the gut communities of Macrotermitinae from all other subfamilies (Fig. 1) and to the similarities between fungus-growing termites and cockroaches (Dietrich *et al.* 2014) (Fig. 1). Two of seven dominant phyla were significantly different in abundances among fungus-growing termites, higher termites and cockroaches, whereas four of seven were significantly different in the comparison with lower termites (Table 2). Nevertheless, the abundance of different phyla was overall significantly different between fungus-growing termites and both higher and lower termites, but not from cockroaches (Table 2). Although Dietrich *et al.* (2014) amplified a nearly identical part of the V3-V4 region of the 16S rRNA gene using different primers, which potentially could affect this pattern, the similarities to cockroaches were evident even at the bacteria genus level. Abundances of core members of the fungus-growing termite microbiota (particularly *Alistipes* and members of the Ruminococcaceae) were on average more similar in abundance to cockroaches than to other Dictyoptera (Fig. 5). Thus, despite the phylogenetic affiliation of Macrotermitinae with other subfamilies of higher termites, they are more similar in overall community structure to the more distantly related cockroach species (Dietrich *et al.* 2014; this study). Another factor contributing to this separation is the low relative abundance of Spirochaetes in cockroaches and the Macrotermitinae (Dietrich *et al.* 2014; this study), which typically dominate the guts of wood-feeding lineages (Hongoh *et al.* 2005; Warnecke *et al.* 2007; Köhler *et al.* 2012), but not soil-feeding higher termites, leaving this phylum overall not significantly different between higher nonfungus-growing and fungus-growing termites (Fig. 1; Table 2).

Although Spirochetes are generally rare in fungus-growing termites, there are substantial differences in their abundance among fungus-growing termite genera, ranging from virtual absence in *Macrotermes* spp. (Hongoh *et al.* 2006; Dietrich *et al.* 2014; this study), to 2–3% in *Pseudacanthotermes* (this study), 6–10% in *Odontotermes* spp. (Liu *et al.* 2013; Makonde *et al.* 2013;

this study), 11–19% in *Ancistrotermes* (this study), and 22–29% in *Microtermes* sp. (Makonde *et al.* 2013; this study). Their function in Macrotermitinae is not clear, but they mainly belong to the *Treponema* I lineage, which comprises several isolates from the hindgut of lower termites. They either have a fermentative metabolism, producing acetate and other products by fermentation of mono- and oligosaccharides or are homoacetogenic (Leadbetter *et al.* 1999; Lilburn *et al.* 2001; Graber *et al.* 2004; Dröge *et al.* 2008). There is evidence that uncultured *Treponema* lineages in higher termites may perform reductive acetogenesis from H_2 + CO_2 (Warnecke *et al.* 2007). The low representation of *Treponema* in Macrotermitinae is consistent with the reduced acetogenesis recorded in the guts of *Macrotermes mülleri*, *P. militaris* and *Pseudacanthotermes spiniger* (Brauman *et al.* 1992). Methanogenesis appears to dominate over reductive acetogenesis as a hydrogen sink in Macrotermitinae (Brauman *et al.* 1992), but the reason for this is still unclear (Brune & Ohkuma 2011; Hongoh 2011).

Variation in the microbiotas of Macrotermitinae members is expected to reflect their ecological differences, where plant diet and *Termitomyces* association probably are the main factors shaping community composition. Although within-termite species variation in gut microbiota composition remains to be elucidated, expectations are that it will be lower than among-species and among-genus variation (cf. Hongoh *et al.* 2005, 2006). Diet has been shown to affect the gut communities of wood-feeding termites (Tanaka *et al.* 2006; Miyata *et al.* 2007; Huang *et al.* 2013), but variation in the diet among the Macrotermitinae is generally poorly understood (Donovan *et al.* 2001; Hongoh 2010). It has been suggested that *Macrotermes* spp., with the exception of *Macrotermes malaccensis*, are primarily leaf litter feeders (Matsumoto 1976; Hyodo *et al.* 2003), that *Odontotermes* and *Ancistrotermes* species predominantly feed on wood, and that *P. militaris* feeds on both leaf litter and wood (Eggleton *et al.* 1996; Hyodo *et al.* 2003). It has also been suggested that the role of *Termitomyces* varies between termite hosts (for a review, see Nobre *et al.* 2011b). Although this remains to be firmly explored, some termite species may mainly gain access to cellulose through the lignolytic activity of *Termitomyces* (Hyodo *et al.* 2000), other species may mainly exploit *Termitomyces* as a protein-rich food source (Rouland *et al.* 1991; Hyodo *et al.* 2000, 2003), while other termite species obtain cellulases and xylanases from *Termitomyces* for the decomposition of plant substrate (Martin & Martin 1978; Rouland *et al.* 1991). Differences in gut communities between termites could, thus, be the result of specific diets or division of symbiont functions.

Although the role of the bacterial community in the breakdown of lignocellulose in the Macrotermitinae remains to be firmly elucidated, metagenomic studies have shed some light on the possible contributions by bacteria to cellulose digestion in wood-feeding termites (Warnecke *et al.* 2007; He *et al.* 2013). Glycosyl hydrolases with predicted cellulase activity in the P3 lumen metagenome of the wood-feeding *Nasutitermes* sp. have been taxonomically binned to the phyla Fibrobacteres and Spirochaetes (Warnecke *et al.* 2007). These phyla, in addition to the TG3 phylum, occur in high abundance in wood-feeding termites belonging to the subfamilies Nasutitermitinae (Hongoh *et al.* 2006; Köhler *et al.* 2012; Mikaelyan *et al.* 2014) and Termitinae (Hongoh *et al.* 2006; Dietrich *et al.* 2014). The fact that these potentially lignocellulolytic bacteria are generally underrepresented in fungus-growing termites suggests that the extent to which the bacterial community takes part in cellulose degradation is limited and further supports the important role played by *Termitomyces* in plant biomass degradation in the Macrotermitinae.

Conclusions

Our study provides the first characterization of gut communities across the phylogenetic diversity of the lineage of higher termites, providing insight into gut community structure within the Macrotermitinae. A set of 42 core bacteria is consistently associated in relatively high abundance with the diversity of fungus-growing termite species. This supports previous suggestions that the termites have remained in need of a functional gut microbiota, despite their alliance with *Termitomyces* as a biomass degrader. The identified core community is distinct from those of the lower and higher nonfungus-growing termites, suggesting gut community adaptations to different nutritional environments in the host gut. Macrotermitinae diets are rich in fungus material, and hence protein, and our analyses support that the shift in diet has played a role in shaping community composition, making fungus-growing termite guts more similar in community structure to those of their distantly related cockroach ancestor. Convergence in gut microbiota composition and function as a consequence of dietary similarities has previously been reported in other insects (Anderson *et al.* 2012), humans and other mammals (Muegge *et al.* 2011) and, most recently, in myrmecophagous mammals (Delsuc *et al.* 2014). Despite community similarity, variation in gut compositions between macrotermitine species and genera remains, which may reflect functional differences in the roles of gut microbes with those of *Termitomyces* and the termite host; consequently, future work to elucidate the link between gut composition and functional roles are needed.

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References

Aanen DK, Eggleton P, Rouland-Lefèvre C *et al.* (2002) The evolution of fungus-growing termites and their mutualistic fungal symbionts. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 14887–14892.

Abdi H, Williams LJ (2010) Principal component analysis. *Wiley Interdisciplinary Reviews: Computational Statistics*, **2**, 433–459.

Anderson KE, Russell JA, Moreau CS *et al.* (2012) Highly similar microbial communities are shared among related and trophically similar ant species. *Molecular Ecology*, **21**, 2282–2296.

Andert J, Marten A, Brandl R, Brune A (2010) Inter- and intraspecific comparison of the bacterial assemblages in the hindgut of humivorous scarab beetle larvae (*Pachnoda* spp.). *FEMS Microbiology Ecology*, **74**, 439–449.

Bignell DE, Slaytor M, Veivers PC, Muhlemann R, Leuthold RH (1994) Functions of symbiotic fungus gardens in higher termites of the genus *Macrotermes*: evidence against the acquired enzyme hypothesis. *Acta Microbiologica et Immunologica Hungarica*, **41**, 391–401.

Bonferroni CE (1935) Il calcolo delle assicurazioni su gruppi di teste. *Studi in Onore del Professore Salvatore Ortu Carboni*.Rome: Italy, 13–60.

Brauman A, Kane MD, Labat M, Breznak JA (1992) Genesis of acetate and methane by gut bacteria of nutritionally diverse termites. *Science*, **257**, 1384–1387.

Brauman A, Dore J, Eggleton P *et al.* (2001) Molecular phylogenetic profiling of prokaryotic communities in guts of termites with different feeding habits. *FEMS Microbiology Ecology*, **35**, 27–36.

Brucker RM, Bordenstein SR (2012) The roles of host evolutionary relationships (genus: *Nasonia*) and development in structuring microbial communities. *Evolution*, **66**, 349–362.

Brugerolle G, Radek R (2006) Symbiotic protozoa of termites. In: *Intestinal Microorganisms of Termites and Other Invertebrates* (eds König H & Varma A), pp. 243–269. Springer Verlag, Berlin Heidelberg.

Brune A (2014) Symbiotic digestion of lignocellulose in termite guts. *Nature Reviews Microbiology*, **12**, 168–180.

Brune A, Ohkuma M (2011) Role of the termite gut microbiota in symbiotic digestion. In: *Biology of Termites: A Modern Synthesis*(eds Bignell D, Roisin Y & Lo N), pp. 439–475. Springer, Dordrecht.

Caporaso JG, Kuczynski J, Stombaugh J *et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, **7**, 335–336.

Cleveland LR (1923) Symbiosis between termites and their intestinal protozoa. *Proceedings of the National Academy of Sciences of the United States of America*, **9**, 424–428.

Delsuc F, Metcalf JL, Wegener Parfrey L *et al.* (2014) Convergence of gut microbiomes in myrmecophagous mammals. *Molecular Ecology*, **23**, 1301–1317.

Dietrich C, Köhler T, Brune A (2014) The cockroach origin of the termite gut microbiota: patterns in bacterial community structure reflect major evolutionary events. *Applied and Environmental Microbiology*, **80**, 2261–2269.

Donovan SE, Eggleton P, Bignell DE (2001) Gut content analysis and a new feeding group classification of termites. *Ecological Entomology*, **26**, 356–366.

Dröge S, Rachel R, Radek R, Konig H (2008) *Treponema isoptericolens* sp. nov., a novel spirochaete from the hindgut of the termite *Incisitermes tabogae*. *International Journal of Systmatic and Evolutionary Microbiology*, **58**, 1079–1083.

Eggleton P, Bignell DE, Sands WA *et al.* (1996) The diversity, abundance and biomass of termites under differing levels of disturbance in the Mbalmayo Forest Reserve, Southern Cameroon. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, **351**, 51–68.

Fisher RA (1932) *Statistical Methods for Research Workers*, 4th edn. Oliver and Boyd, London.

Fraune S, Bosch TC (2010) Why bacteria matter in animal development and evolution. *BioEssays*, **32**, 571–580.

Graber JR, Leadbetter JR, Breznak JA (2004) Description of *Treponema azotonutricium* sp. nov. and *Treponema primitia* sp. nov., the first spirochetes isolated from termite guts. *Applied and Environmental Microbiology*, **70**, 1315–1320.

Hamady M, Knight R (2009) Microbial community profiling for human microbiome projects: Tools, techniques, and challenges. *Genome Research*, **19**, 1141–1152.

Hansen CH, Krych L, Nielsen DS *et al.* (2012) Early life treatment with vancomycin propagates Akkermansia muciniphila and reduces diabetes incidence in the NOD mouse. *Diabetologia*, **55**, 2285–2294.

He S, Ivanova N, Kirton E *et al.* (2013) Comparative metagenomic and metatranscriptomic analysis of hindgut paunch microbiota in wood- and dung-feeding higher termites. *PLoS ONE*, **8**, e61126.

Hongoh Y (2010) Diversity and genomes of uncultured microbial symbionts in the termite gut. *Bioscience, Biotechnology and Biochemistry*, **74**, 1145–1151.

Hongoh Y (2011) Toward the functional analysis of uncultivable, symbiotic microorganisms in the termite gut. *Cellular and Molecular Life Sciences*, **68**, 1311–1325.

Hongoh Y, Deevong P, Inoue T *et al.* (2005) Intra- and interspecific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host. *Applied and Environmental Microbiology*, **71**, 6590–6599.

Hongoh Y, Ekpornprasit L, Inoue T *et al.* (2006) Intracolony variation of bacterial gut microbiota among castes and ages in the fungus-growing termite *Macrotermes gilvus*. *Molecular Ecology*, **15**, 505–516.

Hu Y, Lukasik P, Moreau CS, Russell JA (2014) Correlates of gut community composition across an ant species (*Cephalotes varians*) elucidate causes and consequences of symbiotic variability. *Molecular Ecology*, **23**, 1284–1300.

Huang XF, Bakker MG, Judd TM, Reardon KF, Vivanco JM (2013) Variations in diversity and richness of gut bacterial communities of termites (*Reticulitermes flavipes*) fed with grassy and woody plant substrates. *Microbial Ecology*, **65**, 531–536.

Huse SM, Ye Y, Zhou Y, Fodor AA (2012) A core human microbiome as viewed through 16S rRNA sequence clusters. *PLoS ONE*, **7**, e34242.

Hyodo F, Inoue T, Azuma JI, Tayasu I, Abe T (2000) Role of the mutualistic fungus in lignin degradation in the fungus-growing termite *Macrotermes gilvus* (Isoptera; Macrotermitinae). *Soil Biology and Biochemistry*, **32**, 653–658.

Hyodo F, Tayasu I, Inoue T *et al.* (2003) Differential role of symbiotic fungi in lignin degradation and food provision for fungus-growing termites (Macrotermitinae: Isoptera). *Functional Ecology*, **17**, 186–193.

Inward D, Beccaloni G, Eggleton P (2007) Death of an order: a comprehensive molecular phylogenetic study confirms that termites are eusocial cockroaches. *Biology Letters*, **3**, 331–335.

Jones JA (1990) Termites, soil fertility and carbon cycling in dry tropical Africa: a hypothesis. *Journal of Tropical Ecology*, **6**, 291–305.

Köhler T, Dietrich C, Scheffrahn RH, 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.). *Applied and Environmental Microbiology*, **78**, 4691–4701.

Leadbetter JR, Schmidt TM, Graber JR, Breznak JA (1999) Acetogenesis from H_2 plus CO_2 by spirochetes from termite guts. *Science*, **283**, 686–689.

Lee YK, Mazmanian SK (2010) Has the microbiota played a critical role in the evolution of the adaptive immune system? *Science*, **330**, 1768–1773.

Lehman RM, Lundgren JG, Petzke LM (2009) Bacterial communities associated with the digestive tract of the predatory ground beetle, *Poecilus chalcites*, and their modification by laboratory rearing and antibiotic treatment. *Microbial Ecology*, **57**, 349–358.

Lilburn TG, Kim KS, Ostrom NE *et al.* (2001) Nitrogen fixation by symbiotic and free-living spirochetes. *Science*, **292**, 2495–2498.

Liu N, Zhang L, Zhou H *et al.* (2013) Metagenomic insights into metabolic capacities of the gut microbiota in a fungus-cultivating termite (*Odontotermes yunnanensis*). *PLoS ONE*, **8**, e69184.

Mackenzie LM, Muigai AT, Osri EO *et al.* (2007) Bacterial diversity in the intestinal tract of the fungus-cultivating termite *Macrotermes michaelseni* (Sjöstedt). *African Journal of Biotechnology*, **6**, 658–667.

Makonde HM, Boga HI, Osiemo Z *et al.* (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.

Martin MM, Martin JS (1978) The distribution and origins of the cellulolytic enzymes of the higher termite, *Macrotermes natalensis*. *Physiological Zoology*, **52**, 11–21.

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*, **22**, 153–178.

Meriweather M, Matthews S, Rio R, Baucom RS (2013) A 454 survey reveals the community composition and core microbiome of the Common Bed Bug (*Cimex lectularius*) across an urban landscape. *PLoS ONE*, **8**, e61465.

Meyer F, Paarmann D, D'Souza M *et al.* (2008) The metagenomics RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics*, **9**, 386.

Mikaelyan A, Strassert JFH, Tokuda G, Brune A (2014) The fiber-associated cellulolytic bacterial community in the hindgut of wood-feeding higher termites (*Nasutitermes* spp.). *Environmental Microbiology*, **16**, doi: 10.1111/1462-2920.12425.

Miyata R, Noda N, Tamaki H *et al.* (2007) Influence of feed components on symbiotic bacterial community structure in the gut of the wood-feeding higher termite *Nasutitermes takasagoensis*. *Bioscience, Biotechnology and Biochemistry*, **71**, 1244–1251.

Moran NA, McCutcheon JP, Nakabachi A (2008) Genomics and evolution of heritable bacterial symbionts. *Annual Review of Genetics*, **42**, 165–190.

Muegge BD, Kuczynski J, Knights D *et al.* (2011) Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science*, **332**, 970–974.

Ni J, Tokuda G (2013) Lignocellulose-degrading enzymes from termites and their symbiotic microbiota. *Biotechnology Advances*, **31**, 838–850.

Nobre T, Aanen DK (2012) Fungiculture or termite husbandry? The ruminant hypothesis. *Insects*, **3**, 307–323.

Nobre T, Koné NA, Konaté S, Linsenmair KE, Aanen DK (2011a) Dating the fungus-growing termites' mutualism shows a mixture between ancient codiversification and recent symbiont dispersal across divergent hosts. *Molecular Ecology*, **20**, 2619–2627.

Nobre T, Rouland-Lefèvre C, Aanen DK (2011b) Comparative biology of fungus cultivation in termites and ants. In: *Biology of Termites: A Modern Synthesis* (eds Bignell D, Roisin Y & Lo N), pp. 193–210. Springer, Dordrecht.

R Core Team (2013). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Rouland C, Lenoir F, Lepage M (1991) The role of the symbiotic fungus in the digestive metabolism of several species of fungus-growing termites. *Comparative Biochemistry and Physiology Part A: Physiology*, **99**, 657–663.

Sabree ZL, Hansen AK, Moran NA (2012) Independent studies using deep sequencing resolve the same set of core bacterial species dominating gut communities of honey bees. *PLoS ONE*, **7**, e41250.

Sanders JG, Powell S, Kronauer DJC *et al.* (2014) Stability and phylogenetic correlation in gut microbiota: lessons from ants and apes. *Molecular Ecology*, **23**, 1268–1283.

Sands WA (1960) The initiation of fungus comb construction in laboratory colonies of *Ancistrotermes guineensis* (Silvestri). *Insectes Sociaux*, **7**, 251–263.

Sands WA (1969) The association of termites and fungi. In: *Biology of Termites*(eds Krishna A & Weesner F), pp. 495–524. Academic Press, New York.

Schloss PD, Westcott SL, Ryabin T *et al.* (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, **75**, 7537–7541.

Sharon G, Segal D, Ringo JM *et al.* (2010) Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 20051–20056.

Shinzato N, Muramatsu M, Matsui T, Watanabe Y (2007) Phylogenetic analysis of the gut bacterial microflora of the fungus-growing termite *Odontotermes formosanus*. *Bioscience, Biotechnology and Biochemistry*, **71**, 906–915.

Tanaka H, Aoyagi H, Shiina S *et al.* (2006) Influence of the diet components on the symbiotic microorganisms community in hindgut of *Coptotermes formosanus* Shiraki. *Applied Microbiology and Biotechnology*, **71**, 907–917.

Turnbaugh PJ, Ley RE, Hamady M *et al.* (2007) The human microbiome project. *Nature*, **449**, 804–810.

Warnecke F, Luginbuhl P, Ivanova N *et al.* (2007) Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature*, **450**, 560–565.

Watanabe H, Tokuda G (2010) Cellulolytic systems in insects. *Annual Reviews of Entomology*, **55**, 609–632.

Werren JH, Baldo L, Clark ME (2008) *Wolbachia*: master manipulators of invertebrate biology. *Nature Reviews Microbiology*, **6**, 741–751.

Wong CNA, Ng P, Douglas AE (2011) Low-diversity bacterial community in the gut of the fruitfly *Drosophila melanogaster*. *Environmental Microbiology*, **13**, 1889–1900.

Zhu Y, Li J, Liu H *et al.* (2012) Phylogenetic analysis of the gut bacterial microflora of the fungus-growing termite *Macrotermes barneyi*. *African Journal of Microbiology Research*, **6**, 2071–2078.

S.O. designed the study, performed DNA extractions, prepared libraries, analysed the data, prepared the figures, contributed to the interpretation of the sequencing results, and wrote the first draft of the manuscript. A.M. performed analyses using the curated database, contributed to the interpretation of the sequencing results and to writing the manuscript. T.N. and D.K.A. contributed to designing the study, provided with input to the interpretation of the sequencing results and to the writing. N.A.K. performed fieldwork and termite species morphological identification. L.H.H. and S.J.S. contributed primers, tags, adaptors, and reagents for 454 pyrosequencing and assisted with library preparation. J.J.B. contributed funding for sequencing, input to the interpretation of the sequencing results and to writing the manuscript. A.B. contributed with the curated database, interpretation of the sequencing results, and to writing the manuscript. M.P. designed the study, funded the experimental work, contributed to the interpretation of the sequencing results and to writing the manuscript.

Data accessibility

Clean sequences are deposited in MGRAST under Accession nos 4536054.3-4536062.3.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Relative abundance of the sequences identified and their taxonomic placement (down to genus) are presented for all taxa identified (separate excel file: Otani_TableS1.xlsx).

Table S2 Genus-level taxon contributions to difference between termite gut communities observed in the PCoA analyses (Fig. 2, main paper). Estimates of principal components PC1-PC9 in total, sequence abundances, and taxonomic levels (to genus) are presented for all taxa identified (separate excel file: Otani_TableS2.xlsx).

Table S3 The number of genus-level taxa shared (top) and proportions of communities shared (bottom) in all possible combinations for the nine communities.

Table S4 Two-sample t-test analyses of the relative abundances of taxa assigned to the seven bacterial phyla in fungus-growing termites (this study), cockroaches, lower and higher nonfungus-growing termites (Dietrich *et al.* 2014). Only reads classified to the genus- or subgenus levels were included. Fisher's tests of combined p-values in each combination in the bottom. FGT: fungus-growing termites, HT: higher nonfungus-growing termites, LT: lower termites; significant *P*-values after Bonferroni correction in bold.

Fig. S1 Rarefaction curves of sequence depth generated with R (R Core Team 2013). The curves represent the nine analysed termite gut samples, and each curve shows the number of genus-level taxa as a function of the number of sequenced reads after filtering.

Fig. S2 PCoA plots for pairwise combinations of the first three principal components visualising community dissimilarities (UniFrac analysis).

Fig. S3 PCoA and NMDS plots of Bray–Curtis distances between communities including quality-filtered and classified reads, after running classification-based analysis.

Fig. S4 PCoA and NMDS plots of Bray–Curtis distances between communities including all quality-filtered reads (including the unclassified), after running an OTU-cluster analysis at 97% sequence similarity.