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Methodological Advances

Termites host specific fungal communities that differ from those in their ambient environments

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ABSTRACT

Termites are important plant biomass decomposers. Their digestive activity typically relies on prokaryotes and protozoa present in their guts. In some cases, such as in fungus-growing termites, digestion also relies on ectosymbiosis with specific fungal taxa. To date, the mycobiome of termites has yet to be investigated in detail. We evaluated the specificity of whole-termite associated fungal communities in three wood-feeding termite species. We showed that the whole-termite fungal community spectra are stable over diverse environments, regardless of the host species, and differ markedly from the wood in which they nest. The core mycobiome is similar to that found in other ecologically related insects and consists of a narrow spectrum of common filamentous fungi and yeasts, known for their stress tolerance and their ability to decompose plant biomass. The observed patterns suggest that a number of fungal strains may have a symbiotic relationship with termites, and our results set the stage for future investigations into the interactions between fungi, termites, and their other gut microbiota.

1. Introduction

Dead plant materials are mostly made of lignocellulose, the most common polymer on Earth, which relatively few metazoan taxa are able to significantly decompose (Lo et al., 2003). All key taxa consuming dead plant tissues, such as ruminants, earthworms and insects, largely rely upon a rich microbial consortium, which possess the necessary metabolic pathways for lignocellulose decomposition (Watanabe and Tokuda, 2010; Brune and Dietrich, 2015). Termites process this cellulose far more efficiently than other decomposers (Brune, 2014) and their dominance in tropical ecosystems makes them key players at a global scale (Bignell and Eggleton, 2000; Bar-On et al., 2018; Griffiths et al., 2019). While early branching termite lineages ("lower" termites) feed exclusively on wood or grasses, the "higher" termites (i.e. the crown family Termitidae) consume a variety of plant materials irrespective of decomposition status, and a majority of these taxa are soil-feeders (Jouquet et al., 2006; Krishna et al., 2013). To digest cellulose, termites rely on their own endogenous cellulases (Watanabe et al., 1998), in combination with microbial cellulases in their guts. Lower termites depend primarily on flagellate protozoa, with some contribution by prokaryotes, while "higher" termites lack cellulolytic flagellates completely and depend on bacteria and archaea for

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cellulose decomposition (Brune and Ohkuma, 2011; Hongoh, 2011; Brune and Deitrich, 2015).

In herbivorous or detritovorous insects, both prokaryotes and fungi are generally thought to form core taxa of the gut microbiome, despite the fact that most studies have considered prokaryotes only (Gurung et al., 2019; Ravenscraft et al., 2019). Both types of microorganism can act as nutritional symbionts assisting with digestion, detoxification and essential nutrients synthesis, or as protective symbionts (Dillon and Dillon, 2004; Gurung et al., 2019). Other roles, such as the effect on the host cells physiology and interactions with other microbes can be expected, as is known in mammals (Lai et al., 2018). The core gut mycobiome of wood feeding insects covers a relatively narrow set of ubiquitous yeasts and filamentous fungi such as *Candida*, *Mucor*, *Aspergillus*, *Penicillium*, *Alternaria* or *Trichoderma* (Pérez et al., 2003; Rojas-Jiménez and Hernández, 2015; Ziganshina et al., 2018) and the same taxon spectrum is reported not only in other insects (Moraes et al., 2001; Fredensborg et al., 2020), but also in mammals (Lai et al., 2018).

Associations between termites and fungi have so far been considered in two categories: firstly, interactions that affect the discovery and consumption of food or its nutrient value, but which fall short of mutualism; secondly, the cultivation of fungus-combs (*Termitomyces* spp.) by fungus farming Macrotermitinae (Lenz et al., 1991; Rouland-Lefèvre, 2000). However, apart from a few studies reporting common yeasts and filamentous fungi found in termite guts (Prillinger et al., 1996; Prillinger and König, 2006), the fungi associated with termites are yet to be systematically investigated. The only studies which have compared the microbiota of termite guts and termite ambient environments showed that fungal assemblages of guts differ markedly from nest walls or food nodules in litter and humus feeding termites (Menezes et al., 2018; Moreira et al., 2018). Based on the current knowledge of insect microbiomes, we hypothesized that fungi, which are a neglected part of the termite gut microbiome, form predictable communities and have stable interactions with their hosts.

We compared the specificity of body associated fungal communities (i.e. fungi in gut and on exoskeleton) in three ecologically similar species, *Heterotermes tenuis*, *Coptotermes testaceus* (both lower termites, Rhinotermitidae) and *Nasutitermes octopilis* (higher termites, Termitidae: Nasutitermitinae), which can be simultaneously collected from the same large wood item. We examined the mycobiomes of whole termite bodies as a proxy for termite gut mycobiomes, which enabled us to analyse large sample sizes, necessary for statistical testing. We hypothesized that fungal communities are similar in termites with a similar diet, and more alike in the genera *Heterotermes* and *Coptotermes* compared to *Nasutitermes*, as *Coptotermes* is nested within the genus *Heterotermes* (Bourguignon et al., 2016; Buček et al., 2019). We examined fungal communities using high-throughput sequencing of ITS2 metabarcodes of termite bodies, their food source (narrow termite galleries), and intact control wood near to areas where termites were feeding. The patterns described below are based on repeated samples from the same log, usually of multiple species from the same trunk, which allowed us to test for termite species and colony-level specificity of the associations.

2. Material and methods

2.1. Study site and sampling

The samples were collected in November 2014 in Nouragues Nature reserve (French Guiana; N 04°05′, W 52°41′). Large wood items were inspected for the presence of two “lower” termite species, *Coptotermes testaceus* (Rhinotermitidae) with a preference for sound white wood, *Heterotermes tenuis* (Rhinotermitidae) preferring red-rot wood, and one “higher” termite species, *Nasutitermes octopilis* (Termitidae: Nasutitermitinae), having no clear specialisation for the wood-decomposition degree.

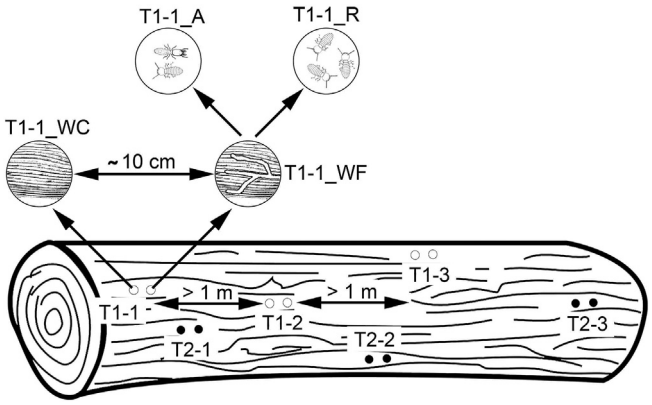

Fig. 1. Sampling scheme. Termites were collected in foraging galleries, and workers and soldiers were used for voucher sample in 80% ethanol (_A), while 10 workers for RNAlater sample (_R). Samples of foraging galleries (_WF) and control wood (_WC; roughly 10 cm from the closest termite gallery) were also stored in RNAlater. Up to three sample sets were collected from the same log, with a distance at least 1 m from each other. If more than a single focal termite (T1 and T2) was found in the same log, both were treated independently.

A single sample set comprised of three samples: (1) 10 workers from a single foraging party (2) their feeding substrate (approx. 1 cm^3 piece of wood containing gallery), and (3) the control sample (approx. 1 cm^3 of wood roughly 10 cm away from the closest termite gallery) (Fig. 1). Two or three sample sets, collected 1 m away from each other, were taken from the single wood log. Visually healthy workers were collected and narrow termite galleries with minimal amounts of frass were selected. Samples were firstly stored in RNAlater® solution at −20 °C within 12 h following collection, and shipped to Prague where they were stored at −80 °C until DNA extraction. In total, 82 samples sets (*Coptotermes*: n = 28, *Heterotermes*: n = 31, *Nasutitermes*: n = 23) originated from 23 trunks were studied. Storage in RNA later® solution caused hardening of termite bodies preventing gut dissection. Thus, as extraction of the intact intestine was impossible, we used whole termite bodies as a proxy for the study of intestinal microbiota.

2.2. DNA extraction and PCR amplification

Total DNA was extracted using Macherey-Nagel NucleoSpin® Soil kit with following modifications. Each termite sample was homogenized together in 500 μL of SL1 Lysis buffer, 100 μL of SX enhancer buffer and two sterilized steel beads (3 mm diameter) using a Mixer Mill MM 400 for 2 min, set on 30 Hz. Sample lysis by using a vortex was shortened to 2 min. The wood samples were mechanically crushed to small pieces, placed in a 2 mL tube with five steel beads, frozen in liquid nitrogen for 1 min and ground in a Mixer Mill Retsch MM 400 for 10 min at 30 Hz; 550 μL of SL2 extraction buffer was added to the homogenized material and the grinding was repeated once more. Sample lysis was extended to 10 min.

PCR amplification of the fungal ITS2 region from DNA was performed using gITS7 (5'-GTGARTCATCGARTCTTTG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Ihrmark et al., 2012; Tedersoo et al., 2015), each of them was barcoded in three PCR reactions per sample. The PCR reactions contained 2.5 μL of 10 × buffer for DyNAzyme II DNA Polymerase, 0.75 μL of bovine serum albumin (20 mg/mL), 1 μL of each primer (0.01 mM), 0.5 μL of PCR Nucleotide Mix (10 mM each), 0.75 μL of polymerase (2 U/μL DyNAzyme II DNA polymerase), and 1 μL of template DNA.

PCR was performed by using an Eppendorf Mastercycler® (Eppendorf AG, Hamburg, Germany) nexus cycler. The PCR cycling parameters were 94 °C—5 min (1 cycle), 94 °C—45 s, 56 °C—35 s, 72 °C—30 s (40 cycles), final extension at 72 °C—10 min. PCR triplicates were combined and purified using MinElute PCR Purification Kit (Qiagen GmbH, Hilden, Germany) according to the manual provided and eluted in 20 μL. Paired-ends amplicon reads were sequenced on Illumina MiSeq sequencer (Illumina Inc., USA) using V2 chemistry producing 2 × 250 bp output.

2.3. Data processing

Raw fungal ITS paired-end sequences were joined using fastq-join software (Aronesty, 2011) and demultiplexed, filtered and trimmed using the pipeline SEED 2 (version 2.1.05) (Větrovský et al., 2018). Low-quality sequences (mean Phred quality score < 30) and all sequences with mismatches in barcodes were removed from the dataset. After the quality filtering, all fungal sequences were extracted from the joined sequences using ITSx (v 1.0.11) (Bengtsson-Palme et al., 2013) to acquire complete ITS2 region. All ITS2 sequences shorter than 40 bp were discarded, yielding a dataset of 3 967 992 fungal ITS2 sequences (length distribution 40—395 bp, avg. 175 bp). The dataset was clustered into operational taxonomic units (OTUs) using UPARSE implementation in USEARCH version 8.1.1861 (Edgar, 2013) with 97% similarity threshold (109 476 fungal chimeric sequences were excluded during this step). A total of 10 742 fungal OTUs (without singletons) were obtained during the clustering step. To reduce the influence of contaminations and minimize the effects of barcode hopping all OTUs with up to 4 reads were discarded, which resulted in 2857 OTUs used for further analysis.

The most abundant sequence from each cluster was used as a representative sequence for taxonomic classification. Fungal sequences were classified based on BLAST best hit against the UNITE database, version 7.2 (Koljalg et al., 2013). The functional guild of each fungal OTU was assigned based on the FUNGuild database (Nguyen et al., 2016). For alpha diversity estimation, all fungal samples were resampled to 909 sequences. Diversity indices were estimated using SEED 2 version 2.1.05. The abundances of sequence reads were plotted on the phylogenetic tree constructed using NCBI molecular data via phyloT (Letunic, 2015) and the iTOL visualisation tool (Letunic and Bork, 2019). Data were deposited in the MG-RAST database under accession number mgp91984 and in NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA639228. Processed data (extracted ITS2 reads) were deposited in the GlobalFungi Database (https://globalfungi.com, Větrovský et al., 2020).

2.4. Statistical analysis

To test the null hypothesis of no difference between termite body, gallery and wood (control) fungal community composition, PERMANOVA analysis (Anderson, 2001) was performed with *adonis()* function of *vegan* package (Oksanen et al., 2018) in R (R Core Team, 2018). Euclidean distance on Hellinger-transformed fungal composition (i.e. Hellinger distance matrix) (Legendre and Gallagher, 2001) was used as response matrix, and sample type (body, gallery or wood) was used as fixed explanatory variable. Since observations were paired within triplets (the three sample types were sampled in each triplet), which, in turn, were nested in logs, the permutations were constrained to occur within triplets, using the variable triplet as blocking factor (or strata). To visualize the results, non-metric multidimensional scaling (NMDS) was performed in two different ways. In the first way, raw community data was ordinated by their fungal composition. This NMDS plot shows all the variability in the dataset. In the second way, community data was first regressed against triplet and log effects (i.e. the effect of spatial variability due to the experimental design was removed from the data) and, then, the residualized distance matrix was ordinated using NMDS as suggested by Anderson et al. (2017). This plot shows the variability in the dataset, once the effect of triplet and log has been taken into account.

To test the null hypothesis of no effect of sample type and termite species on fungal diversity (measured with Chao 1 index, Shannon-Wiener diversity index, and Pielou's evenness), linear mixed effect models were fitted using the function *lme()* from the R package *nlme* (Pinheiro et al., 2018). The interaction between termite species and sample type was fitted as the fixed part of the model, and, a random structure of the form ~1|triplet/log was included in each model to account for the fact that measurements were grouped in triplets, which, in turn, were nested in logs. Tukey *post-hoc* tests were performed using the function *lsmeans ()* of the R package *lsmeans* (Lenth, 2016).

To identify the fungal OTUs contributing to the separation between termite bodies, galleries and wood, partial redundancy analysis (partial RDA) was used (Legendre and Legendre, 2012) for each termite species separately. Separating the communities by termite species allowed checking whether similar OTUs contribute to the separation between sample types in the three termite species. In each RDA, Hellinger-transformed fungal OTU composition was used as response matrix, sample type was used as fixed explanatory factor, and the analysis was conditioned with the effect of the log and triplet. 1% of the OTUs with highest loadings to the ordination axes RDA1 and RDA2 in the three partial RDAs were depicted in triplots (Legendre and Legendre, 2012).

Lastly, to test and quantify the effect of termite species and log identity on fungal mycobiome composition, variation partitioning was performed based on RDA (Legendre and Legendre, 2012). Variation in Hellinger-transformed fungal OTU composition of termite bodies and galleries was partitioned in the effect of termite species and log identity. Since the number of body and gallery samples per species was not equal, *Coptotermes* and *Heterotermes* were randomly subsampled to balance the design, which makes the hypothesis testing more robust to the presence of heterogeneous group dispersions (Anderson and Walsh, 2013). The partial effect of each fraction (i.e. the effect of a fraction —e.g. species— once the effect of the other fraction —e.g. log identity— has been taken into account) was tested using a permutation test in partial RDA results.

3. Results

3.1. Fungal diversity

The diversity of fungal OTUs was significantly higher in termite bodies of all three species than in their galleries and intact wood, and was also significantly different between termite species. The estimated number of OTUs (Chao-1 estimate) in termite body samples, counted from the resampled dataset, ranged from 26 to 221 with an average 92—101 per species. Estimated OTU numbers and diversity indices were at least two times lower in termite galleries and in control wood. The fungal communities from termite bodies were significantly more even than termite galleries and control wood samples (Fig. 2).

3.2. Fungal community composition

The wood control and galleries were dominated by Basidiomycota followed by Ascomycota while there was an obvious shift to the dominance of Ascomycota over Basidiomycota in the termite bodies, with the addition of Mucoromycotina and Chytridiomycota

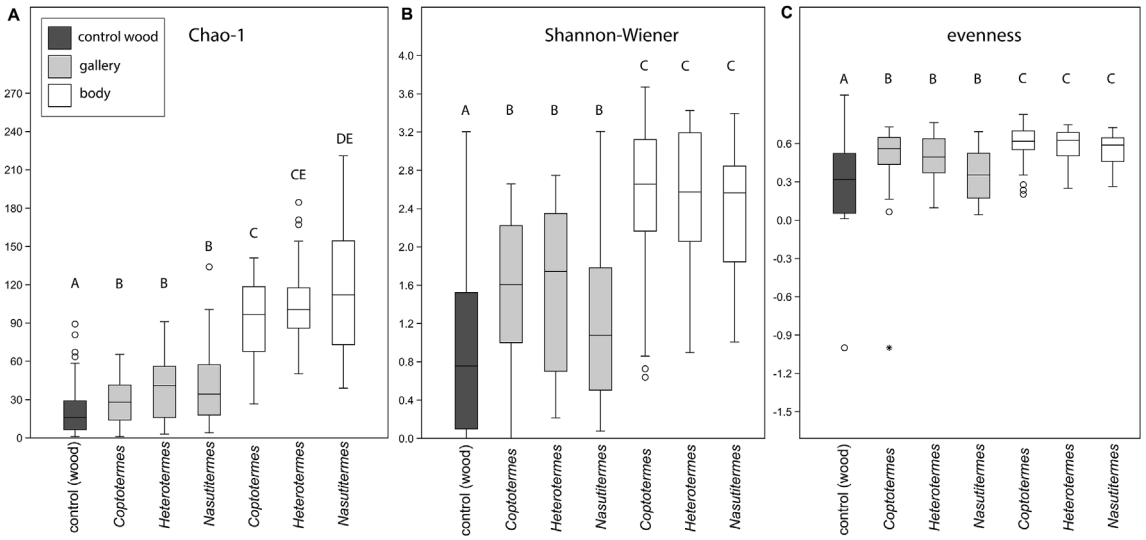

Fig. 2. Alpha diversity indices: Chao-1 index **(A)**, Shannon-Wiener index **(B)** and evenness **(C)** calculated from the fungal OTUs found in termite bodies, their galleries and insect-free wood controls. Data from *Coptotermes testaceus*, *Heterotermes tenuis*, *Nasutitermes octopilis* are shown. Groups sharing a letter are not significantly different (Tukey HSD *post-hoc* tests, $p < 0.05$).

members (Fig. 3A). A significant diversity of fungal taxa unidentified at the phylum level was recovered for all three treatments. At the finer taxonomic scale, 25 fungal orders were most abundant (Figs. 3B and 4). Among the most abundant orders, Mucoromycotina GS23 (artificial group, see Fig. 3 for definition), Eurotiales, Hypocreales, Ophiostomatales and Saccharomycetales were typically associated with termite bodies, whereas Chaetosphaeriales, Auriculariales and partially also Corticiales were associated with wood and galleries. Wood was also marked by the high abundance of Polyporales (Figs. 3 and 4, Supplementary Table 1). Finally, the members of the order Hymenochaetales were abundant in all variants. The majority of the fungal taxa identified to the ecological guild were predicted to be saprotrophs, a combination of mixed trophic modes (mostly saprotrophs and pathotrophs) and pathotrophs. Saprotrophs and pathotrophs were more abundant in termite bodies (frequency of reads in saprotrophs - 39-23%; pathotrophs - 5–11%) than in galleries and wood (22-11%; 0.1–3%). Those taxa belonged mostly to plant pathogens, with the small fraction of insect pathogenic fungi (0.05–0.25%) dominated by *Metarhizium* spp. and *Lecanicillium* spp. (Supplementary Table 2).

Multivariate analysis of the raw OTU dataset did not clearly separate samples by their types, but showed that the intestinal mycobiota of all three termite species is rather homogeneous and similar, in comparison to the very heterogeneous communities colonizing their galleries and wood controls ($k = 3$ dimensions, final stress = 0.24, Fig. 5A). By contrast, once the spatial variability due to the experimental design (i.e. the effect of log and triplet identity) is removed ($k = 3$ dimensions, final stress = 0.25, Fig. 5B), body samples clearly separate from galleries and controls. The NMDS revealed a high stress value indicating that 2D graphical

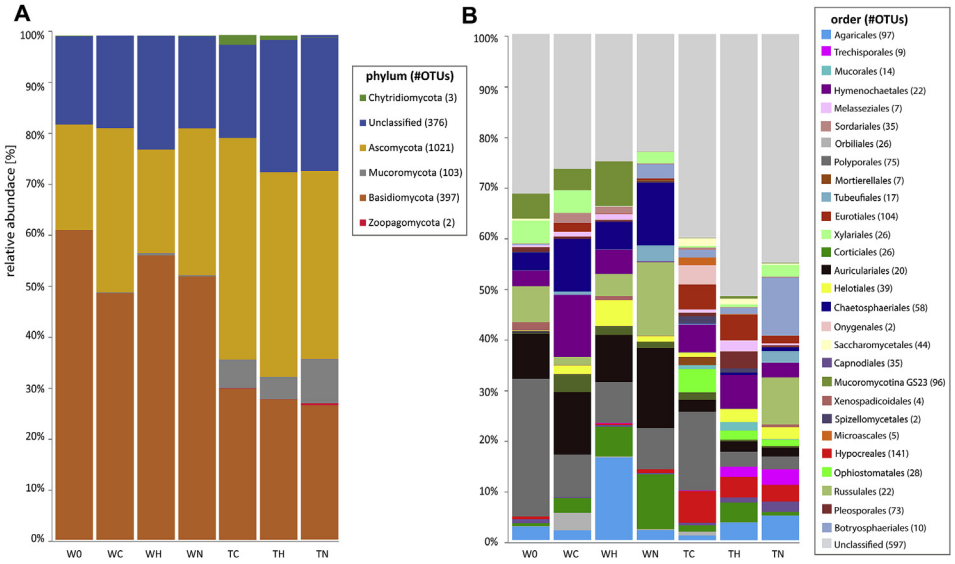

Fig. 3. Relative abundance of sequence reads classified at the phylum **(A)** and order **(B)** level. Only the orders with higher relative abundance (≥1% of reads in at least one sample type) are shown in Fig. 3B. Data from the wood control (W0), bodies (T) and galleries (W) of the termites *Coptotermes testaceus* (C), *Heterotermes tenuis* (H), and *Nasutitermes octopilis* (N) are shown. Artificial order Mucoromycotina GS23 was created for OTU285 (see Table 1) and OTUs withsimilarity ≥95%.

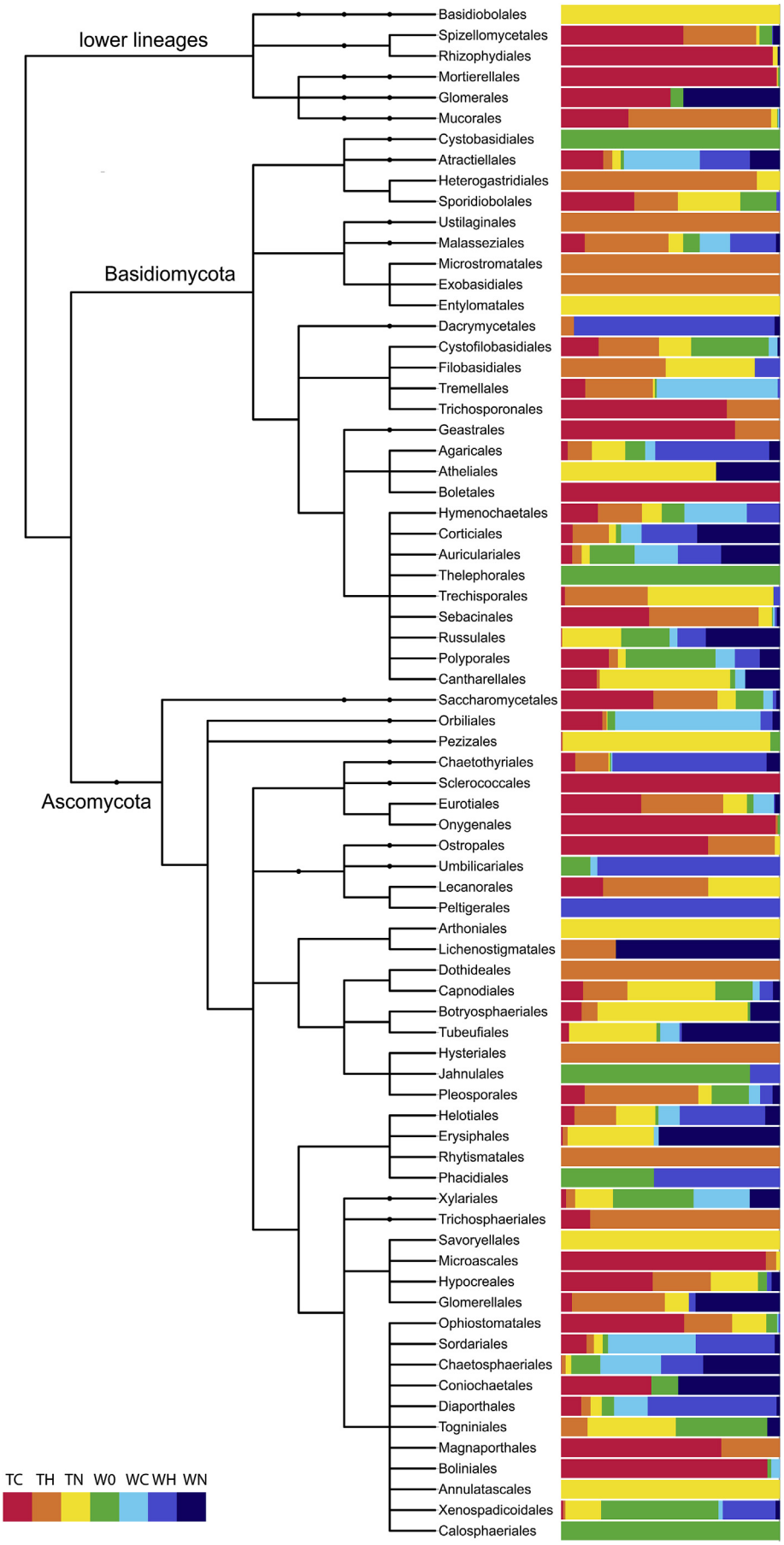

Fig. 4. Relative abundance of sequence reads classified at the order level. Data from the wood control (W0), bodies (T) and galleries (W) of the termites *Coptotermes testaceus* (C), *Heterotermes tenuis* (H), and *Nasutitermes octopilis* (N) are shown. Abundant orders, which reached at least 1% abundance in one fungal community were selected for presentation.

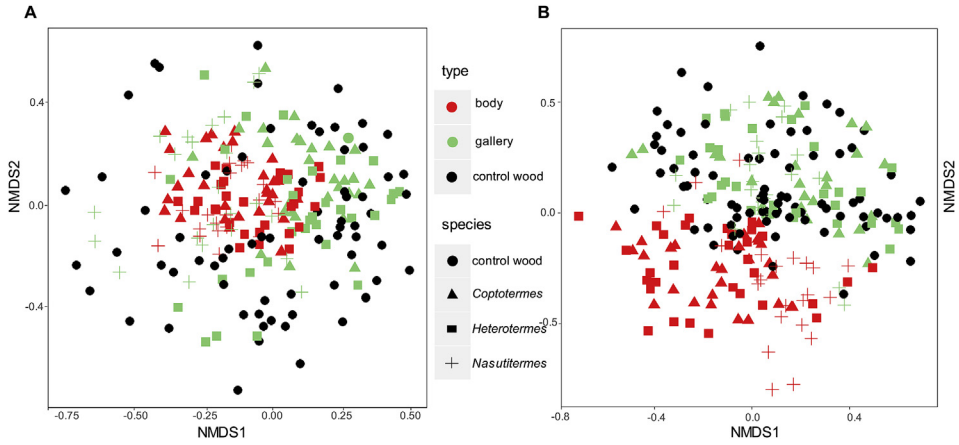

Fig. 5. Non-metric multidimensional scaling (NMDS) ordination of the sampling units by their fungal OTU (≥97% identity) composition based on ITS2 rRNA gene metabarcoding. **(A)** Ordination of raw fungal OTU composition (k = 3 dimensions, final stress = 0.24); **(B)** ordination of residualized fungal OTU composition (i.e. the effect of log and triplet identity removed; k = 3 dimensions, final stress = 0.25). Sample type significantly affects fungal community composition (PERMANOVA: permutations = 999, *P*-value = 0.001, R^2 = 0.044).

representation only roughly corresponds with the underlying data. However, the observed patterns were confirmed by the PERMANOVA analysis which showed that fungal communities from termite bodies were significantly different from galleries and controls (permutations = 999, *P*-value = 0.001).

Constrained RDA analysis with the removed effect of the sampling design revealed a clear separation of samples based on their type in all three termite species. The first axis of RDA (RDA1) separates termite bodies from galleries and controls, whereas the second axis (RDA2) separates galleries from controls (Fig. 6). As opposed to the unconstrained ordination (NMDS, Fig. 5), the constrained ordination (Fig. 6) distinguishes between the fungal compositions of galleries and controls. OTU 12, 20 and 34 are consistently positively associated with termite bodies in all three species. A further 13 OTUs are associated with two termite species (Table 1). The fungal genera linked with termite bodies (i.e. with high negative RDA1 axis loadings, Fig. 6), in all three termite species includes a narrow spectrum of filamentous ascomycetes (*Trichoderma*, *Penicillium, Scytalidium*, *Hawksworthiomyces, Lasiodiplodia*), a few basidiomycete genera (*Malassezia, Phlebia*, *Hyphodontia*, *Corticium, Wrightoporia* etc.), a single but abundant taxon from Mucoromycotina and a chytrid species from the genus *Spizellomyces* specifically associated with *Coptotermes* and *Heterotermes* (Table 1, Supplementary Table 3). Fungal genera linked with galleries include mostly wood saprobes from Basidiomycota (*Resinicium*, *Hyphodontia*, and unidentified genera), the very abundant genus *Chaetosphaeria*, and other wood inhabiting ascomycetes (*Pseudolachnella*, *Orbilia*, *Calonectria*, etc.). Genera linked with wood were *Auricularia*, *Porotheleum* and numerous, mostly unidentified, genera of Polyporales, Auriculariales and Agaricomycetes but also various wood roting ascomycota (*Hypoxylon*, *Kretzschmaria*, *Camarops, Cordana, Chaetosphaeria*) (Fig. 6, Supplementary Table 3).

The fungal community composition of termite bodies and galleries was significantly affected by both termite species and log identity. Total explained variation in gallery mycobiomes (0.118) was more than the half (0.261) of the explained variation in body mycobiomes. Accordingly, the variations explained by only termite species, only log and the shared fraction (i.e. the fraction that cannot be clearly attributed to either species or log) were more than double in termite bodies compared with galleries (Fig. 7).

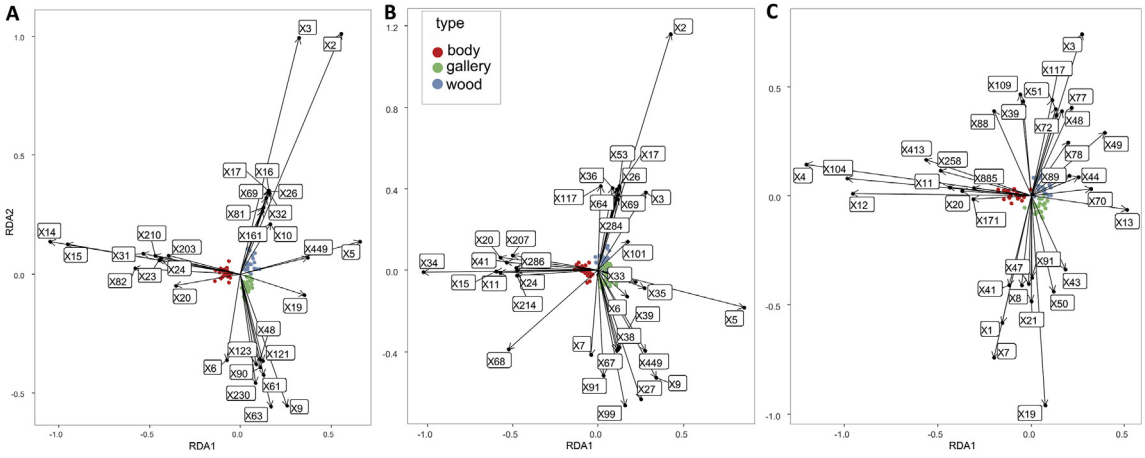

Fig. 6. Partial RDA triplots showing the partial effect (i.e. once the effect of log and triplet identity has been taken into account) of sample type on fungal OTU composition for **(A)** *Coptotermes*, **(B)** *Heterotermes* and **(C)** *Nasutitermes*. Sample type significantly affects fungal community for *Coptotermes* (Permutation test of RDA: permutations = 999, P-value = 0.001), *Heterotermes* (permutations = 999, *P*-value = 0.001) and *Nasutitermes* (permutations = 999, *P*-value = 0.001). One percent of OTUs (labelled as X14 etc.) with the highest fit to RDA1 or RDA2 are depicted (see Table 1 for further details).

Table 1
Fungal OTUs specifically associated with termite bodies. First 30 OTUs with the highest fit to RDA1 axis (see Fig. 6) are presented for each termite species. Taxonomic identity of OTUs is based on the Blast similarity search against NCBI GenBank and UNITE database. Abbreviations: A – Ascomycota, B – Basidiomycota, C – Chytridiomycota, M – Mucoromycotina.

Coptotermes testaceus					*Heterotermes tenuis*					*Nasutitermes octopilis*				
OTU #	RDA axis loadings		Best Hit description		OTU #	RDA axis loadings		Best Hit description		OTU #	RDA axis loadings		Best Hit description	
	RDA1	RDA2	Similarity/Coverage [%]	Taxon		RDA1	RDA2	Similarity/Coverage [%]	Taxon		RDA1	RDA2	Similarity/Coverage [%]	Taxon
14	−1.05	0.14	94.8/100 (KJ654590)	*Phlebia* sp. (B)	34	−1.02	−0.01		unidentified	4	−1.20	0.14	88.1/93.5 (HM162185)	*Podospora* sp. (A)
15	−0.95	0.12		unidentified	15	−0.60	−0.01		unidentified	104	−0.98	0.08	87.1/100 (AB846969)	GS23 sp. (M)[b]
82	−0.58	0.02	99.5/98.5 (HM771021)	*Hawksworthiomyces* sp. (A)	20	−0.58	0.06	91.2/89.5 (AY762623)	*Scytalidium lignicola* (A)	12	−0.96	0.01	100/100 (GU945354)	*Lasiodiplodia citricola* (A)
31	−0.54	0.09	100/100 (AY857228)	*Trichoderma harzianum* (A)	11	−0.57	−0.01	90.4/96.7 (KU214528)	*Arthrographis* sp. (A)	413	−0.56	0.17	97/100 (GU054100)	*Wrightoporia tropicalis* (B)
210	−0.47	0.08	98.3/100	*Spizellomyces punctatus* (C)[a]	41	−0.54	0.04	90.4/76	Auriculariales sp. (B)	258	−0.48	0.12		unidentified
23	−0.45	0.06	(AY997092)	unidentified	68	−0.53	−0.39	(UDB014156)	unidentified	11	−0.44	0.04	90.4/96.7 (KU214528)	*Arthrographis* sp. (A)
24	−0.44	0.06	91.3/44 (UDB028178)	*Hyphodontia pilaecystidiata* (B)	207	−0.50	0.07	99.6/100 (AY743636)	*Malassezia restricta* (B)	20	−0.37	0.02	91.2/89.5 (AY762623)	*Scytalidium lignicola* (A)
203	−0.40	0.08	100/100 (AY154939)	*Trichoderma spirale* (A)	286	−0.48	0.01	92/98 (GQ272617)	*Scytalidium ganodermophthorum* (A)	171	−0.31	−0.02	100/100 (EU401550)	*Trichoderma orientale* (A)
20	−0.35	−0.05	91.2/89.5 (AY762623)	*Scytalidium lignicola* (A)	24	−0.48	0.00	91.3/44 (UDB028178)	*Hyphodontia pilaecystidiata* (B)	885	−0.30	0.04	84.4/100 (AB846969)	GS23 sp. (M)[b]
285	−0.35	0.05	92.9/93.8 (AB846959)	GS23 sp. (M)[b]	214	−0.48	−0.03	87.8/100 (AB846969)	GS23 sp. (M)[b]	183	−0.30	−0.01	90.5/81 (AB846975)	GS23 sp. (M)[b]
286	−0.34	0.04	92/98 (GQ272617)	*Scytalidium ganodermophthorum* (A)	31	−0.45	−0.03	100/100 (AY857228)	*Trichoderma harzianum* (A)	121	−0.27	0.17		unidentified
22	−0.32	−0.17	87.6/72.9 (FJ231021)	*Penicillium curticaule* (A)	210	−0.44	0.12	98.3/100 (AY997092)	*Spizellomyces punctatus* (C)[a]	179	−0.27	0.04	94.8/100 (KT951335)	*Agaricus candidolutescens* (B)
197	−0.32	0.05	99.4/100 (KJ174211)	*Trichoderma koningiopsis* (A)	149	−0.42	−0.01	89.8/90.3 (AB846969)	GS23 sp. (M)[b]	256	−0.26	0.02	86.3/56.7 (KM103946)	Fungi sp. (unidentified)
41	−0.32	0.12	90.4/76 (UDB014156)	Auriculariales sp. (B)	145	−0.34	−0.03		unidentified	118	−0.26	−0.01	88.3/95.7 (UDB013022)	Sordariales sp. (A)
12	−0.32	0.06	100/100 (GU945354)	*Lasiodiplodia citricola* (A)	22	−0.32	0.03	87.6/72.9 (FJ231021)	*Penicillium curticaule* (A)	59	−0.25	0.04		unidentified
154	−0.31	0.02	90.7/100 (GQ272617)	*Scytalidium ganodermophthorum* (A)	629	−0.31	0.00	98.6/100 (KU164491)	*Malassezia restricta* (B)	156	−0.24	−0.07	100/19.5 (GQ280589)	*Calonectria leguminum* (A)
38	−0.30	0.13		unidentified	85	−0.30	−0.07	100/100 (HM770996)	Helotiales sp. (A)	1923	−0.24	−0.05	100/98.8 (EU280098)	*Trichoderma citrinoviride* (A)
28	−0.30	−0.02	86.9/100 (JX857794)	*Corticium* sp. (B)	203	−0.29	0.06	100/100 (AY154939)	*Trichoderma spirale* (A)	6	−0.24	0.01	86/47.6 (DQ826552)	*Resinicium monticola* (B)
34	−0.30	0.04		unidentified	248	−0.28	0.04	100/100	*Boeremia exigua* (A)	32	−0.24	−0.01		unidentified
158	−0.28	0.00	89.2/97.4 (GQ272617)	*Scytalidium ganodermophthorum* (A)	202	−0.27	0.00	(GU237707)	unidentified	229	−0.23	0.03	99.6/100 (UDB014090)	Trechisporales sp. (B)
257	−0.27	−0.02	88.6/100 (KU295549)	GS23 sp. (M)[b]	257	−0.26	−0.02	88.6/100 (KU295549)	GS23 sp. (M)[b]	34	−0.22	0.02		unidentified
152	−0.26	0.03		unidentified	259	−0.26	0.00	100/98.1 (JN626104)	*Penicillium mallochii* (A)	25	−0.22	−0.04	85.6/96.1	*Cordana terrestris* (A)
94	−0.26	−0.22		unidentified	302	−0.26	−0.01	99.5/100	*Verticillium leptobactrum* (A)	172	−0.21	0.03	(HF677173)	unidentified
133	−0.25	0.03	92.9/90.4 (EF127890)	*Hawksworthiomyces lignivorus* (A)	223	−0.25	0.00	(KF472157)	unidentified	199	−0.21	0.04	86/92.6 (KU975068)	*Pseudoproboscispora* sp. (A)

(*continued on next page*)

Table 1 (*continued*)

Coptotermes testaceus				*Heterotermes tenuis*				*Nasutitermes octopilis*				
OTU #	RDA axis loadings		Best Hit description		OTU #	RDA axis loadings	Best Hit description		OTU #	RDA axis loadings	Best Hit description	
	RDA1	RDA2	Similarity/Coverage [%]	Taxon		RDA1	RDA2	Similarity/Coverage [%]	Taxon		RDA1	RDA2	Similarity/Coverage [%]	Taxon
267	−0.25	0.00	94.3/98.1 (AF033470)	*Penicillium sclerotigenum* (A)	946	−0.25	−0.01	93.7/100 (AB846969)	GS23 sp. (M)[b]	82	−0.21	0.07	99.5/98.5 (HM771021)	*Hawksworthiomyces* sp. (A)
30	−0.24	0.03	90.9/43.4 (JX675137)	*Gymnoascus* sp. (A)	12	−0.25	−0.03	100/100 (GU945354)	*Lasiodiplodia citricola* (A)	7	−0.20	−0.74	99/100 (KT224922)	unidentified
509	−0.24	0.03	99.4/100 (DQ109528)	*Trichoderma lieckfeldtiae* (A)	83	−0.25	−0.01	99.3/100 (GQ272617)	*Scytalidium ganodermophthorum* (A)	88	−0.20	0.39	100/100 (HM148090)	*Cladosporium exasperatum* (A)
402	−0.23	0.03	91.1/93.2 (AB846969)	GS23 sp. (M)[b]	122	−0.24	−0.06	99.3/100 (AY273308)	Ascomycota sp. (A)	299	−0.18	0.04	100/19.4 (GQ280589)	*Calonectria leguminum* (A)
399	−0.22	−0.04	90.8/62.5 (AB846970)	GS23 sp. (M)[b]	14	−0.23	0.00	94.8/100 (KJ654590)	*Phlebia* sp. (B)	425	−0.18	0.04	86.6/79.2 (KY687694)	GS23 sp. (M)[b]
207	−0.22	0.05	99.6/100 (AY743636)	*Malassezia restricta* (B)	234	−0.23	0.03	99.3/100 (FR682163)	*Malassezia* sp. (B)	190	−0.18	0.04	88.7/100 (JX545187)	GS23 sp. (M)[b]

[a] – 98% sequence similarity with the type of *Spizellomyces punctatus* NR_111 189.
[b] – no reliable ITS2 sequence similarity to named taxa. The best hits (≤90%) are various *Mucoromycotina* spp. (e.g. HQ406814, LC189046).

4. Discussion

4.1. Termite associated mycobiome

Fungal communities of galleries and intact wood overlapped and differed from termite communities, which also overlapped with each other (Figs. 3–6). The termite mycobiota is likely to be a mix of fungi present on insect cuticular tissues (mostly from the mouthparts and pathogenic fungi present on the body surface), fungi present in the gut, and possibly fungi present internally in other organs or in the haemolymph. In our study, the fraction of insect pathogenic fungi was higher in termites than in their galleries, but their overall abundance was very low and did not contribute to the separation of the studied sample types (Table 1, Supplementary Table 2). Intestinal fungi appear to dominate the termite mycobiome. Whether fungi occur in other internal organs (i.e. haemolymph, gonad rudiments) is unclear. The presence of fungi on termite exterior cuticles could potentially reduce the differences between the termite and gallery communities, due to the fact that termites are in close contact with their galleries. Despite this limitation, we found statistically significant differences between both communities.

In our study, representatives of the Saccharomycetales, Malasseziales, Eurotiales, Hypocreales and Mucoromycota common in whole termites, and much less frequent in galleries and wood (Fig. 3, Supplementary Table 1), can be considered as typical members of termite mycobiome. Two previous studies quantified termite associated fungi using ITS metabarcoding. They found Eurotiales, Trichosphaeriales and Pleosporales (Menezes et al., 2018) together with Hypocreales (Moreira et al., 2018) are associated with guts and much less abundant in surrounding environments. This is in line with our results, including the presence of Trichosphaeriales and Pleosporales, which were rare in our study, but typically present in termite bodies (Fig. 3, Supplementary Table 1).

Yeasts, i.e. species from Saccharomycetales, Malasseziales and Trichosporonales, are the best studied fungi in “lower” termites (Prillinger et al., 1996; Prillinger and König, 2006) and the insect gut in general (Blackwell, 2017; Stefanini, 2018). Genera frequently found in our study, *Candida*, *Debaryomyces*, *Pichia*, *Cryptococcus*, and *Trichosporon*, are known as typical termite gut inhabitants (Prillinger et al., 1996; Prillinger and König, 2006). At the species level (i.e. OTUs with ≥99% similarity) we identified several taxa already known as intestinal symbionts of various insects (e.g. *Candida haemulonis*, *Candida parapsilosis* (Suh et al., 2007; Bozic et al., 2017), *Candida elateridarum* (Suh and Blackwell, 2004), *Malassezia restricta* (Zhang et al., 2003), *Metschnikowia pulcherrima* (Woolfolk and Inglis, 2004) and *Trichosporon insectorum* (Fuentefria et al., 2008) (Supplementary Table 1). Surprisingly, yeasts (with the exception of *Malassezia*) did not contribute to the statistical separation of gut and gallery associated fungal communities, when the effect of sampling design was removed (Fig. 6). This was partially due to the high inter-sample variability of yeast communities, but also because of their consistent occurrence (although in very low abundances) in the galleries.

The statistical separation of the whole termite mycobiota in our study was mostly due to the differences among representatives of ubiquitous genera of plant endophytes and saprobes including *Mucoromycotina* spp., *Trichoderma*, *Hawksworthiomyces* and *Penicillium* (Table 1). Data on termite gut associated filamentous fungi are scarce (for review see König et al., 2006; Prillinger and König, 2006). The genera *Trichoderma*, *Penicillium, Aspergillus* and *Alternaria* (Hendee, 1935; Rajagopal et al., 1979, 1981; Varma et al., 1994; Jayasimha and Henderson, 2007), together with numerous *Mucoromycotina* spp. (Zoberi and Grace, 1990), were already

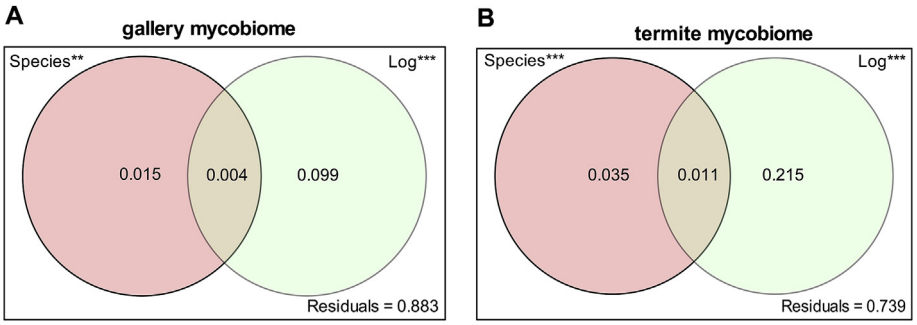

Fig. 7. Venn diagram of RDA variation partitioning of fungal OTU composition in **(A)** termite galleries and **(B)** whole termites. Numbers are adjusted R^2 values. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

reported from termite guts, which corresponds to our results (Table 1). Interestingly, a similar spectrum of genera (i.e. *Penicillium*, *Trichoderma*, *Fusarium*, *Cladosporium*, *Aspergillus, Rhizopus,* and *Mucor*) is also present in the guts of other plant biomass feeders such as isopods (Kayang et al., 1996), *Tenebrio molitor* (Fredensborg et al., 2020), wood feeding beetles (Rojas-Jiménez and Hernández, 2015; Mohammed et al., 2018; Ziganshina et al., 2018), cockroaches (Salehzadeh et al., 2007), bark beetles (Pérez et al., 2003) and grasshoppers (Idowu et al., 2009). It is also worth mentioning the abundant presence of *Spizellomyces* sp., identified in more than 50% of *C. testaceus* and *H. tenuis* samples, but absent in *N. octopilis*. *Spizellomyces* is a genus of zoosporic fungi living in soil, or as plant pollen, or fungal parasites (Wakefield et al., 2010), and its association with termites calls for further studies.

Although previous works studied the presence of various fungi in termites (Prillinger et al., 1996; König et al., 2006; Jayasimha and Henderson, 2007; Santana et al., 2015; Menezes et al., 2018; Moreira et al., 2018), our study is the first large and systematic comparison targeting the fungal communities using a statistically robust dataset of termites, their environments, and their feeding substrates. In agreement with previous studies (König et al., 2006; Prillinger and König, 2006), our results indicate that the termite intestine is inhabited by ubiquitous environmental fungi. We showed that the termite associated community is distinct and relatively homogeneous and stable over diverse environments and termite species, compared with termite galleries and control wood. In addition, termite galleries represent a specific habitat which significantly differs from wood in fungal community composition. This is similar to results found in studies of humus and litter feeding termites, where intestinal fungal communities differed substantially from communities of feeding nodules and/or nest walls (Menezes et al., 2018; Moreira et al., 2018) suggesting that termites possessed a host-defined intestinal mycobiome.

Strong environmental filtering appears to allow a relatively small number of fungi to grow freely and persist inside termites, across different species, after being taken up from the environment (soil galleries used for foraging, or the wood upon which termites feed). Vertical transmission of fungi by termites may also occur, although our results do not provide a clear answer on this. Both modes of symbiont acquisition, or their combination, can result in the observed stability of the intestinal communities across various collection dates, termite populations and species. In addition, the galleries themselves host specific fungal communities, which are more similar to intact wood and less affected by the termite species that form the galleries (Figs. 6 and 7).

Interestingly, the effect of termite species on fungal community composition in whole termites was very low (Fig. 7), which shows that different termites shape their fungal communities in a similar way. This is in contrast to patterns found with bacteria (Colman et al., 2012; Bourguignon et al., 2018; Chouvenc et al., 2018; Menezes et al., 2018; Moreira et al., 2018), which are more host specific. This is partly explained by the fact that many termite-associated bacteria are highly co-evolved vertically transmitted obligate symbionts (of termites or associated protists), whereas most identified fungi are presumably facultative associates, frequently existing as environmental fungi. Higher OTU diversity in whole termites in comparison to galleries and control wood is another feature constantly shared among different termite species (Fig. 2). This pattern is expected if we consider that the intestine itself is highly compartmentalised, which results in an increase in microbial diversity (Mikaelyan et al., 2017).

The core mycobiome of the termite gut is composed of plant biomass decomposers (see below), which are stress tolerant, fast growing and sporulating. It is likely that they are pre-disposed to live in the environment of the termite gut, which is characterized by harsh microaerobic conditions, steep gradients of oxygen and hydrogen, and activity of strong hydrolytic enzymes (König et al., 2006). Furthermore, it is possible that such features allow these fungi to live not only in termites, but also in taxonomically distant insect plant biomass feeders. The apparent stability of the fungal community between different termites could be considered evidence for symbiosis. Although it appears likely that the fungi we identified are able to live and grow in the termite gut, it is also possible that the origin of some strains is from the digested material, but were nevertheless detected using our methods.

4.2. Ecological role of gut associated fungi

Both the presence and the ecological role of fungi in the termite gut have been poorly studied so far. However, fungi have generally not been considered as an important part of the termite holobiont (Slaytor, 1992; Brune and Dietrich, 2015; Peterson and Scharf, 2016) and their presence is usually ignored. It has been shown that the intestinal fungi were not essential for *Nasutitermes exitiosus* survival in the lab experiments (Eutick et al., 1978), but similar studies in other termite species are needed to confirm their facultative status. More insight into their ecological functions has been provided with the transcriptome data. The fungal contigs from nymphoid neotenic (i.e. the developmental stage fed mostly by proteinaceous labial gland secretions) intestinomes in *Reticulitermes* spp. represent 10.2% of the fraction of non-termite origin (Dedeine et al., 2015). Another study showed that 18% of all carbohydrate-active enzymes in *Coptotermes formosanus* transcriptomes were of fungal origin, similar to bacteria (24%) but not to protists (6% only) (Zhang et al., 2012). In *Reticulitermes flavipes* symbiont libraries (gut content only), fungi represent 7% of the non-animal fraction of the reads (protists 71%, prokaryotes 21%) (Tartar et al., 2009). Little is known concerning fungal gut biomass.

In the termites *Zootermopsis angusticollis* and *Neotermes castaneus*, 10^7-10^8 yeast cells per millilitre of gut content were found, which is comparable to the number of flagellates, and similar, or two orders of magnitude lower, than the numbers found in bacteria (König et al., 2006; Prillinger and König, 2006). This suggests that fungi may actively proliferate in termite guts and they might be an important part of the lignocellulolytic machinery as proposed by Zhang et al. (2012).

Ecologically, yeast are typical inhabitants of the insect gut, including termites (Blackwell, 2017; Stefanini, 2018), and they can extracellularly decompose cellulose, hemicellulose and xylans, thus contributing to wood digestion (Schäfer et al., 1996; Prillinger and König, 2006). Interestingly, the dominant fungal strains identified in our study, especially *Trichoderma* and *Penicillium*, are well known for their ability to degrade cellulose, hemicellulose, and lignin, and are often used in biotechnology (de França Passos et al., 2018). Significant lignocellulosic activities have also been reported in *Phlebia*, *Hyphodontia*, *Scytalidium* (Eriksson et al., 2012), *Hawksworthiomyces* (De Beer et al., 2016) and *Lasiodiplodia* (Félix et al., 2018). Such strong enzymatic activities were shown *in vitro* directly in the strains from termites (Tarayre et al., 2015). This information, together with published transcriptomic data, reinforces the idea that, in termites, fungi may contribute to the degradation of lignocellulose and hemicellulose (Tartar et al., 2009). In addition, detoxification ability, which is well known in fungi, was also found in the yeasts from termites (Molnar et al., 2004) and therefore toxin degradation could be another important role of the intestinal fungal symbionts. However, further characterisation of the real contribution of fungi to food-processing in termites still remains to be undertaken.

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Supplementary data

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