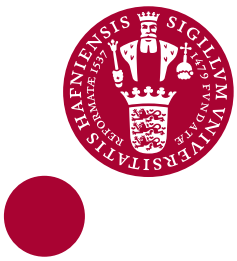

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Can interaction specificity in the fungus-farming termite symbiosis be explained by nutritional requirements of the fungal crop?

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ABSTRACT

Fungus-growing termites are associated with genus-specific fungal symbionts, which they acquire via horizontal transmission. Selection of specific symbionts may be explained by the provisioning of specific, optimal cultivar growth substrates by termite farmers. We tested whether differences in *in vitro* performance of *Termitomyces* cultivars from nests of three termite species on various substrates are correlated with the interaction specificity of their hosts. We performed single-factor growth assays (varying carbon sources), and a two-factor geometric framework experiment (simultaneously varying carbohydrate and protein availability). Although we did not find qualitative differences between *Termitomyces* strains in carbon-source use, there were quantitative differences, which we analysed using principal component analysis. This showed that growth of *Termitomyces* on different carbon sources was correlated with termite host genus, rather than host species, while growth on different ratios and concentrations of protein and carbohydrate was correlated with termite host species. Our findings corroborate the interaction specificity between fungus-growing termites and *Termitomyces* cultivars and indicate that specificity between termite hosts and fungi is reflected both nutritionally and physiologically. However, it remains to be demonstrated whether those differences contribute to selection of specific fungal cultivars by termites at the onset of colony foundation.

1. Introduction

Mutualisms are widespread in nature, with cooperation between species often providing entry into ecological niches that could not support either species alone (Moya et al., 2008). Yet, the interactions between mutualists vary from short-term co-existence to irreversible obligate symbiosis. In addition, there are varying degrees of interaction specificities; i.e., possible combinations of hosts and symbionts (Aanen et al., 2007). Vertical transmission of symbionts generally leads to a high degree of interaction specificity and co-evolution, whereas horizontal transmission typically leads to less specialized associations between symbionts (Bright and Bulgheresi, 2010). Interaction specificity, however, does not only depend on transmission mode. It is often observed that (metabolic) traits of a symbiont are lost because their functions become redundant if the other partner reliably provides the resources (Visser et al., 2010; Ellers et al., 2012). Such reciprocal specialization can favour obligate symbiotic partnerships and foster co-cladogenesis of symbionts, even in the absence of vertical transmission (Aanen et al., 2007).

An intriguing obligate symbiosis is that between a monophyletic group of termites (family Termitidae, subfamily Macrotermitinae) and basidiomycete fungi of the genus *Termitomyces* (Agaricomycetes, Lyophyllaceae), which originated ca. 30 million years ago in sub-Saharan Africa (Aanen et al., 2002; Aanen and Eggleton, 2005; Roberts et al., 2016). Fungus farming enables the termites to utilise food sources they cannot digest themselves, as the fungi convert recalcitrant plant substrates into edible fungal biomass and

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accessible carbohydrates. In return, the termites shelter the fungus from unfavourable abiotic and biotic conditions (Wood and Sands, 1978; Rouland-Lefèvre and Bignell, 2002). There are about 330 described species of fungus-growing termites in 11 monophyletic genera (Aanen et al., 2002; Nobre et al., 2010), all farming *Termitomyces* fungal symbionts (Aanen et al., 2002; Roberts et al., 2016). Even though most termites acquire their symbionts horizontally (Fig. 1A) (de Fine Licht et al., 2006; Aanen et al., 2007; Nobre et al., 2010), there is a degree of co-cladogenesis between the termite and fungus phylogenies; groups of *Termitomyces* associate with specific termite clades (Fig. 1B) (Johnson et al., 1981; Sieber, 1983; Darlington, 1994; Aanen et al. 2002, 2009); yet, within these groups, large differences in interaction specificity are observed. Some are highly specific, e.g., all *Macrotermes natalensis* studied so far associate with members from a single biological species of *Termitomyces*, whereas others have more diffuse co-evolutionary relationships; for example, most species of the genus *Odontotermes* associate with a broad range of *Termitomyces* lineages, which represent multiple species (Aanen, 2006; de Fine Licht et al., 2006; Aanen et al., 2007).

To explain the observed co-cladogenesis and differences in interaction specificity between termite-fungus associations, several studies have suggested that the substrates provided by termites play a role in the selection of a suitable fungal symbiont (Rouland-

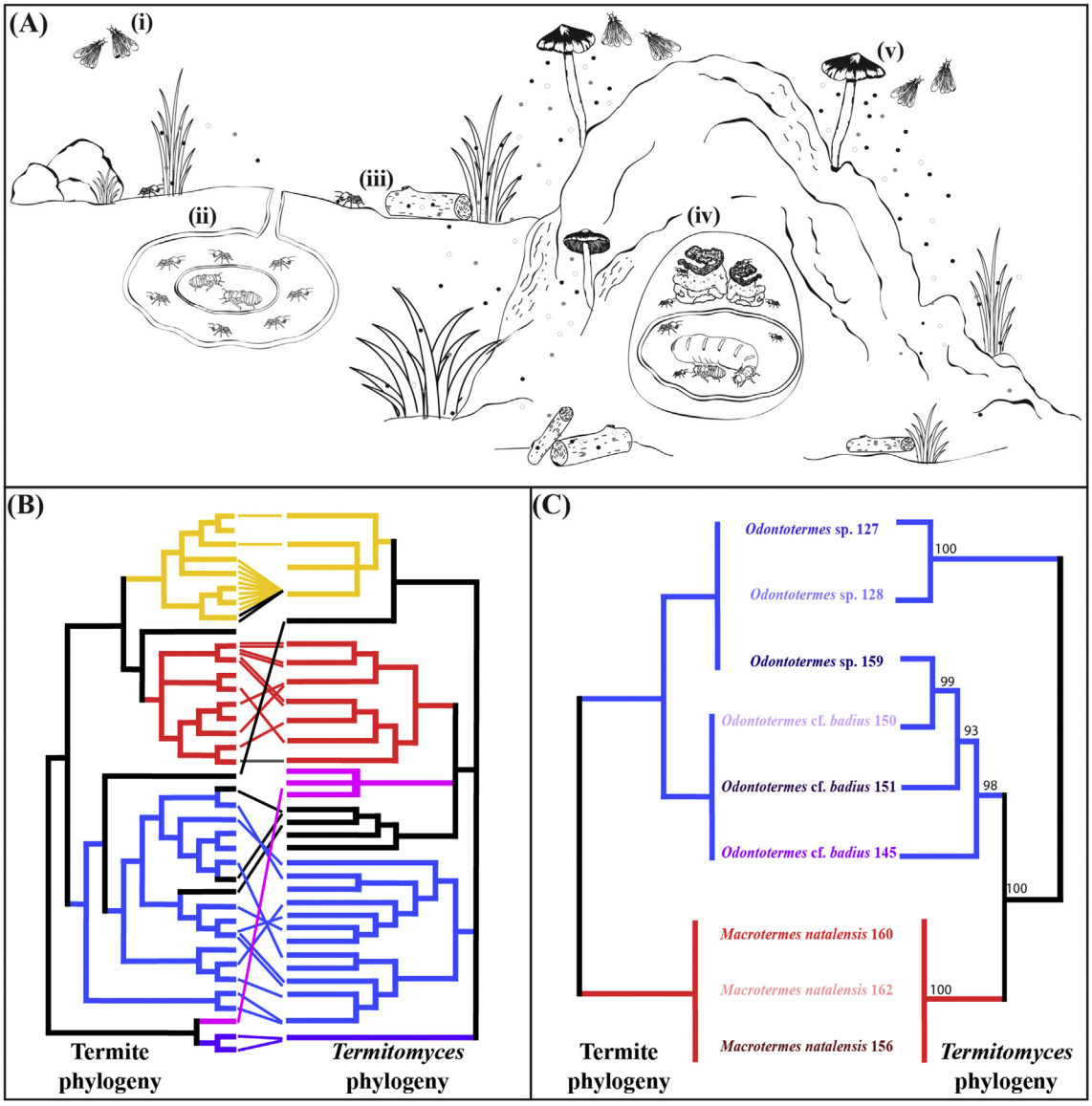

Fig. 1. **(A)** Schematic of fungal/termite life cycle: **(i)** winged male and female termites (alates) leave a mature nest for the nuptial flight. **(ii)** They shed their wings and dig into the ground to establish a new colony. After a few months the first generation of workers will build a chamber around the reproductive pair (former alates, now king and queen). **(iii)** This first generation of workers will leave the nest in order to obtain *Termitomyces* basidiospores from the environment along with plant substrate that will serve as growth substrate for the symbiotic fungus. **(iv)** Frequency-dependent selection between different *Termitomyces* strains assures that mature colonies maintain only a single fungus clone. Once the fungus is established as a fungus comb, it is nourished by workers with continuous plant substrate inoculation. **(v)** In mature nests, the fungus comb can produce fruiting bodies that emerge from the termite mound to release basidiospores into the environment. **(B)** Simplified consensus phylogenetic tree of fungus-growing termite species (left) and their fungal symbionts (right) (modified from Aanen et al., 2002). The lines in the centre indicate associations across termite and *Termitomyces* species; in blue the *Odontotermes* termites and their associated fungi, in red the *Macrotermes* termites and their associated fungi, the other colors represent other fungus-growing termite genera. **(C)** Termite (inferred) and *Termitomyces* (ITS based) unrooted phylogenetic trees using cluster analysis (UPGMA algorithm with Jukes-Cantor distance measurement, and bootstrap support with 10,000 pseudoreplicates) for the three termite species and nine nests used in the study. Sequences were aligned using the CLC Genomics Workbench v9.5.3 (https://www.qiagenbioinformatics.com); gap insertion penalty 10, gap extension penalty 1.0, alignment mode "Very accurate".

Lefèvre, 2000; Nobre and Aanen, 2012). The hypothesis is that the most specific associations have evolved most complementarity in the breakdown of plant biomass. According to this hypothesis, the less specifically cultivated *Termitomyces*, like the ones cultivated by *Odontotermes* spp., are expected to have a broader potential substrate range than very specifically cultivated *Termitomyces*, like the symbiont of *M. natalensis*. In addition, different termite species and genera collect different plant substrates, which could further select for association-specific metabolic capacities in the fungal symbionts (Grassé, 1982; Dangerfield and Schuurman, 2000; Johjima et al., 2006; Soleymaninejadian et al., 2014; da Costa et al., 2018).

Here we explore the extent to which *Termitomyces* performance on different substrates is correlated with interaction specificity in the termite-fungus symbiosis. First, we generate a phylogeny of nine *Termitomyces* isolates to compare to previous work and confirm interaction specificities (Aanen et al., 2002, 2007). Second, we test whether there are qualitative or quantitative differences in *in vitro* cultivar growth performance on 35 carbon sources between *Termitomyces* symbionts of the more specific *M. natalensis* and two less specific *Odontotermes* species. Third, as growth substrates are not expected to differ one-dimensionally (i.e., for carbon source only) between termite species, we use a geometric framework approach to generate nutritional landscapes by which we can visualize *in vitro* cultivar growth performance upon varying carbohydrate and protein concentrations and ratios simultaneously (Lee et al., 2008; Dussutour et al., 2010; Simpson and Raubenheimer, 2012; Shik et al., 2016). Finally, we identify whether the observed growth patterns, separately for the carbon-source and the nutritional-landscape experiments, reflect the interaction specificity between the fungi and their hosts.

2. Materials and methods

2.1. Termite collections and fungal isolations

We studied *Termitomyces* fungi from mature colonies for which selection of the resident fungal cultivar has already taken place. Fungus comb samples were collected from nine fungus-growing termite nests in 2015 at three geographical locations in South Africa (Table 1). Samples were collected from the termite species *Odontotermes* sp. (Od127, Od128 and Od159), *Odontotermes* cf. *badius* (Od145, Od150 and Od151), and *M. natalensis* (Mn156, Mn160 and Mn162), for which the termite species had previously been determined (Otani et al., 2014; da Costa et al., 2018) (Table 1). Mature, nodule-containing parts of the fungus comb were collected, placed into plastic bags and taken to the laboratory. *Termitomyces* fungal nodules were picked from clean parts of the fungus comb (no visible soil particles under a binocular microscope) with a sterile needle and placed on Petri plates with Malt Yeast Agar (MYA: 20 g malt, 2 g yeast extract, 15 g agar in 1 L distilled water). Fungal growth was monitored daily to check the purity of the isolates.

2.2. Termitomyces barcoding and phylogenetic analysis

Termitomyces DNA was isolated using a cetyltrimethylammonium bromide (CTAB) extraction. For isolates from mounds Od128, Od145, Od150, Od151 and Mn162, part of the nuclear ribosomal region, including both internal transcribed spacer (ITS) regions and the 5.8S ribosomal RNA (ITS1, 5.8S and ITS2), were amplified and sequenced using ITS1 and reverse primer ITS4 (White et al., 1990). Because *Termitomyces* is present in termite mounds as a heterokaryon, i.e., with two separate haploid nuclei, the total DNA of each isolate can contain two different copies for each region of the genome, which was the case for at least one of the two ITS regions in the fungal isolates of mounds Od159, Mn156, Mn160 and Od127. Further, if a length mutation exists between the two different copies of a genomic region, Sanger sequencing will fail. Therefore, we used both forward primers ITS1 and ITS3 and reverse primers ITS2 and IST4 (White et al., 1990) to obtain most of the ITS sequences. We obtained both ITS regions, but not the 5.8S region for *Termitomyces* from mound Od159. For Mn156 and Mn160 we obtained ITS1 and part of ITS2, and for Od127, part of ITS1, and part of ITS2. Sequences have been deposited to GenBank (MG283253-MG283261) (Table 1). Sequences were aligned using the CLC Genomics Workbench v9.5.3 (https://www.qiagenbioinformatics.com); gap insertion penalty 10, gap extension penalty 1.0, alignment mode "Very accurate". An unrooted tree was obtained by cluster analysis using the UPGMA algorithm with Jukes-Cantor distance measurement, and bootstrap support was assessed using 10,000 pseudoreplicates (Fig. 1C). Using BLASTn, the ITS sequences were compared to haplotypes obtained in a study of South African *Termitomyces* by Aanen et al. (2007).

2.3. Single nutrient assay: performance on different carbon sources

To determine biomass production of *Termitomyces* strains on different carbon substrates, we used a minimal medium developed for *Schizophyllum commune* (Dons et al., 1979, Supplementary Table 1), supplemented with 300 mg Urea/L, where glucose was replaced by one of 35 carbon sources as described at www.fung-growth.org, without birch wood xylan and oat spelt xylan that were no longer available and with chitin added (de Vries et al., 2017). The use of this specific set allows for comparison to other studies gathered in the FUNG-GROWTH database (e.g., Benoit et al., 2015; de Vries et al., 2017). Sterilised polycarbonate membranes (Profiltra, 0.1 pore size, 76 mm diameter, Almere, The Netherlands) were placed in each Petri dish to facilitate fungal biomass collection

Table 1
Termite species, GenBank accession number for *Odontotermes* spp. colonies previously identified by Otani et al. (2014) (marked with[1]) and da Costa et al. (2018) (marked with[2]), *Termitomyces* isolate codes, GenBank accession numbers for fungal ITS sequences, and geographical locations and their GPS coordinates for where fungus comb samples were collected.

Termite species	GenBank accession numbers for termite identification	*Termitomyces* Isolate code	GenBank accession numbers for *Termitomyces* identification	Excavation location	Excavation GPS coordinates
Odontotermes sp.	KJ4590690[1]	Od127	MG283255	Experimental farm	S25 44.562 E28 15.391
Odontotermes sp.	KJ4590691[1]	Od128	MG283254	Experimental farm	S25 44.544 E28 15.397
Odontotermes. cf. *badius*	MF092801[2]	Od145	MG283258	Experimental farm	S25 45.118 E28 15.525
Odontotermes. cf. *badius*	MF092802[2]	Od150	MG283257	Rietondale	S25 43.666 E28 14.112
Odontotermes. cf. *badius*	MF092803[2]	Od151	MG283256	Rietondale	S25 43.650 E28 14.128
Odontotermes sp.	MF092804[2]	Od159	MG283253	Experimental farm	S25 44.826 E28 15.337
Macrotermes natalensis	NA	Mn156	MG283261	Experimental farm	S25 44.623 E28 15.655
Macrotermes natalensis	NA	Mn160	MG283260	Experimental farm	S25 44.578 E28 15.645
Macrotermes natalensis	NA	Mn162	MG283259	Mookgophong	S24 39.693 E28 47.559

and weighing after incubation. *Termitomyces* strains were cultured for 15 d on MYA before roughly the same amount of fungal material from each strain was harvested and added to each of six Eppendorf tubes containing 750 μL 0.6% saline. The suspensions were subsequently pooled in a 12 mL tube and vortexed. Ten μL of this hypha/spore suspension was used as the inoculum. Three inoculates were placed on one plate; three replicate plates were inoculated per fungal strain per carbon substrate (total of nine replicates per fungus, per carbon source). The plates were incubated for 21–31 d (Supplementary Table 2), after which biomass from each Petri dish was collected, separately and weighed (g wet weight). Contaminated plates were discarded (18% out of 945 plates). Plates without membranes were incubated and photographed for visual inspection (Supplementary Fig. 1). Area could, however, not be evaluated, because of the small number of replicates. Principal Component Analysis (PCA) was performed on the average biomass per strain, per carbon source (35 measurements per strain) in R v. 3.3.2 (R Core Team) using the *FactoMineR* package (Lê et al., 2008). Contamination led to missing data points, for which values were estimated using *missMDA* in R (Josse and Husson, 2016). The carbon sources that contributed the most to the PCA patterns were evaluated by their cumulative percentage of variance explained, loading values, and eigenvalues (Supplementary Tables 3 and 4).

2.4. Geometric framework assay: performance across protein:carbohydrate ratios and concentrations

Termitomyces cultivar biomass formation and radial growth was assessed on media with nine protein:carbohydrate (P:C) ratios (1:9, 1:6, 1:3, 1:2, 1:1, 2:1, 3:1, 6:1, and 9:1) in four different concentrations (8, 32, 56 and 80 g/L protein + carbohydrate) (Dussutour et al., 2010; Shik et al., 2016). The mixtures of carbohydrate and protein were prepared in 250 mL distilled water and 4 g [1.6% (wt:vol)] agar and autoclaved at 121 °C. Carbohydrates were provided by even parts glucose and starch (Sigma-Aldrich, St. Louis, MO, USA). Protein was provided by even parts Bacto peptone, Trypticase peptone, and Bacto tryptone (Sigma-Aldrich, St. Louis, MO, USA). Micronutrients were provided by crushed vitamins added at 2% of the cumulative mass of protein and carbohydrates (Centrum) (modified from Shik et al., 2016) (for full media recipe, see Supplementary Table 5). Sterilised polycarbonate membranes (Profiltra, 0.1 pore size, 76 mm diameter; Almere, The Netherlands) were placed in each plate to facilitate fungal biomass retrieval after incubation.

Prior to inoculation, *Termitomyces* strains were cultured for 20 days on PDA plates, and they were harvested and inoculated as described above, except that each plate was inoculated with only a single inoculum. After 24 days, the Petri dishes were photographed (Supplementary Fig. 2), and contaminated plates excluded (1.3% of the 972 plates). The images were used to measure growth area in ImageJ (NIH Image, v.1.50g). After photographing, fungal biomass was harvested and weighed. To analyse differences in biomass formation for different P:C ratios among fungal strains, a PCA was performed in R v. 3.3.2 (R Core Team) using the *FactoMineR* package (Lê et al., 2008). Cumulative percentage variance, loading and eigenvalues were calculated to establish which ratios of P:C contributed the most to the separation observed in the PCA (Supplementary Tables 6 and 7).

To visualize *Termitomyces* growth, we generated P:C landscapes upon which we mapped variation in fungal area (cm^2) and biomass (g wet weight) after 24 days. We did this using the "*fields*" package in v.2.14.0 R to generate non-parametric thin-plate splines (Lee et al., 2008; Dussutour et al., 2010), setting topological resolution of response surfaces to $\lambda = 0.001$ as a smoothing parameter (Shik et al., 2016). We tested whether colony identity influenced growth, performing GLM analyses (proc GLM, SAS V9.3) for each of the three fungal strains (i.e., fungal strains from nests of a given termite host species). Models for biomass and area included the factors protein, carbohydrate, protein x carbohydrate, protein squared, carbohydrate squared, colony identity, and interactions between colony identity and each of the protein and carbohydrate effects. Colony identity was never a significant factor (Supplementary Table 8), so we generated performance landscapes based on strain-level means. For subsequent interpretation of P:C landscapes, we used least-square regressions based on strain-level means with both linear and quadratic terms to evaluate the main and interactive effects of protein and carbohydrates on performance (Supplementary Tables 9, 10, and 11).

3. Results

3.1. Termitomyces barcoding and phylogeny

All fungal isolates from *M. natalensis* had identical ITS sequences, apart from the occasional SNP between the two nuclei of the heterokaryons, while there were larger differences in ITS sequence and length between fungal isolates from *Odontotermes* spp. All the sequences were compared to sequences available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Sequences of two isolates from *Odontotermes* sp. (Od127 and Od128) only differed in two insertions/deletions, and they were most similar to a fungal strain previously isolated from *Odontotermes latericius* (Aanen et al., 2007). *Odontotermes* sp. (Od159) was most similar to an isolate from *O.* cf. *badius* (Od150) and both were most similar to an isolate from *O. latericius* (Aanen et al., 2007). The other two *O.* cf. *badius* isolates (Od145 and Od151) were most similar to fungal strains previously isolated from the same termite species (Aanen et al., 2007). The placement of the strains in the phylogeny we generated based on our ITS sequences corroborated the phylogeny of the most similar strains generated by Aanen et al. (2007).

3.2. Termitomyces performance across single carbon sources

Contrary to the hypothesis that the less specifically cultivated *Termitomyces* are able to grow on a broader range of substrates, we did not observe qualitative differences between *Termitomyces* strains in growth on different substrates. We did, however, find consistent quantitative differences between *Termitomyces* strains associated with *M. natalensis* and *Odontotermes* spp. The PCA showed that the *Termitomyces* strains associated with *M. natalensis* clustered together and were separated from *Odontotermes* spp. strains (Fig. 2A). Loading values of the PCA indicated that the main contributors to the separation in the first principal component were cotton seed pulp, cellulose, alfalfa meal, arabic gum and soybean hulls (negative loading values), and by D-galacturonic acid, D-glucose, L-arabinose, casein and soluble starch (positive loading values) (Supplementary Table 3). The main contributors to the separation in the second component were D-glucuronic acid and lignin (negative loading values), and apple pectin and beechwood xylan (positive loading values) (Supplementary Table 3). There was no effect of incubation time on biomass formation (correlation analysis: $y = 0.0047x + 0.028$, $R^2 = 0.0043$).

Overall, *Termitomyces* strains grew best on complex substrates such as wheat bran, citrus pulp, rice bran, cottonseed pulp, alfalfa meal and soybean hulls, and on the disaccharide cellobiose (Fig. 2B; Supplementary Table 12). Sugar beet pulp was the only complex substrate with limited *Termitomyces* growth. Among the monosaccharides, D-glucose was the only one that allowed substantial biomass formation across all strains. None of the strains formed substantial biomass on lignin. Also, little biomass was formed on sucrose, even though some biomass was formed on D-glucose and

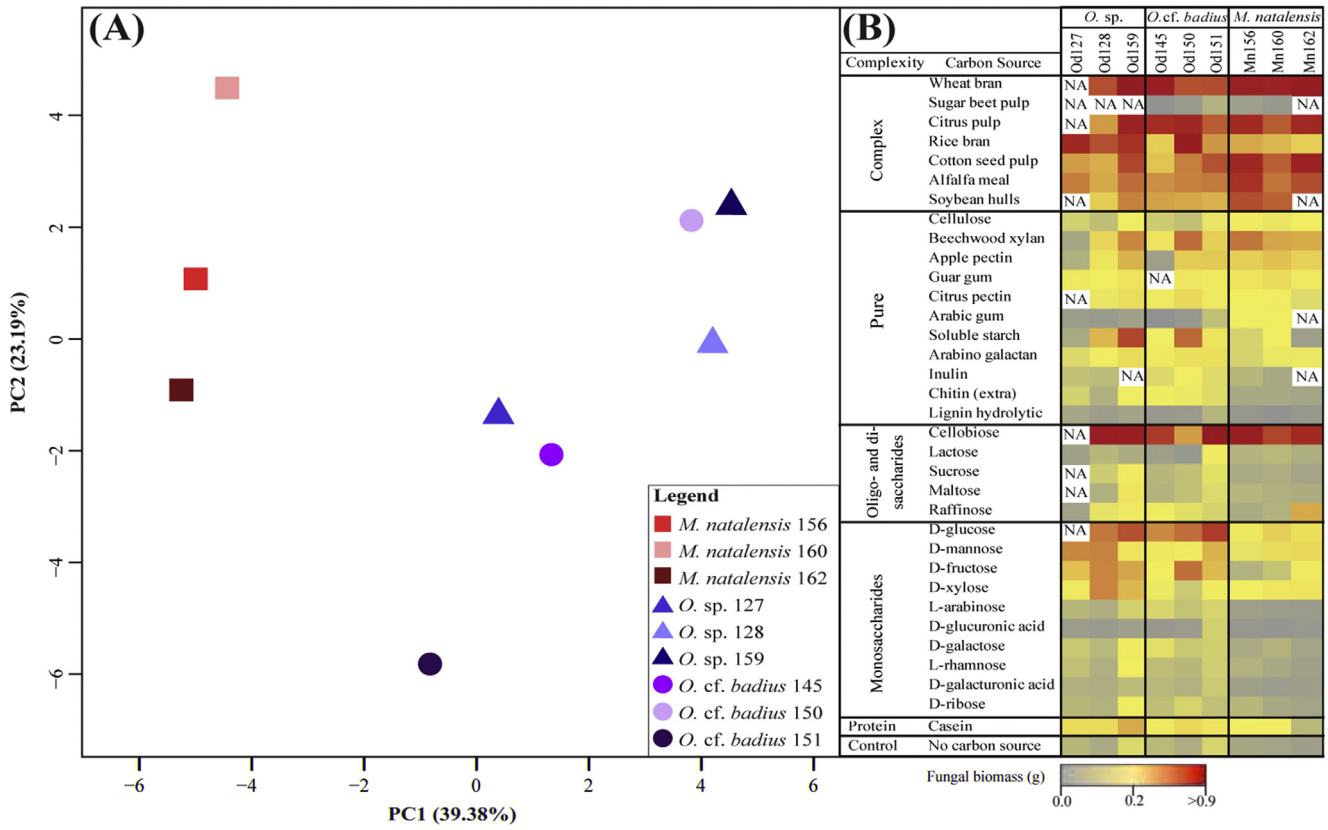

Fig. 2. **(A)** PCA plot of biomass formation patterns on different carbon sources. Each dot represents the average biomass production pattern of a single *Termitomyces* strain (shown in B). **(B)** Heatmap showing average ($n \leq 9$) biomass formation by *Termitomyces* strains on different carbon sources. The colour scale ranges from grey for the lowest values, yellow for intermediate and red for the highest values. NA means that all plates were contaminated, i.e. missing data.

D-fructose, the monosaccharides that build sucrose. *Odontotermes* spp. strains grew slightly better on D-glucose, D-fructose, and chitin than *M. natalensis* strains, while *M. natalensis* strains showed slightly better growth on arabic gum (Fig. 2B).

3.3. Cultivar performance across protein:carbohydrate landscapes

In contrast to the results obtained on biomass formation patterns on different carbon sources, the PCA of biomass on different P:C ratios and concentrations indicated clustering based on termite species (Fig. 3C). Fungal cultivar performance was maximized by specific blends of protein and carbohydrates (Supplementary Tables 13 and 14), with strains exhibiting carbohydrate-biased 'bulls eyes' for maximal biomass production around concentrations of 35 g/L carbohydrates and 20 g/L protein (Fig. 3A; Supplementary Tables 9, 10 and 11). The effects of carbohydrates on fungal biomass production interacted with protein availability (i.e., quadratic terms for C were significant for each of the strains; Supplementary Table 11), indicating that single nutrient assays of cultivar performance (e.g., the carbon source test) need to be interpreted with caution. Visualized on the performance landscapes, the same carbohydrate concentration (i.e., a horizontal line extending from the y axis at 32 g/L) that was associated with the highest cultivar biomass when present in slightly higher abundance relative to protein (i.e., red bullseye at 1:2 to 1:3 P:C) was also associated with low biomass production at higher protein levels (i.e., blue areas to the right of red bullseyes, with diets > 2:1 P:C) (Fig. 3A).

The P:C ratios and concentrations that maximized growth area differed visually from those for biomass formation (Fig. 3A) and statistically, as the quadratic term for P was significant in each of the radial area regressions (but not in the biomass regressions, Supplementary Table 11). Regions of maximal growth area also appeared to differ across *Termitomyces* strains from different termite species (Fig. 3B). The strains from *M. natalensis* showed a P:C diet maximum for radial growth that was comparable to the maximum for biomass formation (between 1:2 and 1:3, and between 32 and 56 g/L) and a second at P:C 3:1 and 6:1 at 8 g/L (Fig. 3B). The strains from *O.* cf. *badius* were similar to *M. natalensis*, while *Odontotermes* sp. strains only displayed one maximum (P:C 3:1–9:1 at 8 g/L) (Fig. 3B).

4. Discussion

4.1. Termite-fungus interaction specificity and its associations with Termitomyces growth

Our phylogeny of fungi isolated from *M. natalensis* and two species of *Odontotermes* revealed patterns of interaction specificity consistent with previous work (Aanen et al., 2002, 2007), demonstrating that *M. natalensis* colonies associate with a single *Termitomyces* species, while symbionts associated with *Odontotermes* spp. are genetically more variable and without one-to-one termite-to-fungus species relationships. Our *in vitro* assays suggest that *Termitomyces* nutritional requirements could contribute to interaction specificity with termite hosts. The patterns of biomass formation on different carbon sources separated *M. natalensis* from *Odontotermes* spp. strains, but not *Odontotermes* spp. from each other (Fig. 2B), consistent with higher specificity in *M. natalensis*-*Termitomyces* interactions. Yet, the *in vitro* growth patterns in our

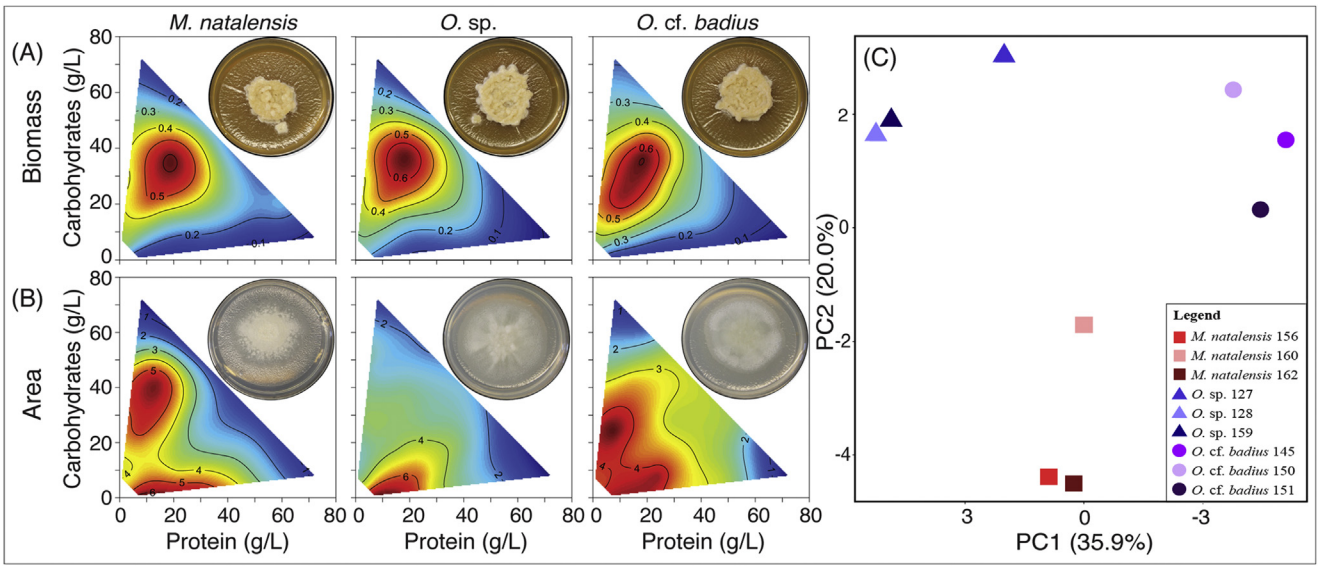

Fig. 3. Nutritional landscapes mapping the performance of *Termitomyces* cultivars on different ratios (1:9 to 9:1 P:C) and concentrations (8–80 g/L) of protein and carbohydrates in nutritionally defined artificial media. **(A)** Red areas indicate P:C blends that maximized biomass production in grams and **(B)** radial growth area in cm^2 and dark blue represents minimum values for these variables. Coloured isoclines increase to maximal dark red values of >0.6 g of fungal biomass in **(A)** and >5 cm^2 fungal area in **(B)** measured after 24 d. Fungal cultivars are from colonies of *M. natalensis* (left), *Odontotermes* sp. (middle) and *Odontotermes* cf. *badius* (right) (n = 3 colonies per species). All response surface regressions were significant (Supplementary Tables 8–11). **(C)** Principal component analysis on the biomass formation on different P:C ratios and concentrations. Each dot represents the average biomass formation production pattern of a single *Termitomyces* strain. Pictures of plates represent the best performance for biomass **(A)** and area **(B)**. For the full results, see Supplementary Fig. 2.

two-factor geometric framework experiment, also separate the strains of the two *Odontotermes* species.

4.2. *Termitomyces* performance of different carbon sources

The termite species included in this study primarily harvest decaying wood and leaf litter (Wood and Sands, 1978), and it was thus unsurprising that all *Termitomyces* strains performed well on complex carbon sources (Visser et al., 2011; Poulsen et al., 2014). Our findings do, however, suggest that *M. natalensis* should preferentially collect leaf litter and dead wood that are rich in cellulose, while *Odontotermes* should preferentially collect substrates richer in mono-, di- and oligosaccharides (Fig. 2B). Substrate preference is generally poorly understood in fungus-farming termites, but variation does indeed exist: fresh leaves (e.g., *Macrotermes mülleri*, *Macrotermes ivorensis, Macrotermes michaelseni*, and *Odontotermes formosanus*; Grassé, 1982; Dangerfield and Schuurman, 2000; Soleymaninejadian et al., 2014), grass stalks (e.g., *Macrotermes bellicosus* and *Macrotermes subhyalinus*; Grassé, 1982), roots (e.g., *M. michaelseni, Ancistrotermes* sp., and *Odontotermes* sp.; Dangerfield and Schuurman, 2000), and mammal dung (e.g., *Odontotermes* sp., *O.* cf. *badius, M. natalensis, M. michaelseni*; Dangerfield et al., 1978; da Costa et al., 2018). Consistently, a recent comparison found higher expression of cellulases in *M. natalensis* than in *Odontotermes* sp. fungus combs (da Costa et al., 2018), and Johjima et al. (2006) found that pectinases and hemicellulases were expressed more than cellulases in *Termitomyces* associated with *Macrotermes gilvus*, which harvests grasses and leaves.

All *Termitomyces* strains formed more biomass on cellobiose than on D-glucose, the building block of cellobiose. This may appear counterintuitive, since the degraded entities that are taken up by the fungus are monosaccharides (Allaway and Jennings, 1970), but is likely because we standardized carbon source concentrations and not carbon content, and cellobiose has more total carbon (and hence higher energetic value) than D-glucose. This appears to be unproblematic for our interpretations, as there was no overall correlation between the number of carbon molecules and biomass formed for the mono-, di- and oligosaccharides for which we could estimate energetic values ($y = 0.003x + 0.038$, $R^2 = 0.019$). Biomass formation is thus more likely associated with metabolic adaptations to certain carbon sources, consistent with the higher expression of enzymes targeting these carbon sources (e.g., cellobiohydrolases) in fungus combs (da Costa et al., 2018). Cellobiohydrolases convert cellobiose to monosaccharides, which likely leads to increased glucose levels in guts of old workers at the final step of plant decomposition (da Costa et al., 2018), and consistent with gut bacteria putatively breaking down and assimilating simple sugars (Poulsen et al., 2014). The potential importance of nutrient co-limitation (evident from the geometric framework experiment) represents a caveat to the above interpretation, since carbohydrate-mediated cultivar growth depends on protein availability.

Termite species forage on different substrates in different geographical locations (da Costa et al., 2018), so *Termitomyces* growth likely varies in response to the availability of substrates in given habitats. Analyses of a *Termitomyces* genome from *M. natalensis* indicate that the cultivar should be able to decompose most complex polysaccharides (Poulsen et al., 2014). This implies that the substrates should not govern symbiont selection alone; however, variation in forage availability between habitats could affect what enzymes are expressed. Further, because multiple fungal symbiont species associate with multiple *Odontotermes* species, genome content and enzymatic capacity variation between symbionts could be larger and allow termite host species to utilise a wider range of substrates. Additional -omics approaches will be needed to fully characterise the standing variation and the relative roles of genome differences vs. gene expression differences between *Termitomyces* species.

4.3. Fungal performance across gradients of P:C blends and termite species-specific farming strategies

The geometric framework provides a powerful graphical

approach for visualizing how diverse foraging organisms from slime molds (Dussutour et al., 2010) to humans (Simpson and Raubenheimer, 2012) prioritize competing nutritional requirements when confronted with imbalanced resources. We used this approach to visualize nutritional landscapes and test for nutritional specificity differences between cultivar strains. While cultivars exhibit nearly identical nutritional P:C targets for maximum biomass production (Fig. 3A), they appear to exhibit strain-specific targets for growth area (Fig. 3B). Thus, if nutrients act as a filter among competing strains in incipient nests, they likely favour cultivars best able to exploit nutrients through rapid radial growth than those able to acquire most biomass over time. This may be driven by heightened starvation responses, with low macronutrient concentrations (especially carbohydrates) inducing extensive radial growth (cf., Boddy, 1999; Boswell et al., 2002; Heaton et al., 2010).

An important next step will be to define nutritional mixtures present in each of the resources harvested by termite colonies in nature. By overlaying these maps of nutrient availability on growth maps, the degree of overlap between the 'realized niche' (nutrients actually provided by termites) and the 'fundamental niche' (resources that maximize cultivar performance) can be evaluated (Raubenheimer, 2011). A reasonable hypothesis is that the substrates available to termite foragers in nature have low overall protein concentrations and low nutritional variability. For instance, the carbohydrate bias of optimal biomass formation may reflect that dead wood can contain less than 0.5% nitrogen (LaFage and Nutting, 1978) and fungus combs contain 1.9% and 41% nitrogen and carbon, respectively (Arshad and Schnitzer, 1987). The P:C cultivar growth has additional eco-evolutionary importance, since the existence of nutritional 'bullseyes' implies that termites may stand to benefit by providing cultivars with a specific blend of nutrients (i.e., to target the fundamental niche of their cultivar). This in turn implies a basis for selection of sensory mechanisms in the termite host, and potentially among termite species growing different cultivar species, to identify and select those nutritional blends.

4.4. Termite colony foundation and forage use may affect fungal cultivar selection

The ecological dynamics by which fungus-growing termites select their symbionts during early phases of colony development remain unknown. One possibility is that the first termite workers selectively pick up *Termitomyces* spores from the species with which they normally associate, along with plant substrate to start the fungal comb. This would, however, require recognition capabilities in the termites (Grassé and Noirot, 1958; Sands, 1960; Aanen et al., 2002). Sieber (1983) demonstrated that provisioning of *Termitomyces* spores to incipient *Odontotermes montanus* and *M. michaelseni* laboratory colonies allowed for the colonization of a primordial fungus comb (comprised of soil) by *Termitomyces*. However, these experiments did not involve testing whether only specific fungal species were collected. Alternatively, multiple *Termitomyces* species may randomly be collected by the first termite workers if spores are brought in with plant forage coincidently. In this case foraged plant material could contribute to drive the subsequent competitive selection within the nest prior to the establishment of a monoculture.

Our findings provide support to the interaction specificity between fungus-growing termites and *Termitomyces* and indicate that specificity between termite hosts and fungi is not only visible from phylogenetic reconstructions, but also reflected physiologically. Differences between fungal symbionts of different hosts could facilitate variation in fungal colonization success of the substrate collected by specific termites in incipient nests, potentially contributing to maintaining termite-fungus interaction specificity if physiological differences cause variable growth during competition between multiple fungal strains at the onset of fungus garden formation.

4.5. Data accessibility

Termitomyces ITS sequences have been deposited in GenBank.

Author contributions

RRDC and SMEV designed the experiments and analyses, collected colonies in the field, isolated fungal strains, carried out the growth assay on different carbon sources, and drafted the first version of the figures, tables, and manuscript; RRDC and JZS designed the P:C experiment, which was carried out by RRDC with analytical help from JZS; SMEV extracted DNA and analysed *Termitomyces* sequences; DKA and MP supervised SMEV and RRDC, respectively, helped design the study, and contributed with comments on analyses, figures, tables, and text. All authors contributed to writing the manuscript.

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Supplementary data

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