Minireview

Towards an integrated understanding of the consequences of fungus domestication on the fungus-growing termite gut microbiota

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Summary

Approximately 30 million years ago (MYA), the subfamily of higher termites Macrotermitinae domesticated a fungus, *Termitomyces*, as the main plant decomposer and food source for the termite host. The origin of fungiculture shifted the composition of the termite gut microbiota, and some of the functional implications of this shift have recently been established. I review reports on the composition of the Macrotermitinae gut microbiota, evidence for a subfamily core gut microbiota, and the first insight into functional complementarity between fungal and gut symbionts. In addition, I argue that we need to explore the capacities of all members of the symbiotic communities, including better solidifying *Termitomyces* role(s) in order to understand putative complementary gut bacterial contributions. Approaches that integrate natural history and sequencing data to elucidate symbiont functions will be powerful, particularly if executed in comparative analyses across the well-established congruent termite–fungus phylogenies. This will allow for testing if gut communities have evolved in parallel with their hosts, with implications for our general understanding of the evolution of gut symbiont communities with hosts.

Introduction

Mutualistic associations allow for partners to gain access to otherwise inaccessible resources or metabolic capacities, and such evolutionary innovation has been a major

force shaping the evolution of life (Sapp, 1994; Gilbert *et al.*, 2012). Symbioses take many forms, ranging from highly specific co-evolved associations, such as between amino acid synthesizing endosymbiotic *Buchnera* associated with aphids (e.g. Baumann *et al.*, 1995; Hansen and Moran, 2011) to complex gut communities composed of hundreds of bacterial phylotypes interacting with each other and their hosts (Hess *et al.*, 2011; Van Horn *et al.*, 2012; Kashyap *et al.*, 2013). The characterization of single members of communities is usually limited because they can rarely be cultured and explored physiologically outside the association with the host, and technologies limit our ability to explore the network of interactions between members within communities in an ecological context (cf. Ohkuma, 2008). Consequently, apart from a few model organisms, such as mice and humans, we know little about the factors shaping the origin, evolution, and short- and long-term dynamics of gut microbiota composition and function.

Although termite guts are among the best studied insect guts (Warnecke *et al.*, 2007; Brune, 2014), our understanding of community functions is generally lacking, in particular regarding assigning specific community members to specific functions. Termites are social cockroaches (Inward *et al.*, 2007), and the ancestral termite gut microbiota was adapted from that of the cockroach ancestor (Dietrich *et al.*, 2014). Subsequent changes in microbiota composition have been shaped by several major transitions in termite evolution. When the ancestor of lower termites and the wood roaches (*Cryptocercus*) acquired cellulolytic flagellates, this was accompanied by a shift to an exclusively wood-based diet (Engel *et al.*, 2009). These cellulolytic flagellates became dominant community members of the lower termites (families Mastotermitidae, Hodotermitidae, Termopsidae, Kalotermitidae, Serritermitidae and Rhinotermitida) (e.g. Cleveland, 1923; Brugerolle and Radek, 2006), complemented by bacteria (Dietrich *et al.*, 2014) (feeding group I as defined by Donovan *et al.*, 2001). Approximately 54 MYA, the common ancestor of the higher termites (family Termitidae) lost these flagellate associations, and their gut

microbiotas became dominated by bacteria (Eggleton, 2006; Bourguignon *et al.*, 2014). Based on gut morphology and content analyses, Donovan and colleagues (2001) distinguished three feeding groups within the higher termites: group II, feeding on dead wood, grass, leaf litter, micro-epiphytes and fungus; group III soil feeders, feeding on organic rich upper soil layers; and group IV soil feeders, containing the true soil-feeding species. These major dietary shifts appear to be associated with changes to the gut microbiotas (Dietrich *et al.*, 2014), suggesting that gut changes have accompanied the extensive dietary diversification and possibly contributed to the ecological success of the extant higher termites (Brune and Ohkuma, 2011; Dietrich *et al.*, 2014).

Approximately 30 MYA, another major transition in the symbiotic lifestyle of termites occurred in the higher termite subfamily Macrotermitinae. This took place in Sub-Saharan Africa and involved the addition of a basidiomycete fungal mutualist in the genus *Termitomyces* (*Agaricomycetes*, *Lyophyllaceae*). The candidate theory of the origin of fungiculture is that the common ancestor of the Macrotermitinae and lower termites in the Rhinotermitida used chewed wood material and faeces to create a sponge-like carton structure (see, e.g., Chouvenc *et al.*, 2013), which attracted fungal growth and became the fungus comb in fungus-growing termites (Eggleton, 2006). The origin of the association with *Termitomyces* was associated with a shift in the composition of the fungus-growing termite gut microbiota (e.g. Hongoh *et al.*, 2006), but only more recent work has elucidated the functional relevance of these changes for the biology of fungus farming. Recent reviews of the termite–fungus association (e.g. Nobre *et al.*, 2011) have, thus, not explicitly been able to address gut microbiota composition and function. This review serves to highlight these recent advances in our understanding of this ancestral shift and discusses research of importance that will allow us to gain a better understanding of complementary fungus–bacteria roles in the symbiosis.

Fungus-growing termites

The termite–fungus association

The termite–fungus association is obligate and mutually beneficial, with the termites relying on the fungus for the breakdown of plant material and as a nutrient source, while the fungus obtains provisioning of plant material, an optimal microclimate for growth, and selective inhibition of competitor and antagonist fungi entering the termite nest (Nobre *et al.*, 2011; Um *et al.*, 2013). The habitats of the symbiosis vary from savannah to rain forest, and the association may contribute up to 20% of carbon mineralization in savannah ecosystems (Wood and Sands, 1978). The association has co-diversified to include more than 330 described termite species in 12 extant genera, although Hypotermes is nested within the genus *Odontotermes*, suggesting that it is unlikely to be a separate genus (Aanen *et al.*, 2002). All genera except *Hypotermes* occur in Africa, while four have colonized Asia and one Madagascar (Eggleton, 2000). Forty species of *Termitomyces* have been described based on sexual fruiting structures (Kirk *et al.*, 2001), but molecular data suggest that morphologically indistinguishable species of *Termitomyces* exist (Frøslev *et al.*, 2003).

Mature fungus-growing termite colonies produce male and female alates (reproductives) that leave their parental nests. In most species, a queen and a king – the royal pair – will establish their new nest without the fungus present (Fig. 1A), and the first foragers will collect *Termitomyces* basidiospores and plant substrate for their growth (Darlington, 1994; Korb and Aanen, 2003) (Fig. 1B). Multiple strains are collected so that a mix of genetically different fungi exist within young colonies, but competitive exclusion through frequency-dependent mechanisms leads to mature colonies maintaining only a single fungal strain (Aanen *et al.*, 2009). Vertical fungus transmission has evolved at least twice: in *Macrotermes bellicosus*, where the king carries a fungal inoculum in his foregut during colony founding, and in the genus *Microtermes*, where the queen brings the fungal inoculum (Korb and Aanen, 2003). Despite predominant horizontal transfer of the fungus between host generations, switches are restricted to being within a relatively narrow fungal clade, so that the evolutionary histories of the Macrotermitinae and *Termitomyces* display phylogenetic congruence, with specific termite genera cultivating specific sets of *Termitomyces* partners (Aanen *et al.*, 2002; Nobre *et al.*, 2011).

The role of Termitomyces in plant decomposition

The association with *Termitomyces* has been attributed to being the main driver for the ecological success of fungus-growing termites. However, the relative importance of *Termitomyces* for lignin, cellulose and hemicellulose degradation has been debated and may differ between termite genera and species (Bignell, 2000; Nobre and Aanen, 2012).

Grassé and Noirot (1958) suggested that *Termitomyces* play a role in lignin degradation to facilitate access to cellulose, and chemical analyses showed that lignin content is reduced within fungus combs in several *Macrotermes* species (Rohrmann, 1978; Hyodo *et al.*, 2000; 2003), but not in other termite genera (Hyodo *et al.*, 2003). The genome of *Termitomyces* associated with *M. natalensis* supports the hypothesis that fungal enzymes to cleave lignin are present (Poulsen *et al.*, 2014). Fungal cellulases and xylanases appear to be
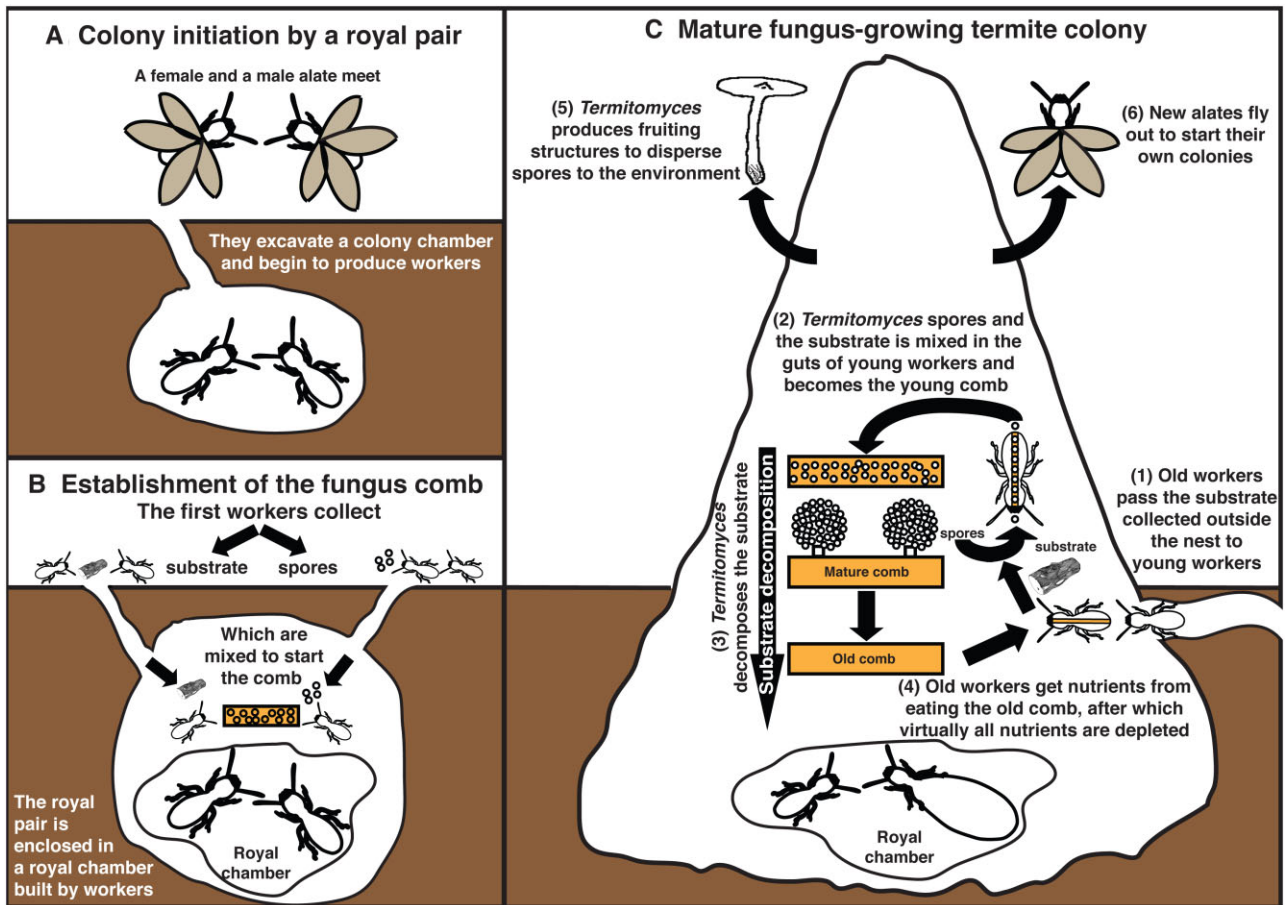
Fig. 1. The life cycle and natural history of fungus-growing termites.
A. Winged males and females (alates) meet during their brief flight and dig into the ground, where they establish the colony without a fungus present (except in *Microtermes* spp. and *Macrotermes bellicosus*, where the fungus is vertically transmitted; Korb and Aanen, 2003).
B. After a few months, the first workers emerge and they construct the royal chamber. The first workers also initiate the fungus comb using basidiospores of *Termitomyces* collected in the environment, along with plant substrate for its growth. Competitive exclusion between genetically different fungi ultimately assures that mature colonies (C) maintain only a single fungus clone (Aanen *et al.*, 2009).
C. In mature colonies, plant substrate is incorporated as follows: Older workers (1) collect substrate from outside the nest and pass this to younger workers, who eat the substrate along with asexual *Termitomyces* spores (2) produced in fungus comb nodules in the mature comb. This substrate–spore mix passes through the termite gut (first gut passage) and is deposited as fresh (young) comb (2). Within the comb, *Termitomyces* break down most plant components, such as cellulose, cellobiose and hemicellulose, and as the comb matures new nodules are produced (3). When the plant substrate is utilized, older termites eat the old comb (4, second gut passage), providing them with nutrition (adapted from Poulsen *et al.*, 2014). In contrast to the within-colony propagation of *Termitomyces*, the fungus is in most termite species transmitted between host generations through the production of spores from fruiting bodies (5). The termite host reproduces sexually through the production of alates (winged prospective queens and kings) that leave the colony (6) to initiate new colonies (for images, see Fig. 2).

important in some (Abo-Khatwa, 1978; Martin and Martin, 1978; Rouland *et al.*, 1988; Rouland-Lefèvre *et al.*, 1991; Matoub and Rouland, 1995; Hyodo *et al.*, 2003; Poulsen *et al.*, 2014), but not all (Veivers *et al.*, 1991) *Macrotermes* species. Similarly, *Termitomyces* from different *Odontotermes* species differ in cellulase expression (Ghosh *et al.*, 1980; Sengupta *et al.*, 1991; Sinha and Sengupta, 1995), and the termites themselves may be the main producers in other *Odontotermes* species (Yang *et al.*, 2004; Deng *et al.*, 2008). Thus, although the first genome of *Termitomyces* from *M. natalensis* showed a diverse set of 201 glycoside hydrolases in 48 families, implying that it can handle most plant components, we continue to have a fragmented understanding of the abilities (and variation in abilities) of *Termitomyces* species associated with different termite species.

The location where fungal enzymes are active has also been debated. The acquired enzyme hypothesis (Martin and Martin, 1978) suggested that *Termitomyces* cellulases and xylanases would complement endogenous termite enzymes within the termite gut, thus providing a direct benefit for the termites through intestinal cellulose breakdown making nutrients of plant origin available. However, fungal enzyme activity does not appear to be consistently high within the guts of different termite species (Abo-Khatwa, 1978; Rouland *et al.*, 1988;

Rouland-Lefèvre *et al.*, 1991; Veivers *et al.*, 1991; Slaytor, 1992; Bignell *et al.*, 1994). The ruminant hypothesis by Nobre and Aanen (2012) is a more likely alternative. This hypothesis suggests that fungal enzymes pass through the termite gut, so that the process of plant decomposition is initiated in the gut, but only completed as the substrate-spore mix is deposited within the fungus comb. This is consistent with the relatively fast first gut passage (Sieber and Leuthold, 1981; see below) and with enhanced concentrations of fungal enzymes in nodules (Martin and Martin, 1978; 1979).

Substrate preparation and comb inoculation

Efficient plant decomposition is obtained through an elaborate sequence of actions shaped through the combination of division of labour in workers of different ages and their associated gut symbionts (Fig. 1C). Older workers collect plant substrate, which is brought back to the nest and passed to younger workers, who eat the crude substrate along with asexual *Termitomyces* conidiospores produced in the mature fungus comb. This mix is deposited as the primary comb, in which *Termitomyces* develop dense hyphal networks and produce the next cohorts of nodules. This first gut passage is fast (Sieber and Leuthold, 1981) and serves to mix the substrate with *Termitomyces* spores to facilitate rapid mycelial growth within the comb (cf. Aanen *et al.*, 2009). Plant decomposition during this gut passage is thus likely to be minimal, but the young workers likely obtain nutrients by ingesting fungus material. Within the comb, *Termitomyces* grow by utilizing plant components, and they produce new nodules with asexual spores until, as the comb matures and the plant substrate is depleted, older termites consume the old comb in a second and final gut passage, which is slower and likely represents the time at which most termite nutrition is realized (Sands, 1960; Nobre *et al.*, 2011; Nobre and Aanen, 2012) (Fig. 1C). After this second gut passage, the final faeces is effectively deprived of organic material (Darlington, 1994). The manner of substrate incorporation, and the central placement of the termite gut in the decomposition process, is observed in the majority of fungus-growing termite species (Nobre *et al.*, 2011). However, workers in some genera, for example *Pseudacanthotermes*, consume the entire fungus comb before building a new one in an empty nest chamber (Rouland-Lefèvre, 2000), which could imply differences in gut microbiota roles.

The compositional shift in the fungus-growing termite gut microbiota

The compositional changes in the gut microbial communities co-occurred with the adoption of *Termitomyces* (Hongoh *et al.*, 2006; Dietrich *et al.*, 2014; Otani *et al.*, 2014) (Fig. 3), although many gut members are shared with other termites and cockroaches. In 2007, Shinzato and colleagues sequenced clone libraries from *Odontotermes formosanus* guts and found that nearly half of the clones were similar to ones reported from other termite guts. This was consistent with findings by Hongoh and colleagues (2006) in *Macrotermes gilvus*, Zhu and colleagues (2012) in *Macrotermes barneyi*, and in a recent study on *Odontotermes* and *Microtermes* (Makonde *et al.*, 2013). However, only more recent use of 16S rRNA amplicon 454-pyrosequencing (Dietrich *et al.*, 2014; Otani *et al.*, 2014) and metagenomics (Liu *et al.*, 2013; Poulsen *et al.*, 2014) have allowed for detailed insight into community structure and function.

Comparative analyses by Dietrich and colleagues (2014) of termite and cockroach guts supported earlier findings that members of the Macrotermitinae associate with a specific set of gut microbes. Otani and colleagues (2014) expanded to include guts from nine species (five genera) of fungus-growing termites and confirmed these findings (Fig. 3). Macrotermitinae gut communities are more similar to each other and to cockroach gut communities than to those of non-fungus-growing termites (Dietrich *et al.*, 2014; Fig. 3), and two main factors contribute to this pattern. First, cockroaches and fungus-growing termite guts are dominated by Firmicutes and Bacteroidetes, particularly the genera *Alistipes*, *Bacteroides*, *Desulfovibrio*, *Clostridium* and *Paludibacter* (Dietrich *et al.*, 2014; Otani *et al.*, 2014). Second, Spirochaetes, which typically dominate wood-feeding higher termite guts (Hongoh *et al.*, 2006; Warnecke *et al.*, 2007; Köhler *et al.*, 2012; Dietrich *et al.*, 2014), are low in abundance in both cockroaches and the Macrotermitinae.

The analyses by Dietrich and colleagues (2014) and Otani and colleagues (2014) indicate that gut communities of termite species belonging to the same genus tend to be more similar to each other than to communities from more distantly related species (Figs 3 and 4). This suggests that termite−*Termitomyces* co-diversification has led to adaptions in gut microbiotas (Figs 3 and 4). Otani and colleagues (2014) identified 42 abundant phylotypes, mainly from the Bacteroidetes and Firmicutes, which were present across the nine termite species, supporting that the shift in gut community composition likely co-occurred with the domestication of *Termitomyces*.

The functional shift in the fungus-growing termite gut microbiota

Natural selection is expected to have optimized the functional roles of gut microbiotas in the combined efforts of integrated gut passages and decomposition with the external fungus comb. Changes in the gut microbiota are

Fig. 2. (A) Mature *Macrotermes natalensis* colony in a South African landscape. (B) The underground fungus comb, in which workers maintain *Termitomyces* (photo by D. K. Aanen). (C) The royal chamber in which the queen and king reside. (D) A *Macrotermes natalensis* royal pair after the ceiling of the royal chamber has been removed. (E) Fruiting structures of *Termitomyces reticulatus* emerging from a colony of *Odontotermes badius* or *Odontotermes transvaalensis* (Wikipedia commons: http://en.wikipedia.org/wiki/Termitomyces).

likely to have been primarily driven by the shift from a strict plant-based to a largely fungus-based diet. The carbohydrate degradation potential of worker gut microbes in *Odontotermes yunnanensis* (Liu *et al.*, 2013) and *Macrotermes natalensis* (Poulsen *et al.*, 2014) has indeed shifted from targeting cellulose and hemicellulose to targeting less complex polysaccharides, consistent with *Termitomyces* being the primary lignocellulose degrader, while gut microbes finalize the decomposition initiated by *Termitomyces* by providing enzymes for complete breakdown of more simple polysaccharides.

The domestication of *Termitomyces* implied that gut communities became exposed to large quantities of fungal cell wall material. The enzymes necessary to break down fungal cell walls are present in both *O. yunnanensis* (Liu *et al.*, 2013) and *M. natalensis* (Poulsen *et al.*, 2014) gut bacteria. If the importance of *Termitomyces* as a food source varies across termite genera, the abundance of

chitinolytic genes, or at the least their levels of expression, should vary accordingly. However, fungilytic enzymes are coded for by the bacterial genera that dominate the subfamily core, suggesting that gut microbes across the termite subfamily target the fungal cell wall and that the fungilytic capacity is ancestral within the termite subfamily.

Towards an integrated understanding of *Termitomyces* and gut roles

Most inferences of the role of *Termitomyces* have been based on *in vitro* growth assays and characterizations of changes in lignocellulose content within the comb, limiting conclusions of what processes are specifically taken care of by *Termitomyces* under natural conditions. All studies support contributions to plant decomposition, but better characterization of *Termitomyces* functions will allow for a more comprehensive understanding of evolutionary
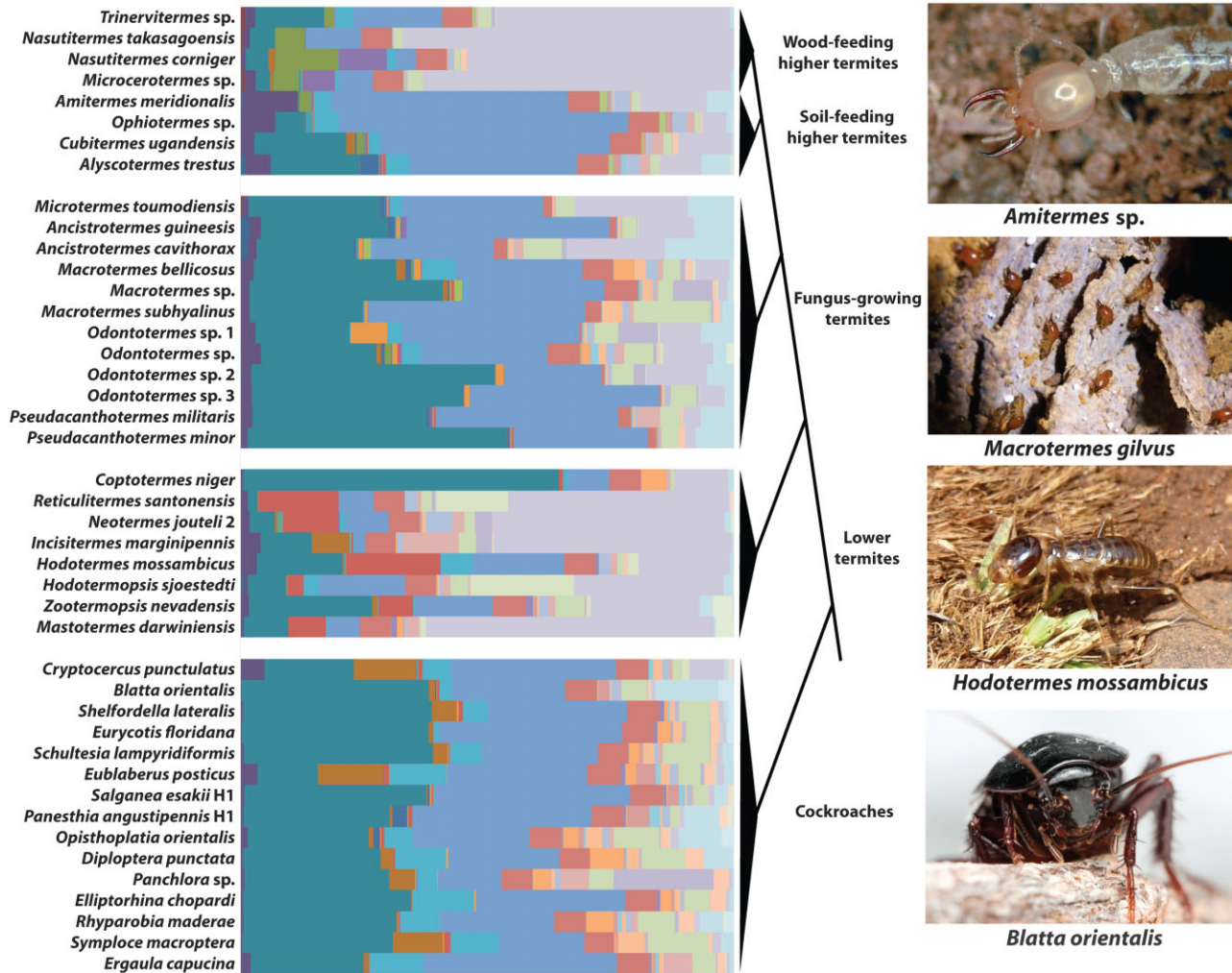
Fig. 3. The evolution of gut microbial compositions in the Isoptera. Class-level gut microbiota composition in cockroaches and termites, based on comprehensive 454-pyrosequencing of 16S rRNA (based on Dietrich *et al.*, 2014; Otani *et al.*, 2014). Isopteran species are listed according to phylogenetic placement (Inward *et al.*, 2007). The dominant classes in cockroaches are Bacteroidia (30.1%), Clostridia 1 (27.7%) and Clostridia 2 (6.4%), in lower termites Spirochaetes (35.6%), Bacteroidia (17.6%) and Clostridia 1 (10.6%), in fungus-growing termites Bacteroidia (30.2%), Clostridia 1 (29.6%) and Spirochaetes (8.3%), in soil-feeding higher termites Clostridia 1 (46.8%), Bacteroidia (10.7%) and Spirochaetes (9.3%), and in wood-feeding higher termites Spirochaetes (59.8%), Clostridia 1 (10.4%) and Bacteroidia (6.%). Images reproduced with permission from Alexander Yelich (*Amitermes* sp.), *Macrotermes gilvus* (www.termiteweb.com), *Hodotermes mossambicus* (Wikipedia commons: http://commons.wikimedia.org/wiki/File:Hodotermes_mossambicus,_c,_Schanskop.jpg) and *Blatta orientalis* (Dmitry Korol).

changes during termite–fungus co-evolution and help establish hypotheses of complementary gut functions. Genome and transcriptome sequencing serve as promising means to characterize *Termitomyces* functions (cf. Wymelenberg *et al.*, 2010; Cairns *et al.*, 2011; Poulsen *et al.*, 2014). By sampling across the diversity of the termite–*Termitomyces* association (Fig. 4), targeted comparative functional analyses of *Termitomyces* can be accomplished.

Connecting next-generation sequencing data with natural history dynamics of termite forage use and comb inoculation will allow us to advance our understanding of the evolution of *Termitomyces*-specific processes of plant

biomass degradation. Coupled with cross-phylogeny analyses of gut microbiota functions, such data have the potential to shed further light on the adaptive significance of gut microbiota changes since the origin of termite fungiculture. Metagenomic approaches have begun to test hypotheses of complementarity, but because only few species have been examined so far, comparative analyses that integrate functional characterization of complementary symbiont actions are needed to establish if co-evolutionary patterns characterize the symbiotic community.

Because *Macrotermes* and *Odontotermes* share the same mode of dual gut passage, with substrate inocula-
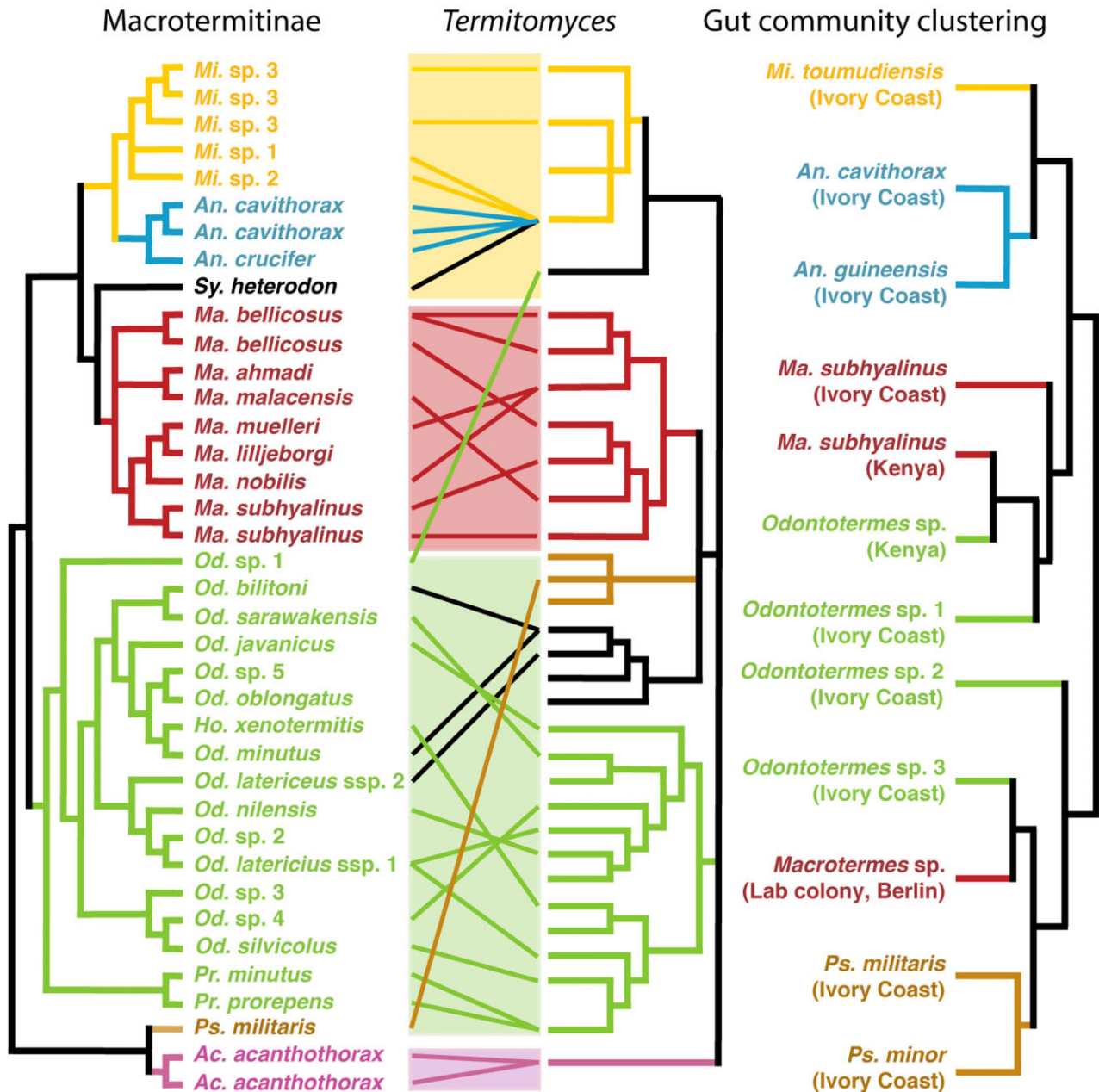
Fig. 4. Patterns of co-cladogenesis in the tripartite symbiosis. Schematic termite–*Termitomyces* co-phylogenies indicate levels of interaction specificity across the evolutionary history of the symbiosis (adapted from Aanen *et al.*, 2002). The right phylogram shows community similarities based on weighted Euclidean distances of the top 100 phylotypes identified using 16S rRNA pyrosequencing of fungus-growing termite worker guts (Ivory Coast species from Otani *et al.*, 2014; remaining species from Dietrich *et al.*, 2014). The cluster analysis is based on datasets using two different primer combinations, which could lead to differences in amplification biases towards or away from specific phylotypes.

tion in younger workers and old comb digestion in older workers, similarities in gut compositions and functions are expected. However, in termite genera where the role of the fungus is less well understood, other functions may apply. For example, Otani and collagues (2014) showed that termite species in the genus *Ancistrotermes*

harbour relatively high abundances of *Fibrobacter* (Fibrobacteres), Termite Cluster I (Fibrobacteres) and 'uncultured 23' (Acidobacteria), which are associated with cellulose breakdown in other termites (Hongoh *et al.*, 2006; Warnecke *et al.*, 2007). Microbial contributions to cellulose breakdown would support the hypothesis that

Termitomyces mainly serve as a nitrogen-rich food source in this termite genus (Rouland-Lefèvre *et al.*, 1991; Hyodo *et al.*, 2000; 2003).

The analyses by Otani and colleagues (2014) allowed for the identification of gut members that were either unique or uniquely abundant in individual termite genera or species, and these differences likely reflect particular adaptive gut functions, shaped by termite-specific metabolic needs, associations with specific *Termitomyces* lineages or specific diets. For example, the relatively unique presence of putatively cellulolytic *Ancistrotermes* gut symbionts implies the potential for shifts in division of labour between *Termitomyces* and lignocellulolytic microbes in this phylogenetically derived termite species. Similar examples of functional adaptions reflected by the presence of lineage-specific bacteria are inevitable, and their exploration will provide novel insight into the functions taken (or given) over by gut microbes from (or to) *Termitomyces*.

Understanding the functional implications of gut passage

Does the microbiota adapt to termite-age and caste-specific gut passages?

Termite sociality provides unique opportunities to tie caste-specific functions with gut composition and functional role, but we know remarkably little about differences in caste-specific gut microbial composition in termites in general. Integrating the roles of castes (minors and majors; workers and soldiers) of different ages and the mode of substrate incorporation will allow for a better understanding of age-specific caste polytheism and functional significance of caste-specific division of labour.

Only two studies have explored gut microbiota differences between castes. Hongoh and colleagues (2006) used Terminal-Restriction Fragment Length Polymorphism (T-RFLP) and 16S cloning of *Macrotermes gilvus* to show that gut communities clustered by termite caste rather than by colony and that termite age influenced gut community composition. Poulsen and collegues (2014) compared the phylogenetic distribution and carbohydrate active enzyme (CAZy) potential of gut microbes from major workers, minor soldiers and a queen in *M. natalensis*. These analyses revealed that major workers and minor soldiers have nearly identical gut compositions and plant decomposition potential, while the queen gut was reduced in microbial diversity and largely lacked CAZymes involved in plant decomposition.

Transmission of gut microbes between host termite generations remains largely a black box. Expectations are that gut microbes are transmitted when one or both of the founders pass gut microbes to the first workers via anal trophallaxis. As the royal pair matures, gut roles likely change, with conceivable effects on community composition, as indicated above for the compositional and functional difference observed in the queen of *Macrotermes natalensis* (Poulsen *et al.*, 2014). We are likely to identify such differences in caste-specific gut microbiotas, and further genomic and metagenomic analyses to examine gut community composition across castes, colonies and termite species are needed to link caste roles to relevant microbiota functions. Because caste differentiation with division of labour has been key in termites and in the eusocial Hymenoptera, this has broad implications for our understanding of the relationship between gut community function and the evolution of eusociality.

Nothing enters the comb before gut passage

Termitomyces are maintained as a monoculture, and it is continuously provided a substrate of decomposing wood for growth. Potential antagonists present within the decomposing plant substrate brought to the nest to manure *Termitomyces* could challenge the association; however, no virulent diseases have been identified. The only specific fungus known to frequently be present is the ascomycete *Pseudoxylaria* (Visser *et al.*, 2009), which does not appear to negatively affect fungus-growing termite colonies (Visser *et al.*, 2011). Other fungi have been isolated from fungus-growing termite combs (Mathew *et al.*, 2011; Um *et al.*, 2013) and termite guts (personal observations), but their possible impact remains unknown. Culture-independent work on gut and comb fungal communities could provide a better understanding of the identity and abundance of putative antagonists, and infection experiments should explore if antagonists have an impact on the success of termite fungiculture.

Because the substrate is not incorporated directly in the fungus comb, but first passes through the termite gut, selective inhibition of non-mutualists during gut passage may preclude the possibility that specialized parasites can enter and exploit the symbiosis. Sterilization, or at least reduction of potential antagonists, within the substrate could provide an adaptive explanation for the lack of specialized diseases. Termite antimicrobial peptides are present in *M. natalensis* (Poulsen *et al.*, 2014), and gut bacteria with antimicrobial properties could also selectively suppress antagonistic fungi (Um *et al.*, 2013, similar to defensive gut bacteria in other insects (Dillon and Charnley, 1995; Forsgren *et al.*, 2010). However, their importance in gut sanitation remains to be fully explored.

Actinobacteria and *Bacillus* (Firmicutes) have been suggested to perform protective functions in fungus-growing termites. Actinobacteria serve as defensive symbionts in many insect associations (for a recent review, see Kaltenpoth and Engl, 2014). Petri plate interaction assays evaluating the antibiotic properties of

fungus-growing termite gut Actinobacteria against *Pseudoxylaria* provided a first indication that these bacteria may act as defensive mutualists (Visser *et al.*, 2012). Carr and colleagues (2012) explored *in vitro* antibiotic properties of a set of Actinobacteria and discovered several novel antibiotics. Thus, targeted empirical examination of Actinobacteria as defensive symbionts in fungus-growing termites is likely to both be ecologically relevant and allow for identification of compounds of biomedical interest. Recent work has established that a clade of *Bacillus* likely suppress non-*Termitomyces* fungi in *Odontotermes formosanus* (Mathew *et al.*, 2011) and *M. natalensis* (Um *et al.*, 2013). *Bacillus* can be isolated from both the termite gut and fungus comb, but more extensive work on their location, antibiotics production and effects of the antibiotics on antagonists is needed. Thus, although specific defensive microbes appear to be present, thorough explorations of their effects on putative antagonists are needed.

Concluding remarks

Gut composition analyses in fungus-growing termites are accumulating, and by capturing 'who is there' we are gaining insight into the shift in the microbial community that coincided with the domestication of *Termitomyces*. However, the application of genomics tools that can address microbial roles has promising potential in fungus-growing termite gut research. Furthermore, functional testing, such as enzyme assays determining microbial contributions to the breakdown of carbohydrates, protein and chitin, is needed to better understand gut community functions. Combining this with a more extensive characterization of *Termitomyces*' role in the association, and the capacities coded for by genes in the termite host, will allow for a better understanding of the significance of combined and complementary adaptations within the symbiosis. Work to elaborate the complementary potential of the tripartite host–fungus–gut microbiota symbiosis should incorporate caste comparisons to establish how gut composition and function is integrated with age- and caste-specific termite roles, and comparative analyses of the ancient and diverse termite–*Termitomyces*–gut symbiosis to inform the dynamics of evolutionary processes that have shaped gut communities since the origin of termite fungiculture millions of years ago.

Acknowledgements

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