As you reap, so shall you sow: coupling of harvesting and inoculating stabilizes the mutualism between termites and fungi

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At present there is no consensus theory explaining the evolutionary stability of mutualistic interactions. However, the question is whether there are general 'rules', or whether each particular mutualism needs a unique explanation. Here, I address the ultimate evolutionary stability of the 'agricultural' mutualism between fungus-growing termites and *Termitomyces* fungi, and provide a proximate mechanism for how stability is achieved. The key to the proposed mechanism is the within-nest propagation mode of fungal symbionts by termites. The termites suppress horizontal fungal transmission by consuming modified unripe mushrooms (nodules) for food. However, these nodules provide asexual gut-resistant spores that form the inoculum of new substrate. This within-nest propagation has two important consequences: (i) the mutualistic fungi undergo severe, recurrent bottlenecks, so that the fungus is likely to be in monoculture and (ii) the termites 'artificially' select for high nodule production, because their fungal food source also provides the inoculum for the next harvest. I also provide a brief comparison of the termite–fungus mutualism with the analogous agricultural mutualism between attine ants and fungi. This comparison shows that—although common factors for the ultimate evolutionary stability of mutualisms can be identified—the proximate mechanisms can be fundamentally different between different mutualisms.

Keywords: fungus-growing termites; host–symbiont conflict; Macrotermitinae; mutualistic symbiosis; *Termitomyces*; transmission modes

1. INTRODUCTION

The existence of mutualisms poses a problem for evolutionary biology (Herre *et al.* 1999). Why should an organism benefit an individual of a different species, if this comes at a short-term cost (Maynard-Smith 1989)? This question is even more relevant if the symbiosis consists of a group of conspecific organisms and a single host. The reason is that in a group of symbionts that are beneficial to the host, genotypes that invest disproportionally in individual reproduction will be selected, even if this comes at a cost for the group of symbionts. This paper analyses the evolutionary stability of the mutualistic symbiosis between fungus-growing termites (subfamily Macrotermitinae, Termitidae, Isoptera) and fungi (genus *Termitomyces*, Basidiomycotina). This 'agricultural' mutualism is obligate for both partners: termites depend on the fungi for food, and the fungi depend on the termites for growth and protection. Despite this reciprocal dependence, reproduction of the two symbiotic partners occurs independently in most species. I address the specific question of how *Termitomyces* fungi distribute the resources made available to them by their termite hosts between returning benefits to the termites and their own individual reproduction. For the first time, a proximate mechanism is proposed on how termites influence this balance, and what ultimate consequences this has for the evolutionary stability of this mutualism.

(a) *Natural history of the mutualistic symbiosis between termites and fungi*

The fungus-growing termites maintain their symbionts on specially constructed fungus combs, which are built from dry, dead plant material that has quickly passed through the gut. The fungus combs are housed in single or multiple chambers inside a mound or dispersed in the soil (Darlington 1994; Aanen & Boomsma 2005). In all genera studied so far, the termite workers consume the nitrogen-rich fungal nodules that grow on the combs. These nodules are unripe mushrooms that are harvested long before they reach sexual maturity (Bathelier 1927; Heim 1977; De Fine Licht *et al.* 2005). Possibly as an adaptation to this early harvesting by termites, nodules contain asexual spores that survive gut passage and serve as inocula for newly constructed comb substrate (Leuthold *et al.* 1989). The continuous seeding with asexual spores allows rapid growth of a new mycelium and of new nodules, which are then consumed again. This mode of propagation is universal for the entire clade, although the details differ between genera (Thomas 1987; Leuthold *et al.* 1989).

In contrast to the asexual fungal propagation within a colony, symbiont transmission between colonies and across generations is sexual and horizontal. Occasionally, sexual fruiting bodies arise from nodules, and grow out of the top of termite colonies (Heim 1977). Those mushrooms are not consumed by the termites and produce sexual, wind-dispersed basidiospores, which form the inoculum of incipient colonies of fungus-growing termites. Most likely, foraging workers of incipient colonies collect *Termitomyces* spores (actively or passively) from their direct nest environment (e.g. Sands 1960; Johnson *et al.* 1981; Sieber 1983; De Fine Licht *et al.* 2005; for an overview, see Korb & Aanen 2003). This transmission mode is ancestral and has been maintained throughout the Macrotermitinae with only two known (independent) exceptions where vertical uniparental symbiont inheritance from a parental colony has evolved (Johnson *et al.* 1981; Aanen *et al.* 2002; Korb & Aanen 2003).
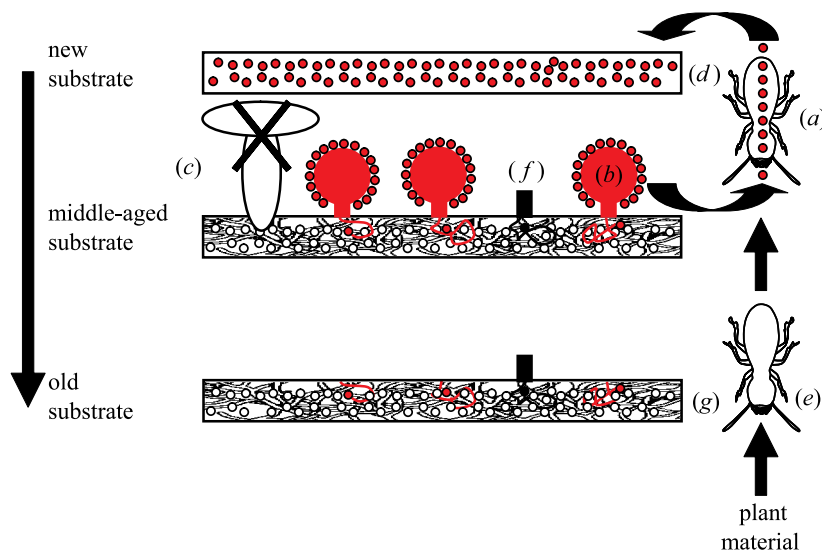
Figure 1. Schematic representation of the propagation of *Termitomyces* fungus within a *Macrotermes* termite colony based on Leuthold *et al.* (1989). Within a fungus garden young workers (*a*) consume nodules (*b*), which are modified unripe mushrooms, so that young termites suppress horizontal transmission (*c*). Nodules contain asexual, gut-resistant spores that are the inoculum for new substrate (*d*) constructed by young workers on top of the existing fungus garden from plant material collected by older workers (*e*). This propagation is associated with bottlenecks, since only a small fraction of the spores in one within-nest generation (shown in red) forms nodules, which produce all the inoculum for the next generation. Furthermore, there is selection for the production of nodules, as any non-nodule-producing mutant (shown in black) will be selected against (*f*). The lowest, oldest fragments (*g*) of fungus garden are consumed by older workers so that there is a continuous turn over of material from top to bottom within a fungus garden.

2. CONFLICTS OF INTERESTS BETWEEN TERMITES AND *TERMITOMYCES*

Conflicts of interests between the mutualistic partners exist at two levels. First, although the termites and fungi are obligately dependent on each other within a colony, horizontal transmission of symbionts across generations implies a decoupling of sexual reproductive interests. The production of inedible, costly mushrooms for horizontal transmission of spores, is not in the termites' short-term interest (Korb & Aanen 2003), whereas the resident fungal symbiont has no interest in the termites producing winged dispersing reproductives instead of workers that would provide them with more growth substrate.

Second, fungi are modular organisms consisting of totipotent cells. Genetic variation within a fungal colony can arise either by mutation or horizontal acquisition of new symbionts. This creates the possibility for competition between genetically different fungal strains for propagation and transmission, selecting for fast horizontal transmission and increased antagonism. The likely trade-off between those competitive traits and the success of the group relative to other groups (Frank 1996; Taylor & Frank 1996) implies that genetic variation creates a conflict between two levels of selection, *viz.* within and between groups of fungal symbionts. Thus, we expect that the termite hosts should minimize symbiont genetic variation if competitive interactions between symbiont strains would reduce group productivity (Frank 1996; Korb & Aanen 2003).

3. CONFLICT RESOLUTION BY WITHIN-COLONY SELECTION FOR FUNGAL PRODUCTIVITY

In fungus-growing termites consumption of nodules is directly coupled with inoculation of asexual spores

growing on those nodules. I propose that this within-nest fungus propagation has three important repercussions for the ultimate long-term stability of the mutualism, one direct and two indirect (illustrated in figure 1). A direct consequence of the within-nest fungus propagation is that termites, by eating the nodules (long before they would develop into ripe mushrooms) actively suppress horizontal transmission in their own best interest. However, the ensuing within-nest propagation of the asexual fungal spores has two additional indirect effects with important ultimate consequences. First, the intra-nest transmission regime is associated with severe recurrent bottlenecks, since only a small fraction of the spores of the previous crop produces all the spores inoculated in the newly formed comb. This will reduce intra-nest genetic variation originating from mutation or from multiple symbiont acquisition and lead to an increase in the average within-nest relatedness (e.g. in figure 1, the entire mass of inoculated spores for the next within-nest crop is produced by just three nodules descending from only three spores inoculated in the previous crop). Second, since harvesting and inoculation are coupled, termites 'artificially' select for high nodule production. If a mutant arises that produces fewer or no nodules, it will automatically be selected against as it will be underrepresented in the inoculum of the next crop (the black spore in figure 1).

4. DISCUSSION

I have proposed that within-nest propagation underlies the evolutionary stability of the mutualism between termites and fungi. An attractive property of this proposed scenario is the immediate pay-off. Termites that consume nodules block the loss of resources that would go to fruiting bodies and immediately benefit by

Table 1. Differences and similarities in between-nest and intra-nest propagation between fungus-growing termites and fungus-growing ants.

	fungus-growing termites	fungus-growing ants
between-nest fungal symbiont transmission mode	sexual, horizontal (with two known exceptions of clonal vertical uniparental transmission)	vertical, clonal, uniparental, occasionally horizontal
intra-nest propagation of fungus	asexual, via spores	asexual, via mycelial growth
number of fungal strains per nest	single-strain monoculture (needs to be confirmed)	single-strain monoculture
how is monoculture achieved?	fungal symbiont undergoes recurrent bottlenecks	vertical uniparental transmission, in combination with active rejection of genetically different clones by resident fungal clone

obtaining food (Darlington 1994). However, besides this direct effect, the consumption of nodules has two additional indirect effects, which I argue have long-term consequences. First, the recurrent bottlenecking of symbionts increases the relative importance of the group component of the symbionts' fitness via kin selection over the individual fitness component (Frank 1996). This would imply that the fungal symbionts have not been selected to invest in antagonistic traits, but this prediction remains to be tested. The few studies that have addressed within-nest variation of fungal symbionts, have found single cultures within single nests (Aanen *et al*. 2002; Katoh *et al*. 2002), consistent with the hypothesis that recurrent bottlenecking within nests reduces fungal within-nest variation. However, more research is needed to confirm this hypothesis, especially for species with multiple separate fungus combs per nest. If migration of workers between combs is limited and fungus gardens are relatively isolated, different *Termitomyces* strains may coexist within a nest but be distributed over different gardens. A second effect of nodule consumption is that continuously 'artificial' selection takes place for the production of nodules. This can be considered as an example that belongs to the category of 'sanctioning' (Denison 2000; West *et al*. 2002), or rather its flip-side, 'rewarding'. Nodule-producing genotypes are rewarded, while non-nodule-producing genotypes are automatically being sanctioned.

(a) *The termites exploit selfish traits of the fungus*
Nodules are modified mushrooms, so their original function is a means of horizontal transmission of the fungus. Asexual spores are rarely found on unripe mushrooms of other basidiomycete fungi (Clémençon 1997), so it is likely that the production of those spores in *Termitomyces* is an adaptation to the symbiosis with termites. Interestingly, therefore, an originally selfish or virulent (*sensu* Frank 1996) trait of the fungi, the production of mushrooms for horizontal spread, is being exploited and even selected for by fungus-growing termites and has become an integrated part of the symbiosis. The resources that—from a termite colony's point of view—would have been lost to the production of fruiting bodies are being redirected into the colony in two different ways: first, as investment into the colony via asexual spores and, second, as food for termites. Notably, suppression of *Termitomyces* fruiting by termites is not

perfect, since horizontal transmission is the regular transmission mode for newly established colonies in most species, and most species of *Termitomyces* irregularly succeed in reproducing (e.g. Heim 1977; Darlington 1994). Data indicate that fruiting usually occurs a few weeks after the termite nuptial flight period (Johnson *et al*. 1981; Darlington 1994). These flights are usually synchronous and may mean that 40% of the colony biomass disperses (Wood & Sands 1978). This implies that a colony may not consume all nodules in this period, which may be the proximate explanation for the occurrence and timing of sexual fruiting of *Termitomyces*.

(b) *A comparison with the attine ant–fungal mutualism*
Also in an analogous agricultural mutualism between attine ants and fungi, the insects cultivate their fungus in single-strain monocultures (Mueller *et al*. 1998, 2005; Poulsen & Boomsma 2005). However, the mechanism whereby this is achieved is completely different from the mechanism for termites proposed here (table 1). The fungi of the attine ants are propagated clonally within nests (without a spore phase), and transmission between generations occurs via vertical, uniparental and clonal inheritance from the parental colony. Vertical transmission minimizes the initial genetic diversity among symbionts, which is to the benefit of the ants. However, occasionally horizontal transmission of fungal symbionts occurs (Bot *et al*. 2001; Poulsen & Boomsma 2005) so that different fungal strains could secondarily colonize a single ant colony. Recently, it has been established that the resident fungal clone in a colony actively rejects genetically different clones, and thereby prevents the coexistence of multiple strains (Bot *et al*. 2001; Poulsen & Boomsma 2005). The proximate mechanism whereby single-strain monoculture is achieved in fungus-growing ants and termites is thus fundamentally different between those convergent examples of agricultural mutualism.

5. CONCLUSION
The present examination of the evolutionary stability of the mutualism between termites and fungi confirms the importance of some previously proposed general factors for the evolutionary stability of mutualisms, such as kin selection within groups of closely related symbionts, and sanctioning of non-mutualistic

symbionts (e.g. Frank 1996, 2003; Herre *et al.* 1999; West *et al.* 2002). However, an important consideration arising from a comparison between two analogous agricultural mutualisms is that those general 'rules' are contingent upon the natural history details of the 'players' in the mutualism.

Aanen, D. K. & Boomsma, J. J. 2005 Evolutionary dynamics of the mutualistic symbiosis between fungus-growing termites and *Termitomyces* fungi. In *Insect–fungal associations: ecology and evolution* (ed. F. Vega & M. Blackwell), pp. 191–211. Oxford: Oxford University Press.

Aanen, D. K., Eggleton, P., Rouland-Lefèvre, C., Guldberg-Frøslev, T., Rosendahl, S. & Boomsma, J. J. 2002 The evolution of fungus-growing termites and their mutualistic fungal symbionts. *Proc. Natl Acad. Sci. USA* **99**, 14 887–14 992. (doi:10.1073/pnas.222313099)

Bathellier, J. 1927 Contribution à l' etude systématique et biologique de termites de l'Indo-Chine. *Faune Colonies Franc* **1**, 125–365.

Bot, A. N. M., Rehner, S. A. & Boomsma, J. J. 2001 Partial incompatibility between ants and symbiotic fungi in two sympatric species of *Acromyrmex* leaf-cutting ants. *Evolution* **55**, 1980–1991.

Clémençon, H. 1997 Anatomie der Hymenomyceten (Anatomy of Hymenomycetes). Teufen: F. Flück-Wirth.

Darlington, J. E. C. P. 1994 Nutrition and evolution in fungus-growing ants. In *Nourishment and evolution in insect societies* (ed. J. H. Hunt & C. A. Nalepa), pp. 105–130. Boulder: Westview Press.

De Fine Licht, H. H., Andersen, A. & Aanen, D. K. 2005 *Termitomyces* sp. associated with the termite *Macrotermes natalensis* has a heterothallic mating system and multi-nucleate cells. *Mycol. Res.* **309**, 314–318. (doi:10.1017/S0953756204001844)

Denison, R. F. 2000 Legume sanctions and the evolution of symbiotic cooperation by rhizobia. *Am. Nat.* **156**, 567–576. (doi:10.1086/316994)

Frank, S. A. 1996 Host symbiont conflict over the mixing of symbiotic lineages. *Proc. R. Soc. B* **263**, 339–344.

Frank, S. A. 2003 Repression of competition and the evolution of cooperation. *Evolution* **57**, 693–705.

Heim, R. 1977 *Termites et champignons*. Paris: Société nouvelle de Èditions boubée.

Herre, E. A., Knowlton, N., Mueller, U. G. & Rehner, S. A. 1999 The evolution of mutualisms: exploring the paths between conflict and cooperation. *Trends Ecol. Evol.* **14**, 49–53. (doi:10.1016/S0169-5347(98)01529-8)

Johnson, R. A., Thomas, R. J., Wood, T. G. & Swift, M. J. 1981 The inoculation of the fungus comb in newly founded colonies of the Macrotermitinae (Isoptera) from Nigeria. *J. Nat. Hist.* **15**, 751–756.

Katoh, H., Miura, T., Maekawa, K., Shinzato, N. & Matsumoto, T. 2002 Genetic variation of symbiotic fungi cultivated by the Macrotermitine termite *Odontotermes formosanus* (Isoptera: Termitidae) in the Ryukyu Archipelago. *Mol. Ecol.* **11**, 1565–1572. (doi:10.1046/j.1365-294X.2002.01535.x)

Korb, J. & Aanen, D. K. 2003 The evolution of uniparental transmission of fungal symbionts in fungus-growing termites (Macrotermitinae). *Behav. Ecol. Sociobiol.* **53**, 65–71.

Leuthold, R. H., Badertscher, S. & Imboden, H. 1989 The inoculation of newly formed fungus comb with *Termitomyces* in *Macrotermes* colonies (Isoptera Macrotermitinae). *Insect. Soc.* **36**, 328–338. (doi:10.1007/BF02224884)

Maynard Smith, J. 1989 Evolution-generating novelty by symbiosis. *Nature* **341**, 284–285. (doi:10.1038/341284a0)

Mueller, U. G., Rehner, S. A. & Schultz, T. D. 1998 The evolution of agriculture in ants. *Science* **281**, 2034–2038. (doi:10.1126/science.281.5385.2034)

Mueller, U. G., Gerardo, N. M., Aanen, D. K., Six, D. L. & Schultz, T. R. 2005 The evolution of agriculture in insects. *Annu. Rev. Ecol. Syst.* **36**, 563–595.

Poulsen, M. & Boomsma, J. J. 2005 Mutualistic fungi control crop diversity in fungus-growing ants. *Science* **307**, 741–744.

Sands, W. A. 1960 The initiation of fungus comb construction in laboratory colonies of *Ancistrotermes guineensis* (Silvestri). *Insect. Soc.* **7**, 251–259. (doi:10.1007/BF02224496)

Sieber, R. 1983 Establishment of fungus comb in laboratory colonies of *Macrotermes michaelseni* and *Odontotermes montanus* (Isoptera Macrotermitinae). *Insect. Soc.* **30**, 204–209. (doi:10.1007/BF02223870)

Taylor, P. D. & Frank, S. A. 1996 How to make a kin selection model. *J. Theor. Biol.* **180**, 27–37. (doi:10.1006/jtbi.1996.0075)

Thomas, R. J. 1987 Distribution of *Termitomyces* Heim and other fungi in the nests and major workers of several Nigerian Macrotermitinae. *Soil Biol. Biochem.* **19**, 335–341. (doi:10.1016/0038-0717(87)90019-8)

West, S. A., Kiers, E. T., Simms, E. L. & Denison, R. F. 2002 Sanctions and mutualism stability: why do rhizobia fix nitrogen? *Proc. R. Soc. B.* **269**, 685–694. (doi:10.1098/rspb.2001.1878)

Wood, T. G. & Sands, W. A. 1978 The role of termites in ecosystems. In *Production ecology of ants and termites* (ed. M. V. Brian), pp. 245–292. Cambridge, UK: Cambridge University Press.