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the globally time-transgressive wave of late Pleistocene extinctions closely tracks human colonization history. Butchered mammoth bones excavated in southeastern Wisconsin date regional human presence to between 14.8 and 14.1 ka (29), coeval with the *Sporormiella* decline at Appleman Lake.

At all sites, the *Sporormiella* decline substantially predated initiation of the Younger Dryas, ruling out abrupt climatic cooling and the hypothesized extraterrestrial impact at 12.9 ka as a cause (9). More generally, the megafaunal declines apparently progressed over several thousand years, given the 1000-year duration of the *Sporormiella* decline at Appleman and the difference in timing between the onset of the *Sporormiella* decline (14.8 ka) and the final extinctions (~11.5 ka) (1). This evidence excludes rapid-extinction hypotheses such as an extraterrestrial impact or a Paleo-Indian blitzkrieg (30).

Our work thus shows close connections among the late-glacial histories of fire, vegetation, and mammalian herbivores and suggests that the loss of a broad guild of consumers contributed to substantial restructuring of plant communities and an enhanced fire regime. The sequence of events at Appleman rules out several hypothesized causes and effects of the megafaunal extinction but does not conclusively resolve the debate over climatic versus human causation (or both) of the North American megafaunal extinctions. However, several promising avenues exist. One is to search for spatial and temporal patterns in the late Pleistocene *Sporormiella* decline (time-transgressive versus synchronous) and to further check its association with vegetation and fire history. Another is to analyze the *Sporormiella* record at sites spanning

the penultimate deglaciation (when humans were absent from North America) and in sites near well-dated records of Paleo-Indian activity. Such analyses should be extended to other continents, to study the ecological effects of the end-Pleistocene extinctions under different contexts of human, climate, and vegetation history (31). By resolving the causes and consequences of the late Pleistocene megafaunal extinctions, such work would address concerns about trophic effects arising from the contemporary widespread declines, extinctions, and restorations of megaherbivores.

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Supporting Online Material

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High Symbiont Relatedness Stabilizes Mutualistic Cooperation in Fungus-Growing Termites

Duur K. Aanen,^{1*} Henrik H. de Fine Licht,² Alfons J. M. Debets,¹ Niels A. G. Kerstes,¹ Rolf F. Hoekstra,¹ Jacobus J. Boomsma²

It is unclear how mutualistic relationships can be stable when partners disperse freely and have the possibility of forming associations with many alternative genotypes. Theory predicts that high symbiont relatedness should resolve this problem, but the mechanisms to enforce this have rarely been studied. We show that African fungus-growing termites propagate single variants of their *Termitomyces* symbiont, despite initiating cultures from genetically variable spores from the habitat. High inoculation density in the substrate followed by fusion among clonally related mycelia enhances the efficiency of spore production in proportion to strain frequency. This positive reinforcement results in an exclusive lifetime association of each host colony with a single fungal symbiont and hinders the evolution of cheating. Our findings explain why vertical symbiont transmission in fungus-growing termites is rare and evolutionarily derived.

Horizontal symbiont transmission is the rule in ancient and ecologically important mutualisms, such as those between plants and mycorrhizas or nitrogen-fixing bacteria. The stability of such interactions is puzzling, because multiple symbiont lineages compete for host re-

sources, and hosts have frequent opportunities to exchange resident symbionts with new and potentially superior lineages (1–3). Here we address the evolutionary stability of an analogous animal-microbial mutualism in the fungus-growing termites (subfamily Macrotermitinae), which coevolved

with a single genus of basidiomycete fungi, *Termitomyces*, while retaining horizontal symbiont transmission in most genera (4).

The termite-fungus mutualism is of major ecological importance in Old World tropical regions for decomposition and mineral cycling (5). The termites cultivate their fungal symbiont in well-protected gardens inside the nests on a substrate (comb) of predigested plant material (Fig. 1A). The mutualistic fungus provides most of the termite food, both directly, when termites eat nodules of fungal material containing asexual spores (Fig. 1B), and indirectly, when they ingest the partially degraded comb biomass later on (6, 7). The symbiosis has a single African rain forest origin, more than 30 million years ago (8–11) and has radiated into 10 extant genera with about 330 described species (12).

No reversals to a solitary life-style are known (4). This is remarkable, as partners in most genera have retained independent reproduction by means

¹Laboratory of Genetics, Wageningen University, 6700 AH Wageningen, Netherlands. ²Centre for Social Evolution, Department of Biology, University of Copenhagen, 2100 Copenhagen, Denmark.

*To whom correspondence should be addressed. E-mail: duur.aanen@wur.nl

of termite swarming and growing mushrooms to disperse sexual spores. New colonies therefore need to acquire their garden symbionts *de novo* by collecting spores from the habitat (13). Similarly to most basidiomycetes, *Termitomyces* life cycles alternate between a homokaryotic and heterokaryotic stage, having one and two genetically different types of haploid nuclei, respectively (14), and fungus garden mycelium is heterokaryotic in all natural colonies studied so far. This implies that incipient termite colonies must be colonized by at least two compatible, and genetically different, sexual spores and that the homokaryotic stage is relatively short-lived [(14–16) and this study].

Following recently developed theory (1, 17, 18), we hypothesized that fungus-growing termites have evolved mechanisms to increase relatedness among symbionts to overcome the genetic heterogeneity of symbionts incurred at the start of a colony cycle. High symbiont relatedness reduces competition among strains and will therefore select for prudent horizontal transmission, causing minimal harm to the host (1). However, high symbiont relatedness needs to serve the short-term interests of both the fungi and the termites to remain stable over evolutionary time (1), and it has remained unclear how that might be achieved.

We used a highly variable intron of the single-copy gene *Elongation Factor 1 alpha* (16) to characterize symbiont diversity in multiple nests of *Macrotermes natalensis* (113 fungal samples collected from 49 different nests), *Odontotermes transvaalensis* (11 fungal samples from seven nests), and *Odontotermes badius* (13 samples

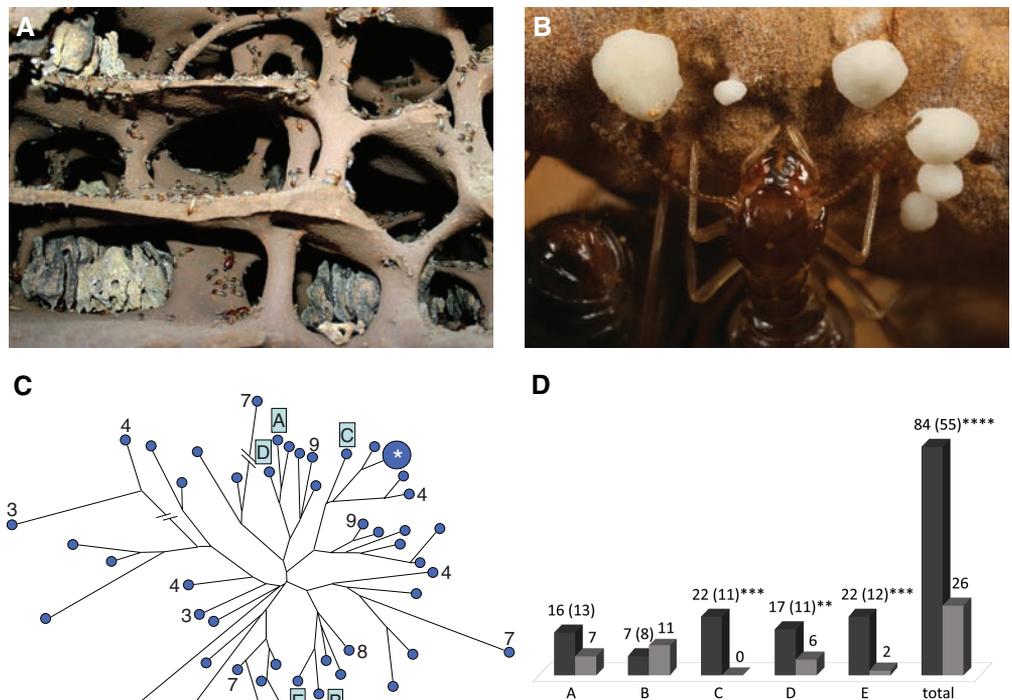
from seven nests) (19). All but 2 of the 49 *Macrotermes natalensis* nests had a genetically unique strain of fungus (Fig. 1C), but we found no variation among multiple samples taken from the same nest. The difference between among- and within-colony diversity was highly significant ($P < 0.00001$) (19), and the detection power for genotypic diversity in a sample of this size was high (19). Similarly, we found genetic variation among symbionts from different nests in the two *Odontotermes* species, but never within nests. It therefore appears that *Termitomyces* is cultivated in single-strain monocultures (4, 20).

We undertook a series of *in vitro* experiments on agar plates to simulate within-nest propagation of *Termitomyces*, using similarly high densities of asexual spores as in natural comb substrate (fig. S1). The propagation cycle starts with termites harvesting modified unripe mushrooms or nodules (Fig. 1B) containing asexual spores (conidia) (21, 22). The spores are ingested, mixed with fragmented plant material, and homogeneously deposited as primary feces to build new layers of fungus comb. We harvested nodules from petri dish cultures and suspended the conidia in sterile water then used this suspension to inoculate fresh agar plates, emulating the termite fecal deposition behavior (fig. S1). The strains used were isolated from five colonies of *Macrotermes natalensis* (Fig. 1C), a species associated with a single species of *Termitomyces* (14–16). To investigate whether competing strains facilitate some form of positive reinforcement that would result in single-strain monocultures, we inoculated mixtures of five strains such

that one strain provided 50% of the spores, while the remaining four strains made up 12.5% each. The results were consistent with positive frequency-dependent selection enhancing the representation of the majority inoculum strain among the asexual spores produced by the next cohort of nodules ($\chi^2 = 30.45$; $df = 1$; $P < 0.0001$; three of the five combinations, $P < 0.01$) (Fig. 1D).

We also compared the yield of asexual spores from monocultures of the five strains with that of mixed cultures with varying strain frequencies in high-density cultures and found that monoculture fungus farming gives a significantly higher yield than mixed cultures (Fig. 2, A and B). Next, we tested whether the scale of asexual spore production is dependent on the extent of successful fusion between clonally related mycelia, because it is well established that successful fusion between basidiomycete heterokaryons only happens when mycelia are clonally related, whereas fusion between genetically different clones is invariably followed by cell death at the interface of interacting mycelia (23). The frequency of successful fusion in a mixture of genetically different strains should therefore be lower than in a monoculture because clonal patches are smaller (fig. S4). However, this would not necessarily increase yield in monocultures, because fungal reproduction is often triggered by contact between genetically different mycelia [reviewed in (24)]. Our experimental results resolved this paradox by showing that average symbiont relatedness is indeed negatively correlated with the number of nodules produced (Fig. 2D), but positively correlated with average nodule size:

Fig. 1. Fungus cultivation by macrotermite termites. (A) The inside of a colony of *Macrotermes natalensis* in South Africa. Fungus gardens are arranged in an interconnected network of chambers, each containing a mass of masticated plant substrate in which *Termitomyces* grows. (B) Close-up of a fungus garden of a *Macrotermes* sp. with multiple asexual fruiting bodies (nodules). The spores survive passage through the termite gut and are mixed with predigested plant material to be deposited as inoculated fresh garden substrate (21, 22). (C) Genetic diversity of *Termitomyces* symbionts associated with *Macrotermes natalensis* in South Africa. Each circle represents a fungal genotype. Only two colonies shared the same fungal genotype [larger circle marked with an asterisk (*)]. No within-colony variation was found in 13 multiply sampled nests (number of samples per nest indicated; $n = 5.9$ samples on average). (D) Experimental demonstration of positive frequency-dependent selection on *Termitomyces* strains in mixtures of asexual spores indicated by letters in (C) with one of them being inoculated at 50% (the x axis label strain and the darker bars) and the others at 12.5% each (pooled in the lighter bars). The expected frequencies of the dominant strain without frequency-dependent selection are given in parentheses. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.



The mixed cultures have about three times as many nodules, but their total spore production is only ~15% of what monoculture nodules produce [(19) and supporting online text] (fig. S4). This shows that coordinated reproduction after successful fusion of neighboring mycelia belonging to the same clone is more efficient because mycelia mergers allow “division of labor” between mycelial growth and asexual reproduction.

The higher yield of *Termitomyces* monocultures relative to mixed cultures is caused by two effects: (i) synergism between successfully fusing mycelia and (ii) lack of supposedly costly cell death in monocultures. In some fungi (e.g., the *Leucoagaricus* associated with fungus-growing ants), cell death after unsuccessful fusion of mycelia is followed by further antagonistic destruction of mycelium on both sides (25), but this effect is not always found when genetically different mycelia of free-living fungi meet [compare (23) and (26)]. When we tested this, we found that there were no visible zones of mycelial antagonism in plated cultures of genetically different *Termitomyces* strains (Fig. 2C). Fungal propagation by termites thus provides a positive-feedback mechanism that leads to monocultures within colonies in spite of promiscuous acquisition of multiple symbionts during colony foundation. This process reinforces the occurrence of genetic bottlenecks, which also happens at each

crop-rotation cycle within colonies (21), and stabilizes the mutualism, as it maximizes the short-term fitness interests of both termites and fungal crops.

Our results show that the comparative study of the convergently evolved fungus-farming systems of the attine ants (27–29) and the macrotermite termites (4, 5, 30) provides insight into the general principles that govern the stability of obligate ectosymbiotic mutualisms. In both systems, the fungal symbionts are reared as single-strain monocultures, in spite of substantial genetic variation among fungal clones across colonies [(14–16) and this study], but this common characteristic is independent of the default symbiont transmission mode (vertical in the ants and horizontal in the termites) [(24) and this study]. Rather than transmission mode, it appears that the lifetime commitment between each farming society and a specific clonal crop is decisive for making both these fungus-farming mutualisms evolutionarily stable. Lifetime commitment is an extreme form of partner fidelity (3) and removes every incentive for cheating by reducing symbiont performance. However, this does not imply that host-symbiont conflict over transmission by independent reproduction cannot be further reduced, so that the occasional secondary evolution of vertical, uniparental symbiont inheritance in two genera of fungus-growing termites is not surprising (13, 19).

Our results imply that the *Termitomyces* farming symbiosis has both a very specific process for mediating partner choice and an extreme degree of partner fidelity. These two mechanisms have featured prominently in theoretical models to explain the evolutionary stability of mutualisms with horizontal, promiscuous partnership exchange (2, 3, 31), but as alternative pathways, rather than causally connected ones. Our study shows that a recurrent process of partner choice can enforce lifetime partner fidelity by direct positive reinforcement, a result that offers general insight into the mechanisms that stabilize promiscuous mutualisms. To appreciate this, it is important to realize that termite colonies with *Termitomyces* symbionts sequester their resource patches for symbiont growth inside the same insect society, so that they also control inoculation. It is the combination of these characteristics that allows positive frequency-dependence to enforce symbiont clonality, in spite of promiscuous acquisition and occasional recolonization by other strains. Lifetime partner fidelity is the inevitable consequence, and this eliminates evolutionary incentives for symbiont cheating (parasitic strains of *Termitomyces* are neither known nor expected).

This unique combination of characteristics is not found in other promiscuous mutualisms such as *Rhizobia* (32), arbuscular mycorrhizae (33), or corals (34). These hosts sequester their compartments for symbionts sequentially during growth and cannot directly control the colonization of new compartments, as the termite fungus farmers can. This lack of host control at the level of single compartments is the crucial difference that allows the persistence of cheating variants. Compartmentalization [reviewed in (35)] has repeatedly been suggested as a possible mechanism to reduce cheating in promiscuous mutualisms, because it induces high local relatedness (1, 17, 18), but our study experimentally confirms the necessity of a direct positive-reinforcement mechanism at the same single-compartment scale to ensure the monopoly of a single symbiont strain.

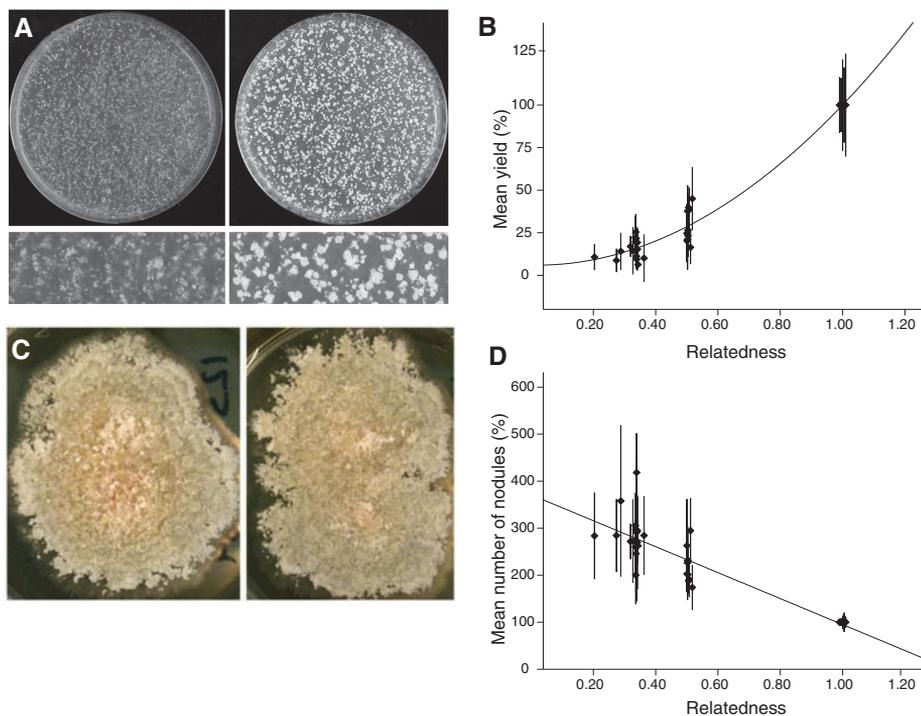


Fig. 2. The effects of symbiont relatedness on fungal spore yield and nodule number. **(A)** Representative examples of monoculture (right) and mixed culture of five strains (left) after we inoculated ~30,000 asexual spores per petri dish; (bottom) close-ups of plates. Normalized yield **(B)** and number of nodules **(D)** (relative to monocultures set at 100%) (19) against symbiont relatedness, the probability that two spores in a mixture are clonally related. Dots are averages of five replicates, bars are 95% confidence limits, and the lines are regressions (quadratic for yield, $R^2 = 0.956$, $P < 0.0001$; linear for nodule number, $R^2 = 0.735$, $P < 0.0001$) (19). **(C)** No direct mycelial antagonism was observed between two genetically different fungal strains inoculated on the same plate (left), relative to two strains of the same clone (right); the figure is a typical example of 21 strains tested in all 231 possible combinations (19).

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Figs. S1 to S4

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Epicontinental Seas Versus Open-Ocean Settings: The Kinetics of Mass Extinction and Origination

Arnold I. Miller^{1*} and Michael Foote²

Environmental perturbations during mass extinctions were likely manifested differently in epicontinental seas than in open-ocean-facing habitats of comparable depth. Here, we present a dissection of origination and extinction in epicontinental seas versus open-ocean-facing coastal regions in the Permian through Cretaceous periods, an interval through which both settings are well represented in the fossil record. Results demonstrate that extinction rates were significantly higher in open-ocean settings than in epicontinental seas during major mass extinctions but not at other times and that origination rates were significantly higher in open-ocean settings for a protracted interval from the Late Jurassic through the Late Cretaceous. These patterns are manifested even when other paleogeographic and environmental variables are held fixed, indicating that epicontinental seas and open-ocean-facing coastlines carry distinct macroevolutionary signatures.

Throughout the global history of Phanerozoic marine life, patterns of diversification and extinction varied substantially among different regions such as paleocontinents (1–4) and latitudinal belts (5). However, the primary geographic and environmental dichotomy recognized among ancient shallow-marine settings is the distinction between epicontinental seas, which covered broad regions of ancient continents during much of the Paleozoic era but began to wane thereafter, and open-ocean-facing coastlines, which became increasingly important through the Mesozoic and Cenozoic eras and rim present-day continents (6, 7). Because they extended over unusually broad areas with water depths typically less than 100 m, epicontinental seas were likely characterized by sluggish circulation in comparison to coastal settings of comparable depths that

faced the open ocean (8, 9), and this may have had important ramifications for taxonomic origination and extinction (6, 10). Potential mass extinction agents were probably manifested differently in the two settings. In epicontinental seas, for example, drops in sea level would have been more lethal because of the rapid subaerial exposure of unusually large expanses of shallow seafloor (11, 12), and biotas there may also have been more susceptible to bottom-water anoxia because of relatively poor circulation and enhanced stratification (13). By contrast, shallow open-ocean-facing settings may have been affected more profoundly in cases where waterborne lethal effects emanated from point sources, such as impacts or volcanic events, the propagation of which may have been inhibited in epicontinental seas because of sluggish circulation.

Here, we contrast the kinetics of extinction and origination in epicontinental seas versus shallow ocean-facing settings during the Permian through the Cretaceous periods, an interval through which both settings are well represented in the fossil and sedimentary records. Global occurrences

of marine genera were extracted from the Paleobiology Database [PaleoDB (14)] for a set of stage-level stratigraphic units that span the study interval. We used global paleogeographic maps (e.g., fig. S1) to demarcate the boundaries of epicontinental seas for each stage (15), and we mapped individual occurrences of marine genera in each stage with respect to these two settings on the basis of their paleogeographic locations. These, in turn, were used to parse the roster of 7868 genera into those with affinities for either epicontinental seas or ocean-facing settings in cases where a statistical preponderance of occurrences for a given genus was located in one regime or the other (15, 16). In all, 3432 genera were assignable on this basis, and, of these, 3418 had first and last appearances that were stratigraphically resolvable to the stage level. Patterns of extinction and origination were then compared stage by stage through the study interval for genera with affinities for each of the two regimes, on the basis of their first and last appearances as depicted in the PaleoDB.

Comparative per capita extinction rates (17) through the study interval (Fig. 1A) document a striking pattern. Whereas there was virtually no difference in average extinction intensity between the two regimes, open-ocean-facing settings exhibited significantly higher extinction rates during the three most profound mass extinctions in the study interval: the end-Permian, end-Triassic, and end-Cretaceous events. This suggests that ocean-facing settings were more susceptible to the agents of extinction in these events.

In contrast, the penultimate stage of the Permian, the Guadalupian, exhibited a significantly higher extinction rate among genera in epicontinental seas than in open-ocean settings. This difference from the subsequent end-Permian event and the two other major extinctions does not demonstrate conclusively that it was caused by a mechanism unique to that interval. Nevertheless, sea-level regression has been implicated in the Guadalupian extinctions because of the loss of major shallow-water provinces (12), and its relation to the suite of catastrophic mechanisms

¹Department of Geology, University of Cincinnati, Post Office Box 210013, Cincinnati, OH 45221, USA. ²Department of the Geophysical Sciences, University of Chicago, 5734 South Ellis Avenue, Chicago, IL 60637, USA.

*To whom correspondence should be addressed. E-mail: arnold.miller@uc.edu