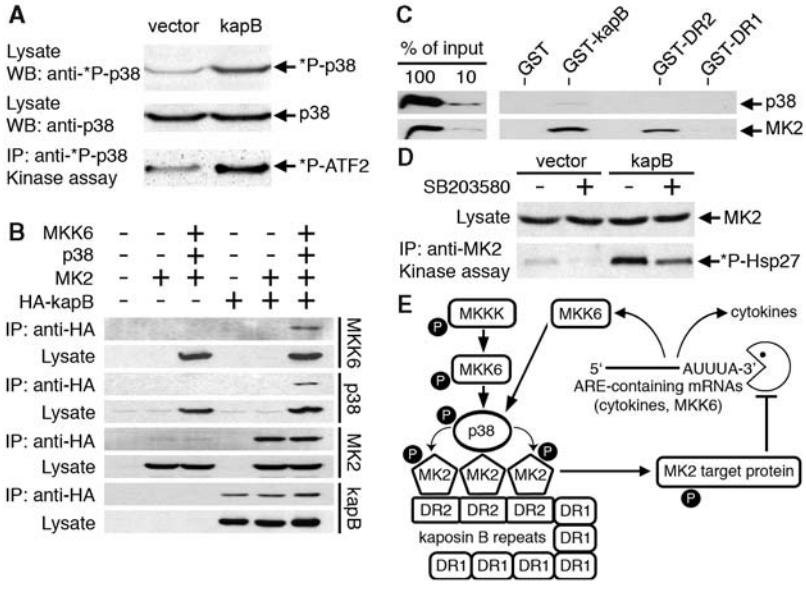

Fig. 4. p38 participates in kaposin B–MK2 signaling complexes. (**A**) HeLa cells were transfected with empty vector or kaposin B expression vector for 30 hours, lysed, and harvested. Portions of the whole-cell lysates were immunoblotted with antibodies to p38 (middle panel) and dual-phosphorylated p38 (upper panel). Dual-phosphorylated p38 was immunoprecipitated from these lysates, and p38 activity was measured with the use of activating transcription factor 2 (ATF2) as a substrate and immunoblotting with antibody to phosphorylated ATF2 (lower panel). (**B**) MKK6 and p38 coimmunoprecipitate with kaposin B–MK2 complexes. 293T cells were transfected with the indicated combinations of hemagglutinin (HA) epitope–tagged kaposin B, MK2, p38, and MKK6 for 48 hours, lysed, and immunoprecipitated with HA mAb. Western blots of the immunoprecipitated material and the whole-cell lysates were probed with polyclonal antibodies to MKK6, p38, MK2, and HA. (**C**) p38 and MK2 proteins (purified from *E. coli*) were incubated overnight at 4°C with the indicated GST-fusion proteins bound to glutathione-Sepharose beads, washed, electrophoresed, and immunoblotted with specific polyclonal antibodies. (**D**) Inhibition of p38 partially blocks kaposin B–mediated increases in MK2 activity. The selective p38 inhibitor SB203580 was added to kaposin B and empty vector transfected HeLa cells for 1 hour, cells were lysed, and whole-cell lysates were immunoblotted with the indicated antibodies. Portions of these lysates were immunoprecipitated with anti-MK2 and assayed for MK2 kinase activity. (**E**) Proposed model of MK2 activation by kaposin B. The reiterated DR2 repeats of kaposin B may bind multiple MK2 proteins in the nucleus, allowing for efficient phosphorylation by p38 MAPK. Phosphorylation of MK2 target proteins results in a blockade in ARE-mediated mRNA degradation, leading to enhanced production of proteins from ARE-containing transcripts. These proteins include cytokines as well as signaling molecules such as MKK6, which in turn could amplify p38 MAPK activation.

activation, leading to further activation of MK2. Much remains to be learned about the mechanisms of MK2 and p38 activation by kaposin B. Binding of MK2 by kaposin B could lead directly to its activation through an induced conformational change; we note that the region on MK2 to which kaposin B binds (Fig. 1D) is consistent with activation via displacement of the inhibitory C-terminal autoregulatory domain of MK2. Bound kaposin B could also shield activated MK2 from the action of cellular phosphatases. As to how p38 becomes activated, because cytokines such as IL-6 can activate the p38 pathway, elevated cytokine release from kaposin B–expressing cells may promote an autocrine or paracrine amplification loop that further enhances p38 activity. Irrespective of its mechanistic details, the finding that kaposin B activates the p38-MK2 pathway forges an important biochemical link between KSHV infection and the enhanced cytokine production that characterizes so many of its associated disease states, and provides a striking example of virus-mediated modulation of mRNA stability.

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24. Dedicated to the memory of Robert Sadler, Ph.D., who discovered kaposin B as a postdoctoral fellow but died tragically shortly thereafter.

Supporting Online Material

www.sciencemag.org/cgi/content/full/307/5710/739/DC1
Materials and Methods
Figs. S1 to S4

28 September 2004; accepted 15 December 2004
10.1126/science.1105779

Mutualistic Fungi Control Crop Diversity in Fungus-Growing Ants

Michael Poulsen* and Jacobus J. Boomsma

Leaf-cutting ants rear clonal fungi for food and transmit the fungi from mother to daughter colonies so that symbiont mixing and conflict, which result from competition between genetically different clones, are avoided. Here we show that despite millions of years of predominantly vertical transmission, the domesticated fungi actively reject mycelial fragments from neighboring colonies, and that the strength of these reactions are in proportion to the overall genetic difference between these symbionts. Fungal incompatibility compounds remain intact during ant digestion, so that fecal droplets, which are used for manuring newly grown fungus, elicit similar hostile reactions when applied to symbionts from other colonies. Symbiont control over new mycelial growth by manurial imprinting prevents the rearing of multiple crops in fungus gardens belonging to the same colony.

Ant fungiculture arose in South America about 50 to 60 million years ago in the ancestor of the New World tribe of fungus-growing (attine) ants (*1*). All extant attine ants (~210 described species in 13 genera) are obligately dependent on this symbiosis,

which mostly involves Lepiotaceaeous fungi (order Agaricales, division Basidiomycota) (*1*–*3*). The symbionts of many lower attine ants are closely related to free-living fungi (*4*), but those of *Atta* and *Acromyrmex* leaf-cutting ants have unique coevolved adaptations as a specialized crop (*5*). The ants provide the fungus with fresh substrate and protection against competitors and pathogens (*6*–*8*), and virgin ant queens carry their mother's symbiont when leaving their colony to mate and disperse (*9*). This vertical transmission of the symbiont is expected to stabilize the mutualism by aligning the reproductive interests of the partners (*10*–*12*). However, horizontal exchange of symbionts may occur during cofounding of colonies or symbiont theft after garden loss (*5*, *13*–*16*).

If hostile interactions between symbionts reduce productivity, the introduction of an alien fungus clone will harm not only the resident fungus but also the ant hosts (*10*, *11*, *16*). Ants in full control could possibly avoid these costs by rearing genetically different symbionts in separate nest chambers, but previous studies have suggested that colonies rear only a single fungus clone (*5*, *16*–*18*). This implies that the ants do not have agricultural practices of exchanging crops, nor do they have multiple crops, which are developments that have been essential in human agriculture (*19*). Here we show that single-crop ant farming is actively imposed by the fungal symbiont.

We used fungus gardens (Fig. 1A) that were cultivated by 18 colonies of two sympatric species of Panamanian leaf-cutting ants, *Acromyrmex echinatior* and *A. octospinosus*. These two ant species have never been observed to hybridize, but their clonal fungi belong to the same genetically diverse clade (fig. S1) (*16*, *20*, *21*). Mycelial incompatibility between fungi from different colonies was assessed by inoculating pairs of fungus that were grown 1.5 cm apart on potato dextrose agar (PDA) medium (fig. S2) (*21*). After 2 months, mycelial compatibility could be scored on a scale from 0 to 3 (Fig. 1B) (*21*, *22*). We also obtained genetic fingerprints of the fungi by using amplified fragment length polymorphism (AFLP) to estimate relative genetic distance (percentage of bands not homologous) between pairs of clones and to confirm that each source colony cultivated a single clone (*21*). The latter was done by AFLP analysis of five to six independently isolated samples of the same fungus garden from each of 10 colonies. Alignment of these profiles showed identical banding patterns within fungus gardens (*21*).

Mycelial incompatibility was highly variable among pairs of clones (Fig. 1B) and was positively correlated with genetic distance (Mantel test: $r = 0.855$, $P < 0.0001$) (Fig. 1C). Average reactions within ant species (1.40 for *A. echinatior* and 1.54 for *A. octospinosus*) were less hostile than between species (1.98). This result corresponded to lower relative genetic distances between fungi within (0.20 for *A. echinatior* and 0.23 for *A. octospinosus*) than between ant species (0.29). An effect likelihood ratio

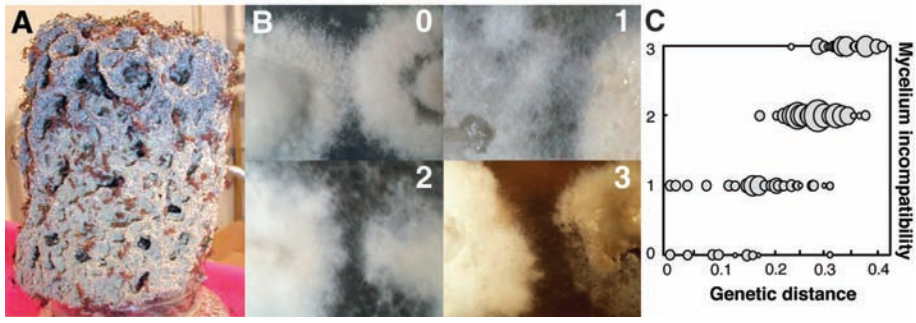

Fig. 1. (**A**) An *A. octospinosus* fungus garden and (**B**) typical examples of the four degrees of mycelial incompatibility between fungus clones in vitro. 0 shows fully compatible clones with no demarcation zone; 1 shows weakly incompatible with a slight, but clearly visible, demarcation zone; 2 shows incompatible clones with a distinct demarcation zone; and 3 shows strongly incompatible clones with a broad demarcation zone and brown coloration of mycelium and/or medium. (**C**) The relation between pairwise degree of incompatibility and genetic distance (171 different combinations, each replicated six times) (fig. S2). The area of each circle is proportional to the number of replicate combinations producing a given result. Controls are not shown, becauses they were all fully compatible (*21*).

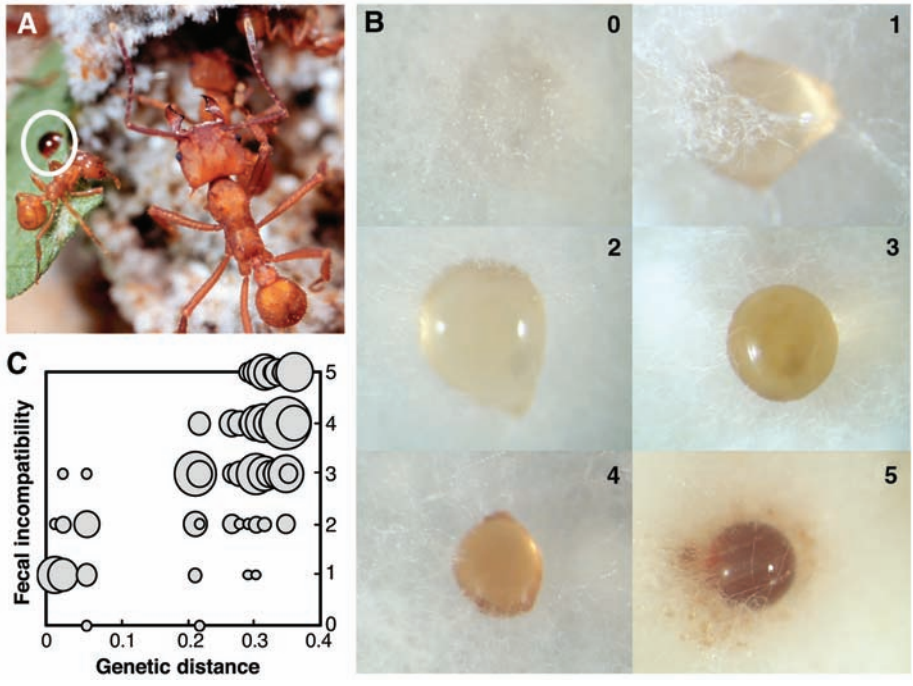

Fig. 2. (**A**) An *A. echinatior* fungus garden with a fecal droplet (encircled) [photo courtesy of Klaus Lechner]. (**B**) Typical examples of the categories of reaction of plated fungi (after 24 hours) (*21*) toward fecal droplets produced by workers from other colonies of *A. echinatior* or *A. octospinosus* from the same site. 0 shows complete absorption indicating full compatibility; 1 shows partial absorption; 2 shows distinct mycelium growth on the droplet but no absorption; 3 shows no mycelial growth on the droplet or in the direct vicinity of the droplet; 4 shows active avoidance of the droplet by fungal hyphae, with droplet turning light to dark brown; and 5 shows complete rejection of the droplet and very dark coloration of both droplet and surrounding mycelium. (**C**) The pairwise degree of incompatibility between fungal fragments maintained by ants and fecal droplets after 24 hours, plotted against genetic distance (garden fragments and fecal droplets from eight different colonies were combined in all possible ways and replicated eight times). Circle area is proportional to number of combinations producing a given result and controls are not shown [67.9% showed full compatibility (0); 30.4% showed partial absorption (1); and 1.7% showed distinct mycelium growth on the droplet, but no absorption (2)] (*21*).

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(ELR) test confirmed that incompatibility is related to fungal genetic distance ($\chi^2 = 2569$, degrees of freedom (df) = 1, $P < 0.0001$) and not an effect of ant species combination ($\chi^2 = 2.199$, df = 2, $P = 0.3330$) (*21*).

Acromyrmex worker ants actively discriminate between fragments of their resident fungus and genetically different symbionts (*16*), which confirms predictions that the ants have a strong short-term interest in maintaining genetically pure gardens (*11*). The ants reject alien fungus fragments for at least 1 week after being deprived of their resident garden, which indicates that avoidance of symbiont competition, at least initially, overrides symbiont replacement behavior, which is in their long-term interest (*16*). This suggests that the weeding behavior of worker ants may be directly linked to fungal incompatibility compounds.

All fungus-growing ants manure newly grown mycelium with their own feces (*23*, *24*). In this process, fungal enzymes, which help break down plant material, pass through the ant gut into fecal droplets (*25*), which are deposited on fresh leaves (Fig. 2A) or directly on the fungus garden, where they are readily absorbed (*2*). If incompatibility compounds remained unaffected by ant digestion, we would expect that fecal droplets secondarily might have become defensive extended phenotypes of the resident symbiont. We tested this idea by examining the reactions of fungus and ants toward fecal droplets from other colonies when the droplets were applied to fungus garden fragments of live experimental colonies (fig. S3) (*21*).

Incompatibility reactions of fungi toward fecal droplets from nonresident ants were scored on a scale ranging from 0 to 5 after 24 hours (Fig. 2B). There was a significant association between the intensity of the reactions and the genetic distance between the resident fungus and the fecal droplet–producing fungus (Mantel test: $r = 0.960$, $P < 0.0001$) (Fig. 2C). ELR testing showed that the magnitude of this effect was not due to ant species combination ($\chi^2 = 1.357$, df = 2, $P = 0.5074$) but only to the genetic distance of symbionts ($\chi^2 = 432.4$, df = 1, $P < 0.0001$) (*21*).

In the same experimental colonies, there was a significant association between the likelihood that ants would remove alien fecal droplets and the genetic distance between the resident and the droplet-producing fungus (Mantel test: $r = 0.721$, $P < 0.0001$) (Fig. 3A) and the droplet-fungus incompatibility reaction (Mantel test: $r = 0.651$, $P < 0.0001$) (Fig. 3B). Weeding behavior around the droplets, which resulted in the accumulation of additional fungal waste material (*8*, *21*), was associated with the same predictor variables [Mantel tests: $r = 0.700$, $P < 0.0001$ (Fig. 3C) and $r = 0.686$, $P < 0.0001$ (Fig. 3D)]. Droplet removal and garbage accumulation were likewise correlated (Mantel test: $r = 0.728$, $P < 0.0001$) (*21*). This suggests that the ants actively discard alien fecal droplets and any mycelium that has been in touch with these droplets.

In a final experiment, we found that initially incompatible interactions between fecal droplets and fungi became compatible when ants were forced to feed on an incompatible alien symbiont for 10 days. The ants' new fecal droplets also became incompatible with their original resident fungus (Fig. 4, B and C) (*21*). An ELR test showed a significant main effect of the ant fungus combination ($\chi^2 = 9.498$, df = 1, $P = 0.0021$) and a significant interaction between the ant fungus combination and time ($\chi^2 = 11.58$, df = 1, $P = 0.0007$) (*21*). Controls did not show significant changes in compatibility, which confirmed that the compounds responsible for incompatibility reactions are exclusively fungus-derived.

The parallel reactions of resident fungi toward alien mycelia (Fig. 1) and fungus-derived fecal droplets (Figs. 2 and 4), and the hostile ant behavior toward alien droplets (Fig. 3) and alien fungus fragments (*16*), show that the host ants and their resident symbiont jointly prevent the introduction of competing fungus clones, which agrees with evolutionary theory on host symbiont conflict over symbiont mixing (*10*, *11*). Incompatibility reactions (brown coloration of mycelium) have also been observed in interactions between the ants' fungal symbionts and pathogenic fungi (*26*, *27*) and probably facilitate the detection of fungal disease. However, the present results suggest that these recognition abilities have been enhanced after fungus domestication, particularly in the higher attine ants (*3*, *5*). Direct assessments by the ants (*16*, *28*, *29*) and the derived recognition mechanism by the fecal droplets (<24 hours; Figs. 2 and 4) are much faster than recognition through mycelial contact, which takes 5.6 days on average (range, 3 to 7 days), even if tufts of alien mycelium are applied directly on mats of plated symbionts (Fig. 1B) (*21*). The chemical nature of the incompatibility compounds remains unknown, but they could be similar to those of free-living basidiomycetes or be derivates of evolutionary-derived enzymes of the symbionts (*30*).

Neither direct recognition of alien symbionts by the ants nor mycelial incompatibility can explain why *Acromyrmex* leaf-cutting ants are restricted to rear a single symbiont clone per colony. Abundant genetic variation between clones is available, and mature nests of *A. octospinosus* and *A. echinatior* are usually compartmentalized with multiple gardens that could keep symbiont strains separated. The present study suggests that

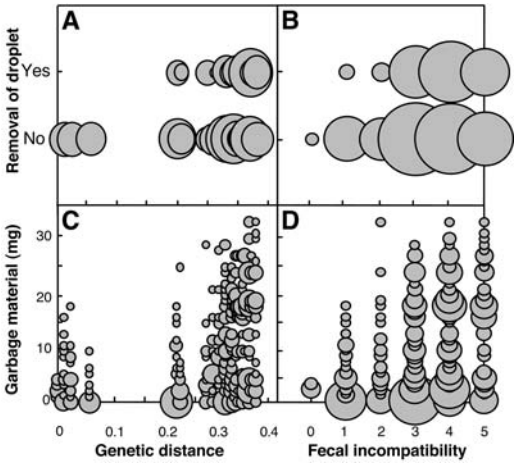

Fig. 3. (**A** and **B**) Fecal droplet removal and (**C** and **D**) amount of garbage (milligrams of fresh weight) deposited 24 hours after application of fecal droplets that were obtained from the same eight-by-eight combinations of fungus gardens and ants used in Fig. 2 (*21*). Data are plotted against genetic distance between the resident and the droplet-producing fungus [(A) and (C)] and the degree of fecal-droplet incompatibility (0 to 5) [(B) and (D)]. Circle area is proportional to the number of combinations producing a given result. Controls are not plotted, because resident fecal droplets were never removed and the average amount of garbage deposited was 1 ± 0.3 mg (mean ± SE), which was substantially less than the mean garbage accumulation of all the test combinations (4 ± 0.4 mg) (*21*).

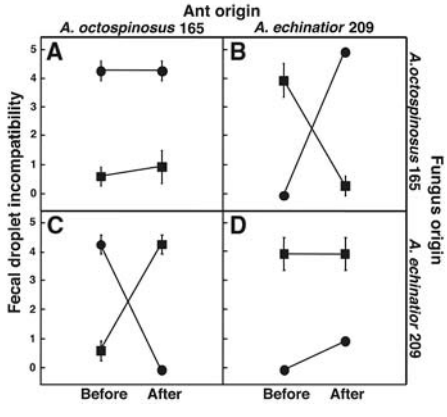

Fig. 4. Fungus incompatibility reactions toward resident and foreign fecal droplets, before and after force-feeding the ants that produced the droplets for 10 days. Two colonies (Ao165 and Ae209), which were initially droplet-incompatible (Fig. 3), were used for reciprocal force-feeding treatments (**B** and **C**) and controls (**A** and **D**). Mean reactions ± SE (n = 3 replicate pairings) displayed by fungus Ao165 (squares) and Ae209 (circles) are plotted.

the ants' manuring practice is the decisive factor that constrains colonies to rearing a single clone of symbiont. Obligate manuring with ant feces allows the resident fungus to control the genetic identity of new gardens in the nest, causing the removal of unrelated fungi before they contribute to ant feeding and the production of compatible fecal droplets (Fig. 4). The manurial imprint of the fungus therefore makes ant agriculture dependent on a single symbiont that is very difficult to replace and impossible to combine with secondarily acquired symbionts.

The manurial imprint of fecal droplets is a clear example of fungal signaling affecting ant behavior and is possibly more important than suggested fungal signaling for directing the choice of food plants by workers to avoid an overdose of toxins (*31*, *32*). Similar manipulative effects of resident fungi toward competing clones may have convergently evolved in the fungus-growing termites, where the fungus substrate is entirely derived from fecal matter (*33*). This could explain why fungus-growing termites also rear single clones of fungus, despite initiating colonies with horizontally acquired fungal spores (*33*, *34*). None of these constraints have ever applied to human agriculture because crops and expertise have been culturally transmitted between tribes from the beginning of human farming (*35*).

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36. We thank B. E. N. Markussen, S. M. Mathiasen, and S. Rønhede for laboratory assistance; D. R. Nash and J. S. Pedersen for detailed statistical advice; D. K. Aanen, S. A. O. Armitage, M. J. Cafaro, C. R. Currie, D. R. Nash, J. S. Pedersen, S. Taerum, and J. M. Thomas for fruitful discussions and comments; the Smithsonian Tropical Research Institute for providing facilities to work in Panama; and the Autoridad Nacional del Ambiente y el Mar for sampling and export permissions. Supported by grants from the Danish Natural Science Research Council and the Carlsberg foundation (J.J.B.) and a Ph.D. stipend from the University of Copenhagen (M.P.).

Supporting Online Material

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Materials and Methods
Figs. S1 to S3
References

25 October 2004; accepted 1 December 2004
10.1126/science.1106688