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Nuclear populations of the multinucleate fungus of leafcutter ants can be dekaryotized and recombined to manipulate growth of nutritive hyphal nodules harvested by the ants

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ABSTRACT

We dekaryotized the multinucleate fungus *Leucocoprinus gongylophorus*, a symbiotic fungus cultivated vegetatively by leafcutter ants as their food. To track genetic changes resulting from dekaryotization (elimination of some nuclei from the multinuclear population), we developed two multiplex microsatellite fingerprinting panels (15 loci total), then characterized the allele profiles of 129 accessions generated by dekaryotization treatment. Genotype profiles of the 129 accessions confirmed allele loss expected by dekaryotization of the multinucleate fungus. We found no evidence for haploid and single-nucleus strains among the 129 accessions. Microscopy of fluorescently stained dekaryotized accessions revealed great variation in nuclei number between cells of the same vegetative mycelium, with cells containing typically between 3 and 15 nuclei/cell (average = 9.4 nuclei/cell; mode = 8). We distinguish four mycelial morphotypes among the dekaryotized accessions; some of these morphotypes had lost the full competence to produce gongylidia (nutritive hyphal-tip swellings consumed by leafcutter ants as food). In mycelial growth confrontations between different gongylidia-incompetent accessions, allele profiles suggest exchange of nuclei between dekaryotized accessions, restoring full gongylidia competence in some of these strains. The restoration of gongylidia competence after genetic exchange between dekaryotized strains suggests the hypothesis that complementary nuclei interact, or nuclear and cytoplasmic factors interact, to promote or enable gongylidia competence.

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INTRODUCTION

Leafcutter ants in the genera *Atta* and *Acromyrmex* cultivate as their main food source monocultures of fungi, called *Leucocoprinus gongylophorus* (Leucocoprini, Agaricales) as sexual morph (Heim 1957; Mueller et al. 2017) or *Attamyces bromatificus* as asexual morph (Kreisel 1972). The ants cultivate their fungus in sheltered gardens (typically underground) and sustain fungal growth in these gardens by supplying their cultivars with substrate for growth (typically finely minced leaves) and nourishment through fecal manuring (Weber 1972; De Fine Licht and Boomsma 2010). Because the ants depend on their fungal garden as primary food source, and because *L. gongylophorus* fungi have so far not been found growing independently of leafcutter ants (Mueller et al. 1998; Pagnocca et al. 2001; Mueller 2002; Bacci et al. 2009; Vo et al. 2009), the leafcutter ant–fungus association appears to be an obligate symbiosis for leafcutter fungi.

Atta leafcutter ants grow their fungi in single-clone monoculture (Mueller et al. 2010) and vertically transmit their fungal strain from one generation to the next. When a virgin leafcutter queen leaves her natal nest for her mating flight and dispersal, she maintains an inoculum of her natal fungal cultivar in a storage pocket in her mouth, then uses this inoculum as a starter culture to initiate the garden in her newly founded nest. Such vertical transmission of clonally propagated fungal strains is typical for all leafcutter ants (Weber 1972; Della Lucia 2011; Marti et al. 2015; Meirelles et al. 2016), and fungal exchange between attine ant nests appears to be constrained by incompatibilities or low fitness of specific ant-fungus combinations (Seal et al. 2012, 2014a, 2014b). However, population-genetic studies revealed occasional horizontal exchange of fungal cultivars between sympatric leafcutter ant species, as well as rare cases of genetic admixture between *L.*

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gongylophorus strains (Adams et al. 2000; Mueller 2002; Mikheyev et al. 2006, 2007, 2010; Mueller et al. 2011a, 2011b, 2017).

Although common and well studied in plants and some animal lineages, polyploidy—the coexistence of multiplied genomes in the same nucleus—is much less frequent and understudied in fungi (Campbell et al. 2016). Of the known polyploid fungi, most derived from evolutionarily recent transitions to polyploidy, and only two fungal polyploids of ancient evolutionary origin are known (*Saccharomyces, Rhizopus*) (Ma et al. 2009; Albertin and Marullo 2012; Shelest and Voigt 2014; Campbell et al. 2016). Among the Basidiomycota, the known cases of polyploidy appear to be of recent evolutionary origins, including both autopolyploid origins (e.g., *Ustilago, Armillaria, Cryptococcus*) and allopolyploid origins (e.g., *Heterobasidion, Armillaria*) (Campbell et al. 2016). Some Basidiomycota can also have diverse populations of differentiated nuclei in multinucleate cells, a state called heterokaryosis. Such multinucleate cells have been observed only a few times in species such as *Phanerochaete chrysosporium* (Alic et al. 1987), *Heterobasidion parviporum* (James et al. 2008), and several species of *Agaricus* and *Termitomyces* (Saksena et al. 1976; De Fine Licht et al. 2005; Dias et al. 2008), as well as *L. gongylophorus* (Scott et al. 2009; Kooij et al. 2015). The number of nuclei present in the vegetative mycelium of these species varies greatly. For example, cytological studies of *Agaricus brasiliensis* determined an average of 5.8 nuclei per cell (Dias et al. 2008), whereas studies of *Agaricus bisporus* revealed as many as 25 nuclei per cell (Saksena et al. 1976). Both polyploidy and heterokaryosis appear as multiallele genotypes in genotyping analyses (e.g., genotyping indicates more than two alleles per locus for a single-strain mycelium), and it is therefore a challenge to determine whether the reason for multiallele genotypes derives from polyploidy (multiple genomes per nucleus), from heterokaryosis (multiple, genetically differentiated nuclei coexist in the same cell), or from both polyploidy and heterokaryosis.

The earliest histological studies of *L. gongylophorus* mycelium revealed that these fungi lack clamp connections (Möller 1893; Hervey et al. 1977) and are multinucleate, with more than two nuclei per cell (Hervey et al. 1977; Mohali 1998). Subsequent microsatellite marker analyses of *L. gongylophorus* from Panama and the USA reported up to five distinct alleles per *L. gongylophorus* strain (Scott et al. 2009; Mueller et al. 2011b), suggesting that either single nuclei are significantly polyploid (i.e., contain duplicated genomes), or that genetically differentiated nuclei coexist in a cell (i.e., cells are heterokaryotic), or that a combination of single-nucleus ploidy and heterokaryotic nuclear populations contribute to multiallele genotypes of *L. gongylophorus*. Kooij et al. (2015) recently reported 7–17 nuclei per cell in fungal symbionts of five species of Panamanian leafcutter ants and that *L. gongylophorus* cells are highly and obligatorily polyploid, with about 5–7 different, heterokaryotic nuclei per cell. Polyploidy and heterokaryosis in leafcutter fungi may boost metabolism (Kooij et al. 2015) or help compensate for evolutionary disadvantages stemming from the apparent absence or rarity of sexual reproduction via meiospore production in leafcutter fungi (Mueller 2002; Mikheyev et al. 2006; Scott et al. 2009; Mueller et al. 2011b, 2017; Kooij et al. 2015). However, leafcutter fungi actually do exhibit low levels of population-genetic admixture (Mueller et al. 2011b), most likely through the occasional exchange of nuclei between anastomosing mycelia (Mueller et al. 2011b), but true recombinatorial exchange of genetic material between nuclei also seems possible. Theory predicts that such rare facultative genetic exchange between differentiated mycelia, rare recombination, or rare ploidy cycling can generate almost the same evolutionary advantages of obligatory sexual reproduction (Kondrashov 1994; Otto and Lenormand 2002).

The multiallelic microsatellite DNA genotypes reported for *L. gongylophorus* (3–5 alleles typical per locus, up to 8 alleles per locus; Scott et al. 2009; Mueller et al. 2011b; Kooij et al. 2015) likely underestimate the true number of coexisting nuclei per cell, for two reasons. First, some nuclei may be genetically similar or identical to each other; and differences between such nuclei would not be readily apparent in the microsatellite marker profiles, as reported by Kooij et al. (2015). Second, microsatellite loci may contain null alleles (nonamplifiable alleles lacking an appropriate primer site), leading to an inherent underestimate of the true number of coexisting nuclei. Despite these potential complications, microsatellite genotyping of *L. gongylophorus* has been useful to advance understanding of population-genetic structure (Mikheyev et al. 2007; Mueller et al. 2011b, 2017), single-strain cultivation per nest (i.e., fungal monoculture; Mueller et al. 2010), symbiont choice (Sen et al. 2010), polyploidy and heterokaryon states (Kooij et al. 2015), quantitative genetics of cold tolerance (Mueller et al. 2011a), as well as genetic admixture between closely related *L. gongylophorus* strains (Sen et al. 2010; Mueller et al. 2011b). Although the evidence for occasional sexual or parasexual reproduction in natural *L. gongylophorus* populations is compelling (Mueller 2002; Mikheyev et al. 2006; Mueller et al. 2011b), it is unclear how genetic material is exchanged between the largely clonally propagated *L. gongylophorus* strains. Interactions between established mycelium and basidiospores (Pagnocca et al. 2001; Mueller 2002; Mueller et al. 2017) or parasexual processes after plasmogamy and exchange of nuclei between nuclear populations in different, anastomosing mycelia (Pontecorvo 1956) are the most plausible possibilities for genetic admixture in *L. gongylophorus* (Mueller et al. 2011b, 2017).

To understand the heterokaryotic genetics of *L. gongylophorus* symbionts, we here (i) develop tools to dekaryotize *L. gongylophorus* fungi (eliminate nuclei from heterokaryotic mycelium); (ii) verify successful dekaryotization with multiplex microsatellite DNA fingerprinting and histological methods; (iii) perform in vitro mycelial confrontation experiments to document plasmogamy or recombination; and (iv) elucidate the genetic basis of an evolutionarily unique morphological feature of *L. gongylophorus*, the so-called gongylidia, which are nutritive hyphal-tip swellings produced by *L. gongylophorus* in clusters (staphylae) to feed the ant hosts (Möller 1893; Hervey et al. 1977; Mueller 2002; De Fine Licht et al. 2010; 2013, 2014; Mueller 2015). These techniques should be useful to further elucidate ant-fungus coevolution, determine species boundaries in mating experiments, and generate dekaryotized mycelium for more cost-effective whole-genome sequencing.

METHODS

Definition of dekaryotization.—We define the term "dekaryotization" as the experimental reduction of the number of genetically differentiated nuclei in a population of nuclei in cells of a heterokaryotic multinucleate mycelium. The term dekaryotization derives from the term "dedikaryotization" used to produce single-nucleus cells from dikaryotic cells (Miles and Raper 1956; McClaren 1970). Because this existing term of dedikaryotization would not apply to the multinucleate fungi cultivated by leafcutter ants (i.e., these fungi are not dikaryotic), we use here the more general term dekaryotization applicable to dikaryotic and multikaryotic mycelium.

***Isolation of* L. gongylophorus *from gardens of* Atta texana.**—We selected three strains of *L. gongylophorus* to develop dekaryotization methods. These three strains were part of a larger collection of *L. gongylophorus* isolated in 2007 from nests throughout the range of *Atta texana*. The strains belong to the T-fungus genotype cluster of leafcutter fungi in the USA, a fungal genotype only cultivated by *A. texana* but apparently not other leafcutter ants (Mueller et al. 2011b). UGM070519-03 was collected from an *A. texana* nest near Graham, Texas (Global Positioning System [GPS]: N33.08, W98.73), UGM070316-05 near Montgomery, Texas (N30.32, W95.58), and UGM070517-01 at the Hornsby Bend Environmental Research Center, Texas (N30.22, W97.65; collection UGM070517-01 was collected from the same nest as UGM060511-01 listed in Mueller et al. 2011b, except UGM070517-01 was collected a year later from the same nest). Isolation protocols followed standard methods for isolating attine cultivars on potato dextrose agar (PDA) medium (Mueller et al. 1996, 1998; Wang et al. 1999; Gerardo et al. 2006).

Dekaryotization and isolation of dekaryotized mycelium.—*Pilot dekaryotization trial.* To dekaryotize *L. gongylophorus*, we adapted methods originally developed for homokaryon recovery of the commercial mushroom *Agaricus bisporus* (Castle et al. 1988; Kerrigan et al. 1992; Zhao and Chang 1993), with the modification that we recovered mycelium regenerating on PDA (2% agar, 0.6 M mannitol; Petri plates of 100 mm diameter, 15 mm depth). We cut a 2-cm^2 agar plug of gongylidia-bearing mycelium from within 5–7 mm of the growth front of 8-wk-old cultures growing on PDA. This mycelial plug was then blended in 20 mL sterile potato dextrose broth (PDB) supplemented with 0.6 M mannitol, using a Waring Commercial Blender 700 model 33BL79 (Waring Products, Torrington, Connecticut, USA). Each blending cycle lasted 10 s. We repeated cycles three times, with a 2-min break between each cycle to avoid overheating the blended liquid. To eliminate larger fragments and enrich the blended liquid for small particulates (i.e., protoplasts or single hyphal cells) while eliminating large particulates (i.e., unmacerated mycelium), we filtered the blended suspension of hyphal fragments through an autoclaved, custom-made column consisting of a 1.2-cm-long plug of cotton packed into the tapering end of a 5-mL plastic pipette tip. The blended liquid was added in aliquots to the column above the cotton plug, and liquid moved by gravity through the plug. For regeneration of mycelium, aliquots of 100 µL of the collected filtrate were plated onto 10 separate 2% PDA plates supplemented with 0.6 M mannitol as osmotic stabilizer. Plates were incubated at room temperature to permit regeneration of hyphal cells and any protoplasts. We did not examine the filtrate microscopically, so we do not know whether regenerating mycelium derived from protoplasts, single cells, minute hyphal fragments, or a combination of these.

Of the three *L. gongylophorus* strains processed, we were successful at isolating regenerating mycelium only from strain UGM070517-01 (no live mycelium grew on the regeneration plates of UGM070519-03 and UGM070316-05). We examined plates every 2–4 d for signs of regenerating mycelium (hyphal growth barely visible with eye), which we then subcultured individually onto fresh PDA plates by cutting out the underlying plug from the original plate and thus avert growth of regenerating mycelium across the original isolation plate. We

recovered regenerating mycelium over the course of 1 mo. In some fungi, homokaryons grow slower than heterokaryons (Kerrigan et al. 1992), and we therefore discarded mycelium that regenerated within 1 wk (visible after 1 wk or earlier) while retaining for further processing only slow-regenerating mycelia (regenerating after 2–4 wk). We subcultured these isolates narrowly from their respective growth fronts at least one more time, but we did not take records to document the exact subculturing history for each individual isolate during this pilot trial aimed at developing basic methods. We obtained a total of 119 isolates in the pilot trial, which we then genotyped at 10 microsatellite loci (methods below, Plex A) to evaluate whether the dekaryotization procedure resulted in reduction of the original multiallelic state of the parental accession UGM070517-01. Because our methods were not yet standardized in this pilot trial, we do not include information from these pilot isolates in the Results section below (instead, we show the preliminary genotyping results from the pilot trial in SUPPLEMENTARY TABLE 1); however, the pilot trial proved valuable to develop methodology used in the rigorously standardized experimental dekaryotization trial.

Experimental dekaryotization trial. Because the pilot trial was successful and produced dekaryotized genotypes that differed from the genotype of the original, multinucleate parental strain UGM070517-01, we repeated the dekaryotization experiment with a fresh isolate of UGM070517-01. After blending and plating using the methods from the pilot trial, we subcultured all regenerating mycelia over 6 wk, including this time also those mycelia regenerating within the first 2 wk. During weeks 1–3, we checked regeneration plates daily under an 80× stereomicroscope, and we excised any regenerating mycelium narrowly to transfer single-strain isolates onto individual PDA plate. During weeks 4–6, we checked plates weekly for late-regenerating (slow-growing) mycelium. We collected a total of 129 accessions in this experimental dekaryotization trial (SUPPLEMENTARY TABLE 2), then genotyped each accession using microsatellite markers (methods below). Observation of mycelial growth of these accessions over several weeks allowed us to categorize growth behavior into four morphotypes (Types 1–4), as explained below and in SUPPLEMENTARY FIG. 1. Several accessions from the experimental trial developed sectors of browning in older cultures. Although such discoloring can also occur in pure strains (U.G.M. and H.D.I., personal observations), we genotyped mycelium from both browning mycelium and from the typical white mycelium, to evaluate whether browning could be the result of incompatibility interactions between genetically differentiated, commingled strains.

Growth measurements.—To compare growth behavior of the 129 accessions of the experimental trial, we recorded (i) the number of days until initial isolation from regeneration plates (i.e., number of days after plating, when the first hyphal growth of a regenerating accession became visible under a microscope) (FIG. 1a); and (ii) initial gongylidia vigor of each isolated accession, defined as the number of days after the first subculturing when a subcultured accession exhibited at least 20 gongylidia under a microscope (FIG. 1b) (data in SUPPLEMENTARY TABLE 6). These two measurements record growth behavior of regenerating accessions immediately after dekaryotization treatment. We terminated observations after 8 wk, and any accessions that did not exhibit any gongylidia growth by the end of 8 wk were recorded as "na" in the "Gongylidia vigor" column of SUPPLEMENTARY TABLE 6 (some of these accessions produced gongylidia when older, or after later subculturing, but not during the first 8 wk).

To compare biomass accumulation of accessions after these first 8 wk, we selected 32 accessions from among the 129 accessions to characterize additional growth measures, including (iii) total area (in mm^3) covered by mycelium growing from a small mycelial tuft on a PDA plate, measured 6 wk after transfer of the mycelial tuft (FIG. 1c); and (iv) biomass (fresh weight) of a mycelial accession attained after 6 wk of growth (harvesting aerial mycelium with a spatula without underlying medium) (FIG. 1d). For these 32 accessions, we used flame-sterilized fine-tipped forceps to subculture a small piece (~1 mm^3) of aerial mycelium from the growth front of 32 established accessions of different genotypes. For each of these 32 accessions, we generated three such replicate plates. We grew cultures at room temperature. Because accessions expanded radially at about equal rate in all directions, we used calipers to measure two edge-to-edge perpendicular diameters of the radial fungal growth, then averaged these two diameters to derive an estimate of radial growth across the culture plate, and then used the average diameter and standard circle calculations to calculate the approximate area (in mm^3). We collected these measurements weekly for 6 wk. After 6 wk, mycelial growth of the three replicates of each accession were carefully scraped off plates with inoculating loops; the mycelial growth of each sample was combined and weighed in a small plastic vial. Two of the accessions had one failed replicate each (the initial small inoculum was not viable, likely because the mycelial bit was cut too small during subculturing); for these samples, we averaged areas and weights only for the two viable replicates of that accession. Researchers processing and weighing mycelia were blind to all information on genotypes and morphotype classifications.

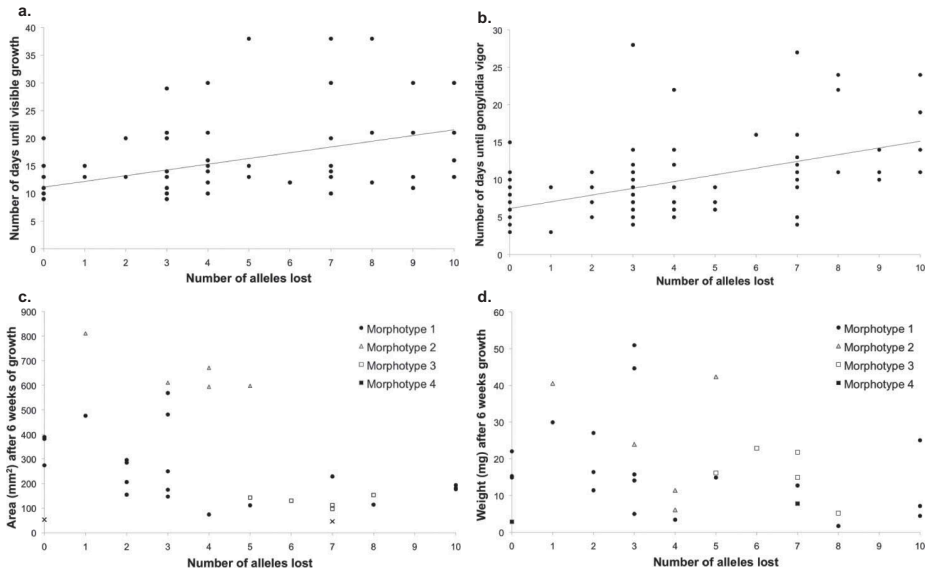

Figure 1. Growth behavior of fungal accessions after dekaryotization treatment. a and b. Growth behavior of a regenerating mycelium for 129 accessions isolated from regeneration plates, for the initial growth when subculturing these isolates immediately after dekaryotization treatment. Regenerating isolates that had lost more alleles as a result of dekaryotization treatment needed more time to (a) develop the first hyphal growth during regeneration ($P < 0.0001$) and (b) develop gongylidia vigor (defined as production of at least 20 gongylidia) ($P < 0.001$). c and d. Growth behavior for 32 dekaryotized accessions grown for 6 wk after several rounds of subculturing. Radial growth (area covered on a plate) was significantly negatively correlated with the number of alleles lost by a genotype ($P = 0.031$). Wet weight was also negatively correlated with the number of alleles lost by a genotype, but this trend was not statistically significant ($P = 0.16$). Overall, the more alleles had been lost by an accession, the poorer the (a) regeneration, (b) gongylidia production during regeneration, (c) radial growth, and (d) possibly biomass accumulation.

Multiplex microsatellite genotyping.—To improve the efficiency of microsatellite genotyping using the polymerase chain reaction (PCR), we first developed two microsatellite multiplex PCRs that amplified, respectively, 10 and 5 of the loci originally developed by Scott et al. (2009) for *L. gongylophorus* fungi. The 15 loci in the two multiplexes (Plexes A and B) were selected because of their high levels of allelic polymorphism and minimal stutter problems (SUPPLEMENTARY TABLES 1 and 2). Some primers in these plexes are identical to those of Scott et al. (2009), but some primers had to be redesigned to generate amplification products of desired fragment sizes. New primers were created with a desired T_m of 59–60 C, a GC content of 40–60%, at least one "GC" clamp, and a designated fragment size range in one of four bin sizes (bin 1: 75–120 bp; bin 2: 130–178 bp; bin 3: 188–205 bp; bin 4: 225–270 bp). Each bin size had two or three loci differentiated by a fluorescent dye (FAM, HEX, or TAMRA) attached to the forward primer 5′ end. SUPPLEMENTARY TABLE 3 lists forward and reverse primer sequences, the fluorescent dye for each primer pair, and primer concentrations optimized for balanced amplification of alleles across all multiplexed loci. During early stages of multiplex-PCR development, we used watchmaker forceps (no. 5) to isolate a small tuft of aerial mycelium from the *L. gongylophorus* growth front, then extracted DNA from this tuft using a 10% Chelex solution, as described in Mikheyev et al. (2007, 2008). After optimizing multiplex-PCR conditions, we found it most time-efficient to add the mycelial tuft directly into the PCR mix and skip this DNA extraction step (i.e., 15 min soaking at 94 C released the DNA, followed immediately by PCR amplification). The optimized microsatellite PCR used the Qiagen Multiplex PCR Kit (Qiagen, Germantown, Maryland, USA) and a 10 μL reaction volume, consisting of 5 μL of 2× Qiagen Master Mix, 1 μL aliquot of primer mix (concentrations for each multiplexed primer are listed in SUPPLEMENTARY TABLE 3), 4 μL of nucleotide-free water, plus a minute mycelial tuft (<1 mm^3) directly added into this mix. We followed the thermal cycling protocol recommended by the Qiagen Multiplex PCR Handbook: 15 min at 95 C (release of DNA from tissue, denaturing of DNA); 35 cycles at 94 C for 30 s, annealing at 57 C for 30 s, and extension at 72 C for 60 s; final extension at 72 C for 10 min. We confirmed DNA amplification by visualization of PCR products under ultraviolet (UV) light in a 2% agarose

gel stained with SYBR safe. For fragment analysis, we mixed 1 µL of PCR product mixed with 8 µL of HiDi (Applied Biosystems, Waltham, Massachusetts, USA) and 1.5 µL of custom-amplified size standards (CASS; ladder of 104R, 156R, 256R, 305R, 424R; each labeled with RoxF; following the methods of DeWoody et al. 2004). We analyzed PCR products on an ABI 3100 Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts, USA) in the Mueller laboratory and scored markers (alleles) using Gene Scan 3.5 and SoftGenetics GeneMarker 1.5 (State College, Pennsylvania).

We genotyped accessions typically only with the first multiplex of 10 loci (Plex A, SUPPLEMENTARY TABLE 3) and if additional marker information was needed also with the second multiplex of 5 loci (Plex B). To verify that dekaryotized accessions showed stable allele profiles (i.e., to rule out that allele profiles changed over time by losing additional alleles or regaining alleles that had been lost as a result of dekaryotization treatment), we repeated genotyping two to four times over a period of several months across multiple subculturing cycles for each dekaryotized accession. Accessions with no missing alleles (i.e., genotypes identical to the parental fungus before dekaryotization treatment) were typically regenotyped only one to two times, whereas accessions that had lost the most alleles during the dekaryotization treatment (e.g., lost 9 or 10 alleles) were regenotyped up to nine times (SUPPLEMENTARY TABLE 6 lists number of regenotyping).

Statistics.—We used regression analysis to explore the relationship between (i) the number of alleles lost by an accession as a result of dekaryotization treatment and (ii) the four growth rate parameters measured for each individual accession (i.e., number of days until visible regeneration; growth vigor at regeneration; area grown and wet mass attained within 6 wk; see Growth Measurements above and FIG. 1a–d).

Testing genetic exchange through recombination or nuclear reassociation between mycelia.—To assess whether genetically stable dekaryotized accessions that had lost alleles could regain alleles trough nuclei exchange or recombination with other dekaryotized accessions, we confronted pairs of accessions on PDA plates. All confronted accessions had been derived through our dekaryotization treatment from the original isolate UGM070517-01; this original isolate showed 40 alleles across the 15 loci screened in our multiplex microsatellite genotyping (see genotypes shown in the first row of SUPPLEMENTARY TABLE 2), whereas each of the confronted accessions had lost between 2 and 6 of these alleles as a result of dekaryotization treatment. The accessions paired in the confrontations were chosen by two criteria: first, several or a large number of alleles were lost in each accession during dekaryotization treatment; and second, the confronted genotypes complemented each other such that exchange of nuclei or parasexual recombination could potentially reconstitute the original genotype of UGM070517-01. SUPPLEMENTARY TABLE 4 lists the accession IDs that we paired in nine confrontation experiments. For a confrontation, we subcultured two agar plugs from the two accessions from their respective growth fronts, then placed these plugs onto a new PDA plate ~1 mm apart (but not touching) to permit rapid contact between the two growing mycelia. We replicated each of these nine confrontations. The confronted pairs were allowed to grow for 16 d, then subcultured three times from the center of the zone where confronted mycelia contacted each other (called below Subculture 1, abbreviated Sub1; 2 × 3 = 6 replicate subcultures per confrontation assay; × 9 assays = 54 Sub1 plates; SUPPLEMENTARY FIG. 2). We allowed these subcultures to grow for 13 d, then subcultured again, but this time from the 12, 6, and 9 o'clock positions along the growth front (called below Subculture 2, abbreviated Sub2; 18 replicates total per confrontation assay; × 9 confrontations = 162 Sub2 isolates). SUPPLEMENTARY FIG. 2 shows the flowchart of the experimental set up and sample sizes in this successive subculturing scheme to generate Sub1 isolates (n = 54 total) and Sub2 isolates (n = 162 total).

We genotyped all Sub1 isolates (n = 6 per confrontation) using both sets of 10+5 multiplexed markers (Plexes A and B). Sub1 replicates are called here "complete recombinants" if all 40 alleles of the original genotype UGM070517-01 were reconstituted in a genetically stable mycelium and persisted across several additional subculturing cycles. Recombinant accessions in which one or several alleles were regained, but the full original genotype was not reconstituted, are called here "partial recombinants." To differentiate between recombination/nuclear exchange versus a physical mixing of two commingled mycelia, we subcultured and genotyped putative complete recombinants for four additional subculturing cycles (SUPPLEMENTARY FIG. 3). To minimize the chance of subculturing two commingled accessions, we subcultured mycelium

with a sharpened needle, cutting the smallest possible portion from the growth front. Finally, after 87 d, we again subcultured from the original confrontation plate and the growth front from the 12, 3, 6, and 9 o'clock positions and any area of unusual morphology (called Sub1-delayed, SUPPLEMENTARY FIG. 2). We reasoned that this additional time ("delayed") could give two commingled strains time to compete with each other, increasing the chance that a pure dominant mycelium could be isolated from the growth front after a total of 87 d.

DAPI staining and counting of nuclei per cell.—To count number of nuclei per mycelial cell, we selected representative dekaryotized accessions for fluorescent staining of nuclei. We used Invitrogen's protocol for 4′,6-diamidino-2-phenylindole (DAPI) counterstaining of nucleic acids for fluorescence microscopy. For each accession, we excised 1-cm^2 pieces of aerial mycelium from representative sectors of healthy cultures, transferred each mycelium into an individual well of a Falcon polystyrene tissue microplates (24 wells/plate; catalog number 08-772-1H; Corning Life Sciences, Tewksbury, Massachusetts, USA), allowed the mycelium to equilibrate in 100 µL phosphate-buffered saline (PBS; pH 7.4; Gibco, Life Technologies, Carlsbad, California, USA) for 2 min, then rinsed each mycelium twice with distilled water. We soaked each sample in approximately 200 µL of a 1:10 dilution of 5 mg/mL DAPI solution, then incubated these in the dark at room temperature for 1 h. We washed postincubation samples twice with PBS to remove excess DAPI. For microcopy, we transferred samples to microscope slides to observe and photograph them with a Zeiss LSM 710 fluorescence confocal microscope (light filters 410–585 nm; Oberkochen, Germany) at the Microscopy Facility of the University of Texas at Austin. We photographed only cells in which nuclei and cell boundaries were unambiguously clear. We processed images using Zeiss ZEN lite 2011 SP1 1.0 (freeware; Zeiss), then edited images with ImageJ (Preibisch et al. 2009). Counts of nuclei per cell were sometimes difficult to determine because nuclei were very close to each other. Specifically, it was sometimes difficult to discern whether two nuclei were stacked on top of each other, two nuclei had just been generated through nuclear division, or a single nucleus was irregularly shaped to incorrectly suggest two overlapping nuclei. To increase reliability of nucleus counts, therefore, two experimenters blindly and independently counted nuclei in each image; if counts disagreed for an image, both experimenters independently reexamined the image until both reached a consensus.

Estimating the number of nuclei/cell from allele profiles of dekaryotized fungal strains.—If we assume that (i) each dekaryotization event (generation of a cell line with less nuclei than the parental strain UGM070517-01) is associated with l nuclei lost from the original set of n nuclei in the parental strain; and (ii) if the frequency of a given allele in the parent is f, then the probability of this allele remaining after dekaryotization follows the combinatorial expression $\frac{\binom{n-f}{n-l}}{\binom{n}{n-l}}$. We can use this probability to calculate the likelihood of the observed data (SUPPLEMENTARY TABLE 2) for each allele for an arbitrary set of parameters under the Poisson distribution, then obtain a maximum-likelihood estimate for the parameter set. We implemented this approach in a custom script in R (R Core Team 2014; SUPPLEMENTARY TABLE 7) separately for each allele that showed presence/absence polymorphism in SUPPLEMENTARY TABLE 2, for a range of n from 1 to 15 that encompassed most of the variation of number of nuclei/cell seen microscopically (FIG. 2a). The 11 alleles that were never lost in the dekaryotization experiment were excluded in this likelihood estimation, because these alleles provide no informative data, and these alleles may even be maintained by selection. The maximum-likelihood modeling is therefore based on the 29 alleles that showed allele loss as a result of the dekaryotization treatment (SUPPLEMENTARY TABLE 2).

RESULTS

Dekaryotization.—Dekaryotization of isolate UGM070517-01 was repeatable between the pilot and experimental trials (SUPPLEMENTARY TABLES 1 and 2). Of the 129 accessions isolated after dekaryotization treatment in the experimental trial, 36.4% (n = 47) showed the full allelic profile of the original isolate UGM070517-01 (i.e., un-dekaryotized mycelium was isolated), and 63.6% (n = 82) had lost at least one allele as a result of the dekaryotization treatment (SUPPLEMENTARY TABLE 2). On average, 4.8 alleles (11%) were lost in the 82 dekaryotized accessions. The

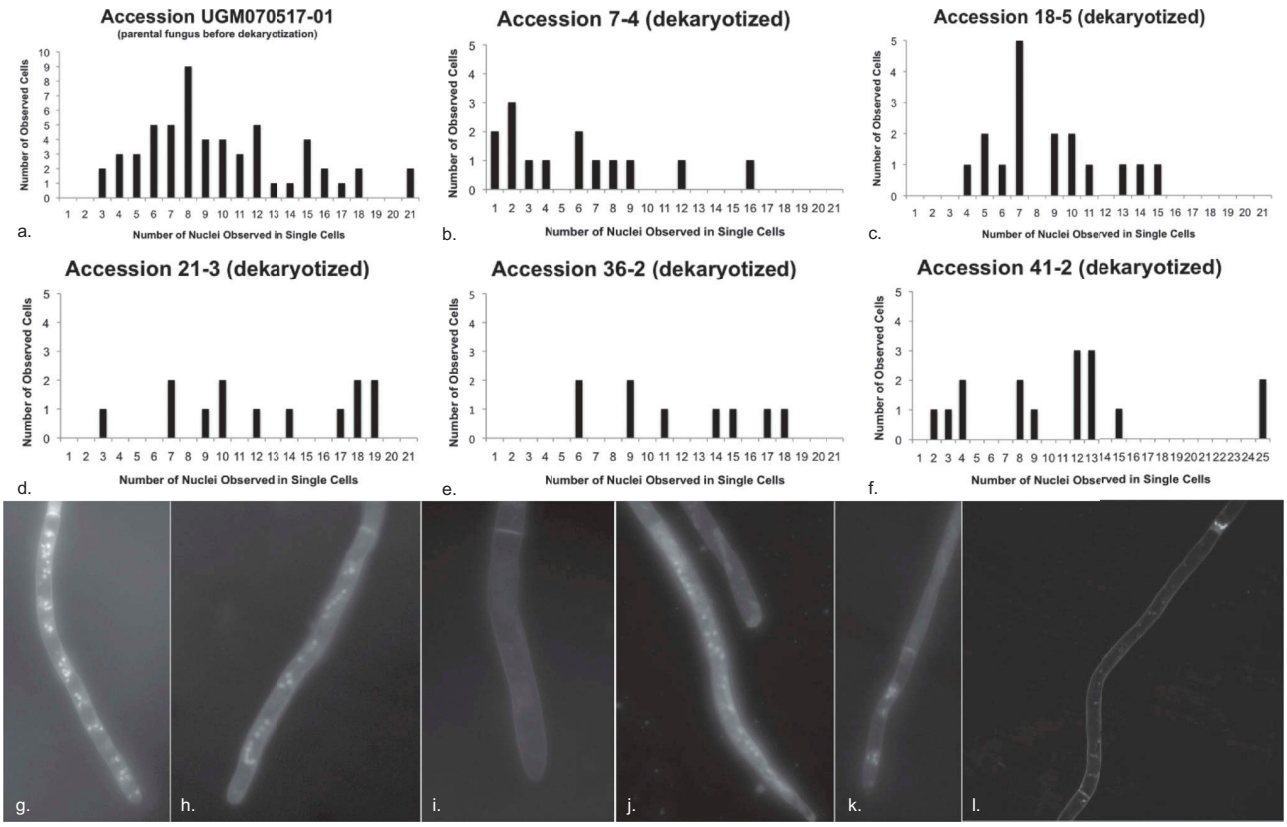

Figure 2. a–f. Distributions of the number of nuclei per cell observed in *L. gongylophorus* mycelium of five dekaryotized accessions and the untreated *L. gongylophorus* isolate UGM0705017-01 from which the dekaryotized accessions were derived. Distributions are shown only for those accessions for which we were able to characterize more than 10 cells (see full data set in SUPPLEMENTARY TABLE 5). g–l. Confocal microscopy images of representative multinucleate cells (additional images are deposited at GoogleDrive; see link in Data Deposition).

maximum number of alleles lost in an accession was 10 alleles from the original 40 alleles present in isolate UGM070517-01 when multiplex genotyping with both Plex1 and Plex2 (SUPPLEMENTARY TABLE 2). At 8 of the 15 loci, a total of 11 alleles were always present (i.e., these alleles were never lost); interestingly, these were the same alleles that were always present (never absent) in a large population-genetic survey of more than 200 *L. gongylophorus* samples collected from leafcutter ants in the USA and Mexico (Mueller et al. 2011b). We found no evidence for a haploid strain among the 129 accessions; that is, we found no dekaryotized accession showing only one allele at each locus. Even in dekaryotized accessions that lost the most alleles, most loci showed more than one allele, although some loci showed only one allele (SUPPLEMENTARY TABLES 1 and 2), suggesting that either (i) haploid strains may not be viable or (ii) single nuclei contained multiplied genomes (i.e., at least some nuclei were polyploid). Repeat subculturing (up to nine times) and multiplex genotyping of dekaryotized accessions revealed that the respective genotypes were stable over time; no additional alleles were lost and no alleles that had been lost as a result of dekaryotization treatment were later regained. Lastly, dekaryotization revealed null alleles at several loci (i.e., no allele amplified at a particular locus in some dekaryotized accessions, but the un-dekaryotized strain UGM070517-01 showed at least one allele at that same locus; SUPPLEMENTARY TABLES 1 and 2), indicating that our microsatellite fingerprints underestimate genetic diversity among the nuclei coexisting in an *L. gongylophorus* cell.

Morphological variation among dekaryotized accessions.—We could distinguish reliably four mycelial morphotypes (called here Types 1–4) for 127 of the 129 accessions growing on PDA medium (SUPPLEMENTARY FIG. 1), and only two accessions exhibited an intermediate morphology. Type 1 grew radially in circular shape, frequently displayed fluffy aerial growth, sometimes developed browning mycelial sectors, and produced abundant gongylidia (hyphal-tip swellings that grow clustered as so-called staphylae, which are harvested by the ants for food;

Weber 1972; De Fine Licht et al. 2014). Type 2 grew radially at a fast rate, was always circular and appressed to the medium, often appeared gray or brown in coloration, produced sometimes a few gongylidia initially after subculturing, but then ceased gongylidia production. Type 3 grew comparatively slower as a dome-shaped dense mycelium, did not initially produce gongylidia, but could eventually show some gongylidia after several weeks or months of growth. Type 4 grew initially as a puffy round ball with abundant gongylidia after subculturing, then slowly spread radially across the plate while continuing to produce gongylidia. Type 4 and Type 1 were the most difficult to distinguish from each other, especially after more than 3 mo of growth. The best age of a mycelium to reveal its morphotype was 1–3 mo after subculturing onto PDA medium. Because Types 1 and 4 produced abundant gongylidia in both younger and older cultures (like the un-dekaryotized parental strain), and because Types 2 and 3 produced far fewer or no gongylidia, we categorize Types 1 and 4 as "gongylidia-competent" and Types 2 and 3 as "gongylidia-incompetent."

On PDA medium, the original fungal accession UGM070517-01 displayed Type 1 mycelial growth, which was also the most common morphotype, found in 75.2% of the 129 accessions isolated after dekaryotization treatment (see "Morphotype" column in SUPPLEMENTARY TABLE 2). The average number of alleles lost, calculated separately for each of the four morphotypes, was 2.5 (Type 1), 2.0 (Type 2), 6.8 (Type 3), and 6.1 (Type 4). Accessions with no observed allele losses were found among Types 1, 2, and 4 (SUPPLEMENTARY TABLE 2). Type 2 morphs were never found with more than five alleles missing. Type 3 morphs always had at least five missing alleles. Types 3 and 4 are genetically the most distinct (i.e., most alleles lost) from the original accession UGM070517-01.

We observed no genotype differences when comparing allele profiles of browning and normal (white) sectors of the same mycelium. Browning sectors therefore do not develop because genetically distinct dekaryotized strains were commingled in the same mycelium, but browning seems a response to possible mutational variation arising in a mycelium, or to unknown physiological factors.

Growth measurements.—We recorded (i) the number of days it took for each regenerating mycelium to become visible on the regeneration plates (FIG. 1a), and (ii) number of days for a subcultured mycelium to show vigorous gongylidia production ("gongylidia vigor" defined as presence of at least 20 gongylidia) after subculturing to a new plate (FIG. 1b). On average, regenerating accessions with missing alleles took longer to show first growth on regeneration plates ($P < 0.0001$, $F = 44.94$, $df = 1, 127$; FIG. 1a) and also took longer to show vigorous gongylidia production ($P < 0.001$, $F = 57.47$, $df = 1, 122$; FIG. 1b) than those accessions that had lost no alleles (i.e., possessing the genotype of the original isolate before dekaryotization treatment).

In a second growth experiment, we selected 32 representative accessions, subcultured and genotyped these several times to document stable genotypes, then measured the area covered by mycelium (i.e., radial growth) and the aerial mycelial wet weight after 6 wk of growth. Area was significantly negatively correlated with the number of alleles lost by a genotype ($P = 0.031$, $F = 5.98$, $df = 1, 30$; FIG. 1c). Wet weight was also negatively correlated with the number of alleles lost by a genotype, but this trend was not statistically significant ($P = 0.16$, $F = 2.05$, $df = 1, 30$; FIG. 1d). Consistent with the above definitions of the growth morphotypes, Type 2 accessions showed the greatest radial growth by area, but not by weight (FIG. 1c and d). Type 3 accessions, which consistently missed at least five alleles, typically showed reduced radial growth and weight compared with the other three morphotypes (FIG. 1c and d). Data for both growth experiments are summarized in SUPPLEMENTARY TABLE 6.

Testing genetic exchange through recombination or nuclear reassociation between mycelia.—In nine confrontation experiments pairing different dekaryotized accessions (SUPPLEMENTARY TABLE 4), we found four cases where the mycelial confrontation generated stable and complete recombinants (i.e., through multiple rounds of subculturing, recombined isolates exhibited all alleles present also in the original strain UGM070517-01 before dekaryotization treatment; SUPPLEMENTARY FIG. 3). This suggests that *L. gongylophorus* strains have the ability to (i) exchange nuclei or recombine parasexually in the laboratory and recover from allele loss after dekaryotization treatment; and (ii) possibly exchange genetic material therefore also under natural conditions in the field (e.g., when two *L. gongylophorus* strains may be cocultivated in a single garden after an ant nest raids gardens of a neighboring nest; Mueller 2002).

Most dekaryotized accessions could be confronted without evidence of vegetative incompatibility. However, although all dekaryotized accessions had

been derived from the same un-dekaryotized fungus, UGM070517-01, some confrontations of dekaryotized accessions appeared vegetatively incompatible (SUPPLEMENTARY FIG. 5). This suggests that complex interactions between multiple genetic factors (e.g., between multiple loci) and possibly also cytoplasmic factors may modulate vegetative compatibility in *L. gongylophorus*, or that cryptic incompatibility between genetic factors can arise through mutation under the long-term asexual propagation of *L. gongylophorus* by the ants. Most interestingly, in some cases the confrontation of two gongylidia-incompetent strains (e.g., a Type 2 and a Type 3 fungus) generated stable recombinants that were gongylidia-competent (SUPPLEMENTARY FIG. 4). The restoration of gongylidia competence in recombinants suggests the possibility that complementary nuclei interact, or nuclear and cytoplasmic factors interact, to facilitate or enable gongylidia competence.

The confrontation experiments also revealed that a specific accession seemed to outcompete the other accession when two paired accession co-occurred in the same mycelium. Four of the confrontation experiments (Mixes 4–8, SUPPLEMENTARY TABLE 4) used the same pairings of accessions (one parent missing six alleles and the other missing four alleles). Genotyping 29 subcultures of the Sub1 isolates derived from these pairings (SUPPLEMENTARY FIG. 3) yielded 21 subcultures (72.4%) matching the dekaryotized strain with four missing alleles, 7 (24.2%) partial or full recombinants between the confronted accessions), and only 1 subculture (3.4%) matching the dekaryotized strain with six missing alleles. The accession missing four alleles therefore was easier to isolate from the contact zone between the paired mycelia, possible because this accession outcompeted the other accession in the confrontation or during the subculturing process. Despite this apparent advantage of specific strains in some of the confronted accessions, the delayed subcultures (isolated from confrontation plates after 3 mo) from the fungal growth front revealed in every experiment (Mixes 1–9, SUPPLEMENTARY TABLE 4) that different genotypes can coexist commingled alongside each other in the same mycelium for at least 3 mo, suggesting that (i) any exchanged nuclei may not have replicated and traveled throughout the entire anastomosing mycelia; and (ii) any competition between confronted strains does not lead within 3 mo to 100% representation of the competitively superior strain and to complete elimination of the competitively inferior strain.

DAPI staining of dekaryotized accessions.—Using fluorescent microscopy, we estimated the number of nuclei in the original accession UGM-0705017-01 and nine dekaryotized accessions (SUPPLEMENTARY TABLE 5; all images used in analyses are deposited at GoogleDrive, see link in Data Deposition). Of the nine dekaryotized accessions, only five grew as sparsely spaced mycelium suitable for easy visualization of single cells (i.e., most of the final data set in SUPPLEMENTARY TABLE 5 derives from these five strains), whereas visualization of single cells for the other four strains was more difficult (i.e., fewer observations for these strains are listed in SUPPLEMENTARY TABLE 5). With very few exceptions, cells of the same mycelium were always multinucleate. The variation of nuclei per cell of the same mycelium was remarkably large (FIG. 2). The original accession UGM-0705017 contained 0–21 nuclei/cell, with an average of 9.4 nuclei, and we observed similar ranges in nuclei/cell in all of the five well-characterized accessions (SUPPLEMENTARY TABLE 5). The spatial distribution of nuclei within the cytoplasm of a cell was typically not uniform; nuclei frequently occurred in pairs or in clusters of four or more nuclei. In a small number of cells of the original fungus UGM-0705017 and once in accession 16-1, nuclei were observed in a chain-like succession in the center of the cell (FIG. 2), possibly because unknown factors force nuclei into the cell center and away from the cell wall. Nuclei varied in size, ranged from spherical to subspherical, with occasional irregularly shaped nuclei (FIG. 2). We observed many cells in which some but not all nuclei appeared to undergo mitosis, suggesting the hypothesis that duplication of nuclei may not be synchronized across all nuclei in a cell. We did not find any clamp connections among a total of 142 cells examined, confirming findings from earlier studies that likewise failed to report clamp connection in *L. gongylophorus* mycelium (Möller 1893; Hervey et al. 1977; Chapela et al. 1994; see also literature reviewed in Mueller 2002).

We observed four apical cell hyphal tips with no visible nuclei, but subapical cells that did contain nuclei (FIG. 2), a phenomenon also seen in the multinucleate fungus *Termitomyces* sp. (De Fine Licht et al. 2005). Two of these cells with no visible nuclei were in the mycelium of the parental strain UGM0705017-01, and one each in the dekaryotized accessions 7-4 and 41-2. Not much is known about nuclear behavior in *L. gongylophorus*, but its nuclei may be able to migrate through septal pores, allowing an anucleate cell to become nucleate eventually, as has been seen in *Agaricus* (Kamzolkina et al. 2006). Some of the anucleate *L. gongylophorus* cells showed unusual cytoplasmic staining, possibly because these cells are older and may have mature vacuoles displacing the cytoplasm (Timothy James, personal communication).

Estimating the number of nuclei/cell from allele profiles of dekaryotized fungal strains.—Maximum-likelihood estimation indicates that the most likely number of nuclei/cell of the parental strain UGM070517-01 prior to dekaryotization was 8 nuclei/cell (SUPPLEMENTARY FIG. 6). This number is the same as the modal estimate of the number of nuclei/cell observed in our microscopic observations (SUPPLEMENTARY TABLE 5).

Data deposition.—Images of *L. gongylophorus* mycelium used in histological analyses are deposited at GoogleDrive: https://drive.google.com/drive/folders/0B_Zr7y_BumzDazhfNHU1MWN1OW8.

DISCUSSION

We developed (i) a simple protocol to dekaryotize multinucleate *L. gongylophorus* fungi cultivated by leafcutter ants; (ii) two panels of multiplexed microsatellite DNA markers (respectively 10 and 5 loci per panel) to track genetic changes during dekaryotization (SUPPLEMENTARY TABLE 3); (iii) phenotyping methods to distinguish mycelial morphotypes among dekaryotized strains that differ in their tendency to produce gongylidia (nutritive structures consumed by the ants; SUPPLEMENTARY FIG. 1); and (iv) genetic methods to combine nuclei from different dekaryotized strains into novel "recombinants."

***Implications for the biology of* L. gongylophorus.**—Mycelium of the parental *L. gongylophorus* accession UGM0705017-01 contained a highly variable number of nuclei per cell (average of 9.4 nuclei/cell, range: 0–21 nuclei/cell; FIG. 1, SUPPLEMENTARY TABLE 5) and showed 40 alleles across the 15 loci screened with our multiplex genotyping (average of 2.7 alleles per locus; range: 2–5 alleles per locus). After one round of dekaryotization, an average of 4.8 alleles were lost in 82 dekaryotized accessions (4.8 alleles are 11% of the total of 40 alleles present across all loci prior to dekaryotization; SUPPLEMENTARY TABLES 1 and 2). We used the frequencies of allele losses to derive a maximum-likelihood estimate for the number of nuclei/cell of the parental strain UGM070517-01. Our estimate of 8 nuclei/cell in the likelihood modeling (SUPPLEMENTARY FIG. 6) is the same as the modal estimate of the number of nuclei/cell observed in our microscopic observations (FIG. 2a) and close to the overall average of 9.4 nuclei/cell of parental strain UGM070517-01 (SUPPLEMENTARY TABLE 5). The average of 9.4 nuclei/cell for our *L. gongylophorus* isolate from the leafcutter ant *Atta texana* from the USA is somewhat lower than the average of 12.5 (± 0.41) calculated by Kooij et al. (2015) for 14 *L. gongylophorus* fungi isolated from eight *Atta* nests and six *Acromyrmex* nests from Panama, suggesting possible regional differences in nuclei/cell of *L. gongylophorus*.

We did not find any haploid strain among the 82 dekaryotized accessions (i.e., we found no dekaryotized accession showing only one allele at each of the 15 loci screened), and the maximum number of alleles lost in a dekaryotized accession was 10 alleles (25% of the 40 alleles present prior to dekaryotization). This suggests that it is either difficult to reduce the large number of coexisting nuclei to one nucleus per cell, haploid strains may not be viable or have poor viability (e.g., heterokaryosis may be beneficial because nuclei with different alleles complement each other), or nuclei contain polyploid genomes. Obligate polyploidy with "5–7 haplotypes on average" per nucleus was inferred by Kooij et al. (2015) for leafcutter fungi from Panama. This high ploidy of *L. gongylophorus* (i) suggests that most of the observed allelic diversity at a locus is due to ploidy compared with allelic diversity between nuclei coexisting in a cell; and (ii) explains why only a limited number of alleles were lost in our dekaryotization experiments.

Of the three *L. gongylophorus* strains that we tried to dekaryotize, we succeeded at isolating regenerating mycelium only from strain UGM070517-01. No live mycelium grew on the regeneration plates of two other strains, and it is unclear why mycelium did not regenerate in these cases. All three strains had been isolated in spring 2007 from nests in central and north Texas, they had been maintained and subcultured in the laboratory under the same conditions, and they had the same Type 1 growth morphology. The three strains belong to the T-fungus genotype cluster, a group of fungi cultivated by *Atta texana* in central, north, and east Texas and in Louisiana, but apparently absent in south Texas and Mexico (Mueller et al. 2011b). Whether some *L. gongylophorus* strains are easier to dekaryotize than other strains is not possible to say from our limited number of dekaryotization attempts.

***Implications for future mycological analyses of* L. gongylophorus.**—Our findings and new methods suggest a series of additional mycological studies to elucidate the biology of *L. gongylophorus* fungi:

(1) To distinguish between the hypotheses explaining why it is difficult or impossible to generate haploid strains (e.g., heterokaryosis may enhance fungal viability; single nuclei are highly polyploid), a future study could (i) generate a far greater number of dekaryotized

accessions (hundreds of accessions may have to be generated and screened to generate eventually a single-nucleus strain), or (ii) reduce the number of nuclei per cell through multiple, successive rounds of dekaryotization treatment. If haploid strains or homokaryons can be generated and nuclei from genetically differentiated strains can be combined in a heterokaryotic mycelium, it may be possible to compare phenotypes of homokaryotic and heterokaryotic strains (e.g., compare tendency to produce gongylidia), paralleling Nobre et al. (2014) who compared spore production of homokaryotic and heterokaryotic mycelia in the termite-cultivated fungus *Termitomyces*. Haploid strains, or strains greatly reduced in genetic diversity in a population of nuclei in a mycelium, may also be useful to test genetic mechanisms determining fungal species boundaries, mating-type-dependent or vegetative compatibilities (James 2015; see also SUPPLEMENTARY FIG. 5), as well as unilateral migration of nuclei (Harder and Aanen 2009) between mycelia of different *L. gongylophorus* genotypes.

(2) Microscopy could elucidate whether our dekaryotization treatment (blending and maceration of mycelium) generates protoplasts (without cell walls) from which dekaryotized hyphae regenerate, or simply separates hyphal cells that differ in number of nuclei (FIG. 2, SUPPLEMENTARY TABLE 5), such that some of the hyphae regenerating from these separated cells show reduced allelic diversity.

(3) Microscopy could also characterize the number of nuclei/cell in dekaryotized strains immediately after dekaryotized treatment (in contrast, we characterized number of nuclei after dekaryotized strains had been maintained alive in the laboratory for 7 y through multiple subculturing cycles). Counting nuclei immediately after dekaryotization treatment could address whether the number of nuclei/cell changes over time, possibly increasing from initially reduced levels to the level of nuclei observed in the parental strain before dekaryotization treatment. Such an increase in number of nuclei/cell over time may exist because the dekaryotized strains that we examined microscopically had approximately the same numbers and ranges of nuclei as the un-dekaryotized parental strain UGM0705017-01 (FIG. 2, SUPPLEMENTARY TABLE 5).

(4) *L. gongylophorus* mycelium shows great variation in number of nuclei per cell (SUPPLEMENTARY TABLE 5), paralleling the variable number of nuclei per cell observed in the termite-cultivated fungus *Termitomyces* (Nobre et al. 2014). This suggests comparative investigations into regulation of heterokaryotic diversity, nuclear compatibilities, and nuclear-cytoplasmic interactions (including interactions between a diverse nuclear population, a diverse mitochondrial population, and possible viruses) that determine mycelial phenotypes of symbiotic fungi cultivated by diverse insect lineages (Mueller et al. 2005).

(5) Because we observed several cases in which a single cell contained nuclei at different stages of mitosis (some nuclei were in the process of dividing, some not), it is possible that *L. gongylophorus* may have the kind of asynchronous nuclear division cycle known for several other filamentous fungi (Gladfelter et al. 2006; Gladfelter and Berman 2009). If *L. gongylophorus* has asynchronous nuclear division, the high nucleus counts that we observed occasionally (SUPPLEMENTARY TABLE 5) may represent cells in which nuclei divided recently, but septa had not yet formed to divide the cells' large populations of nuclei. *L. gongylophorus* fungi may therefore offer unique opportunities to study an evolutionary transition from the presumably synchronized nuclear division in the dikaryotic fungi of lower-attine ants to the asynchronous nuclear division in *L. gongylophorus* symbionts of leafcutter ants.

Implications for future studies of ant-fungus interactions.—Our new dekaryotization and multiplex-genotyping methods can be used for molecular-ecological studies to elucidate ant-fungus interactions:

(6) *Atta* ants cultivate their fungi in monocultures (Mueller et al. 2010), but foreign cultivar strains can enter a monoculture via import of cultivar from other nests, for example when workers of one attine nest steal garden from a neighboring nest (Autuori 1950; Mintzer 1987; Rissing et al. 1989; Cahan and Julian 1999; Adam et al. 2000; Green et al. 2002; Mikheyev et al. 2006), or possibly also because wind-dispersed *L. gongylophorus* spores may enter a nest via workers or substrate (Pagnocca et al. 2001; Mueller 2002; Mueller et al. 2011b, 2017). It may be possible to experimentally insert garden material or spores into a leafcutter nest in the laboratory or field to study the consequences of genetic exchange between genetically differentiated *L. gongylophorus* strains in the presence of the ant farmers.

(7) Because of the possible import of novel *L. gongylophorus* strains into established leafcutter nests, two mechanisms have been considered important in maintaining gardens as monoculture: (i) fungus-fungus

competition leading to dominance of a resident *L. gongylophorus* fungus and (ii) symbiont choice by ants weeding out recombinant or inferior *L. gongylophorus* strains (Mueller 2002; Sen et al. 2010). When different *L. gongylophorus* incocula are offered to leafcutter ants in either a cafeteria-style assay or as a mycelial mix, *Atta* and *Acromyrmex* leafcutter ants can maintain chimeric gardens for months (Sen et al. 2010), suggesting that fungus-fungus competition appears weak, or that the ants are able to maintain a polyculture of intercropped *L. gongylophorus* strains by some unknown mechanisms. However, when mycelia of different *L. gongylophorus* strains are mixed in equal proportions in the absence of leafcutter ant farmers, one fungal strain can easily dominate the mix in short time (R. Sen and U. G. Mueller, unpublished data), suggesting that fungus-fungus competition can be significant under specific conditions. Our new methods permit more refined experiments testing the relative importance of fungus-fungus competition versus symbiont choice exerted by ants in maintaining garden monoculture or polyculture, by confronting in a single garden un-dekaryotized natural strains, or dekaryotized strains, or both.

(8) Because dekaryotized strains differ greatly in growth performance (FIG. 1), and because some dekaryotized strains do not produce gongylidia in vitro, it should be possible to force-switch a leafcutter colony to cultivate a single strain of such a gongylidia-incompetent fungus (e.g., a gongylidia-incompetent Type 3 fungus), then test colony performance (e.g., growth rate, disease resistance, etc.). Such experiments parallel the cultivar-switch experiments with cultivars from *Trachymyrmex* and *Atta* ants conducted by Seal et al. (2012, 2014a, 2014b), except that now different dekaryotized strains of *L. gongylophorus* can be used to test the relative benefits of genetically diverse versus depauperate nuclear population in an *L. gongylophorus* mycelium, as well as test the nutritional roles of gongylidia-incompetent versus gongylidia-competent strains in the evolutionary ecology of the leafcutter ant–fungus symbiosis.

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