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Nutritional niches reveal fundamental domestication tradeoffs in fungus-farming ants

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Abstract

During crop domestication, human farmers traded greater productivity for higher crop vulnerability outside specialized cultivation conditions. We found a similar domestication tradeoff

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Author Contributions

J.Z.S, J.J.B, and W.T.W conceived and designed the study. J.Z.S., E.B.G., M.F., A.J.J.C., D.A.D., and P.W.K. performed field work, collected colonies, isolated fungal cultivars, and performed *in vitro* experiments with fungal cultivars. D.A.D., J.Z.S., P.W.K., and A.J.J.C. extracted DNA and performed DNA barcoding analyses for ants and fungal cultivars. P.W.K., J..Z.S. and J.H. performed the microsatellite analyses. X.A. and J.Z.S. performed statistical analyses. J.C.S. performed phylogenetic analyses. J.Z.S and J.J.B wrote the initial draft of the manuscript. J.Z.S., P.W.K, D.A.D., J.C.S., A.J.J.C, X.A., J.H., W.T.W., and J.J.B contributed to the interpretation of the data and to the editing of subsequent drafts of the manuscript.

Competing Interests

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across the major co-evolutionary transitions in farming systems of attine ants. First, the fundamental nutritional niches (FNNs) of cultivars narrowed during ~ 60 million years of naturally selected domestication, and laboratory experiments showed that ant farmers representing subsequent domestication stages strictly regulate protein harvest relative to cultivar FNNs. Second, ants with different farming systems differed in their abilities to harvest the resources that best matched the nutritional needs of their fungal cultivars. This was assessed by quantifying realized nutritional niches (RNNs) from analyses of items collected from the mandibles of laden ant foragers in the field. Third, extensive field collections suggest that among-colony genetic diversity of cultivars in small-scale farms may offer population-wide resilience benefits that species with large-scale farming colonies achieve by more elaborate and demanding cultivation practices of less diverse crops. Our results underscore that naturally selected farming systems have potential to shed light on nutritional tradeoffs that shaped the course of culturally evolved human farming.

Introduction

Farming evolved by natural selection in several social insect lineages and as a cultural innovation in our human ancestors. Although analogies across these domains need to be phrased carefully, it is reasonable to assume that both types of farming became more sophisticated over time which seems obvious from a crop perspective because our domesticated food plants often only remotely resemble their free-living ancestors. Over thousands of years, humans have continuously selected plant cultivars with nutritionally enhanced or enlarged leaves, roots, fruits and seeds[1,2] which facilitated modern agriculture's ecological expansion[3]. Domestication has also exposed production tradeoffs as increasingly specialized crops came to rely on specific abiotic conditions that farmers needed to provide to match their cultivars' shrinking fundamental niches for moisture, temperature and nutrients[4]. Such tradeoffs were especially pronounced when artificially selected traits ran counter to the naturally selected life histories that previously maximized fitness in wild cultivar ancestors confronting more hostile and fluctuating natural environments[5,6]. As domestication proceeded, culturally informed human farmers managed to push these tradeoffs to extremes. For instance, crops in historically recent eras have been farmed across huge metapopulations even though their vulnerability to herbivores and pathogens has often increased[7], although the strength of such tradeoffs can vary[8]. This geographic expansion has been enhanced by technological solutions that enable farmers to provide their crops with consistently optimized realized niches (e.g. higher doses of pesticides).

The farming systems of ants[9,10], macrotermitine termites[11], and ambrosia beetles[10] required millions of years of natural selection to produce narrow mutualistic co-dependencies[12]. Rather than balancing nutritional needs by farming diverse foods as in large-scale human societies, these insect farmers specialized on clonal monocultures of a limited suite of fungi, like the fungus-farming attine ants that adopted agaricaceous fungal cultivars (Order: Agaricales)[13,14]. Despite this overall difference, ant farmers of diverse attine genera came to rear specialized cultivars with varying degrees of polyploidy[15] and basal metabolic rates[16] that appear linked to transitions to increasingly complex farming strategies. Throughout their shared co-evolutionary histories, fungus-farming ants also evolved specialized fungal enzyme recycling habits to increase crop productivity[17] and powerful antibiotic defences to

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control crop disease[18–21]. These fungicultural innovations have likely been instrumental in the ecological diversification and geographic expansion among the 19 extant attine genera encompassing > 240 species that now inhabit most neotropical rainforests and many drier habitats from Argentina to the northeastern United States[22–25]

Given these historical domestication patterns, we hypothesized that: 1) ant farmers have solved a tradeoff between crop yield and cultivar vulnerability, and 2) this tradeoff hinges upon nutrient availability as the core commodity of crop provisioning and yield. We used the well-established nutritional geometry approach to test this hypothesis, capitalizing on its conceptual and empirical tools for visualizing and modelling tradeoffs that organisms navigate to maintain nutritional homeostasis[26–29]. Our present study builds on an earlier one in which we used nutritional geometry to quantify a related type of nutritional tradeoff in the ant *Mycocepurus smithii*, a representative of the paleoattine clade that is sister to the neoattines that evolved more organizationally-complex farming systems. Colonies of *M. smithii* farm clones of a weakly domesticated fungal cultivar that can maximize growth of edible hyphae in proportion to carbohydrate intake, but with the downside of encouraging cultivars to produce inedible mushrooms that may benefit fungal reproduction to the detriment of farmer fitness[30]. We now expand this nutritional geometry approach across the key evolutionary transitions towards higher organizational complexity in attine farming systems (Fig. 1a) by integrating their nutritional economy with ecological niche theory.

We tested the domestication tradeoff between yield and vulnerability by performing a study with the following three objectives: 1) To quantify and visualize the breadth of fundamental nutrient niches (FNNs)[29,31] when fungal cultivars are grown *in vitro* across artificial nutritional landscapes varying in absolute and relative abundance of protein and carbohydrate macronutrients (Fig. 1b). We used these results to interpret laboratory feeding experiments with nutritionally-defined substrates that tested how ant farmers prioritize macronutrient intake within the performance constraints imposed by their cultivar's FNNs. 2) To examine whether and how ant farmers target their cultivar's nutritional niches while foraging across natural nutritional landscapes in the field (Fig. 1c). We quantified realized nutritional niches (RNNs)[29,31] of naturally collected substrates and assessed their nestedness within the experimentally obtained FNN dimensions of cultivars (Fig. 1d). 3) To assess whether individual small-scale farming colonies within local populations can successfully maintain genetically different cultivars while foraging within a single nutritional landscape. We assess whether small-scale farmers manage cultivar vulnerabilities differently than large-scale farmers that achieve system resilience by cultivating uniform crops of low genetic diversity.

Results

Cultivar FNNs narrowed with full domestication but growth rates did not increase

We first compared cultivar FNNs across three evolutionary stages of crop domestication[14] where we predicted that ant farmers solved the yield-vulnerability tradeoff in different ways. We started with the paleoattine genera *Mycocepurus* and *Apterostigma*, and the genus *Cyphomyrmex* that represents an early branch of the 'lower' neoattines that would later produce the 'higher' neoattine genera described below (Fig. 1A). These three representatives

likely resemble the ancestral fungus-farming attines that arose ~ 60 MYA because all retained small, low-productivity colonies of tens to some hundreds of monomorphic workers and cultivars with undifferentiated hyphae to feed the ant farmers[14] (*but see* reference 32 for a case of a differentiated cultivar). As expected prior to full crop domestication, these cultivars exhibited broad FNNs with hyphal growth maximized across a wide range of imbalanced protein:carbohydrate (P:C) ratios (Fig. 2, Supplementary Table 1). These cultivar FNNs also varied widely among (but not within, Extended Data Fig. 1) cultivar types (Fig. 2), which likely reflects that these farmers have exchanged loosely domesticated fungi with free-living varieties over millions of years[33]. Despite this variation, the agaricaceous cultivars of *M. smithii* and *C. costatus*, as well as the pterulaceous 'coral fungus' *Myrmecopterula velohortorum* reared by *A. dentigerum* [34] all showed continuing or accelerated growth on carbohydrate-biased substrates at high nutritional concentrations (Fig. 2) relative to what can apparently be provided in detrital substrates collected by their ant farmers (*see* discussion of Figure 4a below).

We next measured the FNNs of cultivars in the higher-neoattine genera *Sericomyrmex*, *Mycetomoellerius* and *Paratrachymyrmex* (Fig. 1A) that evolved ~ 27-31 MYA[21,22,35]. The adaptive radiation of this clade coincided with the irreversible domestication of a specialized fungal lineage capable of concentrating nutrients in specialized swollen hyphal tips (gongylidia) bundled into staphylae[36–38]. The extant descendants of these higher neoattines represent an intermediate domestication phase characterized by farming fully domesticated cultivars but without significant changes in foraging substrates[39,40] and colony sizes[16,41] relative to the phylogenetically basal lower neoattines and paleoattines. Consistent with their domesticated crops being derived from a single fungal ancestor, the cultivars of these higher-neoattine farmers had less variable FNNs than the paleoattine and lower-neoattine cultivars, typically maximizing growth on substrates with 8-15 g/L protein and at rather low (8 to 25 g/L) absolute concentrations of carbohydrates (Fig. 2). The narrowed FNNs of these cultivars were confirmed because they often exhibited mortality in petri dish culture when they were provided with suboptimal nutrient mixtures (Extended Data Fig. 2, Supplementary Table 2), consistent with expectations of the hypothesized domestication tradeoff of specialization versus vulnerability.

The leafcutter ants represent the major expansion in scale and organizational complexity of neoattine fungus farming that appeared ~ 18.5 MYA[21,22] (Fig. 1a). The emergence of the leafcutter ants coincided with the use of freshly-cut vegetation from hundreds of plant species[42] to provision massive gardens of the now obligately polyploid[15] staphylae-bearing cultivar *Leucoagaricus gongylophorus* [34] to support tens of thousands of ants in colonies of *Acromyrmex* and up to millions of individuals in colonies of *Atta* [24]. The performance responses of *L. gongylophorus* cultivars isolated from colonies of *Acromyrmex echinatior* and *Atta colombica* resembled those of the other higher-neoattine cultivars (Fig. 2, Extended Data Fig. 1) and also often included steep mortality increases when confined to nutritional blends outside the cultivar's FNN (Extended Data Fig. 2). Thus, the protein-carbohydrate FNN of *L. gongylophorus* cultivars did not appear to have changed in connection to the broader ecological niche of substrate provisioning displayed by the leafcutter ants[42].

The *L. gongylophorus* cultivar of leafcutter ants also exhibited statistically similar *in vitro* growth rates relative to cultivars of the other higher neoattines (Extended Data Fig. 3), which is remarkable because *Atta* foragers in the field provision cultivars with orders of magnitude more substrate mass than any other attine genera[42]. In fact, cultivars of the smallest lower-attine colonies of *A. dentigerum* and *C. costatus* grew faster *in vitro* on a standard potato dextrose agar (PDA) medium known to generally promote high cultivar growth rates[25,43] than all other cultivars tested (Extended Data Fig. 3). This result appears to be analogous to the insignificant changes in growth rate of plant crops during and after human domestication[7] and suggests that accelerated intrinsic cultivar growth rates neither drove nor responded to later increases in scale and organizational complexity of fungus-farming in neoattine ants.

Foraging ants collect nutrients within the FNN dimensions of their cultivars

Ant and human farmers nutritionally provision their cultivars in fundamentally different ways. While humans farm mostly autotrophic plants that need targeted blends of inorganic NPK fertilizers, attine ant farmers maintain heterotrophic fungi that need organic inputs in the form of freshly cut plant material, or scavenged insect frass and other detritus that foragers must gather from their environment (Fig. 1). Fungal cultivars also have no independent resource acquisition via roots, so the ants need to provide crops with most all their nutrients as crude forage[44]. These chemically and physically complex substrates contain the required protein and carbohydrate macronutrients but also water, micronutrients and vitamins, toxins and recalcitrant cell wall fibres[37,45]. We used nutritional geometry tools to cut through this complexity and explore whether and how *in vitro* measures of cultivar FNN breadth constrain the nutritional content of substrates collected by the fungus-farming ants when they forage.

We performed laboratory feeding experiments to measure nutrient-regulation strategies in attine colonies confined to single nutritionally-defined substrates with varying P:C ratios (Fig. 3a). For these experiments, we focused on comparing three attine species representing the main domestication transitions described above: paleoattines (*M. smithii*), higher neoattines (*Paratrachymyrmex cornetzi*), and crown-group neoattine leafcutters (*A. colombica*). We found that the leafcutter ants targeted broader protein dimensions than the two other species (Fig. 3b). Specifically, *A. colombica* foragers collected more of the protein-rich 1:1 P:C substrate than both *M. smithii* and *P. cornetzi* that tightly regulated protein intake to remain at low levels across all P:C substrates (Fig. 3b, Extended Data Fig. 4, Supplementary Table 4). Colonies of *P. cornetzi* and *M. smithii* also had an aversion to substrates with macronutrient ratios above 1:3 P:C, possibly because these blends are associated with increased risks of crop failure (Extended Data Fig. 5), worker mortality (Supplementary Fig. 2a) and reduced total colony biomass (Supplementary Fig. 3a) for both *P. cornetzi* (Supplementary Table 6) and *M. smithii* [30]. The tight regulation of protein intake by foragers of these two species matched the narrow *in vitro* protein FNNs of their cultivars (i.e., the steep declines in growth beyond 15 g/L protein; Fig. 2).

The leafcutter ants did not face a similarly extreme vulnerability-yield tradeoff between starving fungal cultivars of carbohydrates or over-harvesting protein when confined to

nutritionally imbalanced substrates (Fig. 3b, Extended Data Fig. 4, Supplementary Table 4). In fact, *A. colombica* colonies showed greater stability across all P:C substrates tested, remaining stable or increasing in both worker number (Supplementary Fig. 2b, Supplementary Table 6) and colony mass (Supplementary Fig. 3b, Supplementary Table 6). Yet, *A. colombica* colonies also appeared to walk a nutritional tightrope since their foraging levels also varied with substrate macronutrient ratios. Specifically, *A. colombica* workers collected significantly less carbohydrates when confined to the most protein-biased substrates (3:1 and 6:1 P:C; Extended Data Fig. 4). Thus, while *A. colombica* colonies maintained relatively high foraging levels under these protein-biased conditions (Fig. 3b), they still experienced carbohydrate shortfall relative to levels that could maximize cultivar growth on carbohydrate-biased diets. This may be related to the steep *in vitro* decline in growth (Fig. 2) and survival (Extended Data Fig. 2) exhibited by *L. gongylophorus* beyond substrate protein concentrations of 30 g/L, that resembled (but was slightly higher than) the cultivars of *P. cornetzi* that we tested.

Quantifying the RNN dimensions of foraging ant farmers in the field

Targeting FNNs that maximize hyphal growth (Fig. 2) while avoiding inedible mushroom production is a documented nutrient-mediated tradeoff before attine ants fully domesticated their cultivars[30]. However, mushrooms were never produced by plated cultivars of neoattine ants, so we expected them to exhibit more subtle domestication tradeoffs. Analogous to human crops that no longer exchange genes with wild or feral relatives, higher neoattine ant farmers are expected to nutritionally provision their cultivars to optimize resource allocation between somatic hyphal growth and the fraction of this growth to be harvested as edible staphylae with gongylidia. We therefore tested whether and how ant farmers in the field resolved nutrient provisioning vulnerabilities in their crops by estimating colony-level RNNs that could be mapped on the specific FNN dimensions obtained by rearing cultivars *in vitro*.

As before, we compared three attine species representing the transitions from collection of mostly frass—leaf material processed through the guts of insect herbivores (secondary herbivory) in *M. smithii* and *P. cornetzi* and freshly cut vegetation in *A. colombica* (primary herbivory). To assess RNNs, we collected, weighed, and catalogued samples of substrates collected from the mandibles of laden foragers as they returned to their nests, representing 77 hours of total field observations from 22 colonies of *M. smithii* (Supplementary Table 7), 44 colonies of *P. cornetzi* (Supplementary Table 7), and 1 colony of *A. colombica* (Supplementary Table 8). We then nutritionally analysed substrates collected by foragers from the same study populations representing 19 colonies of *M. smithii*, 33 colonies of *P. cornetzi* (Supplementary Table 9), and 1 colony of *A. colombica* (Supplementary Table 8).

We expected colonies of each ant species to forage for natural substrates within the range of the experimentally obtained FNNs of their cultivars, i.e. that their RNNs would not exceed their cultivar's upper physiological tolerances and would tend to converge on the performance maximizing dimensions (dark red) of the nutritional FNNs (Fig. 1c,d). We first found that foragers of *M. smithii* harvested sufficient amounts of protein (horizontal axis) to be able to avoid the cultivar FNN region that would produce inedible mushrooms, matching previously results[30]. However, while these detrital substrates provided a minimal protein

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level, they were also below the higher carbohydrate concentrations (vertical axis) that could accelerate cultivar growth (Fig. 4a). Second, colonies of *P. cornetzi* harvested substrates that yielded an RNN whose protein and carbohydrate amounts fell somewhat below the FNN regions maximizing both growth and staphyla density (Fig. 4b), while avoiding the high-concentration nutritional blends that induced cultivar mortality *in vitro* (Extended Data Fig. 2). Third, the representative *Atta* leafcutter ant colony achieved a comparatively broader RNN with higher maximal concentrations of macronutrients (Fig. 4c). This enlarged leafcutter RNN spanned the distinct FNN peaks for hyphal growth and staphyla production.

Cultivars with variable FNNs co-exist in a single nutritional provisioning environment

Many attine ant species maintain mutualistic associations with several fungal haplotypes[46–48]. This trait also applies to human farming but is puzzling in attine farms that are constrained to rear a single cultivar clone which is usually vertically acquired[12,49]. For instance, if neighbouring colonies of the same ant species farm genetically distinct cultivars with different FNNs, one should expect considerable farmer plasticity to target RNNs that match their cultivar's specific needs. To assess the extent of performance variation across cultivars, we compared two attine species with different levels of fully domesticated crop specificity: 1) the leafcutter ant *A. colombica* as a single ant species (Extended Data Fig. 6) that farms a single species of *L. gongylophorus* cultivar (Fig. 5) and 2) the higher neoattine *P. cornetzi* (Fig. 5, Extended Data Fig. 7, Extended Data Fig. 8) known to farm one of several possible species of fungal cultivar across our study area in Panama[48].

We found that *P. cornetzi* farms five cultivar haplotypes (Fig. 5) with similar FNNs for hyphal growth that also roughly matched those of the *L. gongylophorus* cultivar of *A. colombica* (Fig. 6a) and were consistent with other fully domesticated higher-neoattine cultivars tested (Fig. 2). However, the FNNs for staphyla density among isolates of the single cultivar species of *A. colombica* were much more consistent than the FNNs of the different cultivar haplotypes reared by *P. cornetzi* (Fig. 6a). In particular, the staphyla density FNN of cultivar haplotype 1 was similar to the FNN of *L. gongylophorus* reared by *A. colombica* and protein-biased (from 3:1 to 9:1 P:C) at intermediate nutrient concentrations (20 g/L) (Fig. 6a), while the staphyla-density FNN for *P. cornetzi*'s cultivar haplotype 3 was carbohydrate-biased (1:9 P:C) at high nutrient concentrations (40 to 60 g/L). This cultivar variation extended to intrinsic growth rates viewed across the distinct cultivar haplotypes farmed by *P. cornetzi* (Fig. 6b) although further sampling will be needed to confirm this apparent phylogenetic variation.

Variable FNNs for maximal staphylae density raise questions about the plasticity required by small-scale *P. cornetzi* farmers that found colonies with one specific crop symbiont and are unable to predict what mixture of forage items their habitat patch will provide over time. Such stochasticity may help explain why colonies primarily balance their foraging between two main detrital resources, frass (variable in protein and low in carbohydrates) and wood chips (variable in carbohydrates and low in protein), while also targeting nutritionally variable, but higher total concentration bits of plant material (e.g. flowers, seeds, detrital leaf bits) (Fig. 4b, Supplementary Table 7). Further research will be required to resolve whether

P. cornetzi colonies are actually able to dynamically adjust the cumulative sum of these alternative resources so they would achieve their optimal RNN under most circumstances.

The need for plasticity in cultivar provisioning also became apparent after we mapped spatial colony distributions across higher neoattine species within our 20-m^2 leaflitter plots. This showed that *P. cornetzi* is part of a diverse assemblage of similarly sized higher-neoattine (non-leafcutter) farming species (*Paratrachymyrmex*, *Mycetomoellerius*, and *Sericomyrmex*) in Soberanía Park (Extended Data Fig. 7, Extended Data Fig. 8), with colonies of 4-7 species (mean ± SD: 5 ± 1) co-existing in close sympatry and at high densities of 21 to 66 colonies (mean ± SD: 43 ± 17) per 20 m^2 plot. *P. cornetzi* was the most abundant of these farming ant species with 20 ± 13 (range 4-35) colonies per 20 m^2 (Fig. 6c, Extended Data Fig. 10). At a community scale, most of these fungus-farming ant species share at least some symbionts[48] so that neighbouring colonies separated by < 1 m are within each other's foraging range. Different fungal haplotypes thus coexist within a single local nutritional environment.

Discussion

Towards an ecological explanation of the history and current impact of ant farming

Quantifying nutritional niches of fungus-farming ants reveals a domestication tradeoff between vulnerability and yield. First, weakly domesticated paleoattine and lower-neoattine cultivars tend to express generalist traits reminiscent of free-living fungi. These included broad and variable FNNs that likely matched ecological conditions found in nutritional landscapes available outside regulated nest environments. For instance, their high growth rates at high-nutrient concentrations appear to exceed nutrients available from ant detritus provisioning, and may instead reflect an ability to exploit ephemeral pulses of leaf-litter resources[50]. Second, fully domesticated cultivars of higher-neoattine and leafcutter ants expressed similarly narrowed FNNs, likely reflecting that they are derived from a single fully domesticated *Leucoagaricus* ancestor. The trends observed across attine species generally matched expectations of a domestication tradeoff where increased productivity through specialized nutritional yield benefits (i.e., staphylae with gongylidia) in a narrowed suite of environmental conditions coincided with an increasingly remote resemblance to the cultivar traits that characterized the ancestral free-living fungi.

Our results revealed only minor expansions in FNN breadth of the specialized *L. gongylophorus* cultivar of leafcutter ants compared to the genetically more variable but likewise fully domesticated cultivars of *P. cornetzi*. This result seemed surprising at first because leafcutter ants are remarkably generalist foragers that collect fresh leaves and flowers from hundreds of plant species[42]. However, the broad RNN dimensions of *Atta* colonies that we recorded in the field (Fig. 4c) were consistent with higher degrees of symbiotic resilience when we exposed colonies to nutritionally imbalanced substrates in lab experiments (Fig. 3). Moreover, leafcutter ant workers have evolved many specialized traits enhancing farming performance[18–20,51–53]. Specialized gardener castes may thus help to secondarily adjust the harvested substrates relative to required RNN dimensions when they macerate leaf fragments into a leaf-pulp mixed with glandular and faecal fluids[17–19]. A suite of bacterial symbionts was also recently discovered in leafcutter ant digestive tracts[54] and

fungus gardens[55] that may help to maintain the wide RNN of *Atta* leafcutter ants by providing nitrogen fixation services[55], by recycling excess amino acids such as arginine, or by converting plant-sap citrate into acetate to directly fuel ant metabolism[40].

A range of nutritional solutions to a fundamental domestication tradeoff

Our study focused on three representative attine ant model species varying in colony size and scale of farming to explore: 1) the unique cultivation challenges and opportunities each symbiosis faces, and 2) how each farming system maximizes cultivar performance by mixing nutritionally variable substrates collected from the environment. Our results highlight that there is no single answer to how fungus-farming ants navigate the complex nutritional landscape of a tropical rainforest to target RNNs within the FNNs of their specific cultivars to maximize edible yield. Since the cultivars of paleoattines deliver undifferentiated hyphae as food, *M. smithii* ants need only to mix in sufficient protein via frass provisioning to avoid wasteful mushroom production, which is achievable but likely precludes rapid garden growth[30]. The two more complex farming systems have a differentiated cultivar so that maximizing crop growth (hyphal mass) and edible harvest (staphyla density) have become separate optimization challenges. In the higher-neoattine *P. cornetzi*, opportunities for maximizing yield appear to be somewhat constrained by the nutritional FNN targets that may vary across cultivar haplotypes (Fig. 6a). Some farmers may thus maximize edible yield by collecting more flower petals to acquire extra carbohydrates, while others may benefit from the additional protein available in frass. To detect such dynamic nutritional provisioning strategies, longitudinal sampling of individual colonies will be useful for describing how RNNs change over time, and broader sampling of colonies across habitat types will help determine the resiliency of RNNs among colonies with access to different suites of nutritionally variable substrates.

Our results further showed that the detrital substrates collected by paleoattine and non-leafcutter neoattine ants tend to be nutrient-poor resources, so that farming productivity is likely to be ecologically constrained because their cultivar FNNs peak at higher nutrient concentrations than the ants can usually provide. The expanded RNN of *Atta* leafcutter ants provisions a highly specialized cultivar and appears to have partially overcome such constraints by both being able to handle more diverse macronutrient mixtures and higher absolute macronutrient quantities. The *Atta* cultivar also appeared to be special because it offers the farming ants two distinct macronutrient performance targets – one for hyphal growth (a carbohydrate-biased FNN) and one for staphyla density (a protein-biased FNN). Thus, our results suggest that the RNN expansion achieved by *Atta* leafcutter ants may have allowed a novel form of farming flexibility that could capitalize on high-quality (protein-rich) substrates when available without simultaneously enhancing cultivar growth rate *per se*.

We expect that future work will elaborate on the ecological pathways of fungal cultivar domestication by focusing on nutritional aspects of: 1) the earliest domestication tradeoff at the origin of attine fungus farming between the yield of incipient crops and the costs of abandoning hunter-gathering[16,56], 2) the diversification of farming practices across habitats and biomes[57] as attine lineages diverged and adapted to very different plant substrates[14,22],

and 3) the challenges of maintaining farming homeostasis in response to stochastic environmental variation[25], particularly in large-scale farming systems that evolved quite differently in *Acromyrmex* and *Atta* leafcutter ants. Further progress in answering questions of this kind is feasible because the attine ants are the only insect farming clade where many farming systems varying widely in scale and organizational complexity co-exist within the same ecosystems. This contrasts with the large-scale agricultural systems of humans that have tended to competitively displace subsistence farms in many habitats[58], with the fungus-farming termites where only more organizationally-complex forms of agriculture exist[11], and with the ambrosia beetles that invariably remained small-family cooperative breeders in tunnels that colony foundresses need to excavate in host trees[59].

Methods

Study populations

All attine colonies were harvested from the lowland tropical rainforest at Soberanía National Park, Panama (N 9.13528, W 79.72141) from 28 October 2013 to 10 June 2015, with additional fieldwork conducted from 1 May to 30 June 2019. We located nest entrances of paleoattines, lower neoattines and higher neoattines under leaf litter by placing polenta bait on the ground and following laden workers back to their nests. Leafcutter nests were visible as large established colonies and small dirt mounds of recently founded colonies. Back in the lab at the Smithsonian Tropical Research Institute in Gamboa, we established colonies (see Supplementary Table 5 for demography) in plastic containers with *ad lib* water and ground polenta (or leaves for *Atta*), and acclimated them to lab conditions at 24°C. Ant vouchers were stored at the Smithsonian Tropical Research Institute (STRI) in Panama and fungal vouchers used in barcoding analyses were stored at Kew Gardens in the UK.

Estimating ant cultivar FNNs and their growth performance in vitro

We isolated fungus from 51 colonies of 10 attine species, including *Mycocepurus smithii* (10 colonies), *Apterostigma dentigerum* (3), *Cyphomyrmex rimosus* (1), *C. costatus* (3), *C. longiscapus* (1), *Paratrachymyrmex cornetzi* (13), *Mycetomoellerius zeteki* (2), *Sericomyrmex amabilis* (2), *Acromyrmex echinatior* (3), and *Atta colombica* (13) (Supplementary Table 3). We grew these fungal cultivars on sealed sterile petri dishes containing PDA media (potato dextrose agar, DIFCO) and used them to generate pure fungal stock cultures. We then used PDA as a standard medium to compare cultivar growth rates[25,43] placing 19.6 mm^2 cylindrical plugs of pure culture into separate 60 x 15 mm petri dishes containing 15 ml of PDA. To estimate these intrinsic growth rates, we photographed the plates every 10 days for periods of 25 to 76 days (depending on the overall growth rate of the cultivar; Supplementary Table 3), and then used ImageJ (NIH Image; V 1.49) to measure cumulative fungal growth after 30 days (area mm^2).

We visualized the fundamental nutritional niches (FNNs) of cultivar fungi isolated from nests of 10 attine species (N = 22 colonies), including *M. smithii* (1), *A. dentigerum* (3), *C. rimosus* (1), *C. costatus* (3), *C. longiscapus* (1), *P. cornetzi* (3), *M. zeteki* (2), *S. amabilis* (2), *A. echinatior* (3), and *A. colombica* (3) (Supplementary Table 3). We inoculated fungi in petri dishes containing 12 mL of 36 sterile synthetic agar-based ‘substrate’ treatments

varying in P:C (1:9, 1:6, 1:3, 1:2, 1:1, 2:1, 3:1, 6:1, 9:1) and P + C concentration (8g/L, 20g/L, 40g/L, 60g/L) (n = 8 plates per substrate x dilution treatment, N = 288 plates per colony, N = 6,336 plates). We used growth rates on standard PDA media to set the length of P:C growth experiments for each cultivar (Supplementary Table 3).

Nutritionally-defined media that were used to quantify cultivars FNNs included distilled water and bacteriological agar (1.6% w:v; Amresco, Inc.), carbohydrates as equal parts sucrose (Doradita ® cane sugar) and starch (puriss p.a., from potato, reag. ISO, Sigma-Aldrich), and protein as equal parts bacto-peptone (enzymatic digest of protein, Becton, Dickinson and Company), trypticase peptone (pancreatic digest of casein, BD), and bacto tryptone (pancreatic digest of casein, BD). We also included a crushed multivitamin mixture (Centrum ®) at a concentration of 2% of the mass of protein + carbohydrates. See reference 30 for recipes. These ingredients were mixed with 200 ml distilled water on a stirring plate for 5 minutes and then sterilized by autoclaving at 121°C, yielding a pH of 6.9.

We mapped fungal growth areas across the 36-substrate arrays using the *fields* package[61] in R (3.2.4)[62]. We plotted nutritional landscape contours using non-parametric thin-plate splines and set the topological resolution of response surfaces to $\lambda = 0.001$ as a smoothing parameter. We then used least-square regressions to assess the underlying significance of both linear and quadratic terms (and their interactions) and to verify the interpretation of FNN heatmaps based on fungal growth areas across the 36 protein and carbohydrate substrate combinations. Unless otherwise noted, we present heatmaps based on means of cultivars farmed by each attine species, but also provide all additional heatmaps for individual cultivars sampled from each attine colony in Figure 6A (*P. cornetzi* and *A. colombica*) and in the Extended Data Fig. 1 (all other colonies), as well as the underlying statistical analyses (Supplementary Table 1, Supplementary Table 10).

Each of the higher-neoattine and leafcutter cultivar haplotypes that we tested exhibited mortality on a subset of P:C substrates outside the range of the FNN. Mortality was indicated by inoculation plugs being clear (empty of fungus) or by lack of growth from the inoculation plug onto the P:C substrate by the end of the experiment (Extended Data Fig. 2). We generated heatmaps as described before ($\lambda = 0.01$ for the percentage data) with survivorship averaged across cultivars isolated from nests of a given attine species, standardizing the heatmap colour scale from 0% to 100% survival. We statistically assessed the significance of underlying regressions as described for the growth area heatmaps. This type of mortality response was never observed in the paleoattine or lower-neoattine cultivars tested.

Laboratory no-choice feeding experiments with ant colonies and their natural cultivars

We analysed nutrient regulation strategies of lab-acclimated colonies using nutritional geometry feeding experiments based on agar-based mixtures of protein and carbohydrate macronutrients (1.6g agar/L) at P:C ratios of 1:6, 1:3, 1:1, 3:1, and 6:1, and protein + carbohydrates dilutions of 100 g/L (for *P. cornetzi* and *A. colombica*) (Supplementary Table 11) or 20 g/L (for *M. smithii*)[30]. To evaluate evolutionary trends across a broader dataset than the one collected for the present study, we included some previously published data from a parallel study we performed on *M. smithii* [30]. In our no-choice experiments we

confined colonies to a single P:C substrate for 29 days (*M. smithii*), 39 days (*P. cornetzi*), or 15 days (*A. colombica*). We provide raw pre- and post-experiment colony demography data for colonies in Supplementary Table 5. For standardized comparison across these experiments with different attine species, intake data were analysed for 15 days across all colonies.

We calculated protein and carbohydrate use by the ants from the agar P:C ratios and substrate dry mass loss estimated from dry:wet mass ratios of control agar fragments for each P:C ratio[66]. We weighed colonies on the first (initial colony mass) and last day (final colony mass) of the experiments, and calculated worker mortality rates by collecting all dead workers each time we changed substrates. During the *M. smithii* and *P. cornetzi* experiments, a number of colonies showed signs of crop failure (*i.e.*, elevated worker mortality, living workers ceasing foraging and discarding fungus garden material). For our demographic analyses, we assessed crops as having failed on the day colonies no longer had any fungus garden biomass left.

We performed statistical analyses in R 3.2.4[62], exploring P:C substrate treatment effects on colony behaviour and performance separately for the three ant species (the *M. smithii* data were already similarly analysed in reference 30). We first used general linear models to test for substrate treatment effects on the response variables total macronutrients intake, protein intake, and carbohydrate intake with initial worker number as a covariate. These data were log-transformed prior to analyses to meet the assumptions of normality and homoscedasticity. For both *A. colombica* and *P. cornetzi*, we analysed cumulative substrate use over 15 days. For *P. cornetzi*, we also analysed macronutrient use ‘per day’, with number of days ranging from the full 39 days to any earlier day a specific colony was removed due to crop failure. We next performed a survival analysis (survfit, ggsurvplot) in R using a Cox proportional hazards model to test for substrate treatment effects on crop failure in *P. cornetzi*, including initial fungus garden mass as a covariate. This analysis was not used for *A. colombica* because no colonies exhibited crop failure. To further explore the links between foraging behaviour and colony demography in both *P. cornetzi* and *A. colombica*, we used general linear models to test for effects of P:C macronutrient mixes on changes in worker number and colony mass, analysing the slopes of linear regressions for each colony from values on day 1 to values on the final day of the experiment (day 39 in *P. cornetzi*, day 15 *A. colombica*) or on the day the colony was removed from the experiment due to crop failure (a subset of *P. cornetzi* colonies).

RNNs of fungal cultivars estimated from field-collected substrates

To quantify RNNs for *P. cornetzi* and *M. smithii*, we first focused on cataloguing substrates harvested per unit time (Supplementary Table 7) and then on sampling the specific substrate categories to obtain sufficient biomass for nutritional analyses (Supplementary Table 9). We collected substrate items carried by laden workers returning to nests of 44 mapped *P. cornetzi* colonies from 8AM to 4PM during ~1-hour observation periods (43.7 total hours) (Supplementary Table 7). We collected substrates from 22 colonies of *M. smithii* using the same protocols, with sampling observations spanning over 20 hours (Supplementary Table 7, also see reference 30). Part of the raw biomass data of collected substrates for *M. smithii* and

P. cornetzi were previously published[40] but were here explicitly analysed as RNNs and compared to fungal FNNs (Fig. 4a). These collections occurred during the wet season, a period of high attine activity, from Nov 16 to Dec 31 in 2013 and Jan 2 to Jan 17 in 2014 (Supplementary Table 7). During observation periods, we lay on trash bags next to nest entrances using a headlamp to maximize visibility and carefully grabbed laden foragers with forceps just before they disappeared underground. We collected these substrate pieces in Eppendorf tubes and then dried them at 60°C for 24 hours before classifying them into six categories: insect frass, leaf fragment, wood, flower, seed, and other (miscellaneous plant, insect piece, unknown), and weighing them to the nearest 1 μg on a Sartorius CP2P microbalance. We then calculated the fraction of biomass of each substrate type relative to the total biomass of substrate collected.

For nutritional analyses, we first homogenized individual frass samples collected from 33 colonies of *P. cornetzi* and 19 colonies of *M. smithii* located in the same leaf litter habitats as described above (Supplementary Table 9). We then determined their elemental carbon (%C) and nitrogen (%N) in the lab of Ben Turner (Smithsonian Tropical Research Institute, Panama) with a Flash EA1112 analyser (CE Elantech, New Jersey, USA). The small size of these substrate fragments precluded direct macronutrient-level analyses of their percent protein and carbohydrates. To acquire these macronutrient estimates, we used 30 1-m^2 tarps placed on leaf litter for 24-hour intervals in the same forest in June 2019 to collect a large pooled sample of frass (and other detrital substrates) that fell from the canopy. Back in Copenhagen, we created a 1 g pooled frass sample of each substrate type (previously freeze dried just after collection in Panama using a SP Scientific BenchTop Pro with Omnitronics for 24 hours). We then homogenized these and analysed them for %C (Eurovector CN analyser coupled to an Isoprime isotope ratio mass spectrometer in the lab of Anders Michelsen, University of Copenhagen), as well as % total non-structural carbohydrates (%TNC: water soluble carbohydrates + starch). For %TNC measurements, we analysed 25 mg of frass with a Total Carbohydrate Assay Kit (Sigma-Aldrich) to determine water soluble carbohydrates, and 50 mg of frass with a Total Starch Assay Kit (Megazyme)[67] to quantify starch. We used 5 subsampled replicates for each quantification to calculate a conversion factor (2.59%) to substitute %C with %TNC for the frass and tiny wood fragments in the existing dataset (Supplementary Table 9).

From the elemental N frass data, we estimated crude protein (i.e. including non-available protein bound up by tannins) by multiplying substrate N mass by the standard conversion factor 6.25, following the Felton et al. protocol[67]. We then analysed %TNC in leaf-litter samples of seeds (n = 5) and a flower (n = 1), as well as pooled samples of flowers (n = 5) and tiny wood bits (n = 1) (Supplementary Table 9). In these non-frass samples, we also quantified proteins using a CBQCA Protein Quantification Kit (Molecular Probes) (Supplementary Table 9).

The much larger quantities of fresh vegetative substrates harvested by leafcutter ants required different field collection methods and also facilitated more detailed NIRS nutritional analyses that were not possible for the small amounts of tiny substrate particles harvested from mandibles of paleoattine and higher-neoattine (non-leafcutter) ants. In May 2019, we located an *A. colombica* colony at La Laguna (W 9.11672, N-79.69514) and laid

down on trash bags next to the most active trail, close to the nest entrance. We hand collected a total of 6,868 fragments (40,026 mg dry mass) carried by laden foragers during 3, 1.5 hour periods by two observers (9 total collection hours) between 9AM and 12AM (Supplementary Table 8). These fragments (almost all fresh plant material) were stored in new Ziplock bags every 30 minutes that were promptly transferred to a cooler. Back in the lab, we catalogued the forage fragment samples under a microscope based on the morphology of the veins of leaf fragments, weighed the specific fractions and freeze-dried for 24 hours as described above.

We then stored the samples at -20°C in new Ziploc bags with silica gel until subsequent nutrient and barcoding analyses. We extracted DNA from these samples and identified them using the Internal Transcribed Spacer 1 (ITS1) genetic marker (details in Supplementary methods; NCBI GenBank Accession codes provided in Supplementary Table 8). We then homogenized a subset of dried plant substrates into powder and used near infrared reflectance spectroscopy (NIRS)[67] to estimate the concentrations of total nitrogen, total protein, total non-structural carbohydrate, and starch in all 17 plant substrates sampled (details provided in Supplementary methods, Supplementary Table 12). The pie charts of harvested forage categories reported in Fig. 4 represent relative contributions to the total dry mass of substrate items collected by foraging workers (Supplementary Table 7, Supplementary Table 8). Based on barcoding analyses, we pooled a subset of the 17 samples that were identified as coming from the same plant species which gave a total of 13 plant species in the dataset (Supplementary Table 8). We converted the *in vitro* landscapes used to estimate cultivar FNNs from g/L to percent protein and carbohydrates based on P:C diet recipes to directly compare these data with RNNs based on macronutrient content in the natural forage obtained in the field. For simplicity, only the extreme carbohydrate-biased (1:9 P:C) and protein-based (9:1 P:C) nutritional rails are plotted in Fig. 4 to delineate the range of *in vitro* P:C conditions in which cultivar FNNs were measured.

Obtaining accurate estimates of species diversity of sympatric farming systems

The single species status of *A. colombica* and its fungal cultivar *L. gongylophorus* in central Panama are not in question and the same applies for *M. smithii* except that this paleoattine ant is known to be associated with a series of fungal cultivars[68], some of which were also recognized in our previous study of the nutritional geometry of this species[30]. However, this is much more ambiguous for *P. cornetzi* and their fungal symbionts, because a previous small-scale study[48] showed that there is substantial cryptic diversity not only among the cultivars, but possibly also among the ants[48]. To avoid misinterpretations of our present results due to overlooking (and unjustifiably pooling) cryptic species of ants and cultivars, we did an extensive survey across a large meta-population *P. cornetzi* (54 km^2 of Soberanía Park forest) and within the local populations represented by our six 20-m^2 plots (Extended Data Fig. 10). Within these study plots, we mapped all higher-neoattine nests (*Paratrachymyrmex*, *Mycetomoellerius*, *Sericomyrmex*) by dispersing oat-polenta bait in the leaf litter and following laden workers back to their nests (N = 263 total higher-neoattine nests across all plots, n = 27 ± 3 searching hours per plot), and then collected 2-4 workers from each nest in vials with 95% ethanol. We also sampled workers from 85 additional *Paratrachymyrmex* and *Mycetomoellerius* colonies distributed across Soberanía Park

yielding 297 colonies sampled across these two genera. We similarly sampled 30 *A. colombica* colonies distributed across the Gamboa area.

We then identified the ants using morphological analysis of vouchers, as well as analysis of DNA microsatellite markers and mtDNA barcoding (details for each of these analyses provided in Supplementary methods). Briefly, we used DNA microsatellite analyses to examine the population structure of workers from 28 *A. colombica* colonies (Extended Data Fig. 6) and 297 *Paratrachymyrmex* and *Mycetomoellerius* colonies (Extended Data Fig. 7) using a total of nine variable markers (Ant7680, Ant859, Ant1343, Ant2341, Ant3993, Ant11400, Ant3653, Ant4155, Ant8498)[69]. To supplement these nuclear microsatellite data, we also used DNA barcoding analyses of a mitochondrial marker (~1100 bp of the Cytochrome Oxidase 1 gene), which included ant workers from 147 *Paratrachymyrmex*, 38 *Mycetomoellerius* colonies and 29 *A. colombica* colonies (Supplementary Table 13). A subset of these ants was paired with their fungal cultivars to examine farmer-cultivar association patterns, as we excavated 69 colonies of putative *P. cornetzi* and 29 colonies of *A. colombica* (Supplementary Table 14). For each of these colonies, we collected a clean 2-cm^3 fraction of fungus garden with forceps into vials with 95% ethanol soon after excavation. We identified these samples by analysing mitochondrial DNA (~820 bp of the nuclear large subunit rRNA (LSU) and [~550 bp of the Internal Transcribed Spacer unit (ITS). We used these sequences (plus sequences from an additional *M. smithii* ant and fungal sample) to generate phylogenetic trees (details about tree generation are provided in Supplementary methods).

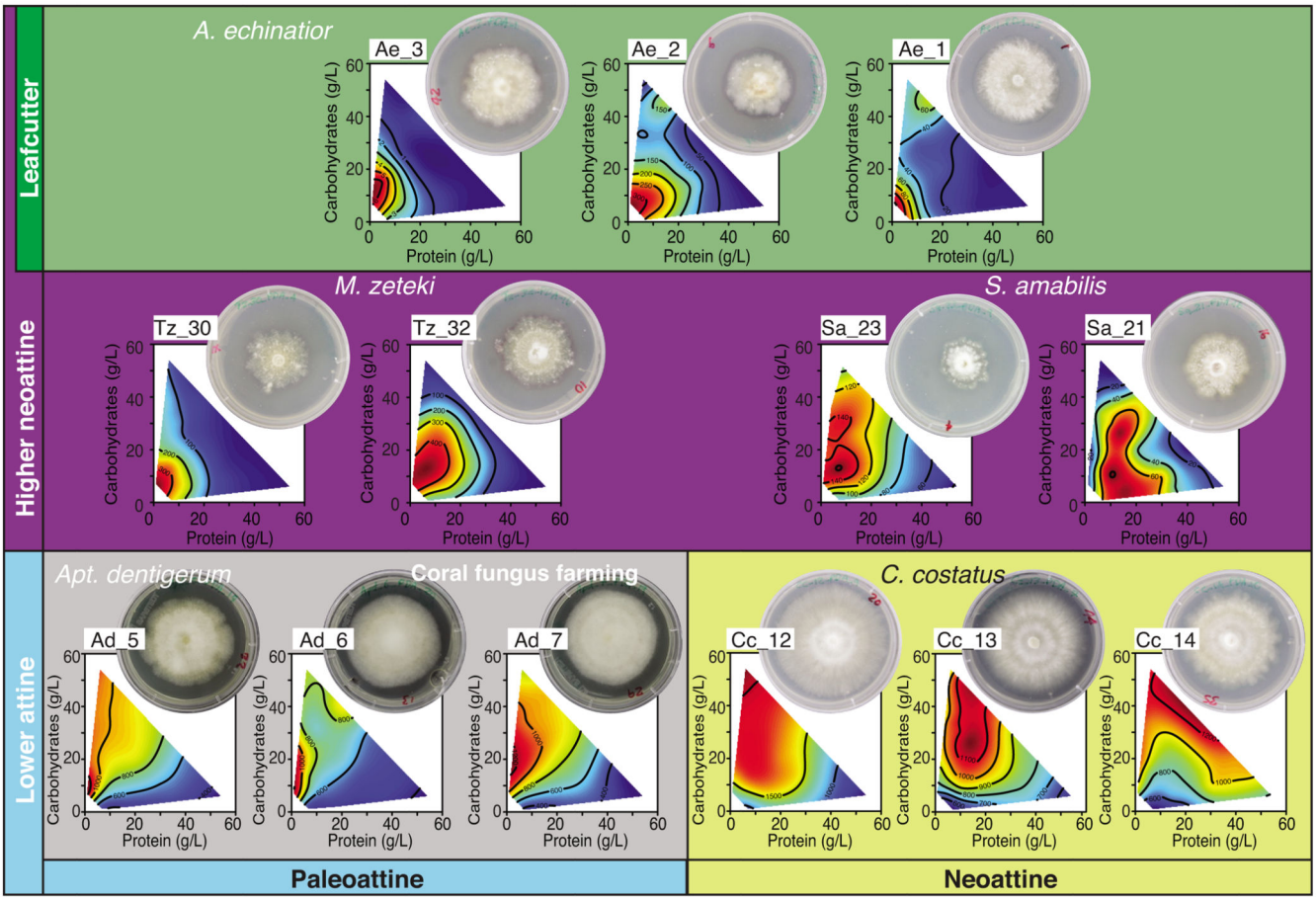
The resulting sequence information and supporting dataset for the 215 ant specimens is named DS-ATTINENG "Nutritional niches reveal fundamental domestication tradeoffs in fungus-farming ants" and has been deposited in the Barcode of Life Data System[70] (BOLD; dx.doi.org/10.5883/DS-ATTINENG). The sequence data is also deposited in GenBank (Accession codes provided in Supplementary Table 13). The sequence information for 99 fungal cultivar samples in Fig. 5 has been deposited in GenBank (Accession codes for LSU and ITS provided in Supplementary Table 14). To further interpret our molecular evidence confirming known species and detecting cryptic species among the collected *P. cornetzi*-like colonies within Soberanía Park, we also explored the biogeographic distribution of this species complex by comparing our sequence data to sequences of 8 Costa Rican and 1 Ecuadorian *P. cornetzi* ant specimen available from BOLD[81] (Extended Data Fig. 8).

Cultivar diversity across colonies, in vitro *FNN variation and growth performance*

We visualized the FNNs of isolated cultivars in Fig. 6A from three colonies of *P. cornetzi* (177605, 177611, 177612) and three colonies of *A. colombica* (177627, 177628, 177626) (Supplementary Table 3). Cultivars were selected prior to determining their haplotype identity, after which the *P. cornetzi* cultivars used in the experiment were identified as haplotype 1 (177605, 177612) and haplotype 3 (177612) (Fig. 6a). Due to general differences in growth rate among cultivar haplotypes on standard PDA medium, these haplotypes were grown on P:C substrates for different lengths of time (Supplementary Table 3). A trained researcher (MF) also estimated staphyla production rate (staphyla number per number of growth days) by visually scanning the high-resolution photos and validating

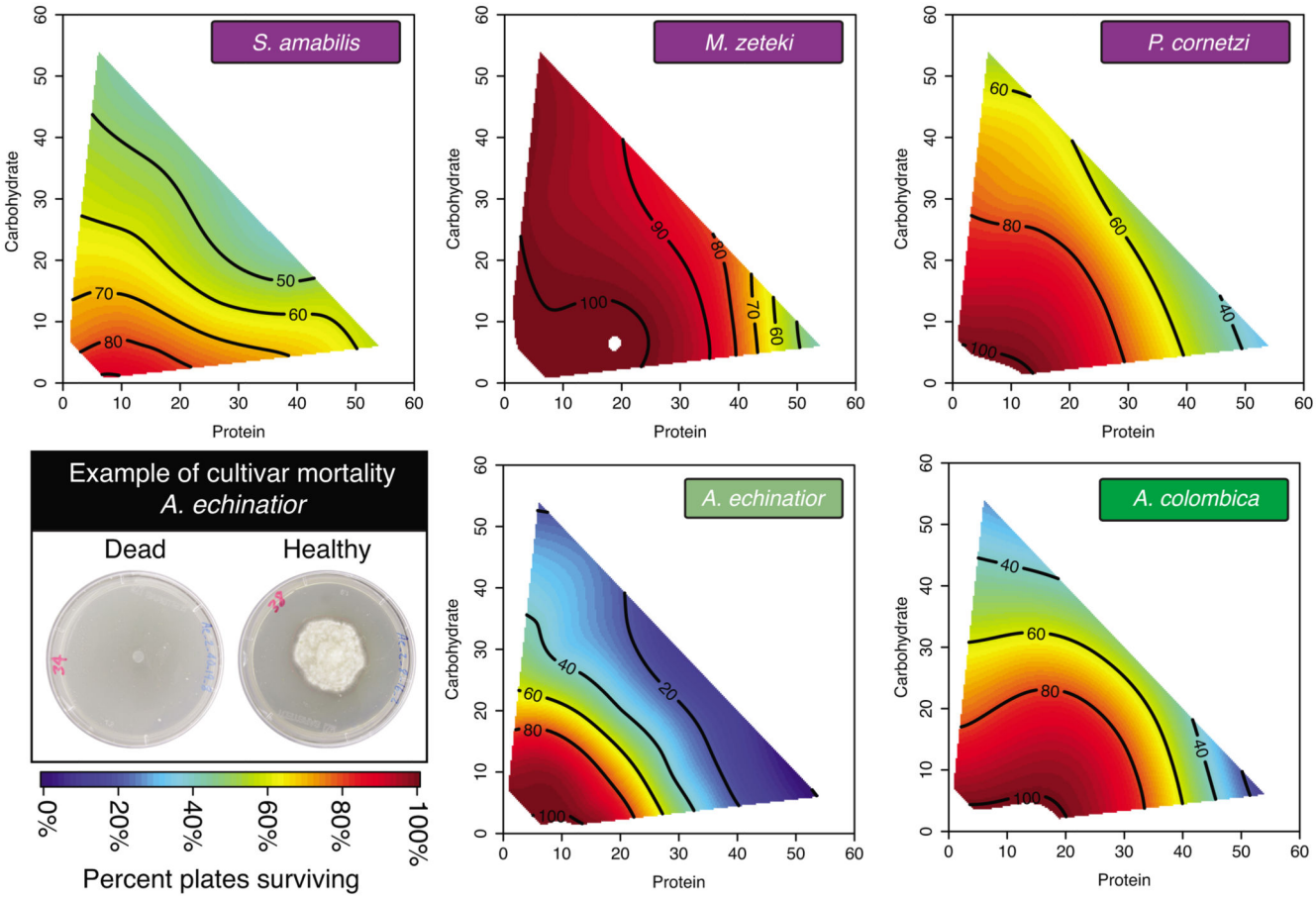
counts by opening a subset of plates and directly counting staphyla (clusters of gongylidia) under a dissecting microscope at 40X magnification.

Extended Data



Extended Data Fig. 1. Cultivars exhibited consistent FNNs for hyphal growth when isolated from different colonies of each attine species, supporting that the heatmaps based on species means (Fig. 2, Fig. 4B,C) accurately represent each cultivar's FNN.

Additional details about how these heatmaps were generated and how they were interpreted are provided in Fig. 2. Heatmaps are provided here for attine species where multiple colonies were sampled. Additional colony-level heatmaps for *P. cornetzi* and *A. colombica* are provided in Fig. 6A. Collection IDs corresponding to experiment IDs were: Ae_3 [177625], Ae_2 [177624], Ae_1 [177609], Tz_30 [177632], Tz_32 [177634], Sa_23 [177623], Sa_21 [177614], Ad_5 [177629], Ad_6 [177630], Ad_7 [177631], Cc_12 [37861], Cc_13 [37862], and Cc14 [37864] (Supplementary Table 3). As in Figure 2, least-square regressions showed that each of the response surface regressions producing the heatmap colour-gradients was significant (Supplementary Table 1).



Extended Data Fig. 2. Cultivars of the higher-neoattine genera *Sericomyrmex*, *Mycetomoellerius*, *Paratrachymyrmex* (purple) and the leafcutter genera *Acromyrmex* (light green) and *Atta* (dark green) often exhibited mortality when confined to media with macronutrient mixtures outside their FNNs.

As shown in the figure legend, dark red indicates 100% survival and dark blue indicates 0% survival (a clear inoculation plug or a failure to colonize the agar plate from the inoculation plug). Percent mortality data were averaged across cultivars isolated from colonies of the same attine species (n = 2, *M. zeteki*; n = 3, *P. cornetzi*; n = 1, *S. amabilis*; n = 3, *A. echinatior*; n = 3, *A. colombica*, Supplementary Table 2). Mortality data were not recorded for the *S. amabilis* colony Sa_23. Small white areas on some of the plots indicate 100% survival.

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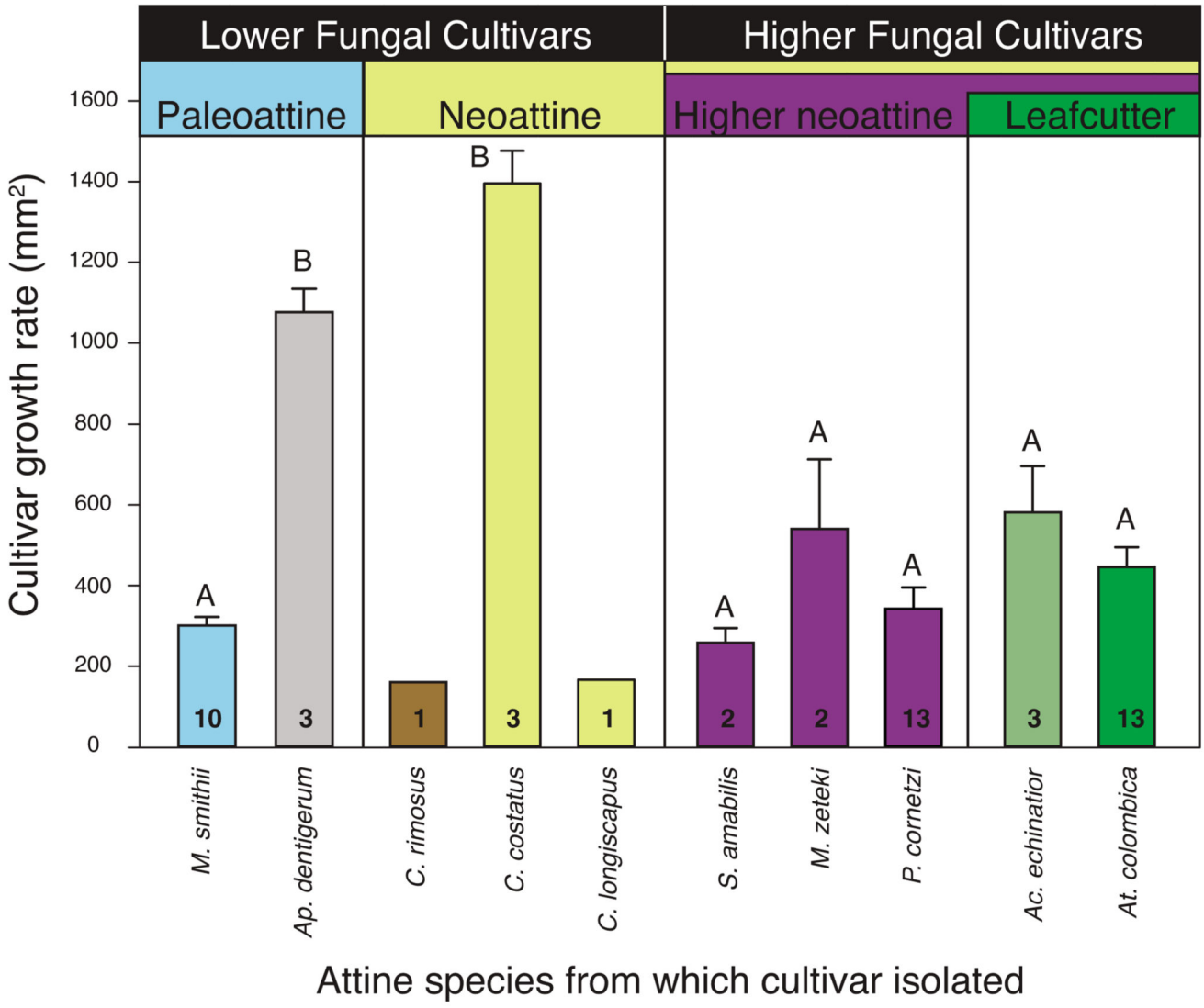
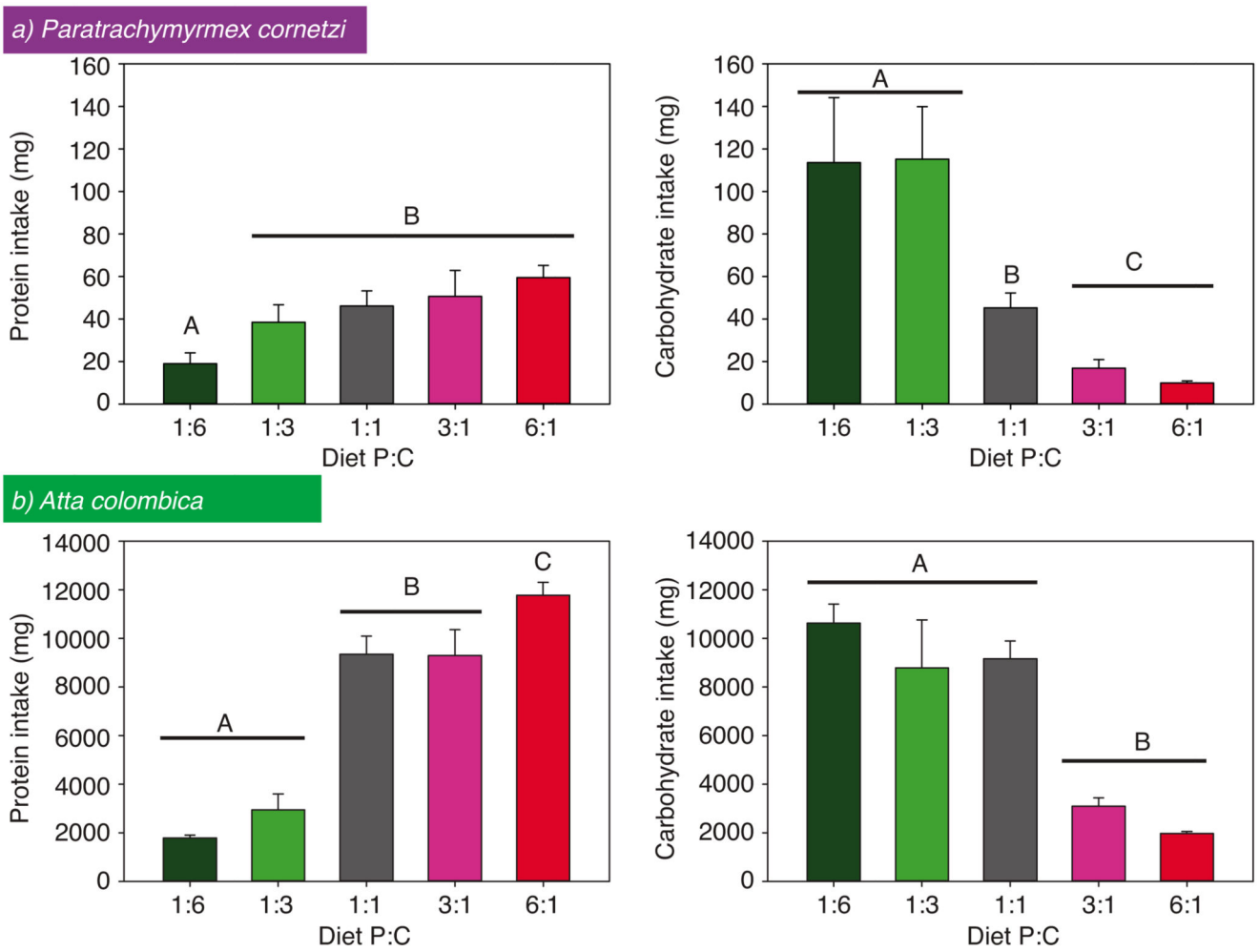


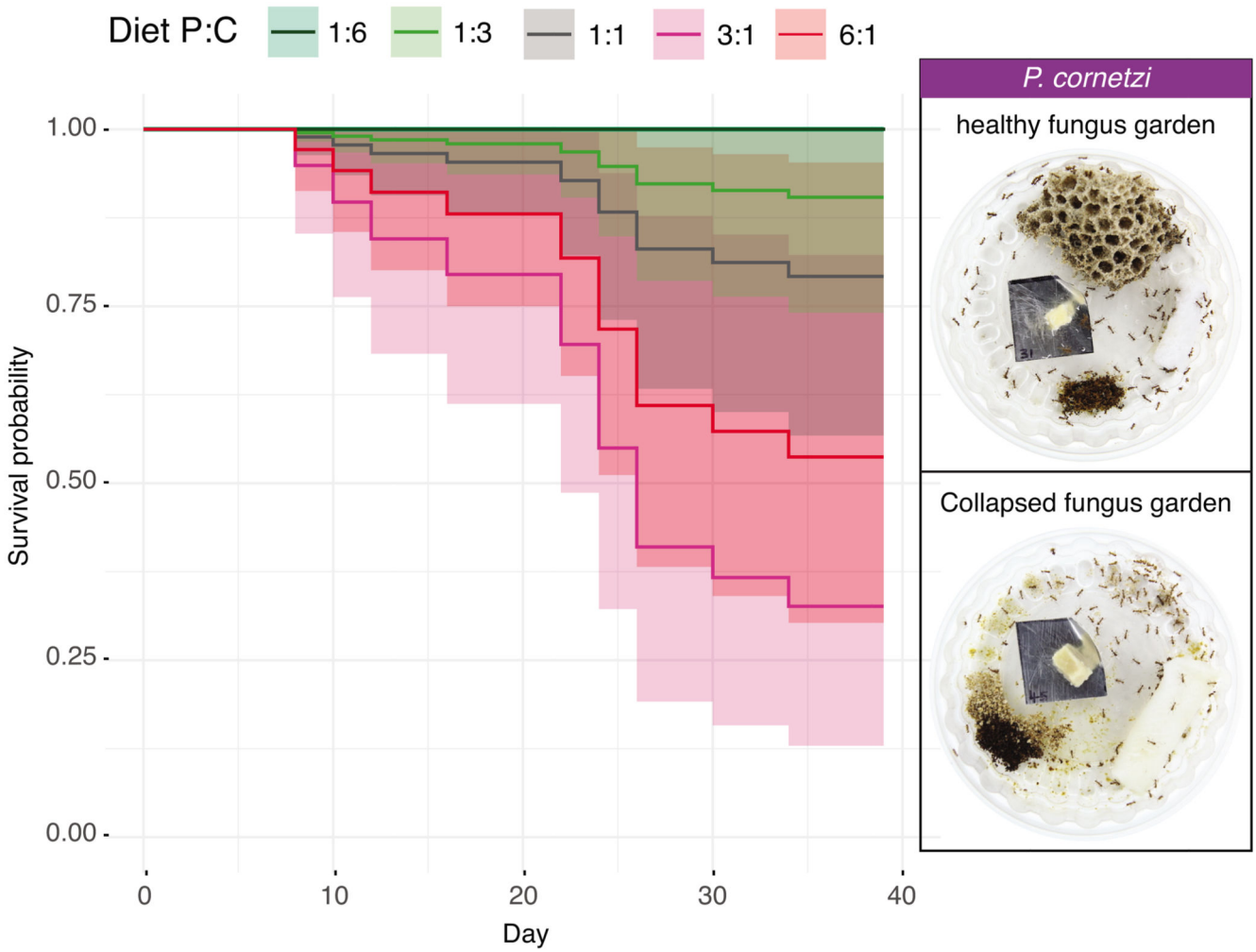
Extended Data Fig. 3. The emergence of large-scale fungus farming by *Atta* leafcutter ants cannot be explained solely by an increase in intrinsic cultivar growth rate.

Coloured bars (corresponding to the farming transitions in the schematic tree of Fig. 1a) represent *in vitro* growth rates of cultivars during 30 days on a standard PDA medium. Vertical lines separate the major farming transitions that distinguish ten attine species sympatrically inhabiting the Panamanian rainforest of Soberanía park (see caption Fig. 1A). ANOVA showed that attine species farmed cultivars with significantly different growth rates after 30-days ($F_{7,41} = 24.42$, $p < 0.0001$) and letters indicate pairwise differences that were significant at $P < 0.05$ in post-hoc Tukey tests. Numbers inside bars indicate numbers of colonies from which cultivars were isolated and used to calculate mean growth rates (+ SE) (Supplementary Table 3). The cultivars of *C. rimosus* and *C. longiscapus* were not included in the statistical analyses because we had no replicate colonies for these two ant species.

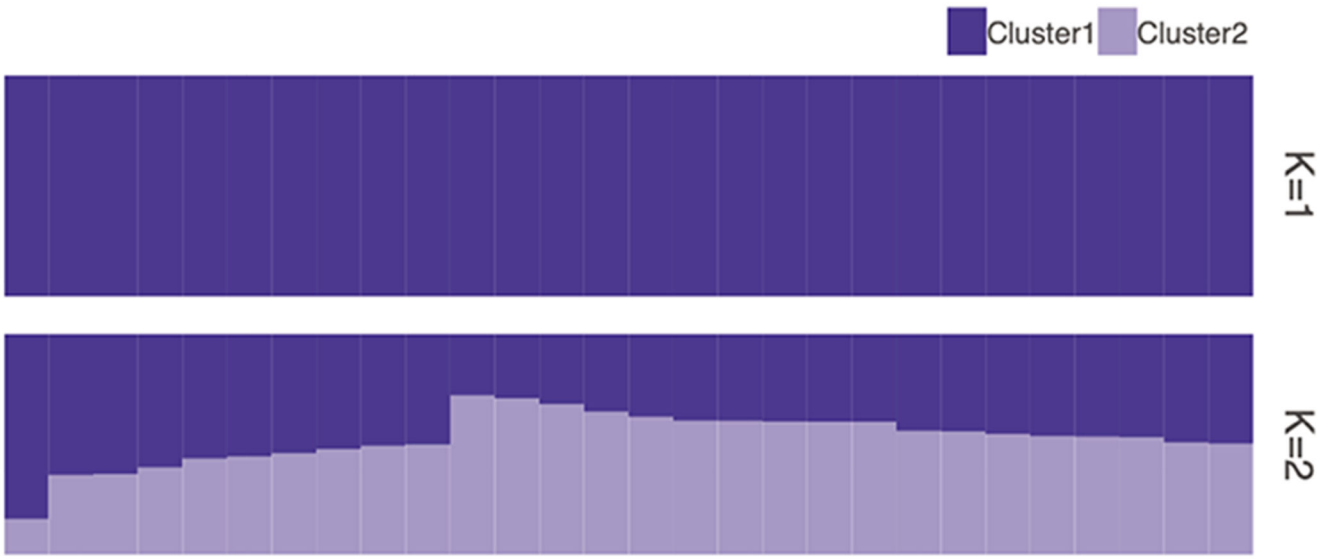


Extended Data Fig. 4. Cumulative amounts of protein and carbohydrates collected over 15 days by whole colonies of a) *P. cornetzi* and b) *A. colombica*.

While colonies of *P. cornetzi* and *M. smithii* [30] avoided protein-biased substrates with ratios above 1:3 P:C, colonies of *A. colombica* collected large amounts of the 1:1 P:C agar that *P. cornetzi* and *M. smithii* avoided. Colonies of *A. colombica* further collected statistically similar levels of carbohydrates on 1:1, 1:3, and 1:6 P:C substrates (Supplementary Table 4). This tolerance of higher protein levels enables *A. colombica* to sustain higher carbohydrate intake levels on 1:1 P:C diets. Letters indicate pairwise differences that were significant at $P < 0.05$ (post-hoc Tukey tests) (Supplementary Table 4). These intake plots (means + SE) provide an alternative single-nutrient presentation of the bi-variate intake data presented in Fig. 3b.



Extended Data Fig. 5. Colonies of *P. cornetzi* increasingly faced crop failure when confined to P:C macronutrient mixtures with excess protein relative to carbohydrates ($\chi^2_4 = 17.9$; p = 0.001). While similar results were observed for *M. smithii* [30], no colonies of *A. colombica* experienced crop failure, even when confined to the same protein-biased substrates. Cultivar survival probabilities were estimated with a Cox proportional hazards model where substrate treatment was the explanatory variable, initial garden mass was a covariate, and days remaining in the feeding experiment was the response variable.



Extended Data Fig. 6. STRUCTURE analyses of 9 microsatellite loci indicated that workers from 28 *Atta colombica* colonies in the Gamboa area of Soberanía park represent a single interbreeding population.

Specifically, the mean log-likelihood was highest at *K=1* (although *ΔK* cannot be assessed at *K* = 1). When *K* was set to two, no individuals could be assigned to a single cluster. This Structure plot included a burn-in of 250,000, MCMC reps of 500,000 and 25 iterations per K.

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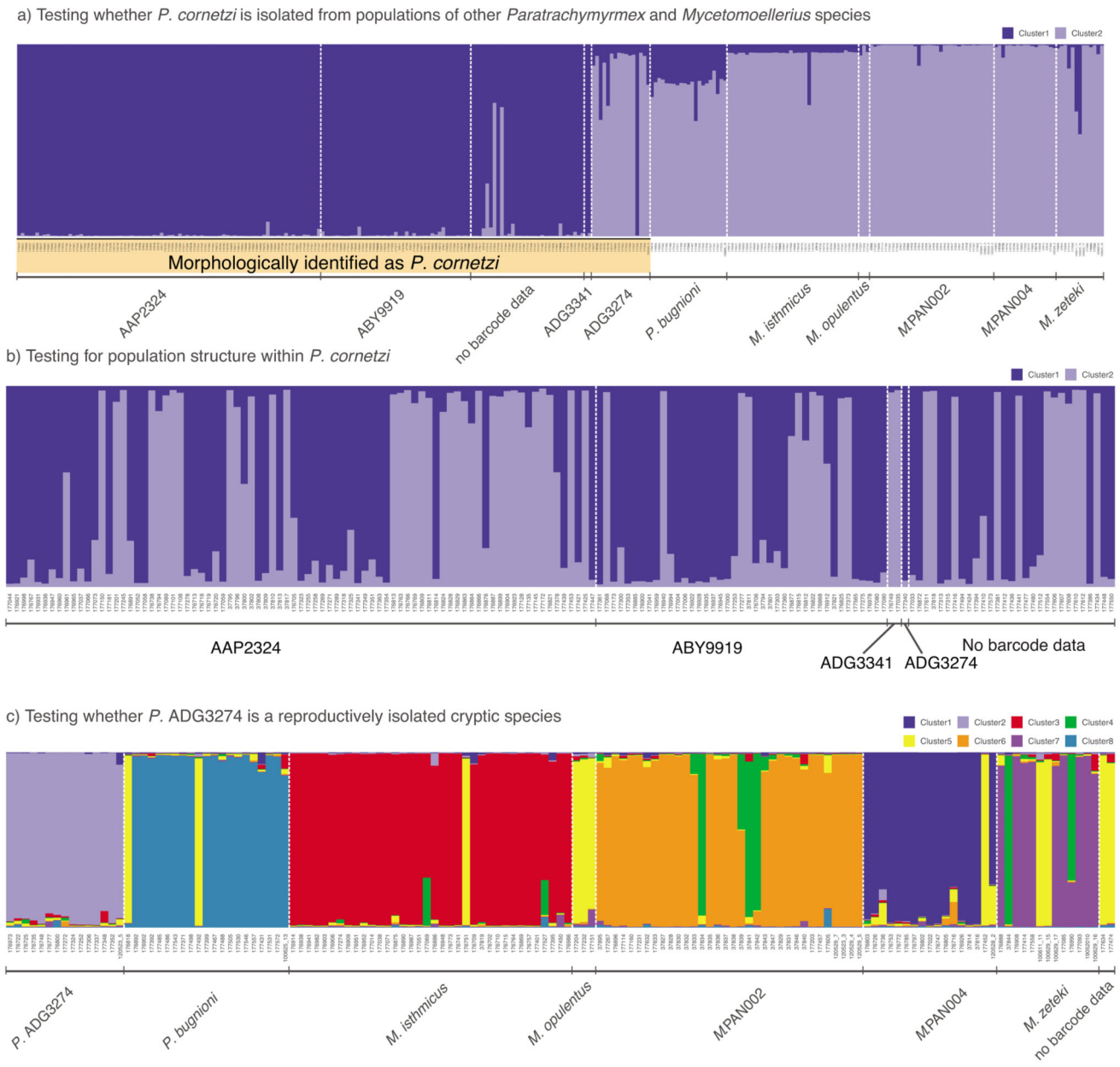
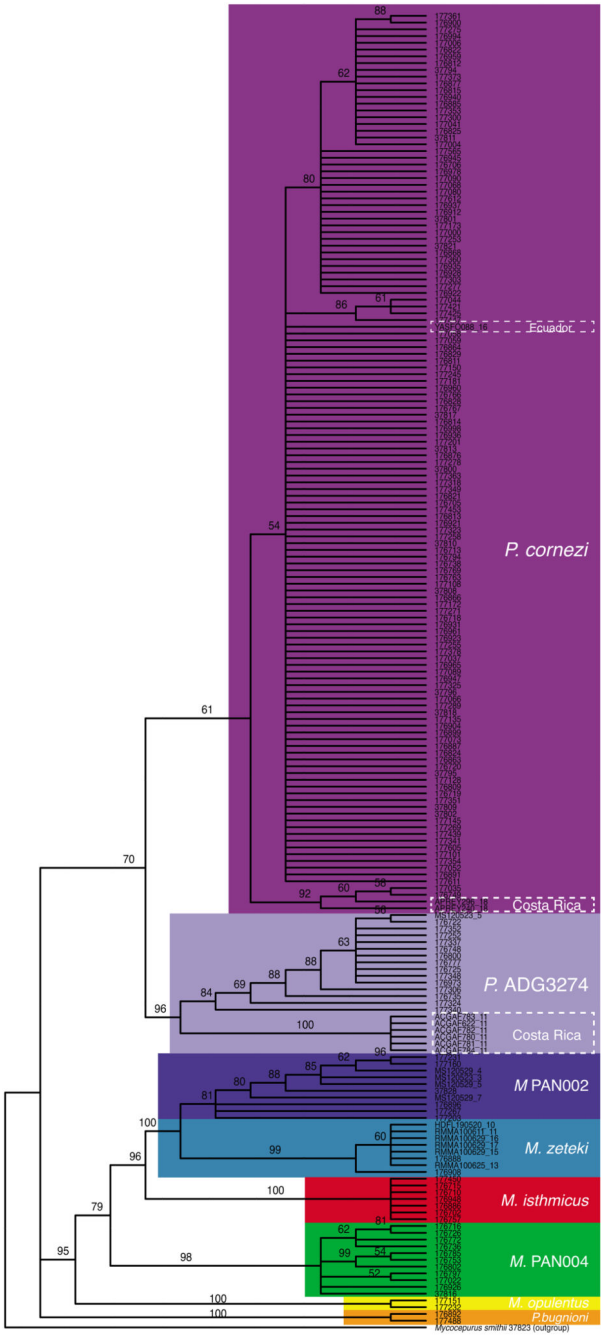


Extended Data Fig. 7. STRUCTURE analyses of nuclear genetic marker data from nine microsatellite loci from 297 *Paratrachymyrmex* and *Mycetomoellerius* workers, from 194 and 103 colonies respectively, indicate that *P. cornetzi* and *P.* ADG3274 represent distinct ant species within the total community of eight species belonging to these two ant genera in Soberanía Park.

a) A STRUCTURE analysis across all samples (N=297) indicated that the most likely population subdivision is two species because K = 2 reached the highest (ΔK) resolution (burn-in 200000, MCMC reps = 5000000, 25 iterations per K). This analysis separated samples that were all previously identified as *P. cornetzi* using morphological characters. It also grouped three Barcode of Life Data System[70] (www.boldsystems.org) haplotypes (AAP2324, ABY9919, ADG3341) separately from the newly recognized cryptic species *P.* ADG3274, that otherwise clustered with the remaining species (light blue). Although ΔK cannot be calculated for $K=1$, the log-likelihood at $K=1$ (-11121.864) was substantially

lower than the one at *K=2* (-9397.616), suggesting that 'no population subdivision' is unlikely, consistent with all other species being morphologically distinct. **b)** A similar STRUCTURE analysis using those individuals assigned to cluster 1 in panel a (N=156) indicated that three barcoded haplotypes (AAP2324, ABY9919, ADG3341) located in the BOLD database represent a single interbreeding population within Soberanía Park called *P. cornetzi*. Specifically, *ΔK* was highest at 2 (1305.79) but the mean log likelihood was similar for K=1 (-3849.092) and K=2 (-3698.860), suggesting that any existing population structure here is weak. The haplotypes also do not separate the dark blue and light blue bars across our sampling sites consistent with failure to reliably assign individual workers to clusters 1 or 2 as expected under high admixture rates. One sample was identified as *P.* ADG3274 by barcoding analysis (177340) (Fig. 5, Extended Data Fig. 8), but could not be reliably distinguished from *P. cornetzi* by this STRUCTURE analysis. **c)** A STRUCTURE plot generated for the remaining (non-*P. cornetzi*) individuals (N = 156), indicated that *K=8* was the most likely subdivision, with little to no admixture between these species. The cryptic species *P.* ADG3274 forms a distinct cluster in this analysis. These genetic species assignments correspond well to species identities obtained by morphological criteria and barcoding (Extended Data Fig. 8) and included four currently named species (*P. bugnioni*, *M. isthmicus*, *M. opulentus*, and *M. zeteki*), and three unnamed species (*M.* PAN004 and *M.* PAN002, and *P.* ADG3274). The ants *M.* PAN004 and *M.* PAN002 were placed in the genus *Mycetomoellerius* [35] since they grouped with *M. isthmicus*, *M. opulentus*, and *M. zeteki* in the CO1 barcoding tree (Extended Data Fig. 8, Supplementary Table 13) and because they exhibited the required morphological characters to justify this decision. This STRUCTURE analysis also suggested there are potentially more than one species within the *M. zeteki* and *M.* PAN002 clusters, and that six stray samples (yellow bars) may also be distinct despite being morphologically grouped similar to *P. bugnioni* (n = 2 samples), *M. isthmicus* (n = 1), *T.* PAN004 (n = 1), and *M. zeteki* (n = 2). Overall, the results of this analysis confirm that perhaps only ~ half of the species are known even in a well-studied insect group in the tropics. The potential taxonomic implications of the *P. cornetzi* and *P.* ADG3274 species complex are beyond the scope of the present study. Samples labelled with "no barcode" were ants that were identified as belonging to *Paratrachymyrmex* and *Mycetomoellerius* using morphological characters but were not included in barcoding analyses.



Extended Data Fig. 8. A majority-rule consensus tree based on COI sequences of 185 *Paratrachymyrmex and Mycetomoellerius* ants supported our microsatellite analyses as the same eight species were recognized as co-occurring across Soberanía National Park.

Three of these species belonged to the genus *Paratrachymyrmex* (*P. cornetzi* [n = 130], *P.* ADG3274 [n = 15], *P. bugnioni* [n = 2]) and five belonged to the genus *Mycetomoellerius* (*M. zeteki* [n = 8], *M. opulentus* [n = 2], *M. isthmicus* [n = 7], *M.* PAN002 [n = 10], *M.* PAN004 [n = 11]; Supplementary Table 13)[22,35] which is sister to the genus *Sericomyrmex*. This tree contains 9 additional public *P. cornetzi* COI sequences deposited at the Barcode of Life Data System database[70] (BOLD, www.boldsystems.org) that supported that the main *P.*

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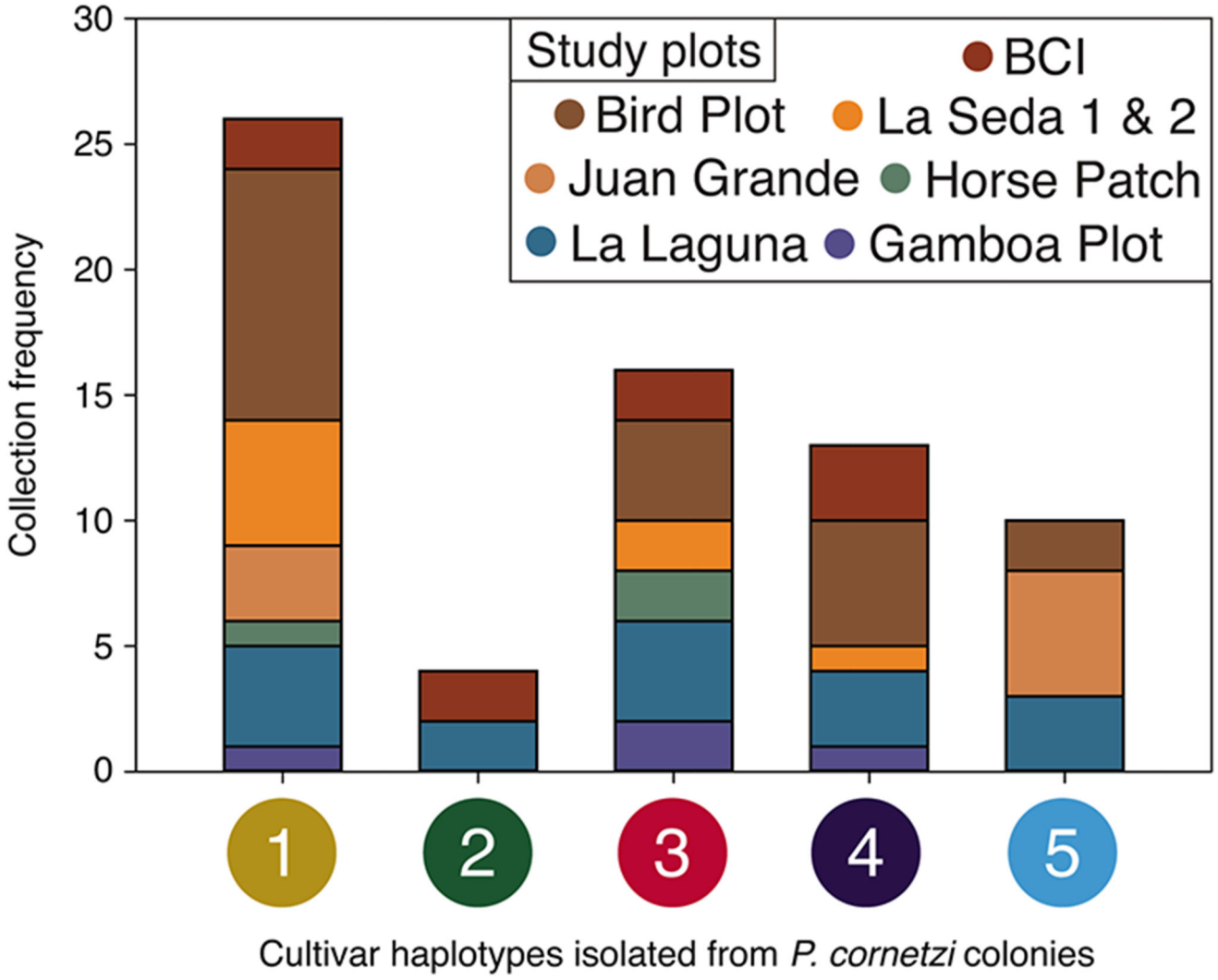
cornetzi haplotype occurs at least from Ecuador to Costa Rica (N = 3 specimens), and that the cryptic *P. cornetzi* haplotype [ADG3274] is distributed at least from the Canal Zone in Panama to La Selva forest in Costa Rica (N = 6 specimens). The tree was rooted by a worker of *M. smithii*.



Extended Data Fig. 9. 9 Each of the five fungal haplotypes cultivated by *P. cornetzi* was widely distributed across Soberanía National Park.

For a map of these sampling locations, see Extended Data Fig. 10, Supplementary Table 14. We combined cultivars from La Seda plots 1 and 2 for this figure since these plots were within 200 m of each other. Four of the five fungal haplotypes that we obtained from these Panama study plots matched cultivar haplotypes sampled from Brazilian colonies of *Paratrachymyrmex* and *Sericomyrmex* by Solomon et al.[35], suggesting broad geographic distributions extending across Central and South America. Additional Soberanía Park sampling localities where no specific plots were assigned (so no labels in Extended Data Fig. 10) included La Laguna (N 9.1196, W -79.6942), Horse Patch (N 9.11990, W -79.70730), and Barro Colorado Island (BCI: N 9.15744, W -79.83523).

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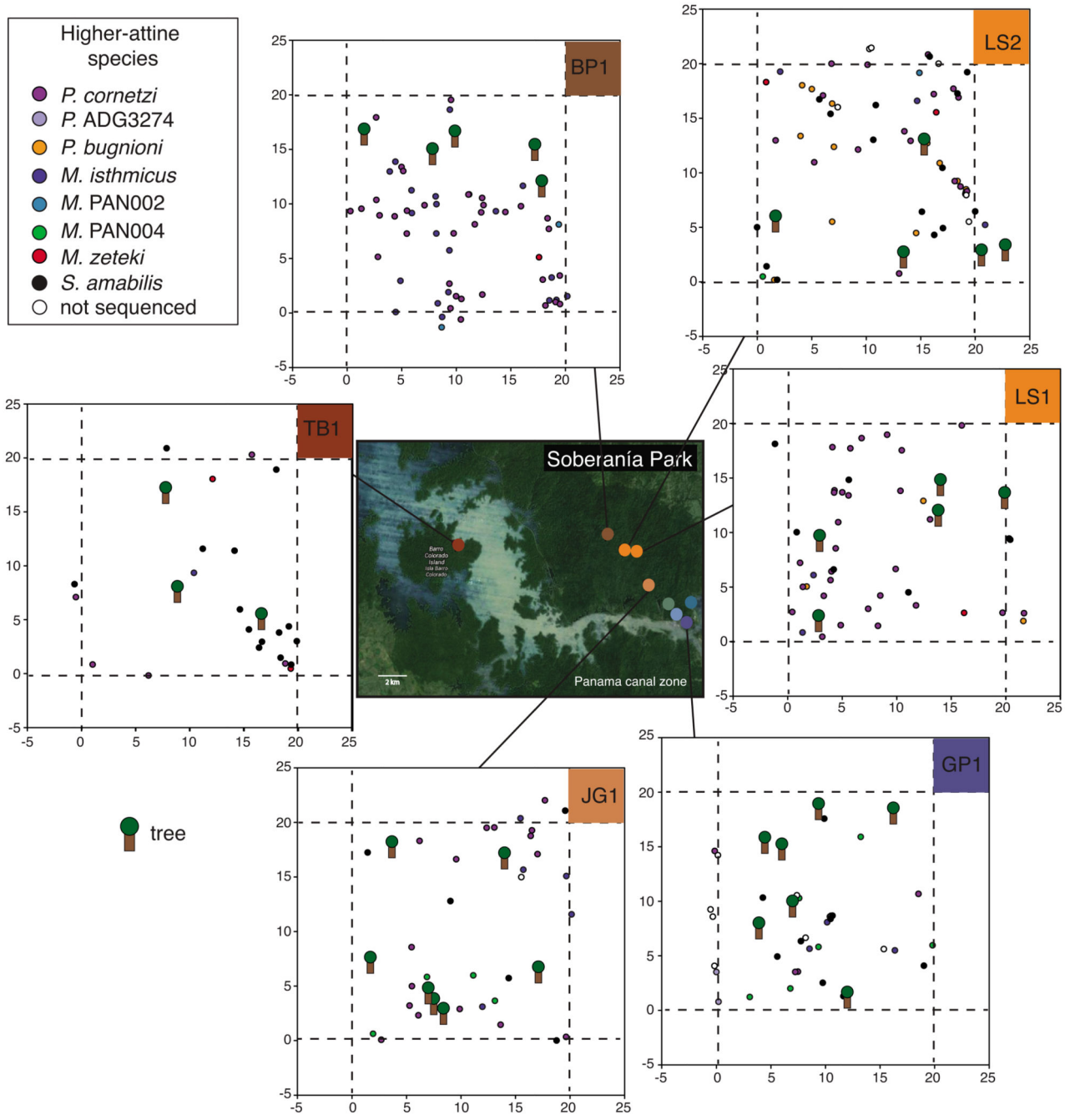

Extended Data Fig. 10. We mapped local-scale higher neoattine colony distributions within the six 20-m^2 study plots distributed across Soberanía National Park.

This totalled 263 colonies (9 species) that were mapped by tracking foraging workers back to their nests after which pinned specimens were screened in microsatellite (Extended Data Fig. 7) DNA barcoding analyses (Extended Data Fig. 8, Supplementary Table 13), as well as with morphological identification. We located colonies by placing bait (polenta) in the leaf litter and observing foragers for 27 ± 3 searching hours per plot (N = 163 total searching hours). The ant *M. opulentus* was recorded within the forest, but not within any of the 20 m^2 plots. Nests were marked by flags at nest entrances, which are inconspicuous holes under the

leaf litter the circumference of a pencil. Within 20-m^2 plots, we observed 43 ± 17 (range: 21-66) higher-neoattine colonies representing 5 ± 1 (range: 4-7) higher-neoattine species. Dashed lines indicate boundaries of 20m^2 plots, and an additional 5 m is provided because some colonies were marked despite occurring just beyond the 20-m^2 plot border lines. Trees > 1 m circumference dbh were also mapped. Plot abbreviations were as follows: BP1: Bird Plot [N 9.16324, W -79.74553], GP1: Gamboa Plot [N 9.11489, W -79.69784], JG1: Juan Grande [N 9.13528, W -79.72141], LS1: La Seda [N 9.15451, W -79.73583], LS2: La Seda [N 9.15624, W -79.73472], TB1: Thomas Barbour [N 9.15744; W -79.83523]). The satellite image of the Panama Canal Zone is from Google Maps (credit to the images data supplier's used in the satellite image (Billeder © CNES / Airbus, Landsat / Copernicus, Maxar Technologies, U.S. Geological Survey, Kortdata © 2020))

Supplementary Material

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Data Availability

The DNA sequences generated during this study are available at NCBI GenBank. This includes the data for: the 215 ant specimens (Accession codes for COI sequences provided in Supplementary Table 13), the 99 fungal cultivar samples (Accession codes for LSU and ITS sequences provided in Supplementary Table 14), and plant sample ITS1 sequences harvested by colonies of *A. colombica* (Accession codes provided in Supplementary Table 8). Ant sequence datasets and supporting information are also deposited in the Barcode of Life Data System[70] (BOLD) under the project titled DS-ATTINENG "Nutritional niches reveal fundamental domestication tradeoffs in fungus-farming ants" (dx.doi.org/10.5883/DS-ATTINENG).

Code Availability

There is no custom code generated for this study. Sequence alignment matrices and newick files used to generate phylogenies in Fig. 5 (both fungal and ant trees) and Extended Data Fig. 8 are available as text files in Supplementary Data 1-3. All code used to generate results will be made available upon request.

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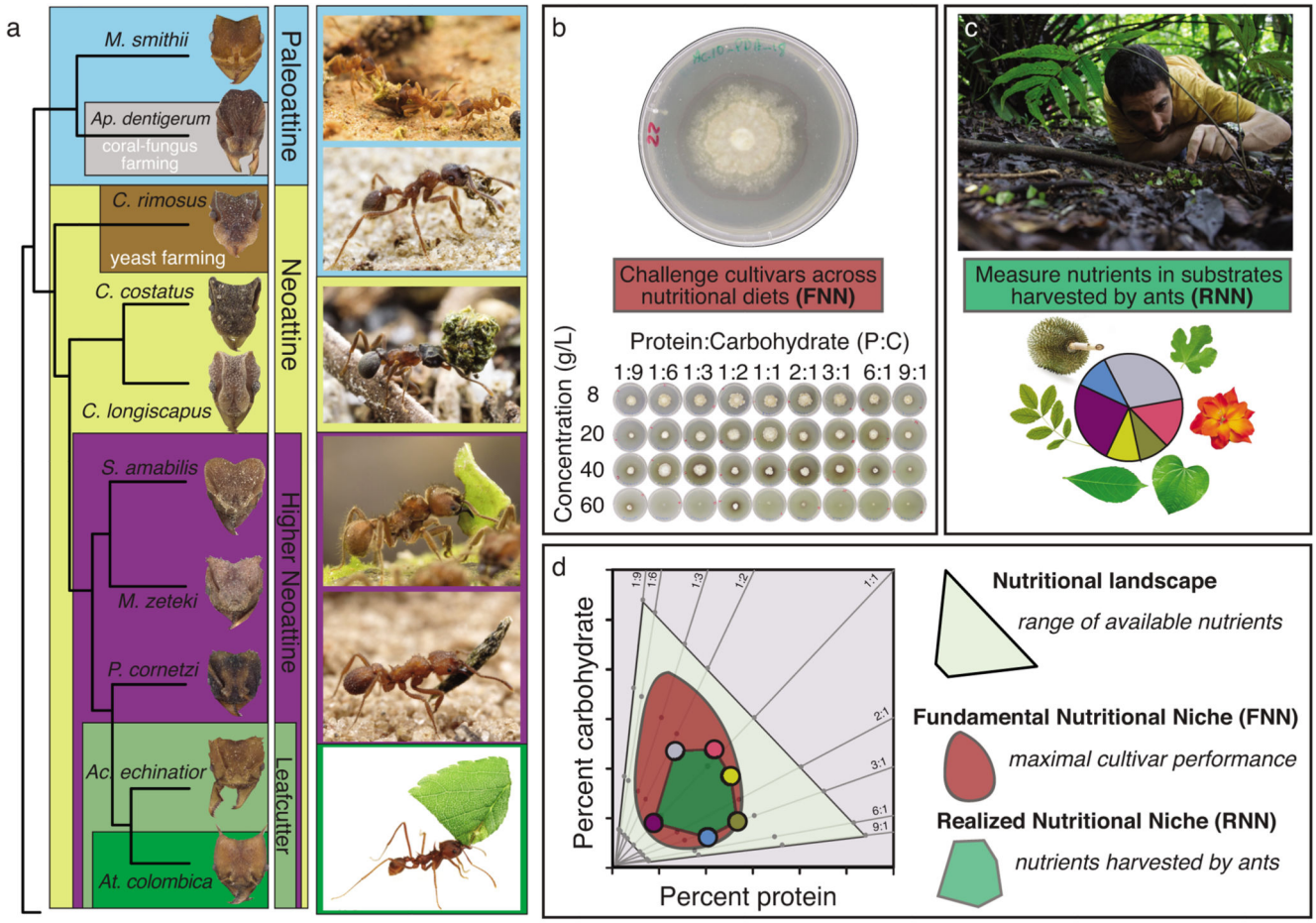

Figure 1. Assessing domestication tradeoffs across transitions in mutualistic farming practice of the fungus-farming ants.

a) Attine ants harvest nutritionally variable detritus fragments in phylogenetically basal genera to freshly cut vegetation in the leafcutter ant crown group (schematic phylogeny[21–23,34,35] restricted to ten representative species co-occurring in Panama). The ants convert this crude forage into nutritional substrates for their fungal cultivars. Farming transitions included: Paleoattines (*Mycocepurus*, *Apterostigma*) that have retained small colonies of 10-100 workers and include a clade within *Apterostigma* secondarily adopting a coral fungus cultivar; Neoattines (*Cyphomyrmex*) that cultivate fungi in a hyphal form and a lineage of yeast farmers; Higher Neoattines (*Sericomyrmex*, *Mycetomoellerius*, *Paratrachymyrmex*) that farm a fully domesticated single lineage of gongylidia-bearing cultivars; Leafcutters including *Acromyrmex* with up to 1,000's of workers per colony, and *Atta* with up to millions of morphologically more specialized workers per colony. **b)** *Defining fundamental nutritional niches* [29,31] (FNNs) of fungal cultivars by quantifying their intrinsic tolerances and nutrient requirements after isolating cultivars from ant colonies and rearing them *in vitro* across substrates varying in concentrations and ratios of protein and carbohydrates. **c)** *Defining realized nutritional niches* [29,31] (RNNs) as the macronutrients that ant farmers offer to their cultivars by sampling and nutritionally analysing substrates collected from the mandibles of returning attine foragers in the field. **d)** *Assessing nutrient-*

provisioning strategies of farmers by overlaying substrate RNNs atop cultivar FNNs. Foraging ants can maximize cultivar performance with a substrate RNN nested inside their cultivar's FNN. In the example provided, the FNN (red bullseye) is derived from a nutritional landscape of 36 diets (small grey circles) spanning nine P:C ratios from 1:9 (carbohydrate biased) to 9:1 (protein biased) along which P:C ratios remain constant (grey lines extending from the origin), but total protein and carbohydrates concentrations increase. The RNN (green polygon) is bounded by nutritional blends of substrates collected by foraging ants in the field, shown here as coloured circles matching substrates in the panel c pie chart. In panel a, ant specimen images are © antweb.org and permission has been granted from Alex Wild (https://www.alexanderwild.com/Ants) to use his ant foraging images. Where possible, we used pictures of the same species represented in this study, but the *Mycetomoellerius* picture represents the closely related *M. tucumanus* [35], the *Apterostigma* picture is of an unknown species of that genus, the *Sericomyrmex* picture is of *S. mayri*, and the *Atta* picture is of *A. cephalotes*. Photograph in panel c is by Sean Mattson.

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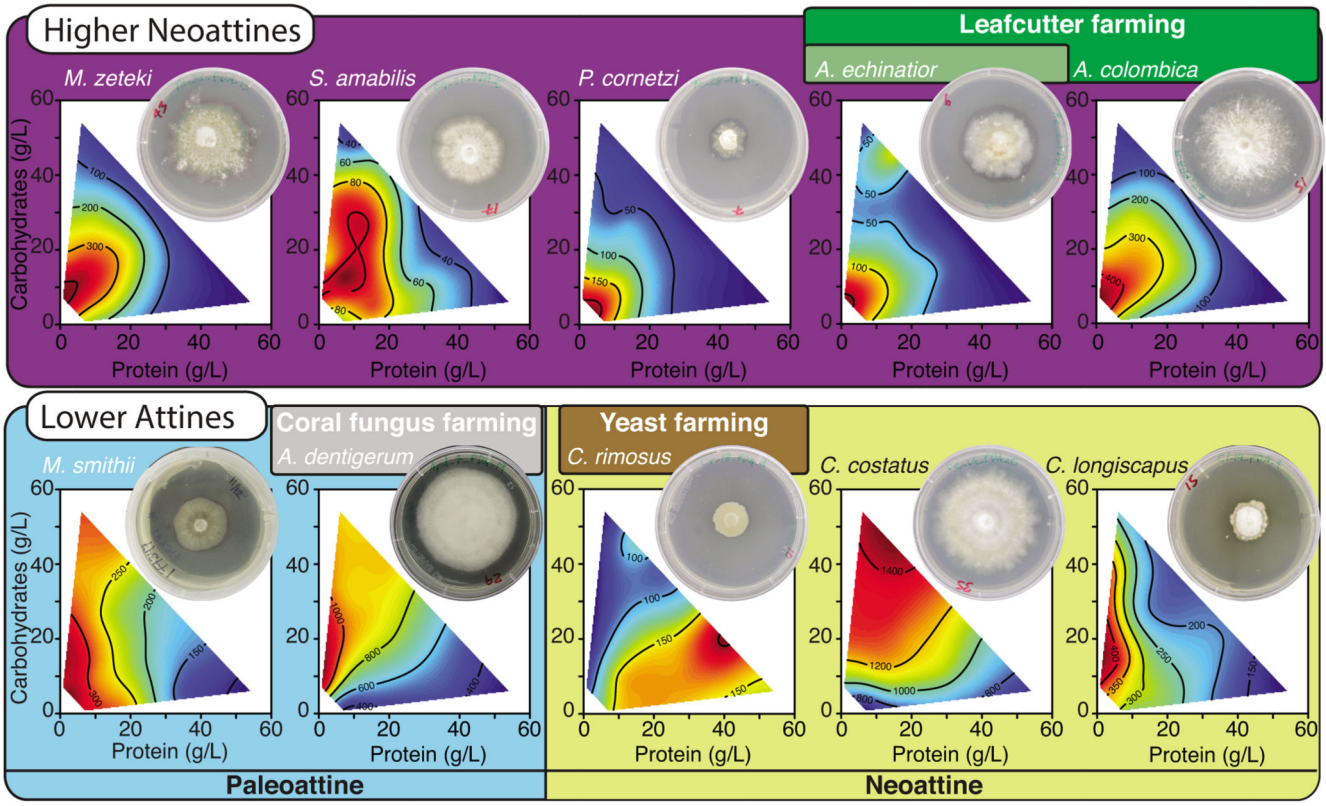

Figure 2. Heatmaps of cultivar growth indicate that domestication by higher-neoattine ants coincided with narrower fungal FNNs than in lower-attine cultivars. This revealed a domestication tradeoff when combined with cultivar mortality data (shown in Extended Data Fig. 2).

Bottom row - *Diverse lower-attine cultivars with variable FNNs*. Top row – *Fully domesticated higher-neoattine cultivars with narrowed FNNs*, showing maximal growth (red heatmap colour) on similar blends of protein and carbohydrates. Whereas dark blue heatmap values in the lower-attine plots indicate slower cultivar growth, dark blue values in the upper higher neoattine and leafcutter plots often indicated cultivar mortality (*see* Extended Data Fig. 2). In each of the ten plots, cultivar growth rates (hyphal area, mm^2) are visualized across 36 experimentally defined artificial media varying in absolute (g/L) amounts and relative (P:C ratio) amounts of protein and carbohydrates[30,60]. As illustrated in Fig. 1D, these substrates spanned nine P:C ratios (1:9, 1:6, 1:3, 1:2, 1:1, 2:1, 3:1, 6:1, 9:1) and four protein plus carbohydrate concentrations (8, 20, 40, 60 g/L). We used the fields package[61] in R[62] to visualize the response surfaces obtained from nonparametric thin-plate splines. Heatmaps are averages of cultivars from three colonies (*Apterostigma dentigerum*, *Cyphomyrmex costatus*, *P. cornetzi*, *Acromyrmex echinatior*, *A. colombica*) or two colonies (*Mycetomoellerius zeteki, Sericomyrmex amabilis*), or were the values from one colony (*M. smithii*, *Cyphomyrmex rimosus*, *C. longiscapus*). FNNs for cultivar growth were consistent when measured across multiple colonies of an attine species, so we assume that the three single colony estimates are representative (Extended Data Fig. 1). We used least-square regressions to assess the underlying significance of linear and quadratic terms (and the linear interaction) across the 36 protein and carbohydrate substrate combinations and to validate

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the interpretation of FNN heatmaps of the dependent variables growth area (Supplementary Table 1) and percent survival (Supplementary Table 2). All response surface regressions producing the heatmap colour-gradients shown here were statistically significant (Supplementary Table 1).

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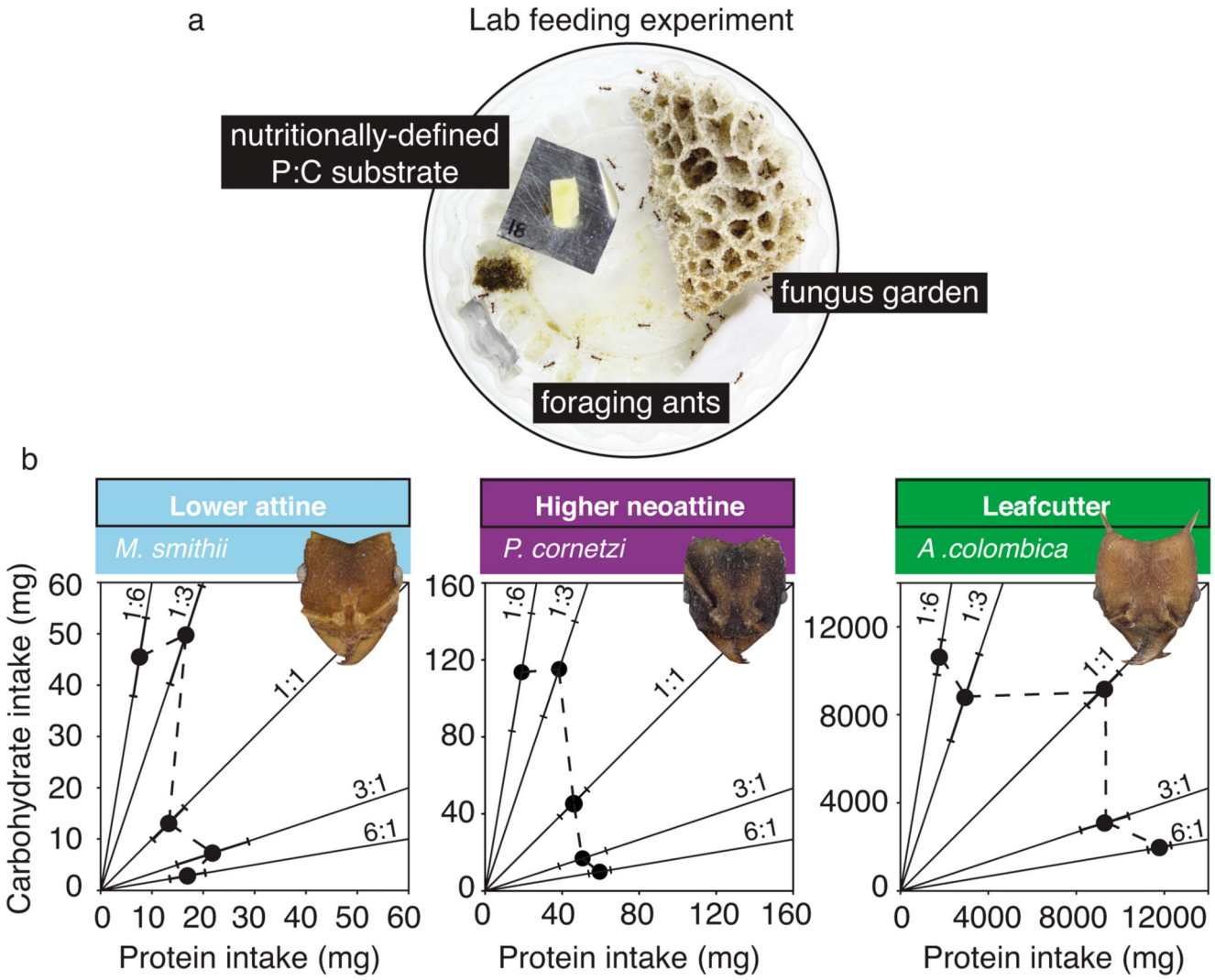

Figure 3. Laboratory experiments with colonies of three representative attine ant species showed that farming ants conformed to their cultivars' FNN dimensions when allowed to forage on single nutritionally-defined substrates.

a) A representative image of an experimental colony of *P. cornetzi*. **b)** Intake levels show that colonies of *M. smithii* and *P. cornetzi* tightly regulated protein concentrations to remain at low levels while allowing carbohydrate levels to fluctuate widely across substrates. In contrast, colonies of *A. colombica* allowed for greater fluctuations of protein intake while sustaining high carbohydrate levels, even when restricted to the 1:1 P:C nutritional rail that *M. smithii* and *P. cornetzi* avoided (*see also* Extended Data Fig. 4). These no-choice feeding experiments were performed on lab-acclimated colonies confined to single nutritional rails of agar-based substrates[63] (Supplementary Table 5) that constrained their intake to specific P:C ratios (1:6, 1:3, 1:1, 3:1, 6:1)[26,30,64]. Dashed lines reflect decisions to over- or under-collect one macronutrient to avoid imbalanced intake of another limiting macronutrient. Mean substrate harvest values ± SE are presented coincident to intake rails[65]. We analysed cumulative intake rates over 15 days of feeding on these substrates (Supplementary Table 4). We present 15-day data for *P. cornetzi* and *M. smithii* to facilitate direct comparison with the

A. colombica results, but the *P. cornetzi* experiment continued for 39 days, yielding long-term results that were consistent with the 15-day data (Supplementary Fig. 1, Supplementary Table 4). The *M. smithii* experiment extended for 29 days and also yielded consistent results[30]. Colonies of *M. smithii* and *P. cornetzi* with collapsed fungus gardens were removed from the experiment on the day they had no remaining cultivar biomass left (Extended Data Fig. 5). In panel b, ant specimen images are © antweb.org

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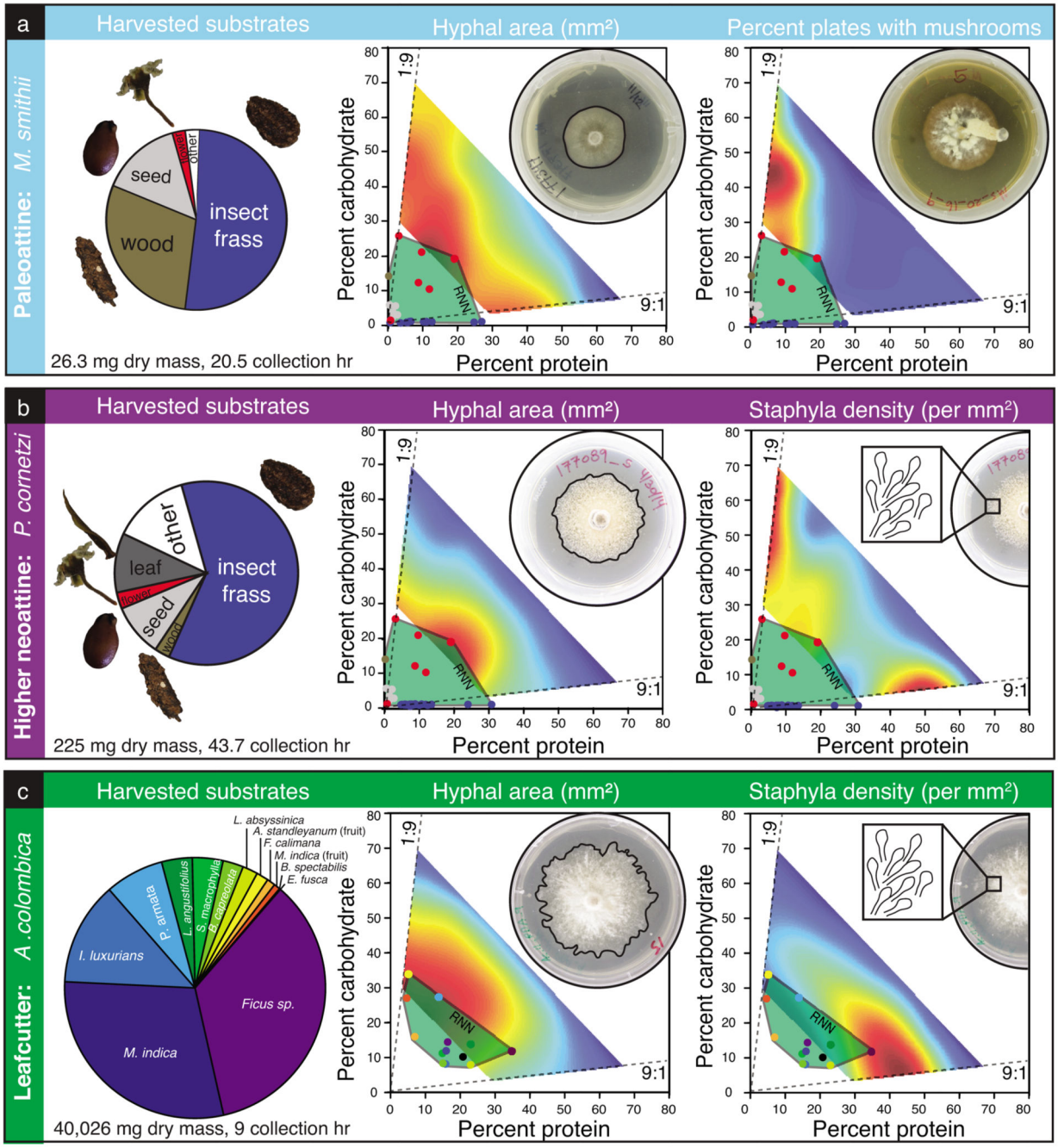

Figure 4. Fungus-farming ants representing different stages of cultivar domestication and organizational complexity navigate nutritional landscapes to harvest RNNs relative to their cultivar's FNNs.

Lying down on the forest floor next to nest entrances, we collected and catalogued tiny substrate bits from the mandibles of laden workers returning to their colonies. Individual substrate types are represented by coloured wedges in pie charts corresponding to coloured circles comprising RNN maps (*see* Fig. 1C,D). **a)** The paleoattine ant *M. smithii* collected 52% insect frass, 29% wood pieces, 14% small seeds, 2% flowers, and 3% other undefined bits of detritus in terms of the proportion of sampled substrate biomass[30] (Supplementary

Table 7). This yielded an RNN enabling the ants to provision cultivars with percent total non-structural carbohydrates (%TNC) ranging from 0.6 to 25.8% while also enabling protein percentages ranging from 0.1 to 27.0% (Supplementary Table 9). **b)** The higher-neoattine ant *P. cornetzi* rears a fully domesticated gongylidia-bearing cultivar, but colonies continue to forage like lower-attine ants, collecting detritus (60.1% insect frass, 10.7% detrital leaf fragments, 9.2% seeds, 3.5% flower pieces, 3.3% wood bits, 13.1% other, Supplementary Table 7). These substrates yielded a RNN with similar dimensions as harvested by *M. smithii*, with %TNC ranging from to 0.6 to 25.8%, and crude protein ranging from 0.1 to 30.84% (Supplementary Table 9). **c)** The leafcutter ant *A. colombica* has a different foraging strategy of primary herbivory focusing on fresh leaves that foragers cut from many plant species (here represented by 13 different vegetative substrates harvested by a single colony, Supplementary Table 8). These substrates range widely in both %TNC (from 7.47 to 33.76%) and in crude protein (from 4.64 to 34.83%) (Supplementary Table 8). Capturing the macronutrient range of different substrate types collected by *M. smithii* and *P. cornetzi* required larger pooled samples of similar substrates collected from leaf litter traps and flower petal samples collected from the mandibles of *A. colombica* that resembled those foraged by *M. smithii* and *P. cornetzi*. Details about sampling and nutritional analyses are provided in the methods and Supplementary Tables 7-9. The slightly larger white areas in the low-concentration nutrient space (lower-left corners) of these fungal FNN heatmaps relative to their representations in Fig. 2 resulted from the conversion of macronutrient units of g/L to percent of dry diet mass within *in vitro* diet recipes. This facilitated comparison of cultivar FNNs and substrate RNNs and did not impact the interpretation of the results. The regressions underlying variation in growth area and staphyla density across P:C substrates for fungal FNN plots were significant (Supplementary Table 1, Supplementary Table 10).

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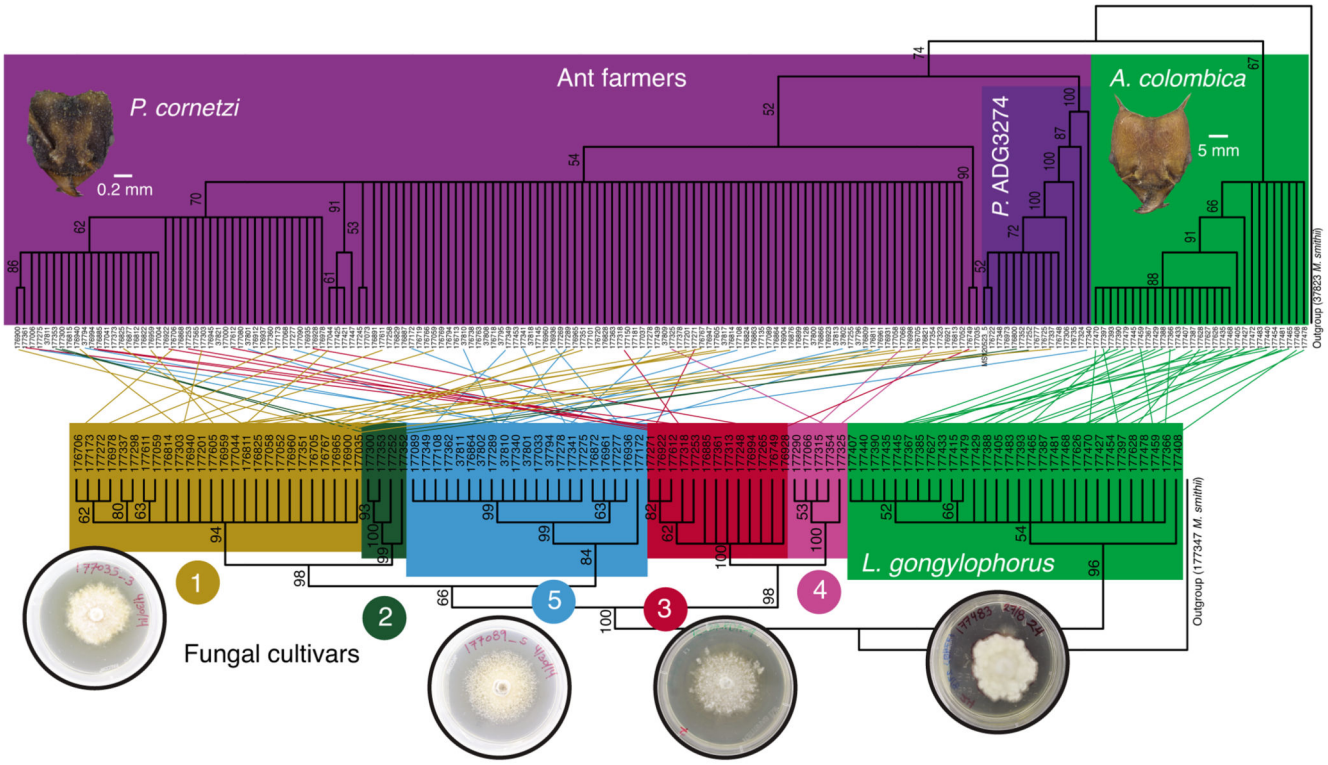

Figure 5. Comparing two fully domesticated farming systems with different operational scales and ecological impacts.

Within Soberanía National Park, the higher-neoattine ant *P. cornetzi* cultivated five haplotypes of related *Leucoagaricus* fungi with bootstrap support indicating that each of the five coloured cultivar clades represents a separate fungal haplotype. Each of these fungal haplotypes was also widely distributed across Soberanía Park (Extended Data Fig. 9) and likely across the neotropics[35]. In contrast, the leafcutter ant *A. colombica* farms only a single cultivar species *L. gongylophorus*. The bootstrap majority consensus barcoding tree based on the Cytochrome Oxidase 1 (CO1) gene for the ants indicated that Panamanian *P. cornetzi* includes a morphologically cryptic species (*hereafter P.* ADG3274) that farms an overlapping diversity encompassing at least three of the five *P. cornetzi* cultivars. The status of *P.* ADG3274 as a distinct cryptic species was supported by microsatellite analyses (Extended Data Fig. 7) and additional barcoding showing that while *P.* ADG3274 is relatively uncommon in our study site it has a regional distribution extending at least to Costa Rica (Extended Data Fig. 8). We do not further consider *P.* ADG3274 in the present study. The bootstrap majority consensus barcoding tree for ants is based on a ~1100 bp section of the CO1 gene and includes 130 colonies of *P. cornetzi*, 15 colonies of *P.* ADG3274, and 29 colonies of *A. colombica* (Supplementary Table 13). The bootstrap majority consensus barcoding tree for fungi is based on a ~820 bp section of the LSU gene and a ~550 bp section of the ITS marker, and includes samples from 69 colonies of *P. cornetzi* and *P.* ADG3274 (fungal haplotype 1 [n = 26], 2 [n = 4], 3 [n = 13], 4 [n = 5], 5 [n = 21]) and 29 colonies of *A. colombica* (Supplementary Table 14). Both ant and cultivar trees were rooted with a *M. smithii* ant or fungus sample. Ant images are © Antweb.org.

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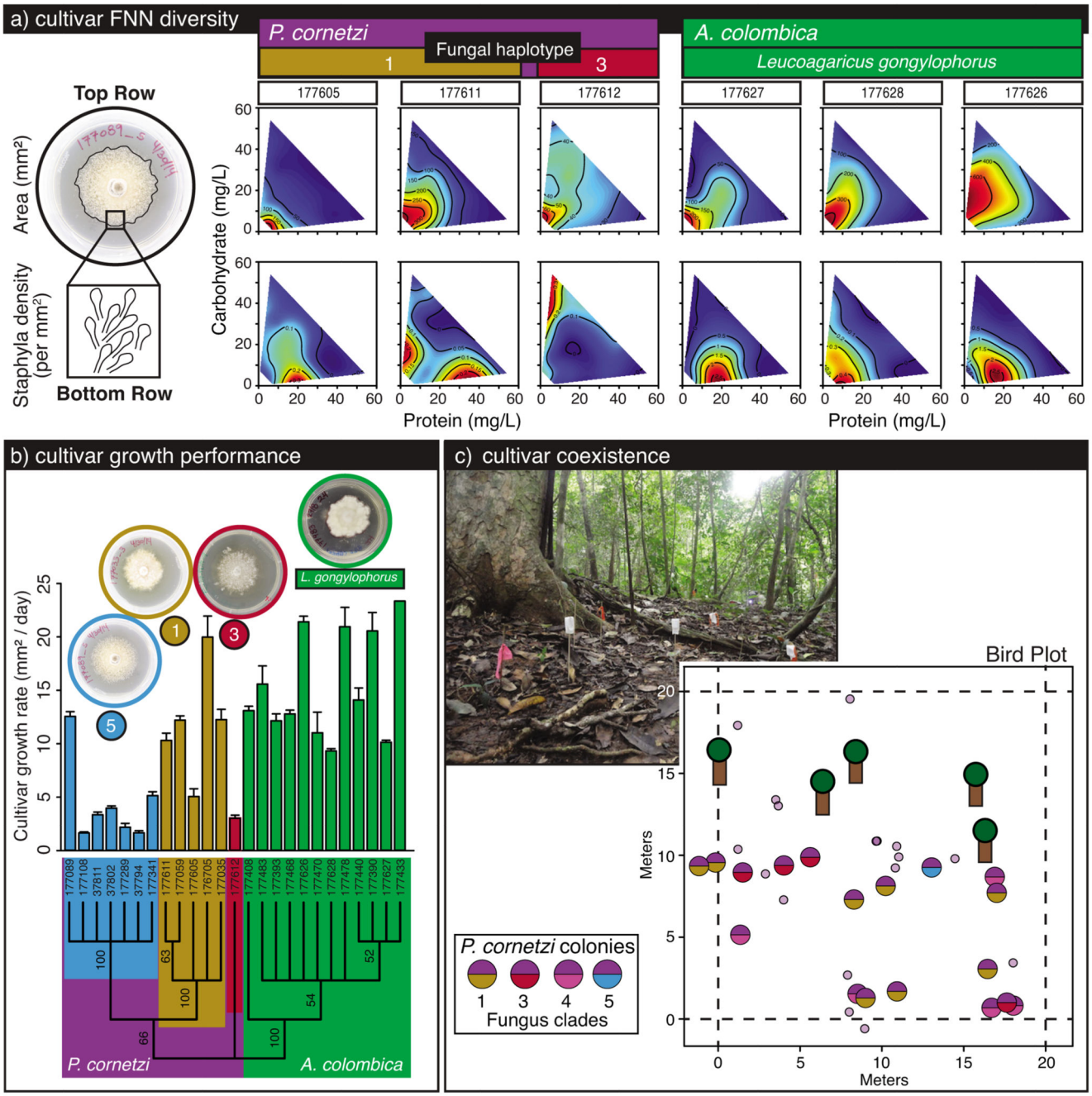

Figure 6. Colonies of *P. cornetzi* co-exist within nutritional foraging environments despite farming different cultivar haplotypes with variable properties of nutrient yield and growth.

a) Fungal cultivars of *P. cornetzi* and *A. colombica* exhibited similar FNNs for maximal hyphal growth (top row). However, *P. cornetzi* (fungal haplotypes 1 and 3) had more variable FNNs for staphyla density (bottom row) while differences across these performance variables were much less variable for the *L. gongylophorus* cultivars of *A. colombica* towards the right. All response surface regressions for fungal growth area (Supplementary Table 1) and staphyla density (Supplementary Table 10) were significant, supporting the interpretation of their response contours. **b)** Testing three of the cultivar haplotypes isolated

from 13 *P. cornetzi* colonies against *L. gongylophorus* cultivars from 12 *Atta* colonies showed that the former grows slower and with greater variation (means + SE of mycelial area per day of growth) on *in vitro* standardized PDA media than the *A. colombica* cultivars ($F_{1,22} = 12.76$, $p = 0.002$). Visual inspection of the growth results plotted on the phylogeny, further suggest strain-specific variation in mean cultivar growth rate, although additional sampling will be needed to provide sufficient statistical power for phylogenetically explicit analyses. **C)** *P. cornetzi* ants farm diverse fungal cultivar haplotypes in sympatry, with four of the five identified cultivars co-occurring within a single 20 m^2 monitoring plot within Soberanía park (Extended Data Fig. 9). Thus, colonies separated by < 1 m often farmed different cultivar haplotypes. Our estimates of local cultivar richness in this example plot are conservative as we sequenced fungal cultivars from a subset of 18 (larger purple semi-circles) of the 35 *P. cornetzi* colonies that we mapped over 23 searching hours. The small light-purple circles indicate *P. cornetzi* colonies where only the ants were genotyped) and the stylized tree symbols indicate trees > 1 m dbh.

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