doi:10.1111/j.1558-5646.2010.00948.x

EVOLUTIONARY TRANSITIONS IN ENZYME ACTIVITY OF ANT FUNGUS GARDENS

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Received May 4, 2009
Accepted December 18, 2009

Fungus-growing (attine) ants and their fungal symbionts passed through several evolutionary transitions during their 50 million year old evolutionary history. The basal attine lineages often shifted between two main cultivar clades, whereas the derived higher-attine lineages maintained an association with a monophyletic clade of specialized symbionts. In conjunction with the transition to specialized symbionts, the ants advanced in colony size and social complexity. Here we provide a comparative study of the functional specialization in extracellular enzyme activities in fungus gardens across the attine phylogeny. We show that, relative to sister clades, gardens of higher-attine ants have enhanced activity of protein-digesting enzymes, whereas gardens of leaf-cutting ants also have increased activity of starch-digesting enzymes. However, the enzyme activities of lower-attine fungus gardens are targeted primarily toward partial degradation of plant cell walls, reflecting a plesiomorphic state of nondomesticated fungi. The enzyme profiles of the higher-attine and leaf-cutting gardens appear particularly suited to digest fresh plant materials and to access nutrients from live cells without major breakdown of cell walls. The adaptive significance of the lower-attine symbiont shifts remains unclear. One of these shifts was obligate, but digestive advantages remained ambiguous, whereas the other remained facultative despite providing greater digestive efficiency.

KEY WORDS: Decomposition, diffuse coevolution, fungus-growing ants, *Leucocoprinus gongylophorus*, mutualism.

The evolution of new functional interactions in mutualisms has often involved strong reciprocal selection (Paracer and Ahmadjian 2000), particularly when a combination of species gained access to previously unavailable niche space, such that rapid adaptive diversification could follow the origin of a key innovation (Ehrlich and Raven 1964; Berenbaum et al. 1996). Over evolutionary time, such dynamic processes are likely to result in periods of stasis alternated with significant further evolutionary elaboration. Stepwise processes of this kind are well documented in obligate nutritional mutualisms that are driven by directional selection for improved efficiency of resource acquisition. Spectacular examples are the termites, where (sub)families are characterized by different assemblies of symbionts (Inoue et al. 2000; Hongoh et al. 2005; Noda et al. 2007; Douglas 2009) and damselfish that cultivate *Polysiphonia* algae as a stable source of food (Silliman and Newell 2003; Hata and Kato 2006). On a much shorter time scale, a symbiotic relationship with an invasive mealy bug may have assisted invasive *Solenopsis* fire ants in their recent expansion in the southeastern United States by supplying up to half of the daily energy requirements of a colony (Helms and Vinson 2002). In all these cases, ecological success is constrained by the range of food items available to the hosts, but is

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enhanced by the synergistic metabolic capacities of the symbiont partner.

Most obligate mutualistic interactions involve intracellular endosymbionts that can be difficult to study (Clark et al. 2007; Moran et al. 2008). Phylogenetic reconstructions of cocladogenesis often indicate some form of coevolution, but the adaptive nature of later evolutionary transitions remains difficult to assess, because in vivo metabolism of endosymbionts cannot be studied in isolation and in vitro rearing is either impossible or highly unlikely to resemble natural growth conditions. These constraints are less problematic in the study of mutualistic ectosymbioses. However, here the interaction itself may be less predictable as geographic mosaics may produce positive coadaptation in some sites and partial (mal)adaptation in other localities due to gene flow and constraints of partner availability (Thompson 2005). The fungus–garden symbionts of ants and termites offer an attractive combination of properties to overcome these difficulties, as they are obligate and can be experimentally isolated from their hosts.

We hypothesized that the attine fungus–garden symbionts have evolved specific modifications in enzyme profiles in conjunction with fundamental shifts in cultivar genotype and substrate used by the ants to manure fungus gardens, paralleling the adaptive modifications of entomopathogenic and phythopathogenic fungi after major shifts in ecology or preferred habitat (Leger et al. 1997). Earlier authors have speculated that specific physiological changes in symbiont function would have to match the digestive capabilities of the ants while maintaining the undamaged transfer of symbiont-derived enzymes through the ant gut to enhance new garden growth via fecal manuring (Bass and Cherrett 1995; Rønhede et al. 2004). Other authors emphasized the necessity of specific enzymes to break down pectin, proteins, and starch in live plant material for the evolutionary derived higher-attine ants (Abril and Bucher 2004; Silva et al. 2006a, 2006b). To date, no comparative studies have addressed whether such transitions have occurred and how substantial they have been. The present study provides such a comparative analysis by investigating whether: (1) the lower-attine ants have fungus garden enzyme activity profiles comparable to free-living agaricaceous fungi, as would be expected from their ongoing acquisition of new fungal symbionts from this clade (Mueller et al. 1998); (2) the shift by some lineages of lower-attine ants to cultivate Clade 2 rather than Clade 1 agaricaceous fungus gardens (Mueller et al. 1998; Mikheyev et al. in press) involved a significant change in symbiont enzyme profiles; (3) the secondarily acquired pterulaceous fungal symbionts of *Apterostigma* express fundamentally different enzyme profiles than the agaricaceous fungi reared by all other attine ants; (4) the symbionts of higher-attine ants changed their enzyme activity in response to the much larger availability of proteins and starch in the live plant material that these ants collect as substrate for their fungus gardens; and (5) the shift in the evolutionary most advanced leaf-cutting ants to rearing a single species of cultivar required a markedly different enzyme profile compared to the diversity of fungal strains cultivated by the other higher-attine ants.

FUNCTIONAL NATURAL HISTORY OF ANT FUNGUS GARDENS

Because reversals to the ancestral "hunter–gatherer" (nonfungus-growing) life style are unknown in the fungus farming ants and termites (Mueller et al. 2005; Aanen and Boomsma 2006), they are good examples of irreversible evolution of novel trait syndromes (Simpson 1953). In ants, fungus farming has allowed the exploitation of plant-based food sources that are unavailable to most other ants (Mueller et al. 1998; Mueller et al. 2001; Currie et al. 2003; Mueller et al. 2005; Schultz and Brady 2008), a life style that arose only once about 50 million years ago (MYA) in South America (Schultz and Brady 2008) when the ancestor of all attine ants started to cultivate a representative of the Agaricales (Basidiomycota; Agaricaceae; formerly Lepiotaceae) (Mueller et al. 1998; Schultz and Brady 2008). In contrast to the fungus-growing termites, which arose as a subfamily (Macrotermitinae) in Africa 24–34 MYA and cultivate only a single genus of *Termitomyces* symbionts (Aanen et al. 2002; Aanen and Eggleton 2005; Mueller et al. 2005), the mutual dependence in the attine partnership was initially asymmetric (Mueller et al. 1998; Aanen and Boomsma 2005). Although the ants became obligately dependent on fungus farming, the fungal cultivars in seven genera that are generally referred to as the lower-attine ants (Fig. 1), comprising both the Paleoattini and four basal genera of the Neoattini (Schultz and Brady 2008) did not become specialized crops. Rather, the fungal crops of these ants appear to have been often acquired de novo from free-living populations, limiting the evolution of fungal adaptations within the symbiosis, if they occurred at all (Mueller 2002; Mueller et al. 2005; Mikheyev et al. in press; Fig. 1). The first cultivation of fungal cultivars is currently believed to have targeted Clade 1 fungi (Mueller et al. 1998; Vo et al. 2009; Mikheyev et al. in press), but many species of lower-attine ants cultivate clade 2 fungi that were domesticated much later (Fig. 1). However, this dichotomy is not always strictly maintained as different colonies of specific ant lineages (e.g., Panamanian *Mycocepurus smithii*) are known to cultivate either Clade 1 or Clade 2 symbionts, whereas other species of the same genus appear to specialize on one of these clades, similar to *Cyphomyrmex longiscapus* always cultivating Clade 1 symbionts and the closely related *C. muelleri* always cultivating Clade 2 gardens (Fig. 1; Chapela et al. 1994; Mueller et al. 1998; Green et al. 2002; Vo et al. 2009). The notable exception is the genus *Apterostigma* where species in the *pilosum*-group cultivate coral fungi of the family Pterulaceae, which are unrelated to the agaricaceous cultivars of all other attine ants

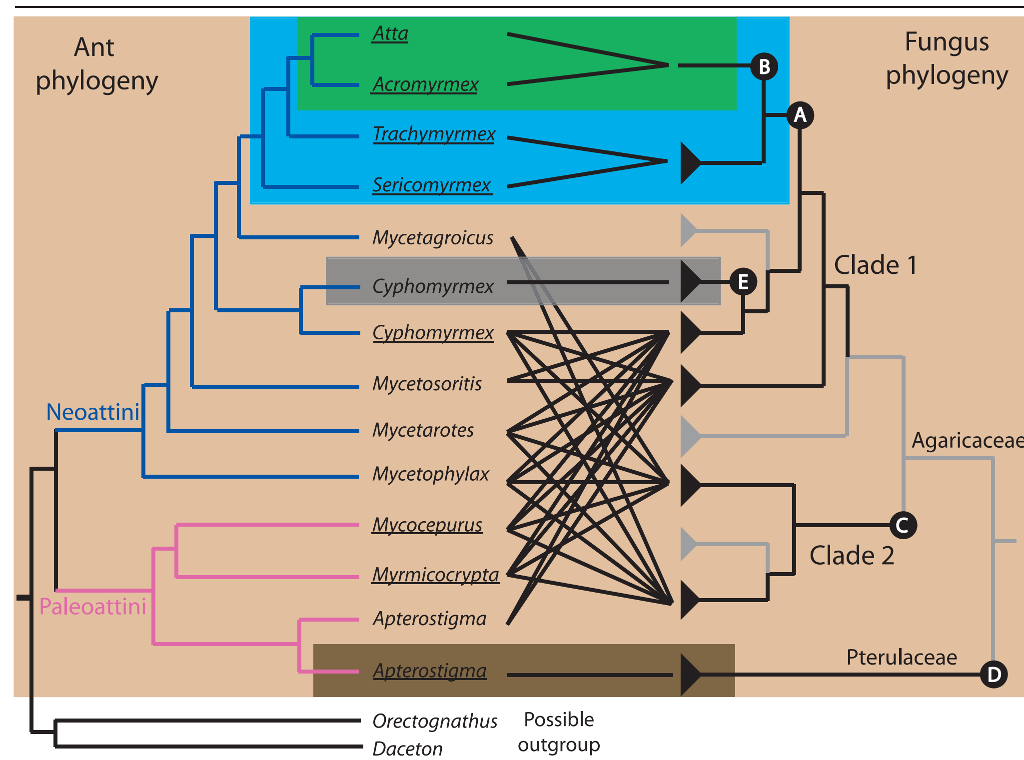

Figure 1. Schematic genus-level phylogeny (underlined genera were used in this study) of the attine ants adapted from Schultz and Brady (2008) combined with the most recent fungal cultivar phylogeny (Mueller et al. 1998; Munkacsi et al. 2004; Mikheyev et al. 2006; Vo et al. 2009; Mikheyev et al. in press), showing the five major groups of fungus-growing ants; green: leaf-cutter farming with freshly cut leaves as fungal substrate; blue: higher-attine farming with freshly shed flowers, insect frass, and soft leaf fragments as substrate; light-brown: lower-attine farming with insect frass, dry plant material, and seed and seed husks as substrate; grey: yeast farming; dark-brown: coral fungus (Pterulaceae) farming with substrates comparable to the other lower-attine genera. Categories of substrate use are based on Hölldobler and Wilson (1990), Mueller (2002), Price et al. (2003) and H. H. De Fine Licht and J. J. Boomsma (unpubl. ms.). The fungal phylogeny consists of two major agaricaceous clades (the original Clade 1 and the secondary acquired Clade 2; Mikheyev et al. in press) and the secondary acquired coral-fungi clade (Villesen et al. 2002). Cultivated symbiont branches are drawn in black, whereas branches of free-living relatives are drawn in gray. Letters in the fungal phylogeny refer to major transitions in fungus farming: (A) toward specialized fungal cultivars with swollen hyphal tips (gongylidia) in the ancestor of the higher-attine ants; (B) toward a single specialized species of symbiont, *Leucocoprinus gongylophorus*, in the ancestor of the leaf-cutting ants; (C) toward the distantly related agaricaceous fungi of Clade 2 in some of the lower-attine lineages; (D) toward completely unrelated pterulaceous fungi in the ancestor of most extant *Apterostigma* ants; (E) toward farming a single cell basidiomycete yeast state rather than a mycelial growth form in the *Cyphomyrmex rimosus* species complex. The cultivar clade information for *Mycetagroicus* is based on Solomon et al. (in press).

(Munkacsi et al. 2004; Villesen et al. 2004; Dentinger et al. 2009; Fig. 1).

About 20 MYA (Schultz and Brady 2008) the so-called higher-attine ants evolved as a monophyletic group in conjunction with the establishment of a farming system based on the permanent domestication of a single fungal symbiont from Clade 1 (Fig. 1, transition A). This symbiont evolved specific nutritious adaptations to being a fungal crop in the form of gongylidia, swollen hyphal tips with a large vacuole filled with nutrients and fungal enzymes that the ants eat and feed to their larvae (Chapela et al. 1994; Schultz and Brady 2008). This transition is thought to have severed gene flow between cultivated fungi and free-living close relatives, so that the mutualism attained the symmetry that characterized the Macrotermitine–*Termitomyces* symbiosis from the very beginning (Aanen et al. 2002; Aanen and Boomsma 2005). Various lineages of fungal symbionts evolved from this transition and now characterize the extant symbionts of the ant genera *Trachymyrmex* and *Sericomyrmex* (Mikheyev et al. 2008; Mikheyev et al. in press). Ultimately one of these lineages became the symbiont of the ancestor of the *Atta* and *Acromyrmex* leaf-cutting ants, which at present primarily cultivate strains of this single species *Leucocoprinus gongylophorus* (Fig. 1, transition B; Silva-Pinhati et al. 2004; Mikheyev et al. 2006; Mikheyev et al. 2007). These two transitions appear to have supported a gradual increase in colony size, resulting in large variation of social complexity across the attine tribe, varying from less than

100 monomorphic workers per colony in paleo- and lower-attines to several millions in *Atta* (Villesen et al. 2002; Price et al. 2003; Hughes et al. 2008; Baer et al. 2009; Fernández-Marín et al. 2009). However, the recurrent transitions from Clade 1 to Clade 2 symbionts in paleoattine and lower-attine ants (Fig. 1, transition C) and the irreversible acquisition of a distantly related symbiont of the family Pterulaceae in a monophyletic section of the paleoattine genus *Apterostigma* (Fig. 1, transition D), did apparently not result in any significant further evolution toward larger colonies and more complex societies (Villesen et al. 2004), so that the adaptive significance of these shifts has remained enigmatic. The collection of woody material such as small twigs and excrements of wood-boring insects is particularly observed in *Apterostigma* (Wheeler 1907; Weber 1958; Price et al. 2003), so that fungal enzyme adaptations to decompose woody substrates may be expected to characterize this transition. A fifth transition (Fig. 1, transition E) concerns the *C. rimosus* species group where the ants cultivate a narrow fungal lineage from within Clade 1 as yeast rather than mycelium while retaining mostly small and inconspicuous colonies (Schultz and Brady 2008; Mikheyev et al. in press). However, closely related free-living fungal strains have also been found among these yeast-cultivars, indicating that secondary exchanges with nonmutualistic fungal populations occur in similar frequencies as in the other paleo- and lower-attine ants (Mueller et al. 1998; Vo et al. 2009). This transition to yeast cultivars will not be further considered here.

The fungus-growing termites with their symmetric diffuse coevolution with a single fungal genus (*Termitomyces*) (Aanen et al. 2002) and almost exclusively monogamous societies (Boomsma 2007) seem to have evolved gradually toward social complexity (Aanen et al. 2007). In contrast, intensive research of more than a decade has now established that the two higher-attine ant transitions toward a narrower range of garden symbionts were sources of major evolutionary innovation that necessitated or were driven by shifts in garden substrate. The lower-attine ants provide their fungus gardens mostly with a mixture of insect-feces, dry plant debris, leaf-litter, and seed fragments (Hölldobler and Wilson 1990; Price et al. 2003; De Fine Licht and Boomsma in review), whereas the *Trachymyrmex* and *Sericomyrmex* higher-attine ants also include considerable amounts of freshly fallen flower petals, soft leaf tissue, and fruits in their "diet" (Fig. 1; Weber 1966; Hölldobler and Wilson 1990; Price et al. 2003). This may have allowed the higher-attine ants to attain an order of magnitude larger colony sizes (Hölldobler and Wilson 1990). The transition in the ancestor of the *Atta* and *Acromyrmex* leaf-cutting ants was even more dramatic. It involved not only an almost exclusive dependence on freshly cut leaves, but also a further increase in colony size of one to two orders of magnitude (Price et al. 2003), considerable differentiation of worker castes (Hölldobler and Wilson 1990, 2008), multiple mating by queens (Villesen et al. 2002; Baer et al. 2009), and a significantly larger investment in disease defense via the metapleural glands (Hughes et al. 2008).

THE CELLULAR MECHANISMS OF FUNGUS GARDEN DECOMPOSITION

Free-living saprophytic fungi first attack cross-linking glycans (hemicellulose) and pectins before initiating degradation of cellulose (Dix and Webster 1995; Radford 2004; Aneja et al. 2006) and the same sequence appears to apply to fungal strains domesticated by attine ants (Schiøtt et al. 2008). Hemicellulolytic glycans cross-link the long unbranched cellulose microfibrils across the secondary cell wall, creating a strong matrix that is highly resistant to enzymatic hydrolysis (Carpita and McCann 2000), whereas pectin is concentrated in the space between leaf cells and functions as a gel-like adhesion ligand (Carpita and McCann 2000). Apparently, the enzymatic degradation of the pectin-material tends to facilitate the decomposition of (hemi)celluloses, as the biochemical changes induced by pectin degradation make cell walls more susceptible to further breakdown by other enzymes (Esquerre-Tugaye et al. 2000). It seems likely that these processes occur more simultaneously when the fungi are domesticated, because the ants actively masticate the leaf substrate into minute fragments (Quinlan and Cherrett 1977; Mueller et al. 2001; Mangone and Currie 2007) before inoculating the garden symbiont. The fungal symbiont of leaf-cutting ants has difficulties penetrating intact leaf surfaces (Erthal et al. 2009), so that leaf-substrate fractioning by the ants is of crucial importance for hyphal growth (Mohali 1998). The pectinolytic activity of the symbiont is likely to play a key role in degrading and weakening the plant cell walls to reach the multilayered crystalline starch granules inside the live plant cells.

Materials and Methods

An important complication of comparative enzyme studies of fungus–garden symbionts is that characteristic enzyme profiles are likely to change rapidly after ant colonies are moved from the field to the laboratory, especially after shifting to laboratory forage that is different from what the ants would have collected as garden substrate in the field. Specifically, plant material that deviates from the natural ant forage can induce large shifts in fungus garden enzyme profiles (P. Kooij, H. H. De Fine Licht, M. Schiøtt, and J. J. Boomsma, unpubl. ms.). This implies that photometric assays standardized to protein content will be quantitatively accurate, but of unclear relevance in what they measure. The alternative is to process samples almost immediately after collection under field conditions, and quantify enzyme activity with methods that are somewhat less precise. For this study, we have chosen to do the latter by extracting total fungus garden enzymes, stabilizing them

in a Tris-buffer, and immediately measuring enzyme activity to minimize proteolysis by internal proteases. We chose to use Tris-buffer because it performed well in pilot experiments, in spite of earlier records that it sometimes inhibits glycosidases (Larner and Gillespie 1956; Dion et al. 1999; Ghalanbor et al. 2008). However, although possible biases can confound studies of absolute enzyme activities, they are less problematic for comparative studies of relative expression patterns (comparison between fungal symbiont types), as in our study.

Samples were collected during May 2007 and April–May 2008 in Parque National Soberanía, Panama (the Gamboa area and forest along Pipeline Road). Initially 12 representative species from eight genera spanning the entire attine phylogeny were collected as whole colonies including all fungus gardens (except for mature *Atta colombica* nests from which only some fungus gardens were dug up). We compared enzyme activities for five polysaccharidases: α-amylase, cellulase, endo-β-1,4-xylanase, endo-protease, and a pectinase in the fungus gardens of all 12 ant species. The fungus gardens of a subset of five species were analyzed more thoroughly for a total of 17 different enzymes.

Total proteins were extracted from the fungus garden by grinding 100 mg fresh garden material with a sterile pestle in eppendorf tubes containing 416 μL 50 mM Tris buffer pH 7.0 (always corresponding to 120 mg fungus garden per 500 μL buffer) after visible ants, larvae, pupae, and eggs had been removed. Extracts were centrifuged at 12,400*g* for 15 min and the supernatant containing crude total protein extracts were immediately used in the enzyme activity assays. The same volume of supernatant was used in each experiment and enzyme assay, which standardized our assays to units of fungus garden mass under the assumption that extraction efficiency across fungus gardens from different species was equal. All fungus gardens were assayed on the day of collection before the colonies were provisioned with food items, so that our data reflect the natural level of enzymes present. We believe that this was also the case for the few *Atta* and *Acromyrmex* colonies that were allowed to rebuild their fungus garden after nests had been extensively disrupted during collection, as we did not provide new leaf substrate before we took samples 1–3 days later. When discernible layers were visible in the fungus garden (reflecting gradients of substrate deposition over time; this is particularly common in *Atta*, *Acromyrmex*, and *Trachymyrmex*) the samples were collected such that they contained material from all layers, so that they were maximally comparable to the samples from species with smaller gardens where almost entire fungus gardens were used for the measurements.

Fungus gardens of five species, representing all major clades of attine ants, *Acromyrmex echinatior* ($N = 9$), *Trachymyrmex* sp. 3 ($N = 9$), *C. longiscapus* ($N = 10$), *M. smithii* ($N = 18$), and *Apterostigma dentigerum* ($N = 12$), were selected for more thorough investigation of activity against 17 specific enzyme substrates. *Trachymyrmex* sp. 3 is an undescribed species that is sympatric with *Trachymyrmex cornetzi* (to which it is likely closely related) in its investigated range in Panama (Fernández-Marín et al. 2004). Most of the colonies were newly collected (a few colonies collected in 2008 were used both in this experiment and the one described above, but with separate extractions) and enzymes were extracted as mentioned in the first experiment.

ENZYME ACTIVITY ASSAYS

Enzyme activity was assayed with Azurine-Crosslinked (AZCL) polysaccharides which are dyed and highly purified polysaccharides that are cross-linked to form a water insoluble substrate (Megazyme, Wicklow, Ireland; Table 1). The enzymes diffuse into the assay media where hydrolysis of AZCL-polysaccharides releases dyed water soluble fragments at a rate that is directly proportional to enzymatic activity (Ten et al. 2004). In tablet form, AZCL can be used in standard test-tube assays where the rate of release is measured spectrophotometrically as the increase in absorbance at 590 nm. However, in powder form these water insoluble substrates can also be embedded in an agarose gel where they are stable for several weeks at room temperature. By making small holes in the agarose gels and placing liquid enzyme extracts in these wells, the enzymes come into contact with the AZCL-substrate particles and release dyed fragments that spread in the agarose plate by diffusion. After precautions to standardize the thickness of the gel plates in petri dishes and the shape and diameter of the wells, the dyed area in the agarose gel (halo) can be used to obtain a quantitative measure of enzyme activity (Hasper et al. 2004; Ten et al. 2004; Schiøtt et al. 2008; Pedersen et al. 2009). Although the AZCL technique has limitations, such as uncertainty about the relationship between enzyme activity and halo area being linear, we chose this technique because: (1) Agar plates can be prepared in advance and used under field conditions. (2) The halo quantifications can also be obtained under field conditions with macro photography. (3) The use of AZCL in plate assays has been verified to be quantitative and produce highly repeatable results making it suitable for large-scale comparisons of specific enzyme activity (Hasper et al. 2004; Ten et al. 2004; Schiøtt et al. 2008; Pedersen et al. 2009). The small standard errors around the specific garden means per enzyme that we obtained confirmed that this method is accurate with the precautions given.

Assay plates were prepared with a medium consisting of 1% agarose, 23 mM phosphoric acid, 23 mM acetic acid, and 23 mM boric acid, mixed, and pH-adjusted for each plate according to manufacturer specifications (Table 1). The medium was heated until the agarose was melted and then cooled to 65°C when 0.1% weight/volume AZCL substrate was added and the medium was poured into petri dishes. After the medium had solidified, wells were made with a c pipette tip to give a diameter of 4 mm. In the first experiment involving all 12 species, 20-μL supernatant

Table 1. The 17 AZCL (Azurine-crosslinked Polysaccharide) substrates used for the enzyme activity assays and the pH values in the assay-plates as per manufacturer's specifications (Megazyme©).

Substrate	pH	Enzyme
Starch		
AZCL-Amylose	4.4	α-amylase
Protein		
AZCL-Casein	7.0	endo-protease
AZCL-Collagen	7.0	endo-protease
Pectin		
AZCL-Debranched Arabinan	4.0	endo-α-1,5-arabinase
AZCL-Rhamnogalacturonan	4.5	Rhamnogalacturonanase
AZCL-Galactomannan	4.5	endo-β-1,4-mannanase
AZCL-Galactan	4.3	endo-β-1,4-galactanase
Cellulose		
AZCL-HE-Cellulose	4.5	cellulase (endo-β-1,4-glucanase)
AZCL-Barley β-Glucan	4.5	cellulase (endo-β-1,3-1,4-glucanase)
AZCL-Xyloglucan	4.5	endo-β-1,4-xyloglucanase
1,3-Glucans		
AZCL-Pachyman	6.0	endo-β-1,3-glucanase
AZCL-Curdlan	6.0	endo-β-1,3-glucanase
Cross-linking Glycans		
AZCL-Xylan	4.7	endo-β-1,4-xylanase
AZCL-Arabinoxylan	4.7	endo-β-1,4-xylanase
AZCL-Dextran	5.0	endo-α-1,6-dextranase
Various		
AZCL-Pullulan	5.0	Microbial pullulanase
AZCL-Chitosan	5.0	Chitosanase

of protein extract was applied to each well. In the second experiment involving only five species, we applied 15-μL supernatant per well. After 22 h of incubation at about 21°C the plates were photographed and the area of the blue halo surrounding the well (a quantitative measure of the amount of enzyme activity; Fig. 2; Ten et al. 2004; Hasper et al. 2004; Schiøtt et al. 2008; Pedersen et al. 2009) was measured using the software program ImageJ version 1.37 (W. Rasband, http://rsb.info.nih.gov/ij/). Negative controls were performed using fungus gardens of laboratory colonies with heat inactivated enzyme extracts (95°C for 20 min) from three colonies each of: *A. echinatior, Sericomyrmex amabillis, C. longiscapus, M. smithii*, and *A. collare*, which never produced any visible coloration. Positive controls (to eliminate the risk of measuring false-negatives) were not performed because: (1) This would have significantly reduced our sample sizes as we had reached the upper limit of plates that could be brought to the field, and (2) We considered this of relatively minor importance because the AZCL assays have been specifically designed by the manufacturer to produce repeatable halos that are proportional to enzyme activity at the concentrations that we applied (Ten et al. 2004; Schiøtt et al. 2008; Pedersen et al. 2009).

Enzyme activities (measured as the halo area in cm^2) were analyzed with analysis of variance (ANOVA) for unequal sample sizes using the GLM procedure in SAS version 9.1 for Windows. Tukey's multiple comparisons test was used to compare means. The differences in enzyme activity were further analyzed using phylogenetically independent contrasts to see whether differences remained significant when coancestry relationships were taken into account. Without this correction for phylogenetic distance, the observed differences may be overestimated, because related taxa are not statistically independent because of their joint evolutionary history (Felsenstein 1985). Ant-specific phylogenetic contrasts were leaf-cutting ants versus all other attine ants, higher- versus lower-attine ants, and pterulaceous versus agaricaceous lower-attine fungus farming. All these contrasts were also calculated using only the Clade 1 cultivating colonies. Independent contrast analyses were evaluated using the Phenotypic Diversity Analysis Programs (PDAP) package for Mesquite version 11 (Midford et al. 2003; Maddison and Maddison 2006). Categorical variables (phylogenetic groupings) were replaced by 0–1 dummy variables to perform the respective regression analyses. The latest available complete phylogeny of the attine ants (Schultz and Brady 2008) was used to infer branch lengths. Species not present in the phylogeny were assumed to be in clades of the closest relatives with branch lengths assumed to be equal to sister species. We used one-tailed tests for the changes in alpha-amylase

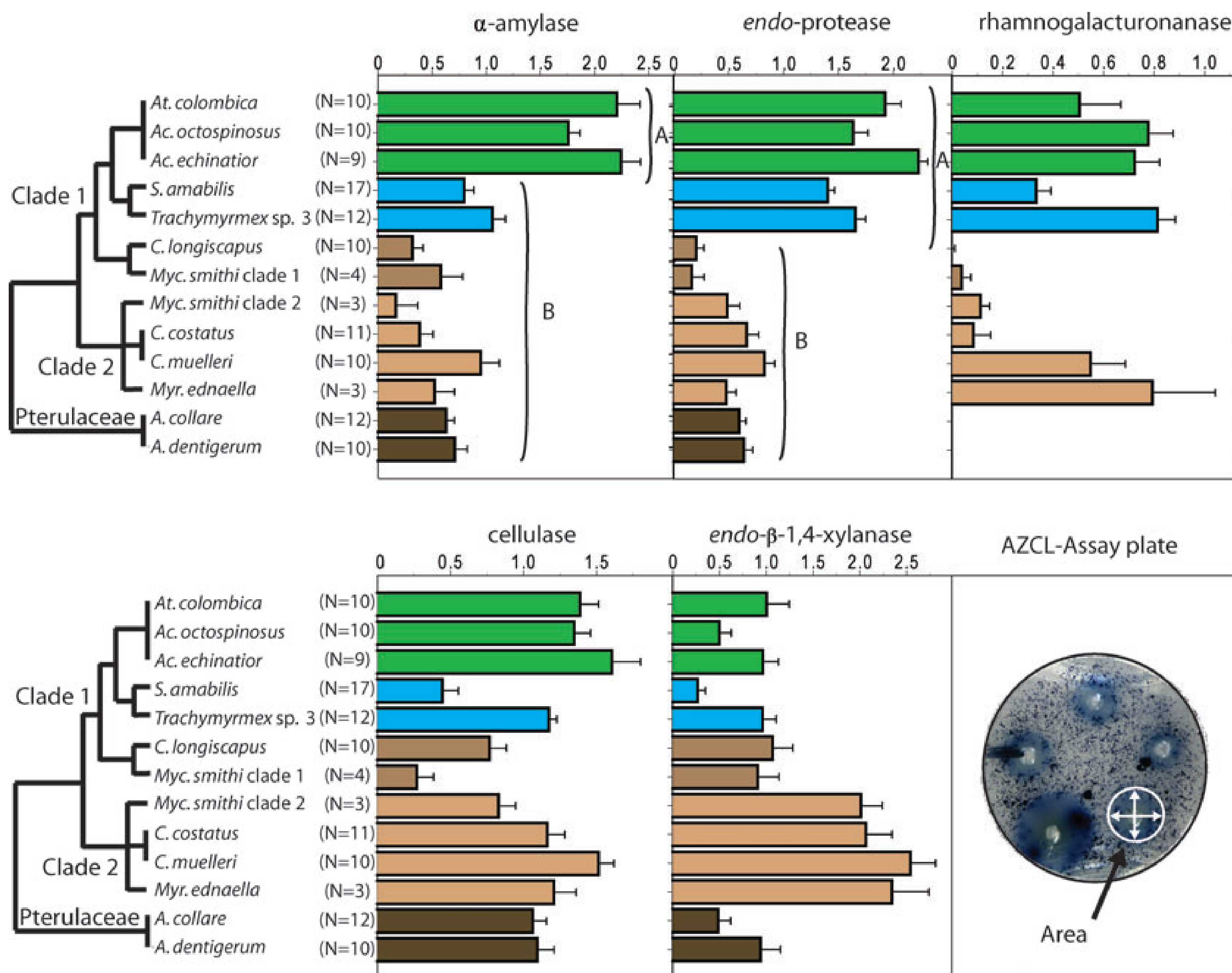

Figure 2. Fungus garden enzyme activity for 12 species of attine ants, 13 types of fungus garden (sample size in brackets), and five key enzymes spanning the entire attine phylogeny (see Table 1; substrate in brackets): endo-protease (Casein), α-amylase (Amylose), rhamnogalacturonanase (Rhamnogalacturonan I), endo-β-1,4-xylanase (Xylan), and cellulase (HE-Cellulose). All five enzyme activities differed significantly between species (endo-protease: $F_{11,121} = 45.53$, $P < 0.0001$; α-amylase: $F_{11,121} = 21.54$, $P < 0.0001$; rhamnogalacturonanase: $F_{11,121} = 11.16$, $P < 0.0001$; endo-β-1,4-xylanase: $F_{11,121} = 13.50$, $P < 0.0001$; cellulase: $F_{11,121} = 9.69$, $P < 0.0001$). Color codes are the same as in Figure. 1, but with Clade 1 cultivating lower-attines represented with a different shade of light brown. Letters represent categories with significant differences in enzyme activity when analyzed with phylogenetically independent contrasts. The lower right panel is a representative example of an AZCL-assay plate.

and endo-protease activity across transitions A and B (Fig. 1) as the known shifts toward including more fresh plant material in the ant forage implied that only increases were expected. All other tests were two-tailed.

We checked whether the measurements that we obtained were in fact only due to the extracellular fractions of enzyme activity. We made separate extractions of gardens of the five main species, by incubating fresh field-collected fungus garden material in a buffer at ambient room temperature for 120 min and measuring enzyme activity in the buffer fraction that exclusively targeted extracellular enzymes. This yielded very similar values as the ones obtained by our earlier extractions, confirming that the activities that we obtained in our larger set of extractions are extracellular, as expected for decomposition enzymes.

GENOTYPING OF CULTIVARS FOR WHICH CLADE STATUS WAS UNKNOWN

DNA was extracted from alcohol preserved pieces of mycelium of *M. smithii* and *Myrmicocrypta ednaella* gardens in either a Cetyltrimethylammonium Bromide (CTAB) solution followed by a phenol–chloroform extraction (Schiøtt et al. 2008), or with a 20% Chelex solution containing proteinase K (Roche©,

Hvidovre, Denmark) and incubated at 65°C for 90 min followed by 99°C for 15 min. Approximately 800 bp fragment of the nuclear large ribosomal subunit (nLSU-rDNA) was amplified with the standard primers LR5 (5′-TCCTGAGGGAAACTTCG-3′) and LR0R (5′-ACCCGCTGAACTTAAGC-3′) (Moncalvo et al. 2000) using the AmpliTaq GOLD polymerase system (Applied Biosystems, Naerum, Denmark). PCR conditions were one cycle of 95°C, 35 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec and a final elongation step of 72°C for 10 min. PCR products were purified and sequenced by Eurofins MWG Operon (Ebersberg, Germany). Sequences are deposited in Genbank with accession numbers FJ895807–FJ895833. The obtained *M. smithii* and *M. ednaella* fungal cultivar nLSU-rDNA sequences where compared with the phylogenies by Mueller et al. (1998) and Vo et al. (2009) with neighbor joining (NJ) and maximum parsimony (MP) analysis as implemented in MEGA version 4 (Tamura et al. 2007), which allowed the colonies to be unambigously assigned to either Clade 1 or 2 (Figs. 1 and 2). In all analyses *M. smithii* is therefore represented twice as it has symbionts in both Clade 1 and Clade 2 (Fig. 2), whereas all *M. smithii* fungus gardens in the larger second experiment were from Clade 1 (Table 2). For all remaining species the fungus cultivar clade affiliation (1 or 2) could be unambiguously inferred from the literature (Mueller et al. 1998; Green et al. 2002; Schultz et al. 2002; Villesen et al. 2004; Vo et al. 2009).

Results

In the first set of experiments all five substrates (α-amylase, endo-protease, rhamnogalacturonase, cellulase, endo-β-1,4-xylanase) differed significantly in enzyme activity across ant species (Fig. 2; $P < 0.0001$ in all ANOVAs). Focusing on transition A (lower- to higher-attine ants) within Clade 1 (Fig. 1), nonoverlapping Tukey's groupings in post hoc analyses indicated a significantly higher endo-protease activity in gardens of higher-attine ants, which was confirmed when using phylogenetically independent contrasts across the entire ant phylogeny ($F_{1,11} = 3.82$, one-tailed $P = 0.0384$) and across the ant genera cultivating only Clade 1 fungi ($F_{1,5} = 8.97$, one-tailed $P = 0.0152$). Pectinolytic enzyme activity (rhamnogalacturonase) was consistently high in the fungus gardens of higher-attine ants (Fig. 2), but not significantly enhanced relative to the lower-attine ants when using phylogenetically independent contrasts for the entire ant phylogeny ($F_{1,11} = 1.00$, $P = 0.3383$). When restricting the analysis to ant genera cultivating only Clade 1 fungi, this difference became more substantial but remained non significant ($F_{1,5} = 5.12$, $P = 0.0732$). Transition B (toward leaf-cutting ants; Fig. 1) involved a significantly elevated α-amylase activity when considering the nonoverlapping Tukey's groupings obtained in post hoc analyses (Fig. 2), a result that was confirmed using phylogenetically independent contrasts, both across the entire ant phylogeny ($F_{1,11} = 4.96$, one-tailed $P = 0.0239$) and in a more restricted analysis across genera cultivating only Clade 1 symbionts ($F_{1,5} = 11.04$, one-tailed $P = 0.0105$). Neither cellulase nor endo-β-1,4-xylanase garden activity was significantly different in fungus gardens across transition A or B (Fig. 1).

Transition C (Clade 1 to Clade 2 in the lower-attine ants; Fig. 1) showed enhanced group-wise activity (i.e., ignoring the ant species identities and excluding the higher-attine ants with their specialized symbionts) in Clade 2 gardens compared to Clade 1 gardens for four of the five enzymes: endo-protease: $F_{1,40} = 24.84$, $P < 0.0001$; cellulase: $F_{1,40} = 23.48$, $P < 0.0001$; rhamnogalacturonanase: $F_{1,40} = 11.00$, $P = 0.0038$; endo-β-1,4-xylanase: $F_{1,40} = 24.57$, $P < 0.0001$. The only exception was α-amylase ($F_{1,40} = 1.67$, $P = 0.2044$). A similar analysis including the species level also indicated a significantly higher activity for endo-β-1,4-xylanase in Clade 2 gardens ($F_{5,40} = 5.31$, $P = 0.0010$), but with overlapping post hoc Tukey's groupings of species similar to all other differences measured (endo-protease: $F_{5,40} = 6.45$, $P = 0.0002$; cellulase: $F_{5,40} = 9.52$, $P < 0.0001$; rhamnogalacturonanase: $F_{5,40} = 12.37$, $P < 0.0001$; α-amylase: $F_{5,40} = 3.22$, $P = 0.0171$). Transition D (from Clade 1 to pterulaceous gardens in lower-attine ants; Fig. 1) showed a significantly higher endo-protease activity in pterulaceous *Apterostigma* gardens relative to Clade 1 gardens (excluding the derived higher-attine gardens) ($F_{3,35} = 9.51$, $P = 0.0001$). Also cellulose activity was enhanced in pterulaceous *Apterostigma* gardens when using a group-wise analysis ($F_{1,35} = 13.45$, $P = 0.0008$), a result that was maintained in species-level analysis, but with overlapping Tukey's groupings ($F_{3,35} = 7.05$, $P = 0.0009$). Endo-β-1,4-xylanase, rhamnogalacturonanase, and α-amylase did not differ in enzyme activity across transition D ($F_{3,35} = 1.99$, $P = 0.1358$; $F_{3,35} = 2.05$, $P = 0.1259$; $F_{3,35} = 2.46$, $P = 0.0802$, respectively, but all trends were in the same direction as the enhanced endo-protease and cellulose activity). Although not explicitly considered as a transition because the same Clade 1 and Clade 2 symbionts were maintained, we note that there was no significant difference between fungus garden enzyme activity across the ancient split between Paleoattini and Neoattini (Fig. 1) for any of the enzymes, when analyzed across the entire phylogeny, after excluding the higher-attine ants (tests not shown).

In the second set of experiments, the independent contrast analysis using the ant phylogeny (now with only five species) confirmed the result of the first experiment. Proteolytic (endo-protease) symbiont activity increased significantly across transition A (casein: $F_{1,3} = 30.32$; one-tailed $P = 0.0059$; collagen: $F_{1,3} = 14.78$; one-tailed $P = 0.0155$). An independent contrast analysis without *A. dentigerum*, that is, on gardens of Clade 1 only, gave similar results (casein: $F_{1,2} = 20.55$; one-tailed $P = 0.0227$;

Table 2. Fungus garden enzyme activity in area/cm^2 for five species of fungus-growing ants. Vertical black bars indicate Tukey's studentized groupings of multiple means in post hoc analyses following ANOVA GLM analysis (SAS version 9.1). No statistics are given for enzymes with activity below the detection limit.

Species	Sample	Amylolytic	Proteolytic		Pectinolytic			
		α-Amylase	endo-Protease (Casein)	endo-Protease (Collagen)	endo-α-1,5-Arabinase	Rhamnogalac-Turonanase	endo-β-1,4-Mannanase	endo-β-1,4-Galactanase
A. echinatior	$N=9$	2.26±0.15	1.82±0.07	1.53±0.17	1.35±0.17	0.71±0.12	1.23±0.11	1.23±0.10
T. sp. 3	$N=9$	1.16±0.15	1.51±0.08	0.94±0.06	0.48±0.04	0.76±0.09	0.21±0.03	0.85±0.08
C. longiscapus	$N=10$	1.04±0.11	0.71±0.07	0.01±0.01	0.47±0.07	0.28±0.07	0.37±0.04	0.79±0.06
M. smithii	$N=20$	0.78±0.06	0.51±0.04	0.09±0.06	0.16±0.04	0.16±0.03	0.15±0.03	0.26±0.04
A. dentigerum	$N=12$	0.81±0.13	0.69±0.08	0.04±0.03	0.10±0.04	0.07±0.04	0.99±0.09	0.52±0.05
ANOVA GLM analysis		$F_{4,55}=26.75$, $P<0.0001$	$F_{4,55}=74.14$, $P<0.0001$	$F_{4,55}=68.40$, $P<0.0001$	$F_{4,55}=39.96$, $P<0.0001$	$F_{4,55}=22.69$, $P<0.0001$	$F_{4,55}=60.76$, $P<0.0001$	$F_{4,55}=32.10$, $P<0.0001$

Species	Sample	Cellulolytic			1,3-Glucanases		Cross-linking Glycans				
		cellulase (Cellulose)	cellulase (β-Glucan)	endo-β-1,4-Xyloglucanase (Xyloglucan)	endo-β-1,3-Glucanase (Pachyman)	endo-β-1,3-Glucanase (Curdlan)	endo-β-1,4-Xylanase (Xylan)	endo-β-1,4-xylanase (Arabinoxylan)	endo-β-1,6-Dextranase	pullula-nase	endo-Chitosanase
A. echinatior	$N=9$	1.46±0.14	1.33±0.13	1.57±0.18	0.07±0.07	0.00±0.00	0.66±0.12	1.17±0.11	0.00±0.00	0.00±0.00	0.00±0.00
T. sp. 3	$N=9$	1.04±0.06	1.07±0.09	1.13±0.09	0.00±0.00	0.00±0.00	0.89±0.18	1.54±0.21	0.00±0.00	0.00±0.00	0.00±0.00
C. longiscapus	$N=10$	1.23±0.08	1.88±0.09	0.85±0.05	0.00±0.00	0.00±0.00	2.08±0.18	2.53±0.15	0.00±0.00	0.00±0.00	0.00±0.00
M. smithii	$N=20$	0.54±0.05	0.97±0.04	0.44±0.03	0.00±0.00	0.00±0.00	1.55±0.11	1.80±0.10	0.00±0.00	0.00±0.00	0.00±0.00
A. dentigerum	$N=12$	1.05±0.09	1.35±0.15	0.54±0.08	0.00±0.00	0.00±0.00	0.84±0.13	1.10±0.12	0.00±0.00	0.00±0.00	0.00±0.00
ANOVA GLM analysis		$F_{4,55}=19.99$, $P<0.0001$	$F_{4,55}=13.95$, $P<0.0001$	$F_{4,55}=32.57$, $P<0.0001$	-	-	$F_{4,55}=16.63$, $P<0.0001$	$F_{4,55}=16.81$, $P<0.0001$	-	-	-

collagen: $F_{1,2} = 9.42$; one-tailed $P = 0.0458$). In contrast to a nonsignificant result in the first experiment, endo-β-1,4-xylanase activity now decreased across transition A, marginally significantly so for xylan ($F_{1,3} = 7.36$, $P = 0.0730$), but significantly when only Clade 1 gardens were included in the analysis ($F_{1,2} = 4.73$, $P = 0.0413$; Table 2). It therefore remains somewhat ambiguous whether xylanase activity decreases or remains constant across transition A. Pectinolytic activity could now be analysed for four enzymes rather than one in the first experiment. One of these, rhamnogalacturonase, showed a significant increase in activity across transition A ($F_{1,3} = 52.77$; $P = 0.0054$). The three others showed a significant or marginally significant increase in activity across transition B (endo-α-1,5-arabinase: $F_{1,3} = 56.44$; $P = 0.0049$; endo-1,4-β-galactanase: $F_{1,3} = 7.01$; $P = 0.0771$; endo-1,4-β-mannanase: $F_{1,3} = 10.68$; $P = 0.0470$). Similarly, amylolytic symbiont activity (α-amylase) was significantly enhanced ($F_{1,3} = 97.76$; one-tailed $P = 0.0011$) across transition B as in the first experiment. Proteolytic (endo-protease) symbiont activity also increased across transition B, but this difference was only significant in ANOVA, but not using independent contrasts (casein: $F_{1,3} = 2.86$; one-tailed $P = 0.0948$; collagen: $F_{1,3} = 5.14$; one-tailed $P = 0.0541$). A marginally significant increase across transition B was also observed for endo-β-1,4-xyloglucanase ($F_{1,3} = 7.86$, $P = 0.0676$).

Differences across transition C (Fig. 1; Clade 1 toward Clade 2) could not be analyzed in this second experiment as only Clade 1 and pterulaceous cultivars were included. The analyses across transition D (from Clade 1 toward pterulaceous gardens) did not confirm the results of the first experiment as endoprotease and cellulolytic activity were not significantly enhanced (Table 2). Also cellulolytic (cellulase) enzyme activities, now measured for three enzymes, did not change across transition D (Table 2), as they did in the first experiment. Pectinolytic endo-1,4-β-mannanase activity increased substantially (to a similar level as in *Acromyrmex* leaf-cutting ants), but this difference was not significant ($F_{1,20} = 2.84$, $P = 0.1081$). Endo-arabinase and endo-1,4-β-galactanase activity remained as low as for fungi associated with *M. smithii* and *C. longiscapus* (Table 2). In contrast to the first experiment (Fig. 2), enzyme activities against cross-linking glycans (endo-β-1,4-xylanase) were higher in the Clade 1 lower-attines than in the pterulaceous fungus gardens (see ANOVA in Table 2).

The endo-1,3-β-glucanases (on AZCL-Curdlan and AZCL-Pachyman, Table 2), an endo-1,6-β-dextranase, a pullulanase, and an endo-chitosanase were not analyzed as the activities were negligible. As in the first experiment, there were no differences in any enzyme activity between gardens of paleo-attine and neo-attine ants in analyses spanning the entire attine phylogeny and in more restricted analyses involving only Clade 1 gardens of lower-attine ants (tests not shown).

Discussion

THE EXTRACELLULAR ENZYME PROFILES OF ATTINE SYMBIONTS AND FREE-LIVING AGARICACEOUS FUNGI

The similarity in overall enzymatic profiles between lower-attine gardens and free-living saprophytic agaricaceous fungi (Leger et al. 1997) matches the mix of substrates that the ants collect to manure their fungus gardens, which resemble the normal substrates of saprotrophic fungi (Fig. 1; Deacon 2006). This similarity, which mainly concerns the endo-xylanases and glucanases, has likely facilitated exchanges between domesticated symbionts and the large pool of related free-living fungal strains, as has been documented in previous studies (Mueller et al. 1998; Vo et al. 2009). It is also consistent with the view that these symbionts did not evolve major specialized functions or other adaptations to a symbiotic life (Vo et al. 2009), in contrast to the fungal cultivars of the higher-attine ants. Fungus farming in lower-attine ants therefore appears to be merely an extension of natural saprotrophic decomposition in leaf litter habitats, but controlled and likely accelerated by the provisioning, care, and microbial management of the ants.

The activity profiles of intact fungus gardens show that several enzymes have been specifically modified in response to the agricultural ecology of their farming ants (Fig. 2). These enzymes are not necessarily all derived from the fungal symbiont, however, as they could potentially originate from the ants or their larvae, or from other microorganisms in the garden matrix (Febvay and Kermarrec 1983; Erthal et al. 2004, 2007; Erthal et al. 2009). The latter may include bacteria and yeasts (Carreiro et al. 1997; Rodrigues et al. 2008), some of which have been shown to degrade protein, gelatin, starch, and cellulose in gardens of *Atta sexdens* (Bacci Jr. et al. 1995). When measuring activity profiles of entire fungus gardens, it is not possible to determine from which organisms the focal enzymes originate. Although enzyme production is not necessarily proportional to microbial biomass, we believe that most of the enzyme activity that we measured in the field collected fungus gardens stems from the fungal cultivar, which is known to produce many plant degrading extracellular enzymes, and which comprises the largest microbial biomass in a garden (Abril and Bucher 2004). The single explicit test of this hypothesis that is currently available did indeed characterize one of these enzymes, an endo-xylanase, as being unequivocally produced and extracellularly secreted by *L. gongylophorus* cultivated by the leaf-cutting ant *A. echinatior* (Schiøtt et al. 2008).

THE POSSIBLE ADAPTIVE SIGNIFICANCE OF EVOLUTIONARY TRANSITIONS OF FUNGAL SYMBIONT

Our study found evidence for modified enzyme activity profiles of fungus gardens across all four major transitions in attine ant

farming that were recognized by Schultz and Brady (2008), but the observed patterns in the higher-attines (transition A and B) are easier to interpret than those in the lower-attines (transition C and D, Fig. 2, Table 2). The transition from lower- to higher-attine agriculture (A, Fig. 1) involved an elevation of endo-protease activity, almost certainly as a modification to ant foraging behaviour that mostly targets fresh plant material (fallen leaves, fruits, and flowers), rather than dead plant material as in the lower-attine ants (Fig. 1). Transition A represents the most crucial evolutionary innovation of symbiont function, that is, the emergence of fungal gongylidia as a synapomorphy for all extant higher-attine ant symbionts (Boyd and Martin 1975; Rønhede et al. 2004). We thus hypothesize that high endo-protease activity and a matching substrate of fresh plant material were decisive innovations for making the ant–fungus mutualism obligatory (i.e., symmetrical) for both parties (Mueller et al. 1998; Aanen and Boomsma 2006).

Endo-protease levels became even higher across transition B (toward leaf-cutting farming) and are accompanied by a profoundly higher activity of α-amylase and some pectinases, whereas enzyme activity toward cross-linking glycans tended to decrease or remain similar (Fig. 2, Table 2). These results support the hypothesis that leaf-cutting ant gardens primarily use the protein and starch of the fresh leaf substrate that the ants provide (Abril and Bucher 2004; Silva et al. 2006a), and invest relatively less in degrading the structural cell wall components except for partial pectin degradation to facilitate access to the inner plant cell contents. This is consistent with in vitro garden cultures of leaf-cutting ant fungi growing faster and producing more enzymes on starch than on cellulose substrates (Gomes de Siqueira et al. 1998; Silva et al. 2006b). Similar growth tests have not been done systematically with lower-attine cultivars. However, the interpretation of such test results would be more difficult because genetic diversity of cultivated fungi is high (Mueller et al. 1998) and the expression of enzymes to degrade specific parts of plant cell walls quite possibly depends on several substrates being present simultaneously (Cooke and Rayner 1984).

The ancestor of the about 45 recognized extant species of *Atta* and *Acromyrmex* leaf-cutting ants evolved 8–12 MYA (Schultz and Brady 2008). This considerable radiation of the ants was not accompanied by a similar radiation of their symbiont, and whatever variation may have emerged appears to have been annihilated by a single lineage sweeping through all leafcutter ants by horizontally transmission only 2–4 MYA (Mikheyev et al. in press). As a result, all extant leaf-cutting ants appear to cultivate a single fungal species, *L. gongylophorus* (Silva-Pinhati et al. 2004; Mikheyev et al. 2006; Mikheyev et al. 2007). Our present comparison between this single cultivar lineage and the other Clade 1 symbionts shows that the leaf-cutting ant cultivar has a unique enzymatic profile, which appears to be ideally suited to degrading freshly cut plant material.

Our most surprising result came from the analyses of enzyme profiles across transition C, the recurrent switch in many lower-attine ants from cultivating Clade 1 to Clade 2 agaricaceous symbionts and vice versa (Fig. 1). Decomposition activity patterns of Clade 2 cultivars were significantly higher (Fig. 2) for all categories of enzymes except α-amylase. As the latter enzyme seems unlikely to be of much practical significance for symbionts that use dead plant material as substrate (where other microorganisms have already decomposed the starch), this pattern suggests that the secondarily acquired Clade 2 fungi are superior symbionts across the lower-attine ant genera. This raises the interesting question, why not all lower-attines have shifted to rearing Clade 2 symbionts, as horizontal transmission would appear common enough for every lineage to have access to these alternative symbionts, in spite of vertical transmission being the norm (Von Ihering 1898 as referenced in Wheeler 1907; Weber 1972). We hypothesize that the answer is to be found in enzyme activity trade-offs with quantitative life-history traits such as a lower growth rate, a narrower stress tolerance, and/or a lower resistance to disease. Explicit field and laboratory tests to assess which of these trade-offs apply, and under what conditions, would both be highly rewarding and feasible as some lower-attine ants rear both types of symbiont across sympatric colonies (Mueller et al. 1998; Vo et al. 2009).

Transition D (Fig. 1) to cultivating pterulaceous (coral) fungi (Munkacsi et al. 2004; Villesen et al. 2004) in the ancestor of the *A. pilosum* species group is difficult to explain from our comparative analyses, as we found no clear indication for specific enzymatic advantages of cultivating pterulaceous fungus gardens (Fig. 2, Table 2). Endo-protease and cellulase activities in these *Apterostigma* gardens appear higher than in Clade 1 gardens of lower-attine ants (Fig. 2), but these differences disappeared in more detailed analyses (Table 2). It may be that our survey missed some specific enzymes that would be particularly useful in degrading the wood chips that *Apterostigma* workers are known to collect occasionally (Villesen et al. 2004). However, it seems unlikely that such enzymes would be completely missing in the agaricaceous symbionts, because *Myrmicocrypta* workers also occasionally include wood chips in the substrate of their agaricaceous gardens (Murakami and Higashi 1997).

In contrast to transition C, transition D has apparently been irreversible, as no secondary reversals to agaricaceous fungi are known in the monophyletic *Apterostigma* section (*pilosum*-group) that rears pterulaceous fungi (Villesen et al. 2004). The radiation of this farming life style suggests a considerable degree of coadaptation between the ants and their alternative cultivars, but no significant net efficiency benefits, as pterulaceous *Apterostigma* societies do not seem more advanced than those of their sister clade rearing agaricaceous symbionts (Villesen et al. 2004). As in transition C, it thus seems likely that the functional explanation for transition D needs to be sought in fungal life-history traits

other than enzyme profiles. Similar to other representatives of this clade, the two *Apterostigma* species studied here (*dentigerum* and *collare*) have hanging fungus gardens underneath stones or logs or in small soil crevices (Villesen et al. 2004), a garden phenotype that is never observed in any other attine ant (Schultz and Brady 2008). Specific disease resistance, desiccation and temperature tolerance, or detoxifying properties may therefore be interesting candidate traits for further comparative studies of *Apterostigma* fungus gardens.

THE FUNDAMENTAL INEFFICIENCY OF LEAF-CUTTING ANT FUNGUS FARMING

In general, foliage and grass feeding insects do not digest cellulose, which is often attributed to low activity/efficiency of cellulases in their guts (Douglas 2009). However, fungus-growing ants do not have this constraint as the initial digestion takes place outside their bodies by a garden symbiont. It is therefore remarkable that fungus gardens have not become better adapted to degrading plant cellulose. During the first about 30 million years of attine ant evolution, the fungal crops remained essentially unchanged as they did not become reproductively isolated from their free-living relatives and could thus not readily evolve adaptations specific to symbiosis with the ants (Mueller et al. 2001; Mueller 2002). Our results confirm that only the evolution of the higher-attine ants (about 20 MYA) and the leaf-cutting ants (about 10 MYA; Schultz and Brady 2008) were associated with novel enzymatic symbiont functions that specifically target the stored protein and starch in live plant cells. At the same time, our results indicate that no evolutionary advances in the attine ant farming symbiosis seem to have ever involved an enhanced efficiency in decomposing the cell wall material. Instead, cellulolytic activity in fungus gardens remained similar throughout the phylogeny (Fig. 2, Table 2), and the glycan targeted enzyme functions, which made the first free-living agaricaceous fungi suitable as fungal crops, in fact decreased in relative importance when the mutualism became symmetrically committed and more advanced (Fig. 1). We are aware that our samples come from a restricted area outside the centre of attine ant biodiversity in South America, but as several Brazilian studies have earlier raised doubts about the cellulose degrading capacities of leaf-cutting ant fungi (Abril and Bucher 2002), we believe that our results are likely to reflect general tendencies for the entire clade of fungus-growing ants.

Whereas free-living fungi can only access new resources by extending hyphae into neighboring substrate, their domesticated relatives reared in nests of lower-attine ants have foragers that actively transport new substrate to the fungus garden mycelium. This implies that the cost of foraging likely trade-offs with the benefits of continued exploitation of substrate already obtained. Following this line of reasoning, even lower-attine ants may have some optimum of incomplete substrate degradation where it pays to bring in new substrate rather than continuing the process. All available data of forage acquisition in attine ants indicate that the supply side of this trade-off shifted considerably across the transitions (A and B) toward higher-attine and leaf-cutting farming (De Fine Licht and Boomsma, in review). This may well have implied that social evolution in the higher attine ants was driven primarily by the occupation of novel foraging niches, rather than by more efficient exploitation of the ancestral niches. Our study indicates that, once the vast resources of freshly shed (higher-attines) and still attached fresh leaves (leaf-cutting ants) had become accessible for foragers, fungus garden resource acquisition became focused on the most valuable intracellular compounds of fresh plant material while becoming more wasteful with regard to the less valuable components of leaves. This would explain why colonies of *Atta* leaf-cutting ants are renowned for their large underground compost chambers (Hölldobler and Wilson 1990) or, in some species, surface waste piles (Hart and Ratnieks 2002) where they accumulate expended garden material and where bacterial communities degrade cellulose (Bacci Jr. et al. 1995; Wirth et al. 2003).

Hardly using the plant cell walls while concentrating degradation on the leaf pectins and the even more valuable intracellular resources implies that disproportionally large quantities of leaves are needed to sustain an *Atta* colony (Abril and Bucher 2004). This may have been a decisive factor for *Atta* colonies to become major enhancers of nutrient cycling in all ecosystems where they occur (Wirth et al. 2003; Herz et al. 2007). The production of excessive amounts of waste in turn necessitated elaborate hygienic waste management practices (Bot et al. 2001; Hart and Ratnieks 2002) and induced adaptive radiation in additional eukaryote symbionts that depended on the ant waste without being parasites of the ant societies (Hölldobler and Wilson 1990; Hughes et al. 2008).

ACKNOWLEDGMENTS

The authors thank D. K. Aanen, M. Poulsen, and three anonymous reviewers for helpful comments on an earlier version of this manuscript, the Smithsonian Tropical Research Institute, Panama, for providing logistic help and facilities to work in Gamboa, and the Autoridad Nacional del Ambiente y el Mar (ANAM) for permission to sample ants in Panama and to export them to Denmark. H. Fernández-Marín, G. Bruner, and J. Broch helped in the field and Lene Lange suggested the use of the AZCL method. We were supported by the Danish National Research Foundation (HHDFL, MS, JJB), the Carlsberg Foundation (MS), and the National Science Foundation Award DEB-0639879 to UGM.

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Associate Editor: P. Abbot