Reciprocal genomic evolution in the ant–fungus agricultural symbiosis

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The attine ant–fungus agricultural symbiosis evolved over tens of millions of years, producing complex societies with industrial-scale farming analogous to that of humans. Here we document reciprocal shifts in the genomes and transcriptomes of seven fungus-farming ant species and their fungal cultivars. We show that ant subsistence farming probably originated in the early Tertiary (55–60 MYA), followed by further transitions to the farming of fully domesticated cultivars and leaf-cutting, both arising earlier than previously estimated. Evolutionary modifications in the ants include unprecedented rates of genome-wide structural rearrangement, early loss of arginine biosynthesis and positive selection on chitinase pathways. Modifications of fungal cultivars include loss of a key ligninase domain, changes in chitin synthesis and a reduction in carbohydrate-degrading enzymes as the ants gradually transitioned to functional herbivory. In contrast to human farming, increasing dependence on a single cultivar lineage appears to have been essential to the origin of industrial-scale ant agriculture.
**Results**

**Sequencing data and phylogenetic analyses.** At around 300 Mb, the five newly sequenced attine ant genomes were of fairly standard size and composition compared with published attine and other ant genomes18–20 (Supplementary Tables 1–19 and Supplementary Figs 1–7). The dikaryotic fungal symbiont of the lower attine *C. costatus* was assembled into a draft genome with a relatively large size of ca. 126 Mb (Supplementary Tables 5 and 7), whereas we obtained genome-wide transcriptomes for the cultivars of the higher attine ants as their increasing degrees of polyketotic chimerism (functional polyploidy)14 precluded accurate genome assembly (Supplementary Table 7).

Phylogenies based on 2,795 and 1,075 one-to-one orthologues for the ants and their cultivars, respectively, provided four novel insights (Fig. 1 and Supplementary Figs 8–11). First, the fungal phylogeny (Fig. 1, grey branches, node 2) indicates that the leucocoprinaceous cultivar clade arose simultaneously with the farming ants ca. 55–60 million years ago (MYA, see also refs 5,11), although this node has a considerable confidence range of 44–72 MYA. Second, domestication of the higher attine cultivars that produce staphylae with gongylidia occurred ca. 30 MYA (node 3 and 4), earlier than indicated by previous studies (ca. 20–25 MYA22). Third, the leaf-cutting ants arose ca. 15 MYA (node 9) rather than ca. 10 MYA5. Fourth, the single cultivar species, *Leucoagaricus gongylophorus* grown by *Atta* and *Acromyrmex* leaf-cutting ants, originated subsequent to the origin of its farmers (nodes 11 and 9, respectively) from a fungal lineage cultivated by *Trachymyrmex* ants. This finding confirms that leaf-cutting ants horizontally acquired a replacement cultivar after *Atta* and *Acromyrmex* had diverged (node 9)21.

**Attine ant genome evolution.** Attine ant genomes show very high rates of structural rearrangement. No animal lineage for which multiple genomes are available has experienced faster rates of synteny loss than the fungus-growing ants, including all non-attine ants that have been examined, which show similar levels of genome rearrangement to other insects20 (Fig. 2a,b: attines versus other ants, Mann–Whitney *U*=0.22, *P*=0.0093; all differences with other lineages *P*<0.0001; Supplementary Fig. 12 and Supplementary Table 20). Many attine ant gene families contracted at the origin of fungus farming, suggesting that the new specialized lifestyle made some ancestral genes obsolete (Fig. 2c and Supplementary Figs 13 and 14). In contrast, the ancestral branch of the evolutionarily derived *Atta* leaf-cutting ants shows many gene family expansions, including 129 novel genes with no significant homology to known genes and no indication of horizontal gene transfer from microorganisms, consistent with previous findings19. These gains indicate that substantial new genetic material became available for recruitment during the recent evolution of these industrial-scale farming societies.

The arginine biosynthesis pathway is known to be absent in the evolutionarily derived leaf-cutting ants18,19, but our comparative analysis (Fig. 2d and Supplementary Figs 15 and 16) indicates that this deficiency probably originated in the earliest attine farmers with the loss of the argininosuccinate lyase gene that encodes the final enzymatic step in arginine biosynthesis. Demise of the penultimate argininosuccinate synthase gene appears to have been secondary, as pseudogenized sequence fragments can still be identified in several genomes (Fig. 2d). Arginine biosynthesis deficiency may have precluded independent life,
consistent with the lack of known reversals to a hunter–gatherer lifestyle in the attine subtribe. As arginine is the most nitrogen-rich naturally occurring amino acid, it may be a suitable vector for transferring nitrogen from fungal symbionts to the farming ants. Two gene families with potential links to energy metabolism were found to be expanded in all attine ants: Tom70 genes that encode mitochondrial import proteins and Nardilysin, which was previously identified as expanded in A. echinatior and has been linked to protein complex formation in the mitochondrial citrate cycle (Supplementary Fig. 13 and Supplementary Table 21). We also found increased dN/dS ratios among many energy metabolism-related genes in the higher attine ants (Supplementary Tables 22 and 23). These changes may reflect genomic responses to documented reductions in metabolic rate following the origin of fungus farming and persisting throughout the lower attine ants, only to be reversed again to normal ant levels with the irreversible domestication of staphylae-producing cultivars ca. 30 MY later.

Fewer carbohydrate degradation genes in domesticated crops. Lower attine cultivars are loosely domesticated symbionts that are likely to be capable of living apart from ants or to exchange genes with close free-living relatives; thus, conditions for ant-fungus coevolution did not become unambiguously favourable until a cultivar lineage committed to genetically isolated long-term vertical transmission ca. 30 MYA (Fig. 1). This event coincided with the first use of fresh plant material as garden substrate and, thus, we compared genome-wide changes in carbohydrate-degrading potential of attine cultivars and the related free-living Agaricales fungi Coprinopsis cinerea, Agaricus bisporus, and Schizophyllum commune. Among these farmed and free-living fungi, the C. costatus cultivar has the most substantial carbohydrate-degrading repertoire (Fig. 3a and Supplementary Data 1), consistent with the recruitment of highly versatile decomposers by early farming ants once they became obligately dependent on their fungus gardens to convert dead plant material into food. However, the number of carbohydrate-degrading
enzymes in truly domesticated, staphylocae-producing cultivars is consistently reduced (binomial test, \( P < 0.0014 \)) across three of the six CAZy classes (encoding auxiliary activities, carbohydrate esterases and glycoside hydrolases; Fig. 3a). Clustering analysis confirmed that the \( C. \) costatus cultivar and the free-living fungi have similar CAZy profiles, and that fully domesticated cultivars share a distinctly different biodegradation potential (Fig. 3b and Supplementary Data 1).

The fully domesticated higher attine cultivars have significantly fewer lignin-degrading genes than the \( C. \) costatus cultivar (binomial test, \( P < 1.6 \times 10^{-3} \)), indicating that secondary cell walls of vascular bundles, wood and bark became a marginal foraging priority after irreversible cultivar domestication (Fig. 3c). Key genes encoding proteins with the ligninase domain (IPR001621) are absent across higher attine cultivars (Supplementary Table 24), although synteny of the up- and downstream genes of the \( C. \) costatus cultivar and \( A. \) bisporus is maintained in domesticated cultivars (Fig. 3d). The \( C. \) costatus cultivar gene is a triple tandem repeat, possibly compensating for the absence of a second ligninase gene present in \( A. \) bisporus (Supplementary Table 25). The overall reduction in CAZymes and loss of lignin-degrading potential probably prevented independent saprotrophic life for the truly domesticated cultivar and is consistent with \( T. \) septentrionalis, \( A. \) echinatior and \( A. \) colombica foragers primarily targeting soft leaves and petals, and \( A. \) foragers avoiding the lignin-rich midrifs of leaves that they otherwise harvest entirely\(^{23} \) (Fig. 3e,f). These findings match the maintenance by \( A. \) foragers of large waste heaps\(^ {24} \) consisting mostly of old fungus and recalcitrant cell wall material\(^ {12,25,26} \).

**Crop and ant genomes coevolved to produce and digest chitin.**

The cells of the \( L. \) gongylorrhous cultivar of \( A. \) and \( A. \) echinatior leaf-cutting ants contain substantial amounts of chitin, which is degraded by chitinolytic enzymes that are produced in abundance by the ant labial glands\(^ {27} \). The genomic basis for this adaptation appears to be parallel evolutionary changes in fungal pathways...
related to chitin synthesis and digestion of chitin by the ants. Genes encoding chitinase and β-hexosaminidase were positively selected in the ancestral attine ant (Fig. 4a; likelihood ratio test (LRT), P<0.05; Supplementary Tables 26–28 and Supplementary Data 2), consistent with early adaptation to fungivory. The positively selected sites (nine in the β-hexosaminidase and four in the chitinase) are mostly located on the protein surfaces (Supplementary Figs 17 and 18) and their messenger RNAs are highly expressed in the ant labial glands (Fig. 4b). The inferred isoelectric points of the proteins match earlier direct measurements and are significantly higher than those of orthologous proteins in non-farming myrmicine ants (Fig. 4c; phylogenetic analysis of variance (ANOVA), P<0.03; Supplementary Tables 28 and 29). These changes in charge properties probably optimize functionality in the ant foreguts, which are known to have increased pH levels.

The attine ant chitinase has lost a carboxy-terminal domain often associated with binding to the peritrophic matrix of the insect gut (Fig. 4d), consistent with selection on this protein to become soluble in the labial gland fluid. A single additional amino acid site was positively selected in the ancestor of the higher attines and the average chitinase residue weight in the attine cultivars is completely lost (Supplementary Table 24). Loss of function of this enzyme leads to enhanced chitin synthesis and cell walls of increased thickness in ascomycete fungi, suggesting
that the increased volumes and masses of higher attine ant gardens may be enabled by fortified cell walls.\cite{27,30}

**Discussion**

The results of our study shed considerable new light on the evolution of ant agriculture. First, based on the most probable divergence-date estimate, fungus farming may have originated shortly after the Yucatan impact that caused the major Cretaceous-Tertiary extinction 65 MYA and before the early Eocene climatic optimum 50–55 MYA (Fig. 1). Second, farming probably became irreversible when the ants lost the arginine biosynthesis pathway, relying instead on predictably present symbionts to supply this amino acid (Fig. 2c). Third, despite the evolution of modified chitin-processing genes that facilitated the digestion of fungal food (Fig. 4), early attine lineages remained constrained to rearing small, slow-growing gardens until a single cultivar became irreversibly domesticated ca. 30 MY after the origin of subsistence agriculture. Fourth, genetic isolation of this cultivar promoted coevolution with the farmers, producing not only specialized ant-feeding organs and six new genus-level lineages of ant farmers but also more massive fungus gardens and a gradual shift in decomposition profile towards active functional herbivory, ultimately resulting in the loss of an ancestral ligninase domain. As the genomes and transcriptomes that this study adds to the public domain span the main evolutionary transitions across ant fungus farming, we expect future research to clarify additional symbiotic adaptations associated with transitions from simpler to more elaborate levels of fungus farming.

Our confirmation of the secondary acquisition of the ancestor of extant *L. gongylophorus* <10 MYA (\cite{ref. 21}; this study) strongly suggests that crop innovation was critical to the establishment of industrial-scale agriculture in *Atta* and, to a lesser extent, in *Acromyrmex* (Fig. 1), even though we found relatively little evidence for substantial genome- or transcriptome-wide changes in the ancestral lineages that gave rise to the leaf-cutting ants and *L. gongylophorus*. We hypothesize that the main factor underlying the ecological dominance of the leaf-cutting ants may have been that this novel cultivar was a genetically chimeric polyploid\cite{14}, a trait that commonly characterizes modern asexually propagated human-domesticated plants, but that is highly unusual for fungi. It thus emerges that the journeys of both ants and humans towards industrial-scale agriculture included long prior histories of subsistence farming that preceded specialization on genetically isolated crop varieties. However, although ant agriculture continued as a mutualistic symbiosis characterized by gradual reciprocal modifications and a single superior cultivar lineage with little genetic variation across the clones maintained by sympatric colonies, human agriculture proceeded by cultural evolution. Artificial selection by humans drove much faster domestication rates in a multitude of diverse cultivars\cite{15,17}, accompanied by, at least so far, relatively modest reciprocal modifications of our genomes.\cite{31}

**Methods**

**Biological material and sequencing.** Queenright colonies of *C. costatus*, *T. zeteki* and *T. cornetti* were collected in Gamboa, Panama, and maintained in the lab on a diet of polenta, oatmeal and bramble leaves at 25 °C and 60–70% relative humidity (RH). For *A. colombica* and *T. septentrionalis*, ants from single colonies were collected from Gamboa, Panama and from Apalachiola National Forest, Tallahassee, FL, USA, respectively. Fungal cultures were obtained by incubation on potato dextrose yeast-extract agar plates containing streptomycin or, for *T. septentrionalis*, potato dextrose agar plates containing streptomycin + penicillin followed by propagation in liquid potato dextrose agar medium. Samples that were not immediately processed were stored in RNAlater at ~80 °C. DNA and RNA was then extracted using QIAGEN kits or standard extraction protocols (see Supplementary Information for full details). Sequencing libraries were insert sizes ranging from 200 bp to 10 kbp were generated for the genomic DNA using standard procedures, whereas 200 bp fragments were used for complementary
DNA sequencing libraries. All libraries were paired-end sequenced on an Illumina HiSeq 2,000 platform with read lengths of 100 bp for small insert sizes, 49 bp for large insert sizes, and 100 bp for the DNA barcoding library. Queen insemination data were obtained from 6 colonies using 4 polymorphic microsatellite markers and for T. septentrionalis by using 4 microsatellite markers to genotyped ca. 10 workers from 10 field colonies made available by Jon Seal, University of Texas at Tyler.

Assembly and annotation. Genomic sequencing reads were filtered to remove low-quality reads and PCR duplicates, and then assembled using SOAPdenovo (v2.04). Using Orthomcl V.2.09 (ref. 42). Homologous relationships among sequences were determined using Blastp with an e-value cutoff of 10−5 to remove contaminant (bacterial or bacterial + fungal) sequences. Cut-offs were then used for the most distant outgroup taxon. Phylogenetic inference was performed using the same method as described for genome-based annotation above.

Gene family analyses. Genes from the seven attine ant and five other ant genomes (Solenopsis invicta, Pogonomyrmex barbatus, Camponotus floridanus, Linepithema humile and Harpegnathos saltator), as well as three outgroup insects (Apis mellifera, Drosophila melanogaster and Nasonia vitripennis) were clustered into gene families using OrthoMcl V.2.09 (ref. 42). Homologous relationships among sequences were determined using Blastp with an e-value cutoff of 10−5 and an alignment length cutoff of 50% of the gene length followed by clustering by MCL. Only gene families found in single copies in all species (2,795) were used for phylogenetic inference (see below).

Once significant relationships among gene families of attine ants and the two closest, sequenced outgroups (S. invicta and P. barbatus) were determined based on pairwise reciprocal best Blastp (e-value < 10−5) hits. Groups of orthologous genes were combined based on pairwise orthologous relationships, resulting in 7,443 one-to-one ant orthologue groups. Orthologue groups for the cultivars were similarly merged to produce a unique, composite, orthologous set of orthologue groups. This resulted in 1,075 one-to-one orthologue groups, which were used to build the fungal phylogeny (see below).

Codon-based alignments of groups of one-to-one orthologous ant genes were generated with PRANK v.1.20716 (ref. 43) and low-scoring sites masked using an empirically determined SiteDrop (ref. 19). Codon alignments were modelled with PAML45 version 4.7, using models with two to four distinct dn/ds ratios. Model likelihoods were compared with log-ratio tests and false discovery rate (FDR) correction to assess significance. Alignments that showed significant increases in likelihoods were compared with log-ratio tests and false discovery rate (FDR) corrections. The most significant alignments were subjected to the Hypergeometric test with an FDR-corrected p-value cutoff: 10−6. Candidate outlier families were manually checked, resulting in the identification of candidate outlier families that were not included in the analysis (see above and were verified using HMMER60 searches with the potentially lost IPR domain profiles against six-frame translations of all transcriptomes, as well as the CAZy, SwissProt and Interpro databases).

Phylogenies. Protein sequences of 2,795 (ants) or 1,075 (fungi) single-copy gene families were aligned using MUSCLE65 with default parameters, converted into coding sequence (CDS) alignments and concatenated in Geneious v7.0 (ref. 50), resulting in a data matrix consisting of 1,886,151 amino acid sites and 13 taxa (ants) or 825,886 amino acid sites and 8 taxa (fungi). The concatenated matrix was analysed under the parsimony criterion in PAUP* V.4.0a140 (ref. 51) using a heuristic search and 100 random-taxon-addition replicates for the ants and an exhaustive search for the fungi, in each case resulting in a single optimal tree. Using this maximum-likelihood tree as a reference tree and the 2,795 (1,075) loci as the input, the most possible partitioning analysis was conducted in PartitionFinder v.1.1.1 (ref. 52) in which all possible protein model were considered and compared (models = all protein) under the Bayesian Information Criterion using the hclust search algorithm, resulting in a scheme consisting of 132 (19) partitions. These partitions and model were employed in a maximum-likelihood analysis in RAxML 7.7.7 (ref. 53), resulting in a best tree with topology identical to the maximum-parsimony topology. The partitions and models were also employed in maximum-likelihood bootstrap analyses in RAxML consisting of 1,152 pseudoreplicates using the -s (through search) bootstrap option, resulting once again with the same topologies with high bootstrap values.

We inferred divergence dates for the maximum-likelihood tree using the penalized likelihood approach implemented in r8s v.1.7 (ref. 54). For the ant dating analysis, the bee outgroup A. mellifera was excluded and two nodes in our tree were calibrated with fixed ages based on the results from a large-scale diversification analysis of 19 (ref. 42). Healy Myrmecinae that employed a total of 27 fossil calibrations across 251 species55. The two calibrated nodes in our tree correspond to (a) the most recent common ancestor (MRCA) of C. costatus and its sister group and (b) the MRCA of P. barbatus and its sister group. Three separate analyses were conducted, using the mean, 5% minimum credibility interval and 95% maximum credibility interval, respectively, characterized by the total number of IPR domain profiles against six-frame translations of C. costatus (196, 33.8) MYA and node b (95.4 (82.106.00) MYA). For the fungal dating analysis, the most distant outgroup taxon S. commune was used to root the tree, providing estimates for branch lengths descended from this root node and subsequently excluded from the analyses. We applied a fixed age calibration to the node corresponding to the MRCA of the outgroup Apis and its sister group using the results from a previous study55, a procedure similar to another diversification date analysis of lepidopteraceae cultivars56. We conducted three separate analyses using different fixed ages for this node. These fixed ages were obtained from previous age estimates for this node from Geml et al.57. Thus, we conducted analyses using the mean age (73 MYA) and derived confidence ranges using the 5% minimum age (55 MYA) and the 95% maximum age (91 MYA) calibrations.

Ant genome synteny and arginine biosynthesis pathway loss. Pairwise genome synteny was determined using anattine, among 5 other sequenced ants, among 12 fruit flies, 8 primates, 22 birds and 16 mosquito genomes. Pairwise orthologous genes were identified based on reciprocal best Blastp hits as described above. Synteny blocks were then defined as containing at least five contiguous orthologous genes and were extended across gaps of no more than 4 genes. No more than 5 gene inversions in total were allowed in any pairwise syntenic block. Blocks of synteny between P. barbatus were assed using a roll-off neighboring and lized process and rates of synteny loss were calculated accordingly as 1−0.10/T, where T is divergence time (in millions of years) and 0.1 was used to incorporate the most recent common ancestor (MRCA) of the outgroup Apis and its sister group using the results from a previous study55, a procedure similar to another diversification date analysis of lepidopteraceae cultivars56. We conducted three separate analyses using different fixed ages for this node. These fixed ages were obtained from previous age estimates for this node from Geml et al.57. Thus, we conducted analyses using the mean age (73 MYA) and derived confidence ranges using the 5% minimum age (55 MYA) and the 95% maximum age (91 MYA) calibrations.

Fungal CAZY and interpro analyses. Protein sequences of attine cultivars and three outgroup fungi (C. cinerea v1.0, A. bisporus v.2.0 and S. commune v.2.0) were matched against the CAZY database (v2013)59 using BLASTp, requiring full-length alignment of the query with an e-value < 10−6 and identity > 50%. These matches were then subjected to BLAST against a library of individual CAZY module sequences and HMmer searches66 using specific models for each CAZY module family, requiring both methods to yield the same match. The 'MSA' output for the CAZY outgroup was evaluated using binomial probabilities assuming equal count distributions.
of surrounding genes using manual BLAST searches against the A. biporus (H97 v2.0) and L. gongylophorus (AcD2 v1.0) genome sequences.

Positive selection. Positive selection was assessed using PAML (v4.6)45 branch-site models on the orthologue group alignments, using three different starting values for kappa and omega. We required an FDR-corrected P-value < 0.05 from the LRT test and at least one site with a Bayes Empirical Bayes probability > 0.95, and manually checked alignment quality around inferred positively selected sites. Significant differences in mean gene expression levels were determined with a QX200 ddPCR system (Bio-Rad) using TaqMan probes. The two genes encoding Ribosomal Protein L18 (RPL18) and TATA-binding protein, with the Genbank accession numbers XM_011064584 and XM_011062766, respectively, were used as housekeeping genes, to normalize the expression levels across samples. Primers and probes were designed using the Primer3Plus68 and PCR efficiency Calculator69 web interfaces, and are shown in Supplementary Table 29. PCR reactions were run on a Bio-Rad S1000 Thermal Cycler with a final concentration corresponding to 5 ng m/C0 M, respectively. Each reaction contained 1 of template per reaction, and 

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Author contributions

J.J.B., G.Z., T.R.S. and W.T.W. conceived of the study. M.S., T.R.S. and C.R. collected data. S.N., G.Z., T.R.S. and W.T.W. provided logistic help and facilities to work in Gamboa and the Autoridad Nacional del Ambiente y el Mar (ANAM) gave permission to sample and export ants. S.G.B. and T.R.S. provided support to work in Panama. The Visitor’s Office of the Smithsonian Tropical Research Institute provided logistic help and facilities to work in Gamboa and the Autoridad Nacional del Ambiente y el Mar (ANAM) gave permission to sample and export ants from Panama.

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