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Evolutionarily advanced ant farmers rear polyploid fungal crops

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Leucoagaricus;
Basidiomycota;
fungus-growing ants.

Abstract

Innovative evolutionary developments are often related to gene or genome duplications. The crop fungi of attine fungus-growing ants are suspected to have enhanced genetic variation reminiscent of polyploidy, but this has never been quantified with cytological data and genetic markers. We estimated the number of nuclei per fungal cell for 42 symbionts reared by 14 species of Panamanian fungus-growing ants. This showed that domesticated symbionts of higher attine ants are polykaryotic with 7–17 nuclei per cell, whereas nonspecialized crops of lower attines are dikaryotic similar to most free-living basidiomycete fungi. We then investigated how putative higher genetic diversity is distributed across polykaryotic mycelia, using microsatellite loci and evaluating models assuming that all nuclei are either heterogeneously haploid or homogeneously polyploid. Genetic variation in the polykaryotic symbionts of the basal higher attine genera *Trachymyrmex* and *Sericomyrmex* was only slightly enhanced, but the evolutionarily derived crop fungi of *Atta* and *Acromyrmex* leaf-cutting ants had much higher genetic variation. Our opposite ploidy models indicated that the symbionts of *Trachymyrmex* and *Sericomyrmex* are likely to be lowly and facultatively polyploid (just over two haplotypes on average), whereas *Atta* and *Acromyrmex* symbionts are highly and obligatorily polyploid (ca. 5–7 haplotypes on average). This stepwise transition appears analogous to ploidy variation in plants and fungi domesticated by humans and in fungi domesticated by termites and plants, where gene or genome duplications were typically associated with selection for higher productivity, but allopolyploid chimerism was incompatible with sexual reproduction.

Introduction

Polyploidy, the possession of more than two sets of chromosomes, is widespread in flowering plants (Masterson, 1994; Otto & Whitton, 2000), with 15% of speciation events having likely been facilitated by genome duplication, a figure that is even higher (31%) in ferns (Wood *et al.*, 2009). Polyploidy may be advantageous when it increases functional heterozygosity and helps masking phenotypic effects of deleterious recessive alleles, but this may come at costs of chromosome loss during mitosis, spindle irregularities and other imbalances during meiosis, and lower epigenetic stability (Comai, 2005). Polyploidy is rare in metazoans (Wertheim *et al.*, 2013), suggesting that the costs are usually prohibitive when reproducing via a unitary germ line and a single set of sexual organs, rather than via vascular somatic modules that each produce flowers or sporangia. However, it is now well documented that major early 'bauplan' innovations in the metazoan lineage were preceded by genome duplications (Cañestro, 2012).

Polyploidy is rare in filamentous fungi, but this may be primarily related to fungal cells not being haploid or diploid in the normal sense, but having one (monokaryon) or two (dikaryon) genetically different haploid nuclei per cell, with just a few exceptions in unicellular

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yeasts and the honey fungus, which occasionally and predominantly have diploid nuclei, respectively (Rizzo & May, 1994; Carvalho *et al.*, 1995). In the Basidiomycota, monokaryotic hyphae emerging from germinating sexual basidiospores are thus analogous to gametophytes in vascular plants, as the sexual cycle cannot be completed until two monokaryons merge to form a dikaryon. When such contact is genetically compatible, the nuclei of the parental monokaryons distribute themselves evenly throughout the hyphal system so that all cells end up having both types of nuclei that will henceforth be transmitted jointly in somatic cell divisions. However, mitochondria do not migrate so that most cells contain only one type of mitochondrial DNA, implying that transmission of mtDNA is uniparental (Aanen *et al.*, 2004). This process makes basidiomycete dikaryons functionally analogous to diploid vascular plant sporophytes (Raper, 1955; Ellingboe & Raper, 1962).

Because the two 'mated' haploid nuclei that comprise a dikaryon remain physically autonomous throughout vegetative mycelial growth, nuclear parity may need to be actively reinforced with every cell division to ensure that nuclei retain equal probabilities of taking part in sexual reproduction whenever mycelia will initiate the formation of sexual organs. The mushroom-producing Basidiomycota that mostly have dikaryotic hyphae have resolved this challenge by evolving clamp connections in most lineages, that is specialized bridges between the new cell, which has received a copy of one nucleus, and the old cell that is about to become separated by a new cell wall (Niederpruem *et al.*, 1971). This septum forms simultaneously in the clamp connection where it separates the two copies of the second nucleus, so one of them is predictably returned to the old cell (Moore *et al.*, 2011; Nieuwenhuis *et al.*, 2013). Once a basidiomycete dikaryon has formed, incompatibility mechanisms will normally exclude entry of additional nuclei from other monokaryons or dikaryons unless they are genetically identical or very closely related (Adaskaveg & Gilbertson, 1987; Wilson, 1991; Marçais *et al.*, 2000).

In a recent genome sequencing project of the basidiomycete fungal symbiont of the leaf-cutting ant *Acromyrmex echinatior* (Nygaard *et al.*, 2011), we found strong indications for more than two haploid genomes in a single mycelium, that is for a genetic makeup analogous to some form of chimeric polyploidy (De Fine Licht *et al.*, 2013). This was consistent with earlier findings (Mohali, 1998; Scott *et al.*, 2009; Mueller *et al.*, 2011) and one of the reasons for an assembled draft genome remaining unavailable. The leaf-cutting ant crop symbiont, *Leucoagaricus gongylophorus*, is an evolutionarily highly derived representative of a fungal genus that generally lacks clamp connections (Vellinga *et al.*, 2003). The ancestor of all extant attine ants started farming leucocoprineaceous fungi in underground gardens ca. 50 MYA (Mueller *et al.*, 1998, 2005; Schultz & Brady, 2008), but it took ca. 25–30 million years until a single lineage was irreversibly domesticated, and evolved specialized hyphal tips (gongylidia) to feed the ants (Quinlan & Cherrett, 1978, 1979; Bass & Cherrett, 1995) while losing genetic exchange with free-living relatives (Mueller *et al.*, 2005; Schultz & Brady, 2008; De Fine Licht *et al.*, 2010, 2014). This gave rise to four genera of so-called higher attine ants that all rear cultivars belonging to this clade of gongylidia-bearing crop fungi, of which the most advanced *Atta* and *Acromyrmex* leaf-cutting ants arose only ca. 10–12 MYA (Schultz & Brady, 2008; Mikheyev *et al.*, 2010).

We tested the hypothesis that domestication might have induced functional crop polyploidy in the attine ant cultivars. To resolve this question, we (i) estimated the average number of nuclei per cell across the fungal symbionts of 14 species (eight genera) of Panamanian attine ants, comparing higher attine crop fungi that produce staphylae with gongylidia as ant food with lower attine symbionts that lack these symbiotic organs, (ii) mapped nuclei-per-cell numbers on phylogenetic symbiont trees that were constructed from sequence data and microsatellite genotypes, (iii) estimated the number of haploid genomes per mycelium for each of the higher attine crop symbionts that had more than two nuclei per cell and (iv) evaluated the likelihood that nuclei in polykaryotic mycelia continued to be haploid as in normal dikaryons or had evolved some form of chimeric polyploidy. In the former case, multiple genomes would likely reside in additional haploid nuclei, whereas each nucleus in a mycelium would have similarly enhanced genetic diversity and homogeneously polyploid nuclei in the latter case (Fig. 1).

We realize that polyploidy terminology is usually based on integer duplication of chromosome numbers

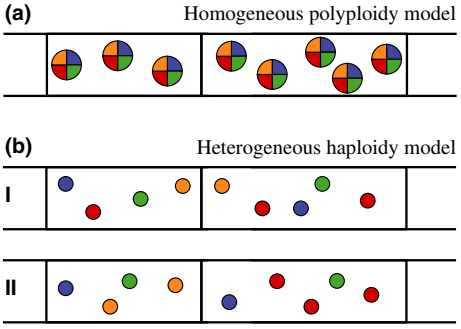

Fig. 1 Hypothetical extreme distributions of genetic variation within and among nuclei of basidiomycete fungi (without clamp connections) within and among cells. (a) Homogeneous polyploidy: each nucleus is equally polyploid and contains all genetic variation of a mycelium. Each cell also has an equal mix of haplotypes even if the number of nuclei may vary somewhat between cells in the same mycelium. (b) Heterogeneous haploidy: all nuclei are haploid and genetic variation occurs between them. Cells all contain either the complete set of genetically diverse nuclei (I) or a subset, so that some genetic differentiation between cells exists (II).

within a nucleus, which does not appear to apply in these asexual ant fungi where haplotype number varies continuously. However, we felt that using ploidy terminology would allow more precise descriptions of the implications of our findings than alternatives such as chimerism or mosaicism, which are about mixing of cells rather than haplotypes. We use the term 'autopolyploidy' when diploid (dikaryon) genetic variation is maintained in spite of the number of cell nuclei increasing well beyond two, and we use the term 'allopolyploidy' when mycelia with polykaryotic cells have enhanced ($n > 2$) genetic variation consistent with some form of somatic hybridization between multiple strains. We occasionally also use the term 'chimeric' as adjective or adverb to emphasize higher than diploid genetic variation.

Materials and methods

Fungal sampling and counting nuclei

Fungal symbionts of attine ants were isolated on potato dextrose agar (PDA) from 42 laboratory colonies collected in Panama from 2004 to 2012. Samples included representatives of the different evolutionary stages of ant fungus farming (see Table S1 for details on species and laboratory nest numbers), such that we had 25 samples of higher attine ants rearing domesticated gongylidia-bearing fungi and 17 samples of lower- and paleo-attine ants rearing fungi without obvious adaptations to being crops (Mueller *et al.*, 1998; Schultz & Brady, 2008). Plates were maintained at ca. 25 °C, and DNA was obtained with 5% Chelex extractions (Walsh *et al.*, 1991) when sufficient biomass had been produced.

Small pieces of mycelium from each colony were stained with DAPI on a microscope slide, after which we counted the number of nuclei per cell in five randomly chosen cells under a fluorescence microscope. For analysis, the data were grouped in five categories that are generally recognized as characterizing the evolutionary diversification and progression of attine fungus farming: basal leucocoprineaceous agriculture, pterulaceous coral fungus agriculture, leucocoprineaceous yeast agriculture, basal higher attine-domesticated agriculture and advanced leaf-cutting agriculture (Schultz & Brady, 2008; Mehdiabadi & Schultz, 2010). Differences in number of nuclei per cell were subsequently analysed with a general linear model in SAS with colonies as random factor nested within species, species nested within genera and genera nested within the five categories of fungus farming.

Genetic analyses

To confirm that fungi isolated from attine fungal gardens were the same as those cultivated by the fungus-growing ant hosts and to verify whether the two known clades of basal leucocoprineaceous crops (Clade 1 and Clade 2) were both represented (Mueller *et al.*, 1998; Schultz & Brady, 2008; Mehdiabadi & Schultz, 2010), we amplified and sequenced two conserved regions: internal transcribed spacer (ITS) and nuclear large subunit rRNA (LSU) (see Appendix S1 for primers and PCR conditions). All PCR products were sequenced at BGI Europe in Copenhagen, and sequences were deposited in GenBank (see Table S1 for accession numbers). When sequences were of unsatisfactory quality due to multiple divergent copies with small length mutations, we cloned PCR products in pCR4-TOPO using the TOPO TA cloning method (Invitrogen, Carlsbad, CA, USA) and resequenced them. A phylogenetic tree similar to Mueller *et al.* (1998)was reconstructed by applying maximum likelihood calculations with 500 bootstrap replicates, using MEGA5.1 software (Tamura *et al.*, 2011).

As we were finding multinucleate cells throughout the higher attine ant symbionts, we decided to screen these fungi for allelic variation at ten microsatellite loci: A128, A1030, A1132, A1151, B12, B447, C101, C126, C647 and D115 (Scott *et al.*, 2009) (see Appendix S1 for PCR conditions). These markers were specifically designed for *Atta*, *Acromyrmex* and *Trachymyrmex* symbionts and did not amplify in the symbionts of the lower attine genera. A phylogenetic tree was reconstructed using pairwise F_{ST} values based on the scored alleles and subjected to neighbour-joining analysis with 500 bootstrap replicates using POPULATIONS 1.2.32 software (Langella, 2001). To control for cross-contamination and possible binding problems at primer sites, we amplified all microsatellite loci for two representative samples (an *Acromyrmex* symbiont, Ae372, and a *Trachymyrmex* symbiont, Tcor002, for which all loci could be amplified) and subsequently sequenced one representative PCR product for each locus at Eurofins Genomics in Ebersberg, Germany. This showed that all loci that had amplified for each of these fungal samples produced the expected repeat sequence. Multiple base pair differences in the microsatellite flanking regions were subsequently compared to homologous sequences in the unassembled draft genome of an *A. echinatior* symbiont (De Fine Licht *et al.*, 2013). This showed that both the *Acromyrmex* and *Trachymyrmex* symbiont sequences were amplified correctly in spite of significant overall base pair differences ($t_9 = -2.3174$, $P = 0.023$) due to primers having been derived originally from either an *Acromyrmex* or a *Trachymyrmex* symbiont.

Estimating the number of haploid genomes

The number of marker loci successfully amplified from each cultivar varied between 4 and 10 (six on average in *Trachymyrmex* and *Sericomyrmex* symbionts; always 10

in leaf-cutting ant symbionts), but the sequence chromatograms often showed more than two allelic peaks, indicating that polykaryotic mycelia had more than the default dikaryotic number of haplotypes, particularly in the leaf-cutting ant symbionts. If we would have had a marker locus with close to infinite allelic variation, the observed number of alleles would have provided an exact estimate of the genetic diversity of polykaryotic mycelia. However, such ideal markers do not exist, so we had to rely on the more limited cumulative detection power of the moderately variable amplifying marker loci to estimate the likelihood of not detecting genetically different haplotypes (because alleles are either identical by descent or by chance) before arriving at an unbiased extrapolation estimate of the mean number of haplotypes per mycelium.

As we did not have a priori knowledge of how genetic variation in polykaryotic cells is distributed over nuclei, we analysed the data against two opposite null models, one assuming that all nuclei remained haploid as in the ancestral dikaryotic state and another assuming that all nuclei in a mycelium were homogeneously polyploid (Fig. 1). For each polykaryotic symbiont mycelium, we then plotted the number of observed alleles for each amplified marker locus against the mean number of nuclei counted and calculated regression slopes for both groups of symbionts to evaluate whether they were correlated. This gave approximate indications of the distribution of genetic variation across nuclei, as significantly positive correlations (with a maximum slope of one when each nucleus would contribute a new distinct allele) would be consistent with the heterogeneous haploidy model, whereas absence of correlations (zero slopes) would be consistent with the homogeneous polyploidy model (Fig. 1). Nuclei numbers in polykaryotic cells were normalized by subtracting the two standard dikaryon nuclei and further adjusted to have a baseline number of 2.12 nuclei because cells were sometimes observed after division had likely been initiated (an error rate estimated from the dikaryotic lower attine symbionts; see Results). Allele numbers were normalized by subtracting 1+H from each locus-specific count, where H is the expected heterozygosity per locus, so that 1+H is the null-expectation count for a dikaryon. ANCOVA was used to evaluate heterogeneities among slopes and intercepts of regressions of adjusted allele numbers on adjusted nuclei numbers.

Next, we inferred that under the heterogeneous haploidy model, positive correlations between allele number and nuclei number should follow regressions through the origin, of which the slopes should be increasing functions of locus-specific heterozygosity. In contrast, slopes are expected to be zero under the polyploidy null model, so that the mean allele number per locus at the average number of nuclei (the normalized intercept) should be an increasing function of locus-specific heterozygosity. Heterozygosities were calculated separately for symbionts of the basal *Trachymyrmex* and *Sericomyrmex* higher attines and the evolutionarily derived *Atta* and *Acromyrmex* leaf-cutting ants and corrected for finite sample sizes using the expression $\Sigma p^2{}_i = (N\Sigma y^2{}_i - 1)/(N - 1)$, as derived by Pamilo (1993) for estimating another kind of haplotype diversity, where N is the number of colonies, y_i is the frequency of the ith allele in the population, and $\Sigma p_i{}^2$ is the sample size corrected heterozygosity estimate.

To evaluate the heterogeneous haploidy model, we plotted the regression slopes of allele number on nuclei number against locus-specific expected heterozygosity based on observed allele frequencies, setting three slightly negative slopes to the minimal possible value of zero. Similarly, we evaluated the homogeneous polyploidy model by plotting the normalized intercepts against locus-specific expected heterozygosity. As slopes forced through the origin could only vary between zero and one, we used logistic beta regression after weighing each data point by its standard error, to obtain an overall estimation of the number of haplotypes that we should have detected with a putative single marker locus with infinite allelic variation (H = 1). The normalized intercepts were not constrained by a maximal value but increased disproportionally with heterozygosity, which necessitated that final curve fitting of this overall relationship was performed with log-transformed y-values. All analyses were performed in R using the standard 'STATS' package (R Core Team, 2013) and the beta-regression function 'betareg' (Cribari-Neto & Zeileis, 2010). The comparative slope and intercept functions finally produced an estimate of mycelium ploidy by extrapolation to the maximal heterozygosity of one for a putative marker locus with infinite allelic variation. Locus B12 was not variable for the *Trachymyrmex* and *Sericomyrmex* symbionts and was thus omitted from these analyses.

Results

The obtained ITS and LSU sequences were compared to the NCBI GenBank database using blastn, which showed that all sequences had highest similarities with previously sequenced fungal symbionts from the same or similar attine ant species. Consistent with previously published results (Mueller *et al.*, 1998; Munkacsi *et al.*, 2004; Vo *et al.*, 2009; Mikheyev *et al.*, 2010), the fungus-garden samples clustered as distinct symbiont clades, with all leaf-cutting agriculture symbionts forming one clade, all other gongylidia-bearing symbionts forming another clade and all lower attine leucocoprineaceous symbionts appearing as basal branches when using the closely related human-domesticated mushroom *Agaricus bisporus* as out-group (Fig. 2a). Microsatellite markers evolve much faster than ribosomal sequences (Sia *et al.*, 2000) and showed not only a

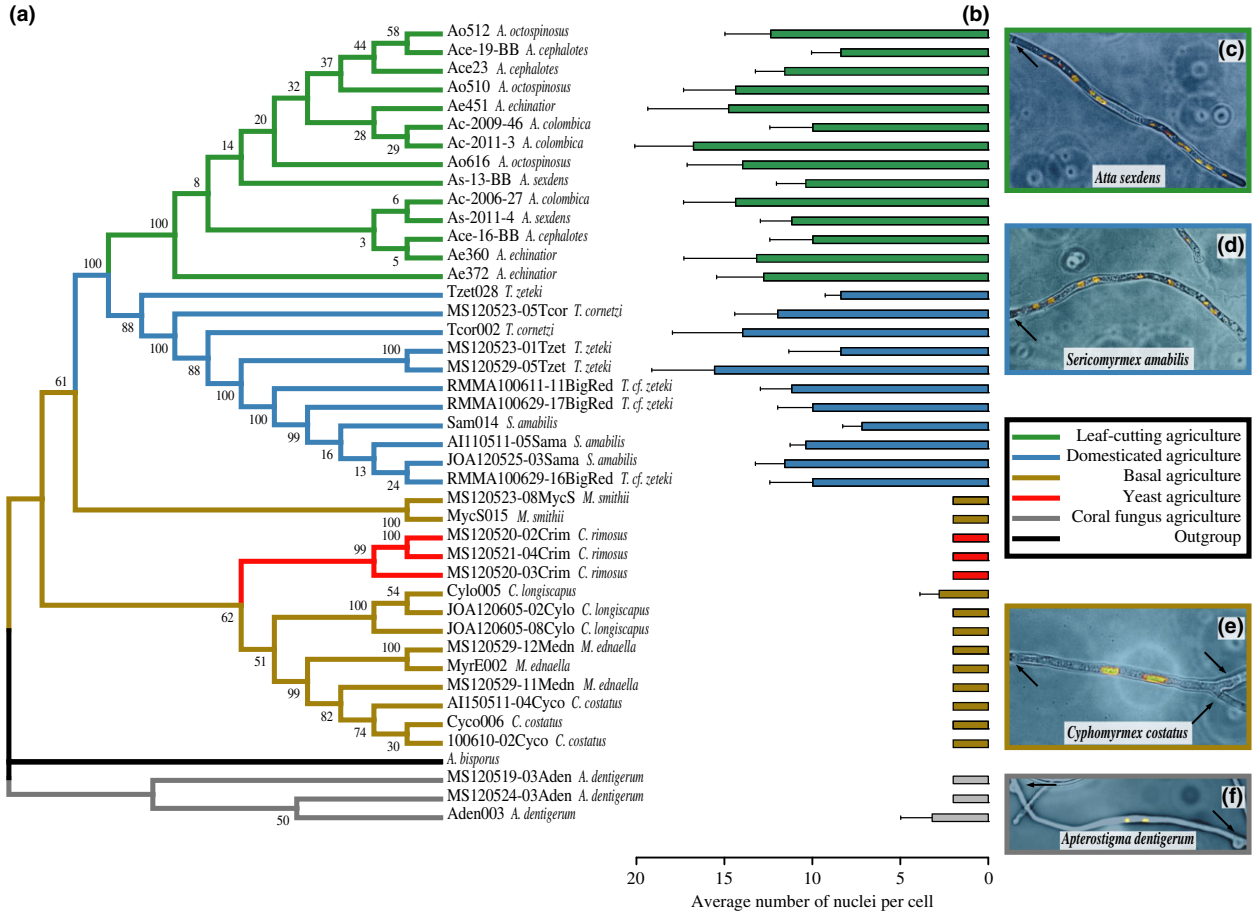

Fig. 2 Ancestry, diversification and mean number of nuclei per cell in garden symbionts of 14 Panamanian fungus-growing ants belonging to eight genera. (a) Phylogenetic tree of attine ant fungal symbionts based on ITS and LSU sequences and maximum likelihood analysis with 500 bootstrap replicates and *Agaricus bisporus* (ITS: JX684008.1, LSU: AY635775.1) as out-group (black branch). Different levels of agriculture are presented in different colours: coral fungus agriculture (grey), basal leucocoprineaceous agriculture (yellow), leucocoprineaceous yeast agriculture (red), domesticated agriculture (blue) and leaf-cutting agriculture (green). Numbers at nodes are percentages consensus support, and colony identification numbers and ant species names are given next to branches (see Table S1 for full Latin names). Because *Apterostigma dentigerum* grows a coral fungus unlike the leucocoprineaceous fungi of the other attine ants, its tree was generated in a separate analysis with the same *A. bisporus* out-group. (b) Histogram of the mean (± SD) number of nuclei per cell of the fungal symbionts, showing a clear separation between the three basal levels in the phylogeny (grey: 2.40 ± 0.29 nuclei per cell for coral fungus agriculture, yellow: 2.07 ± 0.05 for basal leucocoprineaceous agriculture, red: 2.00 ± 0.00 for leucocoprineaceous yeast agriculture) and the two advanced levels of gongylidia-bearing fungus farming (blue: 10.80 ± 0.43 for *Trachymyrmex* and *Sericomyrmex* domesticated agriculture, green: 12.46 ± 0.41 for *Atta* and *Acromyrmex* leaf-cutting agriculture). Representative pictures of stained nuclei are given towards the right: (c) *Atta sexdens* (14 nuclei per cell), (d) *Sericomyrmex amabilis* (8 nuclei per cell), (e) *Cyphomyrmex costatus* (2 nuclei per cell) and (f) *Apterostigma dentigerum* (2 nuclei per cell). Arrows indicate the septae that separate the cells.

clear split between the gongylidia-bearing fungi reared by *Atta* and *Acromyrmex* on one hand, and those reared by *Trachymyrmex* and *Sericomyrmex* on the other (Fig. S1), but also between *Atta* and *Acromyrmex* symbionts, a differentiation that was not resolved by the ribosomal markers. This indicates that there is no gene flow between these fungal clades at our study site in Panama and that these symbionts are also not transmitted horizontally across leaf-cutting ant species belonging to different genera (see also Kooij *et al.*, 2014).

Practically, no variation was found in the number of nuclei per cell across the basal leucocoprineaceous and the pterulaceous coral fungus lineages. We always observed two nuclei, with only two slightly higher counts (*Cyphomyrmex longiscapus* [Cylo005] and *Apterostigma dentigerum* [Aden003]) that were likely due to hyphal tips being observed while cells were dividing so that nuclei were already duplicated but a new septum had not yet formed. Mapping the number of nuclei per cell on the branches of the phylogenetic tree produced a clear pattern, with all lower- and paleo-attine symbionts,

coral fungus symbionts and leucocoprineaceous yeast symbionts having two nuclei per cell, and all higher attine ant symbionts having considerably higher numbers (7–17) of nuclei per cell (Fig. 2b). The sharp transition coincided with the origin of the fungal staphylae, the uniquely modified clusters of swollen hyphal tips (gongylidia) whose only known purpose is to be eaten by the ants. Statistical analyses showed that the number of nuclei per fungal cell differed between domesticated agriculture (including advanced leaf-cutting agriculture) on one hand and basal, yeast and coral fungus agriculture on the other (blue and green vs. other colours in Fig. 2a: $F_{4,27} = 74.46$, $P < 0.0001$), but not between symbionts of the ant genera within each of these farming types ($F_{4,27} = 1.64$, $P = 0.1941$) or between species-level symbionts within ant genera ($F_{6,27} = 1.71$, $P = 0.1560$) (see also Fig. S2).

Maximum microsatellite allele numbers per locus varied between 2 and 10 (Table S2), and all loci produced higher allele number scores than the dikaryotic expectation of 1+H for the *Atta* and *Acromyrmex* leaf-cutting agriculture symbionts, but not for the domesticated agriculture symbionts of the remaining *Trachymyrmex* and *Sericomyrmex* higher attine ants. ANCOVA of the ten locus-specific regressions of allele numbers on nuclei numbers showed that slopes were not significantly different from zero, neither individually (Fig. S3) nor collectively (domesticated symbionts: $F_{10,47} = 1.179$, $P = 0.329$; leaf-cutting symbionts: $F_{10,120} = 0.557$, $P = 0.846$), but that the ten intercepts (Fig. S3) were significantly different (i.e. statistically heterogeneous) for the leaf-cutting symbionts ($F_{9,120} = 18.421$, $P < 0.0001$) but not for the domesticated symbionts of *Trachymyrmex* and *Sericomyrmex* ($F_{9,47} = 1.042$, $P = 0.422$). Nonsignificant slopes were mostly slightly positive in the domesticated symbionts (eight positive vs. two negative in Fig. S3), but there was no such bias for the leaf-cutting symbionts (five positive and five negative slopes in Fig. S3), suggesting that the homogeneous polyploidy null model is more likely to be correct in the latter group than in the former.

We continued to analyse both models of Fig. 1 to evaluate how sensitive ploidy extrapolations would be to the way in which genetic variation is distributed across nuclei in polykaryotic symbionts, because real-life variation may be intermediate. We plotted both the ten intercepts of the homogeneous polyploidy model (Fig. S3) and the 10 slopes obtained with the heterogeneous haploidy model (Fig. S4) against locus-specific heterozygosity (Fig. 3). Inferred ploidy (under)estimates based on intercepts (Fig. S3) increased monotonously with locus-specific heterozygosity in the leaf-cutting symbionts (Fig. 3a), leading to an extrapolation estimate of 5.7 (95% CL 4.6–7.1) for an ideal genetic marker with H = 1. However, this increase was very small for the domesticated symbionts of *Trachymyrmex* and *Sericomyrmex* symbionts (Fig. 3b), where we obtained an extrapolated H = 1 ploidy estimate of 2.3 (95% CL 1.6–4.1), confirming that most mycelia may in fact have two nuclear haplotypes, whereas only some have additional haplotypes.

When using the heterogeneous haploidy model based on slopes through the origin (Fig. S4), we obtained a similar monotonic increase of ploidy (under)estimates with locus-specific heterozygosity as for the homogeneous polyploidy model (Fig. 3c), leading to an extrapolated H = 1 ploidy rate estimate of 0.41 ± 0.01 (95% CL 0.38–0.43) for leaf-cutting ant symbionts. This corresponds to 41% of the putative haploid nuclei in excess of the dikaryon default value representing genetically distinct haplotypes, producing a H = 1 ploidy extrapolation of 6.9 haplotypes (95% CL 6.6–7.2). Applying the heterogeneous haploidy model to the domesticated *Trachymyrmex* and *Sericomyrmex* symbionts again produced much more shallow curves (Fig. 3d) and a ploidy rate estimate of 0.04 ± 0.02 (95% CL −0.01–0.08) corresponding to a H = 1 ploidy extrapolation of 2.4 (95% CL 1.9–2.8). The latter slope was marginally significantly different from a null model assuming that additional nuclei were always identical copies of two distinct haploid nuclei ($z = -1.652$, $P = 0.049$). However, the lower ploidy confidence limit being below 2 for the domesticated *Trachymyrmex* and *Sericomyrmex* symbionts in both models suggests that not all of these mycelia had more than two haplotypes.

Discussion

Our results allowed us to infer that the polykaryotic mycelia reared by leaf-cutting ants are highly and obligatorily polyploid (n = 5–7) and that the equally polykaryotic symbionts of *Trachymyrmex* and *Sericomyrmex* appear to be lowly and facultatively polyploid (n = 2–3). It also appeared that all nuclei in the leaf-cutting ant symbionts were likely to be equally polyploid. This result is gratifying because there is no mechanism such as clamp connections to maintain the same number of genetically diverse haploid nuclei across somatic cell divisions. The alternative of heterogeneous haploidy would thus be evolutionary unstable, as has been argued for arbuscular mycorrhizal fungi (Pawlowska & Taylor, 2004), so symbionts of *Atta* and *Acromyrmex* leaf-cutting ants have apparently managed to avoid such instability. Whether the low and facultative polyploidy of the *Trachymyrmex* and *Sericomyrmex* symbionts represent such an unstable situation remains unclear.

Crop domestication and polyploidy for higher mutualistic productivity and robustness?

The emergence of polykaryotic crop mycelia with gongylidia ca. 20–25 MYA correlates with nearly an order of magnitude increase in scale of symbiotic

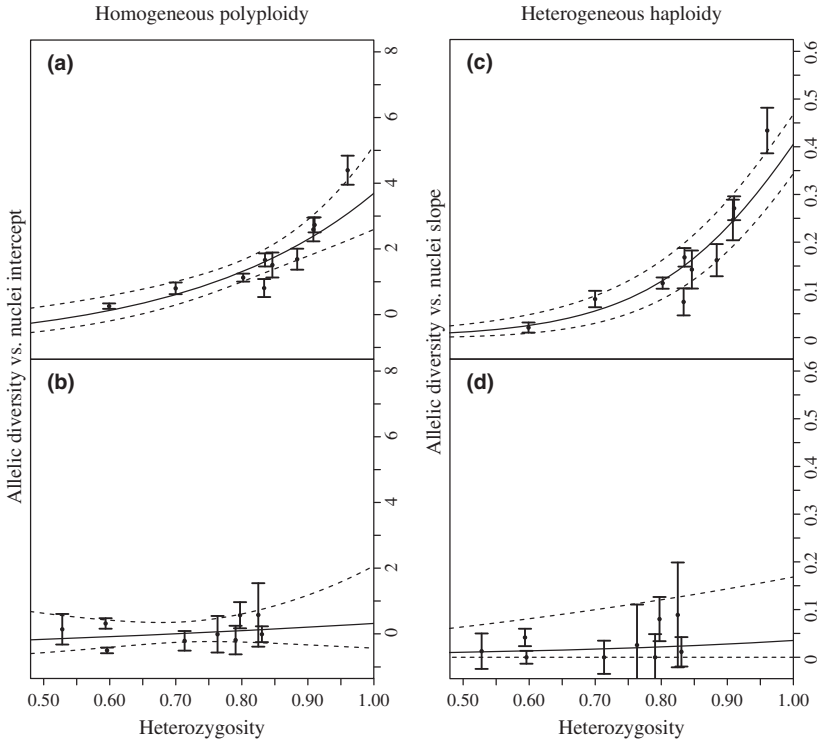

Fig. 3 Regression intercepts (means ± SE) for the locus-specific increases in the scored number of alleles (*y*-axes a and b panel; see Fig. S3) and regression slopes (means ± SE) for the locus-specific increases in the scored number of alleles with increasing numbers of counted nuclei per cell (*y*-axes c and d panel; see Fig. S4) plotted against the expected locus specific heterozygosities for *Atta* and *Acromyrmex* leaf-cutting agriculture symbionts (a, c) and *Trachymyrmex* and *Sericomyrmex* domesticated agriculture symbionts (b, d). The fitted regression lines for the homogeneous polyploidy model are as follows: (a) $\log(y) = -0.8762+1.548x$ ($R^2 = 0.8026$) and (b) $\log(y) = -0.2785+0.3978x$ ($R^2 = -0.05161$). The fitted logistic beta-regression lines for the heterogeneous haploidy model are as follows: (c) $p(x) = e^{-8.53+8.15x}/(1+ e^{-8.53+8.15x})$ (Pseudo $R^2 = 0.8716$) and (d) $p(x) = e^{-5.77+2.46x}/(1+ e^{-5.77+2.46x})$ (Pseudo $R^2 = 0.0171$). Almost all means had standard errors that overlapped with the 0.05–0.95 quantiles (dashed lines).

productivity, as colony sizes of farming ants increased from a few hundred workers in the lower attines to maximally ca. 2000 in *Trachymyrmex* and *Sericomyrmex* higher attine ants. The subsequent evolution of leaf-cutting agriculture with obligatorily polyploid crop fungi was associated with a further increase in scale of farming by 1–3 orders of magnitude (Schultz & Brady, 2008; Baer *et al.*, 2009; Mehdiabadi & Schultz, 2010; Riveros *et al.*, 2012). We have summarized these overall trends in Fig. 4 and Table S3, using previously published data on colony size and caste polymorphism (Kempf, 1965; Murakami *et al.*, 2000; Schultz *et al.*, 2002; Baer *et al.*, 2009; Fernandez-Marin *et al.*, 2009; Evison & Hughes, 2011; Fernandez-Marin *et al.*, 2013; Ferguson-Gow *et al.*, 2014; and RMM Adams, H Fernández-Marín, K Giampoudakis and J Sosa-Calvo personal observations). This shows that colony size, but not worker caste polymorphism, increased across the first transition, whereas both increased more massively across the second transition. The first cultivar transition (to mostly just polykaryotic cells) may have been driven by growth-rate advantages related to gene dosage, reminiscent of autopolyploid hybridization in human crop plants, a change that may remain compatible with normal sexual reproduction (Udall & Wendel, 2006). However, the second transition is highly unlikely to allow sexual reproduction, consistent with observations of mushroom formation from higher attine gardens being extremely rare (see e.g. Pagnocca *et al.*, 2001). All attine ants suppress fungal primordia, and mushrooms that may mature in colonies of higher attines have to our knowledge not been confirmed to produce viable spores under laboratory conditions where they are initiated somewhat more frequently (Fisher *et al.*, 1994; Mueller, 2002; PW Kooij, M Schiøtt and HH de Fine Licht personal observations).

The transition to polykaryotic cells in domesticated, gongylidia-bearing symbionts may have helped the higher attine ants to abandon the brown decomposition food chain (Shik & Kaspari, 2010) to stepwise become functional herbivores. This change was initiated in the *Trachymyrmex* and *Sericomyrmex* lineages

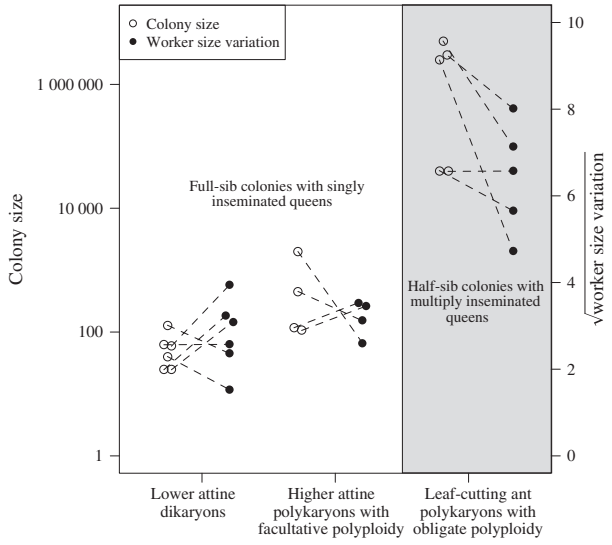

Fig. 4 Colony size (log number of ant workers; open circles), and 'somatic' division of labour among workers [approximated by the square root of worker size variation as used by Ferguson-Gow *et al.* (2014) and others; closed circles] for the three levels of fungal symbiont ploidy across the attine fungus-growing ants. Details on the comparative data are given in Table S3). Data on single and multiple insemination of queens follow Villesen *et al.* (2002).

that use modest amounts of soft fresh leaves and flower petals to nourish fungus gardens, and was followed by leaf-cutting agriculture where the ants almost exclusively use fresh plant material (Weber, 1972; Hervey *et al.*, 1977; De Fine Licht & Boomsma, 2010). We hypothesize that the challenge of progressing from leaf-litter forage to fresh leaves that are actively defended by secondary plant compounds (De Fine Licht *et al.*, 2013) may have been easier to meet with polyploid polykaryotic symbionts than with functionally diploid polykaryons, as the former are likely to express a more diverse spectrum of complementary enzymes and biochemical pathways (Otto & Whitton, 2000). The simultaneous expression of multiple resistance factors against the specialized garden parasite *Escovopsis* may have been another advantage of polyploid polykaryons (Yek *et al.*, 2012). The extent to which the second cultivar transition to leaf-cutting herbivory was driven by evolutionary changes in the ants or by the acquisition of a novel clade of crop symbionts 2–4 MYA (Mikheyev *et al.*, 2010) remains unknown. However, our present results underline that crop innovation has been a generally important factor in shaping extant leaf-cutting ant societies, as ploidy elaborations correlate both with the scale of farming (colony size) and with somatic division of labour (worker size variation) in the ant colonies rearing the crops (Fig. 4).

Comparative data on scale of farming, (a)sexuality and polyploidy of crops

Whereas polyploidization in human crop plants often appears to have predated loss of sexual reproduction (Comai, 2005), the attine crop fungi had a long history of asexual propagation before polyploidy evolved, making it reasonable to ask what general selective advantages may drive such developments and what the order is in which transitions take place. In the paragraphs below, we review the best-known convergent domestication events to alleviate the problem that our results on attine ants represent a unique evolutionary event that does not allow a formal phylogenetically controlled comparative analysis. We compiled a data set on five other comparable symbioses with eukaryotes maintained by humans, insects or vascular plants to evaluate whether co-evolutionary selection for enhanced productivity has had consistent effects on symbiont sexuality and ploidy, and to what extent that outcome depended on the host benefitting from the somatic or sexual tissues of the symbiont (Table 1). These data remain too heterogeneous for formal comparative analysis, but they reveal a number of interesting insights in the connections between polyploidy and selection for cultivar productivity by farming hosts.

Polyploidy is the rule in almost all modern human plant crops with a consistent domestication history (Table 1). If sex is maintained in perennial crops, it is often for making vigorous stem and root systems on which desired varieties are grafted, such as in *Coffea arabica* where diploid stems are propagated through seeds and polyploid hybrid (sub)species propagated through cuttings and grafting. Exceptions usually indicate lack of real domestication, such as nut trees that were human dispersed but otherwise not really domesticated. A few plant crops that humans rear for their somatic tissues (leaves or roots) have also remained diploid (Table 1). Seeds are always a nuisance in any plant crop that humans rear for fleshy fruits, so there asexuality has almost invariably been imposed after significant and sometimes high degrees of genome duplication had been achieved by artificial selection (Meyer *et al.*, 2012). The three cases of domestication of basidiomycete fungi (Table 1) provide interesting contrasts. As discussed in the previous section, the attine ant fungi are reared purely for their somatic mycelia, consistent with ant farmers suppressing sex even in dikaryotic lower attine symbionts where sex and meiosis should not have been compromised. This is because symbiont sex is a waste of resources for ant farmers when vegetative vertical transmission secures symbiont propagation across generations. However, when mushrooms are the direct focus of domestication, losing sex would be less likely. A handful of basidiomycete fungi are human domesticated and reared on an industrial scale where artificial selection for high mushroom

Table 1 Overview of known human, insect and plant mutualisms with multicellular symbionts that have clear characteristics of obligate unilateral or bilateral domestication listing whether, as far as known or reasonably inferred: (i) selection for higher productivity must have occurred, (ii) such selection targeted sexual or somatic tissues/organs, (iii) sexual reproduction was maintained, and (iv) obligate (chimeric) polyploidy evolved. Genetic studies of human and insect symbioses with ascomycete fungi appear not to have been performed, so these cases are only mentioned as footnotes.

Mutualistic symbioses with farming or similar domestication characteristics	Fungal symbiont taxon	Selection for higher productivity*	Tissue/organ targeted by natural/artificial selection for higher productivity	Crop/ symbiont sex maintained?	Obligate polyploidy evolved	References
Modern human farming for plant fruits/flowers†	Angiosperms	Yes	Sexual	No	Yes	Varoquaux *et al.*, 2000; Udall & Wendel, 2006
Human farming for plant roots or leaves‡	Angiosperms	Yes	Somatic	Yes	Yes (No)	Adams & Wendel, 2005; Udall & Wendel, 2006;
Human farming for fungal mushrooms§	Basidiomycota	Yes	Sexual	Yes	No	Sonnenberg *et al.*, 1988; Molitoris *et al.*, 1996
Termite fungus agriculture	Basidiomycota	Yes	Somatic	Yes	No	Nobre *et al.*, 2014
Leaf-cutting ant fungus agriculture¶	Basidiomycota	Yes	Somatic	No	Yes	Present study
Arbuscular mycorrhiza symbionts of plant roots	Glomeromycota	Yes	Somatic	No	Yes	Pawlowska & Taylor, 2004; Lin *et al.*, 2014

*Typical result is autopolyploid genome duplication in plants and polynucleate cells in basidiomycete fungi.

†Polyploidy appears to be universal under prolonged domestication; our internet search did not find exceptions; and stem and root systems may still be propagated sexually, but fruit- or flower-bearing shoots are propagated asexually by cutting and grafting.

‡Polyploidy is the rule, but there appear to be a few exceptions; sex is normally maintained (e.g. many auto- and allopolyploid biannuals such as carrots, cabbages and asparagus, which are harvested before plants flower).

§This record is representative for four other human domesticated basidiomycete fungi that are farmed on an industrial scale (Chang, 2001). Three of these (Oyster mushroom, Enokitake, Shiitake) have normal dikaryotic mycelia with clamp connections and the fourth (Straw mushroom) lacks clamp connections and has polynucleate cells (see text for details). In addition to basidiomycete fungi, humans also farm some filamentous Ascomycota, such as *Penicilium camemberti* and *P. roqueforti*, but no information on genetics could be found and sporulation may or may not occur, so we have not included these inconclusive records.

¶A number of other ants farm filamentous Ascomycota for structural reinforcement of carton nests, runway galleries, or lining of domatia with as yet unclear function; sporulation may or may not occur, but ploidy has apparently not been studied (Schlick-Steiner *et al.*, 2008; Voglmayr *et al.*, 2011). Also ambrosia beetles farm filamentous Ascomycota, which are asexual but unlikely to have been selected for higher productivity in comparison to the large scale fungus farms of attine ants and macrotermitine termites (eg. Ploetz *et al.*, 2013).

production has been imposed (Chang, 2001). Three of these (*Flammulina velutipes*, *Lentinus edodes*, *Pleurotus ostreatus*) have retained diploid dikaryotic cells, but also have clamp connections (Kang, 2004; Chen, 2005; Okamoto *et al.*, 2010), which likely preclude polynucleate cells from evolving. The fourth, *Volvariella volvacea*, is an Agaricales mushroom that lacks clamp connections and has polynucleate cells, but whether its wild sister species had dikaryotic cells is unknown and neither has it been investigated whether this species has evolved chimeric allopolyploidy (Chang & Ling, 1970; Chiu, 1993). The champignon or button mushroom, *Agaricus bisporus*, that we list in Table 1 as a representative case belongs to a genus that mostly lacks clamp connections and that has species with various levels of autopolyploid gene duplications, with polyploids having shorter growth seasons (and thus faster growth) and being less likely to have clamp connections (Molitoris *et al.*, 1996). This suggests that several genera within the Agaricales had pre-adaptations for becoming a successful human crop. It is therefore perhaps no surprise that all crop fungi domesticated by attine ants and macrotermitine termites also belong to the Agaricales.

The fungus-growing termites have independently gone through a similar development towards polykaryotic crops, but as far as known without evolving chimeric functional polyploidy (Nobre *et al.*, 2014). Moving beyond two haploid genomes per *Termitomyces* mycelium has likely been constrained because termite fungus gardens need to have regular sexual reproduction via mushrooms on top of termite mounds that produce and disperse recombined haploid spores to be acquired by the first workers of newly founded colonies (Korb & Aanen, 2003; Aanen *et al.*, 2009). Polyploid elaborations that would make sexual reproduction less effective could therefore probably not evolve in *Termitomyces* [in spite of the genus lacking clamp connections; (De Fine Licht *et al.*, 2005)], whereas such constraints did not apply in the attine ants who propagate fungal symbionts vertically, asexually and uniparentally (Mueller, 2002). However, this constraint might have disappeared in some derived fungus-growing termites

[the genus *Microtermes* and at least one species of *Macrotermes* – *M. bellicosus*; (Aanen *et al.*, 2002)] that switched from horizontal acquisition of sexual spores to somatic vertical transmission of *Termitomyces*. These termite lineages stopped producing mushrooms, so it would be interesting to investigate whether forms of chimeric polyploidy might have arisen here. However, the active selection argument that applies in leaf-cutting ants, based on a higher variety of expressed enzyme and detoxification pathways to meet challenges of herbivory, would seem less likely here, as fungus-growing termites rarely process live plant material (Wood & Thomas, 1989; Donovan *et al.*, 2001).

The final, and possibly most interesting, comparison is between the allopolyploid symbionts of *Atta* and *Acromyrmex* leaf-cutting ants and the similarly chimeric polykaryotic mycelia of arbuscular mycorrhiza that are obligate mutualists (albeit with occasional parasitic tendencies) of vascular plant roots (Table 1). These Glomeromycota fungi are an ancient asexual lineage of basal filamentous fungi, and also for this lineage it has remained unclear whether (i) nuclei represent multiple genetically distinct haplotypes (see eg Kuhn *et al.*, 2001; Hijri & Sanders, 2005) or forms of pervasive polyploidy for most if not all nuclei (Pawlowska & Taylor, 2004; Lin *et al.*, 2014) and (ii) sex occurs at all or just very rarely (Sanders, 2011). Given that arbuscular mycorrhiza have existed for hundreds of millions of years and that chimeric polyploidy in the crop symbionts of leaf-cutting ants evolved ca. 10–12 MYA, it is perhaps not surprising that intermediate steps are still extant as crop fungi of *Trachymyrmex* and *Sericomyrmex*. However, both developments seem old enough to suggest that chimeric polyploidy may have been an effective antidote against the unavoidable accumulation of deleterious mutations via Muller's ratchet because functional heterozygosity is always high (Comai, 2005). If further study would confirm that the *Atta* and *Acromyrmex* leaf-cutting ants maintain crop symbionts that evolved robustness against the accumulation of deleterious mutations, it might help explain the large ecological footprint of these ants. An earlier study indicated that fungal clones reared by leaf-cutting ants have retained the capacity for recombination (Mikheyev *et al.*, 2006), but this does not prove that sex via sporulating mushrooms can still take place, because reshuffling of genetic material in fungi is also possible via somatic recombination (Pontecorvo, 1946), a possibility that students of arbuscular mycorrhiza also keep open (Kuhn *et al.*, 2001; Pawlowska & Taylor, 2004; Lin *et al.*, 2014).

As indicated in footnotes to Table 1, there are a number of other insects that maintain symbioses with filamentous Ascomycota, but these appear to mostly be for reinforcement of nest structures rather than eating. Some Ascomycota have also been domesticated by humans in connection to cheese making, but too little is known about their ploidy and the likelihood of positive selection for higher productivity to discuss them further. A good example is the black truffle, *Tuber melanosporum*, which appears to be polykaryotic (Kües & Martin, 2011), but the large scale domestication of this fungus has so far failed. Other Ascomycota are farmed by ambrosia beetles. At present, it seems unlikely that higher ploidy levels have evolved here and neither does it seem probable that there has been selection for higher productivity or chimeric polyploidy as ambrosia beetle colonies tend to be small and short-lived because they are excavated in dead wood (Ploetz *et al.*, 2013). The only exception (for longevity, not for colony size) is the eusocial species *Austroplatypus incompertus*, which excavates tunnels in the stems of live *Eucalyptus* trees (Kent & Simpson, 1992). As far as data are available, the overall comparisons of Table 1 thus suggest that obligatorily symbiotic fungal lineages (i.e. those with symbiotic organs that preclude independent life) that could be maintained asexually had the potential to independently evolve polynucleate chimerism reminiscent of allopolyploidy in plant crops, but that domesticated sexual basidiomycetes only evolved polynucleate cells without further chimeric elaborations.

Chimeric genetic diversity analogies beyond symbiont domestication events

Positively selected forms of chimerism also characterize immune defence systems such as the large haplotype diversity in the expression of MHC alleles in otherwise diploid vertebrates (Potts & Wakeland, 1993). The evolution of obligate multiple queen-mating in hymenopteran eusocial insects is an analogous case, as increased haplotype diversity among worker offspring of single colony-founding queens is known to reduce colony-level vulnerability towards infections in honeybees and attine ants, whereas such evidence is lacking in lineages with facultative multiple insemination of queens (reviewed in Boomsma, 2013). It is intriguing that our estimate of 5–7 fungus garden haplotypes having merged into a single allopolyploid symbiont in *Atta* and *Acromyrmex* colonies represents a similar degree of chimerism as the collective haplotype diversity of the farming leaf-cutting ants themselves, which are offspring of a single diploid queen inseminated by minimally two but up till ca. 10 unrelated haploid males. In contrast, the *Trachymyrmex* and *Sericomyrmex* sister lineages whose fungal crops have only modest and facultative tendencies towards ploidy beyond 2 have also retained full sibling colonies founded by singly mated queens (Fig. 4). It thus appears that the directional coevolution between the higher attine ants and their domesticated crop fungi has produced several mutual adaptations to enhance symbiotic productivity including bilateral chimerism.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Comparison between the fungal symbiont phylogenetic tree based on ITS and LSU sequences using Maximum Likelihood with 500 bootstrap replicates (left) and the tree based on microsatellite F_{ST} values using neighbour-joining with 500 bootstrap replicates (right).

Figure S2 The mean number of nuclei per cell was significantly different for the five different types of fungus farming, with the gongylidia-bearing symbionts of the higher attine ants (both the blue *Trachymyrmex* and *Sericomyrmex* bars and the green *Atta* and *Acromyrmex* leaf-cutting ant bars) having consistently higher numbers of nuclei per cell than the nongongylidia-bearing symbionts reared by the paleo- and lower attine ants (brown and grey), including the yeast growers (red) ($F_{4,27} = 74.46$, $P < 0.0001$).

Figure S3 Intercept estimations of regression plots for the number of alleles observed per symbiont mycelium against the average number of nuclei per cell for domesticated *Trachymyrmex* and *Sericomyrmex* symbionts (blue) and *Atta* and *Acromyrmex* leaf-cutting agriculture symbionts (green).

Figure S4 Slope estimations of regression plots for the number of alleles observed per symbiont against the average number of nuclei per cell for each of the amplified microsatellite loci for domesticated *Trachymyrmex* and *Sericomyrmex* symbionts (blue) and *Atta* and *Acromyrmex* leaf-cutting agriculture symbionts (green).

Table S1 Overview of the colonies used in this study with corresponding GenBank accession numbers and

the best matches with GenBank for internal transcribed spacer (ITS) and nuclear large subunit (LSU) rRNA sequences.

Table S2 Allelic scores for each of the ten microsatellite loci in our screening of 25 domesticated and leaf-cutting agriculture symbionts reared by the higher attine ant genera *Trachymyrmex*, *Sericomyrmex*, *Acromyrmex* and *Atta*.

Table S3 The Panamanian attine ant species for which data on colony size (mean number of workers in mature colonies) and worker caste polymorphism (the percentage of head width variation compared to the mean; square root transformed in Fig. 4) were available in addition to our present assessment of three ploidy classes of the fungal symbionts (lower attine dikaryons, higher attine polykaryons, leaf-cutting ant chimeric polykaryons).

Appendix S1 PCR conditions.

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