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Landscape genomics of an obligate mutualism: Concordant and discordant population structures between the leafcutter ant *Atta texana* and its two main fungal symbiont types

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Abstract

To explore landscape genomics at the range limit of an obligate mutualism, we use genotyping-by-sequencing (ddRADseq) to quantify population structure and the effect of host–symbiont interactions between the northernmost fungus-farming leafcutter ant *Atta texana* and its two main types of cultivated fungus. Genome-wide differentiation between ants associated with either of the two fungal types is of the same order of magnitude as differentiation associated with temperature and precipitation across the ant's entire range, suggesting that specific ant–fungus genome–genome combinations may have been favoured by selection. For the ant hosts, we found a broad cline of genetic structure across the range, and a reduction of genetic diversity along the axis of range expansion towards the range margin. This population-genetic structure was concordant between the ants and one cultivar type (M-fungi, concordant clines) but discordant for the other cultivar type (T-fungi). Discordance in population-genetic structures between ant hosts and a fungal symbiont is surprising because the ant farmers codisperse with their vertically transmitted fungal symbionts. Discordance implies that (a) the fungi disperse also through between-nest horizontal transfer or other unknown mechanisms, and (b) genetic drift and gene flow can differ in magnitude between each partner and between different ant–fungus combinations. Together, these findings imply that variation in the strength of drift and gene flow experienced by each mutualistic partner affects adaptation to environmental stress at the range margin, and genome–genome interactions between host and symbiont influence adaptive genetic differentiation of the host during range evolution in this obligate mutualism.

KEYWORDS

Atta texana, BEDASSLE, environmental cline, intergenomic epistasis, mutualism, population structure

1 | INTRODUCTION

Range expansions can be impeded or facilitated by mutualistic or competitive interactions between species (Gilman, Urban, Tewksbury, Gilchrist, & Holt, 2010; Lavergne, Mouquet, Thuiller, & Ronce, 2010; Urban, 2011). In mutualisms, codependencies between mutualistic partners can facilitate range expansion, for example when association with a symbiont increases the niche breadth under which a host

can exist (Afkhami, McIntyre, & Strauss, 2014; Douglas, 2009; Nobre, Eggleton, & Aanen, 2010). Mutualisms can also impede range expansion, for example when a symbiont tolerates a narrower window of environmental conditions (e.g., temperature) compared to the conditions tolerated by a host (Bronstein, 1989; Dixon et al., 2015; Hume et al., 2016), and thus the range of a host is determined by its mutualistic partner. Generally, the importance of species interactions for either facilitating or restricting range adaptations of a host are less well understood than range-limiting adaptations driven by abiotic range-limiting factors, such as temperature or precipitation (Schoville et al., 2012; Sexton, McIntyre, Angert, & Rice, 2009; Urban, 2011).

Much work on the evolution of range-limiting species interactions has been theoretical (Case, Holt, McPeek, & Keitt, 2005; Gilman et al., 2010; Holt, Barfield, Filin, & Forde, 2011; Lavergne et al., 2010; Norberg, Urban, Vellend, Klausmeier, & Loeuille, 2012; Urban, 2011), primarily because it is difficult to test adaptation to range-limiting factors (Angert et al., 2011; Hoffmann & Sgrò, 2011; Holt, 2003). Empirical work has focused mostly on range-limiting competitive and antagonistic interactions (Cunningham, Rissler, & Apodaca, 2009; Hellmann, Prior, & Pelini, 2012; le Roux, Virtanen, Heikkinen, & Luoto, 2012), and less so on range-limiting mutualisms, such as plant–pollinator, plant–microbe or insect–endobacteria mutualisms (Bronstein, 1989; Bronstein & Patel, 1992; Moeller, Geber, Eckhart, & Tiffin, 2012; Smith et al., 2011; Stanton-Geddes & Anderson, 2011; Thompson & Rich, 2011). Adaptations that alter range limits are least understood for mutualisms with vertical transmission of a symbiont (e.g., plant–endophyte or insect–endobacteria mutualisms), where the two partners function as an integrated unit, and fitness of the association is determined by genome-by-genome interactions (intergenomic epistasis; Wolf, 2000; Heath, 2010) and sometimes by complex host–symbiont interactions, such as host–symbiont conflict (Mueller, Gerardo, Aanen, Six, & Schultz, 2005; Mueller, 2002; Wade, 2007).

As populations expand into new habitat, the action of selection, drift and gene flow can influence adaptive potential and leave a distinct footprint on the genome (Excoffier, Foll, & Petit, 2009; Sexton, Hangartner, & Hoffmann, 2014; Sexton et al., 2009). Successive founding events at an expanding range edge, for example, can result in strong genetic drift over a large geographical area, reducing both genetic diversity and efficacy of selection as new populations at the range edge adapt to novel environmental conditions. Gene flow from the interior of the range can reconstitute genetic diversity lost in populations at the range edge, but gene flow can also swamp adaptive variation accumulating at range limits (Holt, 2003; Kirkpatrick & Barton, 1997). In a mutualism, the evolutionary forces acting during a range expansion could be similar or different for each mutualistic partner, potentially affecting the colonization of new habitat.

Here, we examine a range expansion of an ant–fungus mutualism to evaluate the effect of host–symbiont interactions on genetic differentiation relative to the effects of two abiotic factors, temperature and precipitation (Figure S1). We couple genotyping-by-sequencing (ddRADseq; Peterson, Weber, Kay, Fisher, & Hoekstra, 2012) of the leafcutter ant *Atta texana* with microsatellite-marker genotyping of *Leucocoprinus gongylophorus* fungi cultivated by the ants for food, to elucidate how range expansion affects population structure in each mutualistic partner and genome-wide differentiation in the ant host. We expected population-genetic structure to be similar between the ants and the fungus if the dominant evolutionary processes operating during the range expansion are similar for each partner in the mutualism, as might be predicted for the obligate leafcutter mutualism in which the life cycles of the mutualistic species are inextricably linked and the ants codisperse with their fungal cultivars. We also expected ant–fungus genotype combinations and climate to be correlated with genome-wide differentiation if symbiotic interactions and abiotic factors influenced ant evolution during the range expansion.

2 | MATERIALS AND METHODS

2.1 | Study system

The leafcutter ant *A. texana* is the northernmost representative of its genus (Bacci et al., 2009), ranging from the USA–Mexico border region in northeast Mexico to northern Texas and western Louisiana west of the Mississippi River (Figure S1; Mueller, Mikheyev, Hong, et al., 2011; Mueller, Mikheyev, Solomon, Mikheyev, Solomon, & Cooper, 2011). The closest relatives of *A. texana* are the Mexican *Atta mexicana* and the Cuban *Atta insularis* (Bacci et al., 2009; Solomon, Bacci, Martins, Gonçalves Vinha, & Mueller, 2008). The three *Atta* species diverged from each other before the last glaciation, presumably in southern North America. Following the end of the last glaciation about 11,000 years ago, *A. texana* is thought to have expanded northward from ancestral populations in northeastern Mexico (Mueller, Mikheyev, Hong, et al., 2011; Mueller, Mikheyev, Solomon, et al., 2011). The first published observations of *A. texana* in Louisiana and eastern Texas documented a widespread presence of *A. texana* in counties at or near the current northeastern range limits in Louisiana (Jones, 1917; Smith, 1939; Snyder, 1937; Walter, Seaton, & Mathewson, 1938). Moreover, the current range limits of *A. texana* in Louisiana (Dash, 2004; Mueller, Mikheyev, Hong, et al., 2011; Mueller, Mikheyev, Solomon, et al., 2011) are essentially the same limits that were already recognized 80 years ago by Smith (1939), indicating that range expansion of *A. texana* was halted towards the east by the shallow water table of the Mississippi River basin (nests drown in areas of regular flooding) and halted towards the north by low winter temperatures (Mueller, Mikheyev, Hong, et al., 2011). This combined historical–biogeographical information suggests a conservative estimate for the northeastward expansion of *A. texana* from southern populations sometime between 11,000 and 100 years ago (an estimated 20–2,000 ant generations, assuming 5–10 years for an ant nest to reach peak reproductive output).

Atta texana leafcutter ants cultivate a fungus called *Leucocoprinus gongylophorus* (Mueller et al., 2017, 2018), which is obligately dependent on the ants (i.e., the cultivars are not known to live independently from the ants; Mueller, Rehner, & Schultz, 1998; Mueller, Schultz, Currie, Adams, & Malloch, 2001; Mueller, 2002; Vo, Mikheyev, & Mueller, 2009) and clonally propagated within nests and also from

maternal to offspring nests (Marti, Carlson, Brown, & Mueller, 2015; Mikheyev, Mueller, & Abbot, 2006, 2010; Mueller, Scott, Ishak, Cooper, & Rodrigues, 2010). *Leucocoprinus gongylophorus* cultivated by *A. texana* is polyploid (multiple genomes per nucleus) and multinucleate (multiple nuclei per cell), with an observed average of 9.4 nuclei per cell for one fungus from *A. texana* (range 3–21 nuclei per cell; Carlson et al., 2017). The exact ploidy of *L. gongylophorus* is not known (probably 3–7; Kooij, Aanen, Schiøtt, & Boomsma, 2015; Carlson et al., 2017), ploidy appears variable between *L. gongylophorus* strains, and the number of nuclei per cell is variable in a mycelium (range 3–21 nuclei per cell; Carlson et al., 2017). Although *L. gongylophorus* clones are occasionally transferred between nests of sympatric leafcutter ant species through little-understood mechanisms (Mikheyev, Mueller, & Abbot, 2006, 2010; Mikheyev, Mueller, & Boomsma, 2007; Mueller, 2002; Mueller et al., 2017; Rodrigues, Mueller, Ishak, Bacci, & Pagnocca, 2011), each leafcutter nest appears to cultivate only a single *L. gongylophorus* clone (Mueller et al., 2010; Sen, Ishak, Kniffin, & Mueller, 2010), but communities of additional mutualistic, commensal and pathogenic microbes grow alongside the main cultivar and affect the function of gardens of *A. texana* (DeMilto, Rouquette, Mueller, Kellner, & Seal, 2017; Meirelles et al., 2016, 2015; Rodrigues, Cable, Mueller, Bacci, & Pagnocca, 2009; Rodrigues et al., 2011; Seal & Mueller, 2014; Sen et al., 2009; Shik et al., 2014). A comprehensive population-genetic analysis of *L. gongylophorus* from *A. texana* found two main genotype clusters of cultivated fungi (so-called M-fungi and T-fungi), which are distributed in sympatry across the range of *A. texana* (Mueller, Mikheyev, Solomon, et al., 2011). Because genetic admixture between M- and T-fungi occurs at low frequency (so-called admixed fungal genotypes; Mueller, Mikheyev, Solomon, et al., 2011), these two genotype clusters represent distinct clone-lineages of the same fungal species, *L. gongylophorus*.

Atta texana does not exist sympatrically with other leafcutter species throughout its range in Texas and Louisiana (Mueller, Mikheyev, Hong, et al., 2011; Mueller, Mikheyev, Solomon, et al., 2011; Rabeling, Cover, Johnson, & Mueller, 2007), so *L. gongylophorus* cultivars cannot be exchanged between different sympatric host species, whereas at other locations in the leafcutter range different sympatric leafcutter species can exchange cultivars between nests (Mikheyev et al., 2007; Mueller et al., 2017, 2018; Silva-Pinhati et al., 2004). The absence of sympatric leafcutter species therefore simplifies analyses of ant–fungus coevolution in *A. texana* (Mueller, 2015), including analysis of possible species-specific adaptations stemming from genome–genome interactions of specific ant–fungus combinations (so-called intergenomic epistasis; Wolf, 2000; Heath, 2010; Wade, 2007).

The range of *A. texana* is thought to be limited by low temperatures in north Texas, low precipitation in west Texas and a shallow water table to the east along the Mississippi Valley in Louisiana (Mueller, Mikheyev, Solomon, et al., 2011). Leafcutter ants can protect their fungal gardens against some temperature fluctuations (Mueller, Mikheyev, Hong, et al., 2011), but at their northern range limits the highest soil temperatures in winter (≈15°C) occur at depths below 5–10 m, whereas more shallow depths, where gardens are maintained, are colder (5–15°C) in winter (Mueller, Mikheyev, Hong, et al., 2011). Consequently, fungiculture in leafcutter populations at their northern range limits must operate throughout winter at temperatures that would critically compromise survivorship of *L. gongylophorus* associated with tropical leafcutter ant species to the south. In an experiment testing for cold tolerance and desiccation resistance in *L. gongylophorus*, cold-adapted strains occur at northern sites across the range of *A. texana*, and cold-susceptible strains tend to occur at warmer southern sites in the Rio Grande Valley at the USA–Mexico border (Mueller, Mikheyev, Hong, et al., 2011). In contrast, Mueller, Mikheyev, Hong, et al. (2011) did not find significant regional differences in desiccation resistance among strains.

2.2 | Sample collection and processing

For genotype-by-sequencing using ddRADseq (Peterson et al., 2012), we used *A. texana* workers from 111 nests collected across Texas and Louisiana (Table S1; Mueller, Mikheyev, Solomon, et al., 2011). Mesosomas from three large workers per nest were washed three times in 100% ethanol to clean the integument, crushed with a sterile pestle in liquid nitrogen, then extracted with the Qiagen DNeasy kit. We digested 240–320 ng of *A. texana* DNA with NlaIII and EcoRI-HF (NEB) and prepared the ddRAD libraries following Peterson et al. (2012) using "flex" barcoded adaptors. We used one male to generate a draft reference genome, which was assembled using ABYSS (Simpson et al., 2009). Methods for constructing ddRAD libraries and the reference genome are available in the Appendix S1. Libraries were sequenced with Illumina Hiseq 4,000 (ddRAD; 2 × 150 bp) and Illumina Hiseq 2,500 (draft genome; 2 × 100 bp) devices. The RADseq information is available in NCBI BioProject PRJNA395768, and the draft genome of *A. texana* is available at NCBI as accession QEPB00000000.

Leucocoprinus gongylophorus samples from 117 *A. texana* nests (including the above 111 nests) had previously been genotyped with 12 microsatellite loci as described in Mueller, Mikheyev, Solomon, et al. (2011). This fungal data set included 48 nests with so-called M-fungi (sensu Mueller, Mikheyev, Solomon, et al., 2011), 63 nests with T-fungi, three nests with C-fungi and three nests with fungi that were admixed between these distinct fungal types. Because M- and T-fungi comprise about 95% of the fungi known to be cultivated by *A. texana* across its range (Mueller, Mikheyev, Solomon, et al., 2011), and because sample sizes of three ant nests cultivating C-fungi and three ant nests cultivating admixed fungi are insufficient for the below population-genomic analyses of the ants, we included only ant samples from the 111 nests with the abundant M- and T-fungus types (N = 48 and 63, respectively) in the analyses evaluating genetic differentiation between ants cultivating either M- or T-fungi. For population-genetic analyses of the fungi, our analyses used 79 variable markers distributed across the 12 fungal microsatellite loci (2–10 alleles per locus for this multinucleate, polyploid fungus). The 79 alleles across 12 loci are more than in typical microsatellite marker analyses, and adequate for elucidating population structure across the range of *A. texana*. Previous studies have shown that

microsatellite markers and single-nucleotide polymorphisms (SNPs) give similar estimates of population structure when using a sufficient number of polymorphic microsatellite loci or SNP loci (Glover et al., 2010; Haasl & Payseur, 2011; Liu, Chen, Wang, Oh, & Zhao, 2005).

All ant samples were processed blind (Kardish et al., 2015) with respect to fungal genotype (M- or T-fungus) cultivated by the ants.

2.3 | Bioinformatics

We used STACKS 1.40 (Catchen, Amores, Hohenlohe, Cresko, & Postlethwait, 2011) to demultiplex the ddRAD sequences and aligned reads to the *A. texana* genome with BWA MEM version 0.7.12 (Li, 2013), retaining only reads that mapped in a perfect pair. We genotyped samples using SAMTOOLS MPILEUP/BCFTOOLS version 1.3.1 (Li et al., 2009), specifying an MAPQ score >20, maximum read depth of 500, mapping quality adjusted to 50, BAQ disabled and the consensus calling model. We removed sites with individual read depths <15, genotyping quality <20, and sites genotyped in <80% of individuals using VCFTOOLS 0.1.15 (Danecek et al., 2011). We used only SNPs from biallelic loci in analyses. Single-nucleotide polymorphisms were thinned to a maximum of one per 100 kb to remove linked sites.

2.4 | Spatial analysis of principal components

Spatial principal component analysis (sPCA) is a model-free method that incorporates spatial relationships among samples into a PCA to infer genetic structure among individuals or populations (Jombart, Devillard, Dufour, & Pontier, 2008). To implement sPCA, we randomly chose one ant per nest and specified a Gabriel graph to define spatial connectivity (Figure S2a) using the ADEGENET package (Jombart & Ahmed, 2011) in R version 3.2.1. We visually inspected screeplots to determine how many sPCA axes to retain for analysis, using an abrupt decrease in eigenvalues as our criterion (Jombart et al., 2008). To accommodate the apparent variation in ploidy among *L. gongylophorus* individuals (Carlson et al., 2017), we estimated the ploidy level for each individual as the maximum number of microsatellite alleles counted across any of the 12 loci. Because *L. gongylophorus* fungi of *A. texana* are multinucleate and polyploid (Carlson et al., 2017), we did not calculate standard population-genetic parameters (heterozygosities, *F*-statistics, etc.) for the fungi and treated all alleles as dominant markers as recommended for polyploids by Falush, Stephens, and Pritchard (2007). To assess whether global and local structures were present in our data, we implemented the global.rtest and local.retest functions in the ADEGENET package (Jombart & Ahmed, 2011). We visualized the results by plotting the sPCA scores onto a map of Texas and Louisiana using GGMAP (Kahle & Wickham, 2013).

2.5 | Genetic diversity

We assessed the relationship between genetic diversity and geography in *A. texana* by regressing heterozygosity on longitude and separately also on latitude. *Leucocoprinus gongylophorus* fungi exhibit interindividual variation in ploidy, so we used allele richness as a measure of genetic diversity. Allele richness is highly correlated with heterozygosity for microsatellite markers—for example, the review by Eckert, Samis, and Lougheed (2008) calculated a correlation of $r = 0.81$ for estimates from 15 published studies—and thus serves as an acceptable proxy for genetic diversity. We generated subsets of *L. gongylophorus* samples grouped by their estimated ploidy level (see Section 2.4 above) to prevent confounding comparisons of allele richness among individuals that could be solely due to ploidy. We used Spearman rank correlation to compare allele richness with longitude and latitude because allele richness could not be transformed to meet the assumptions of linear regression. We visualized results using the R package GGMAP (Kahle & Wickham, 2013).

2.6 | Cluster-based analysis of population structure

We assessed population-genetic structure in *A. texana* using ADMIXTURE (Alexander, Novembre, & Lange, 2009) to complement the sPCA. Unlike sPCA, ADMIXTURE clusters samples using a maximum-likelihood-based population-genetic model that assesses the contribution of *K* ancestral populations to each individual genome. To determine the *K* for the analysis, we applied ADMIXTURE's implementation of cross-validation ($n = 5$ folds, $K = 1$–5) and selected *K* from the model with the lowest error. Admixture proportions were then plotted using the maps package in R (Becker, Wilks, Brownrigg, & Minka, 2016) to visualize genetic structure across the range of *A. texana*.

2.7 | Contributions of cultivar genotype and climate to genetic variation in *A. texana*

We used BEDASSLE (Bradburd, Ralph, & Coop, 2013) to assess whether genetic differentiation among *A. texana* nests is associated with three factors: temperature, precipitation and fungal genotypic cluster (M- or T-fungi; see above). We downloaded mean temperature and precipitation for the years 1998–2010 from the National Centers for Environmental Information (http://ncdc.noaa.gov) and assigned the fungal genotype cluster for each nest as inferred already in an earlier microsatellite-marker analysis (a total of 79 variable markers across 12 highly polymorphic microsatellite loci; Scott, Kweskin, Cooper, & Mueller, 2009; Mueller, Mikheyev, Solomon, et al., 2011). We initiated two replicate Markov chain Monte Carlo (MCMC) runs of the overdispersion model using the BEDASSLE package in R version 3.2.1. Each run used a different random seed value to ensure independence, and each run included ~10 million MCMC iterations (after removing the first 20% of iterations for burn-in), with parameter values sampled every 5,000 generations. Model convergence was evaluated using graphical functions implemented in the BEDASSLE package.

We evaluated model fit by comparing naturally observed F_{ST} values with 100 posterior predictions drawn randomly from the MCMC iterations. The effect size of each ecological parameter (α_E) relative to isolation by distance was then calculated by dividing their posterior distributions by the posterior distribution of the effect size of geographical distance (α_D) on genetic differentiation; this step permits

comparison of effect sizes for different ecological factors (e.g., temperature, precipitation) to each other using the same units of IBD (isolation-by-distance), as explained by Bradburd et al. (2013) and Weber, Bradburd, Stuart, Stutz, and Bolnick (2017). We calculated the highest posterior density credible intervals for each ecological factor (Plummer, Best, Cowles, & Vines, 2006) and interpreted those not overlapping zero as statistically significant. Finally, we calculated mean posterior estimates for within-population allelic correlations (ϕ), and then converted these to *F*-statistics (analogous to inbreeding coefficients) following Bradburd et al. (2013). We used a linear regression to test for an association between fungal type and *F*-value.

3 | RESULTS

Using genotyping-by-sequencing ddRADseq methods, we identified 4,003 SNPs in *A. texana* leafcutter ants collected from 111 nests covering the entire range of this ant species. A screeplot of the eigenvalues in the ant sPCA indicated three positive eigenvalues (representing global structure) for retention (Figure S2b). Plotting the spatial and variance components of these eigenvalues reinforced that these three axes were distinguishable from the rest of the eigenvalues, and thus were suitable for interpretation (Figure S2c) (Jombart & Ahmed, 2011). Negative eigenvalues (representing local structure) were relatively small (Figure S2b), suggesting weak local structure in the data. Formal tests using MCMC simulation confirmed that genetic variation was significantly associated with global spatial structure ($p < 0.001$) but not with local spatial structure ($p = 0.99$), and we therefore analysed only global structures further.

The fungus microsatellite-marker data set (Mueller, Mikheyev, Solomon, et al., 2011) of *L. gongylophorus* fungi cultivated by the same 111 nests of *A. texana* included 79 variable markers distributed across 12 loci (2–10 alleles per locus for this multinucleate, polyploid fungus). We identified one positive eigenvalue for retention in the analysis (Figure S3a,b), and as in the ant data set, little evidence of local spatial structure in the screeplots (Figure S3a). Markov chain Monte Carlo simulation confirmed that genetic variation was significantly associated with global ($p = 0.003$) but not local structures ($p = 0.49$). We thus only focused on global structures in the remaining analyses.

3.1 | Genetic structure of *A. texana* ants

Spatial genetic structure among nests was different between the ants and their fungi. The first sPCA axis in the ants revealed a broad cline spanning from the Rio Grande at the USA–Mexico border (older populations of the range expansion) to Louisiana (Figure 1a). This cline probably results from a strong effect of IBD (Figure S4) on genetic variation in the ants. A concurrent decline in heterozygosity was also evident from the Rio Grande to Louisiana (Figure 2a). We found a negative relationship between heterozygosity (range: 0.0005–0.26) and latitude (β [95% CI] = −0.011 [−0.014, −0.008], $R^2 = 0.33$), and between heterozygosity and longitude (β [95% CI] = −0.007 [−0.010, −0.003], $R^2 = 0.11$). Together, these results indicate that genetic drift (serial founder events) reduced genetic variation in the ants as they expanded their range northeast, and gene flow had insufficient time to erode the genetic signature of this range expansion.

The second and third sPCA axes revealed additional genetic structure. The second sPCA axis differentiated a cluster of nests along a corridor from north Texas to east Texas (Figure S5a), while the third sPCA axis differentiated a cluster of nests in central Texas (Figure S5b).

ADMIXTURE, which relies on an evolutionary model to assign admixture proportions, revealed similar results to the sPCA. The cross-fold validation estimated $K = 3$ as the most likely number of clusters in the data (Figure S6a). The first cluster consisted of nests in southwest and central Texas, the second of eastern and north Texas, and the third of eastern Texas/Louisiana (Figure S6b). Qualitatively, these divisions correspond to the patterns revealed in the first sPCA axis (Figure S2b,c), and probably result from ADMIXTURE partitioning the spatially autocorrelated genetic variation generated by IBD into discrete clusters (Bradburd, Coop, & Ralph, 2018; Meirmans, 2012).

3.2 | Genetic structure of *L. gongylophorus*

Spatial PCA of the fungi revealed a patchwork of genetically similar cultivars distributed across the range (Figure 1b). This is in agreement with Mueller, Mikheyev, Solomon, et al. (2011), who documented a similar pattern when analysing the same data set using a model-based approach (STRUCTURE). To assess population-genetic structure within the two main fungus types that had been identified by Mueller, Mikheyev, Solomon, et al. (2011), called M-fungi and T-fungi, we partitioned the data by fungus type and conduced separate sPCAs on each type. We found a cline in population structure similar to that of the ants in the M-fungi, extending from southern to northeast Texas (Figure 1c). The second sPCA axis for M-fungi showed a weaker cline from west to east across Texas (Figure 1d). In contrast, population structure in the T-fungi was dominated by a large population spanning most of Texas (Figure 1e), with no evidence of a south-to-northeast cline seen in the ants (Figure 1a), while the second sPCA axis for T-fungi showed a west-to-east cline stretching across Texas to Louisiana (Figure 1f).

Allele richness decreased with longitude and latitude in putative triploid fungal samples (longitude: Spearman's rho = −0.70, $p < 0.001$; latitude: rho = −0.53, $p < 0.001$, $n = 48$; Figure 2b), but not in putative tetraploids (longitude: rho = −0.35, $p = 0.3$; latitude: rho = −0.26, $p = 0.5$, $n = 10$; Figure 2c) or putative pentaploids (longitude: rho = 0.14, $p = 0.3$; latitude: rho = −0.29, $p = 0.1$, $n = 60$; Figure 2d). The decline in genetic diversity in triploid samples was due to a cluster of genetically identical genotypes with low allelic richness in northeast Texas (Figure 2b). Partitioning the data into M- and T-fungi did not reveal any additional clines in genetic diversity (Figure S7).

3.3 | Contributions of fungal genotype and climate to genetic variation in *A. texana*

We found that temperature, precipitation and fungal genotype-cluster (M- or T-fungi) were all significantly associated with genetic

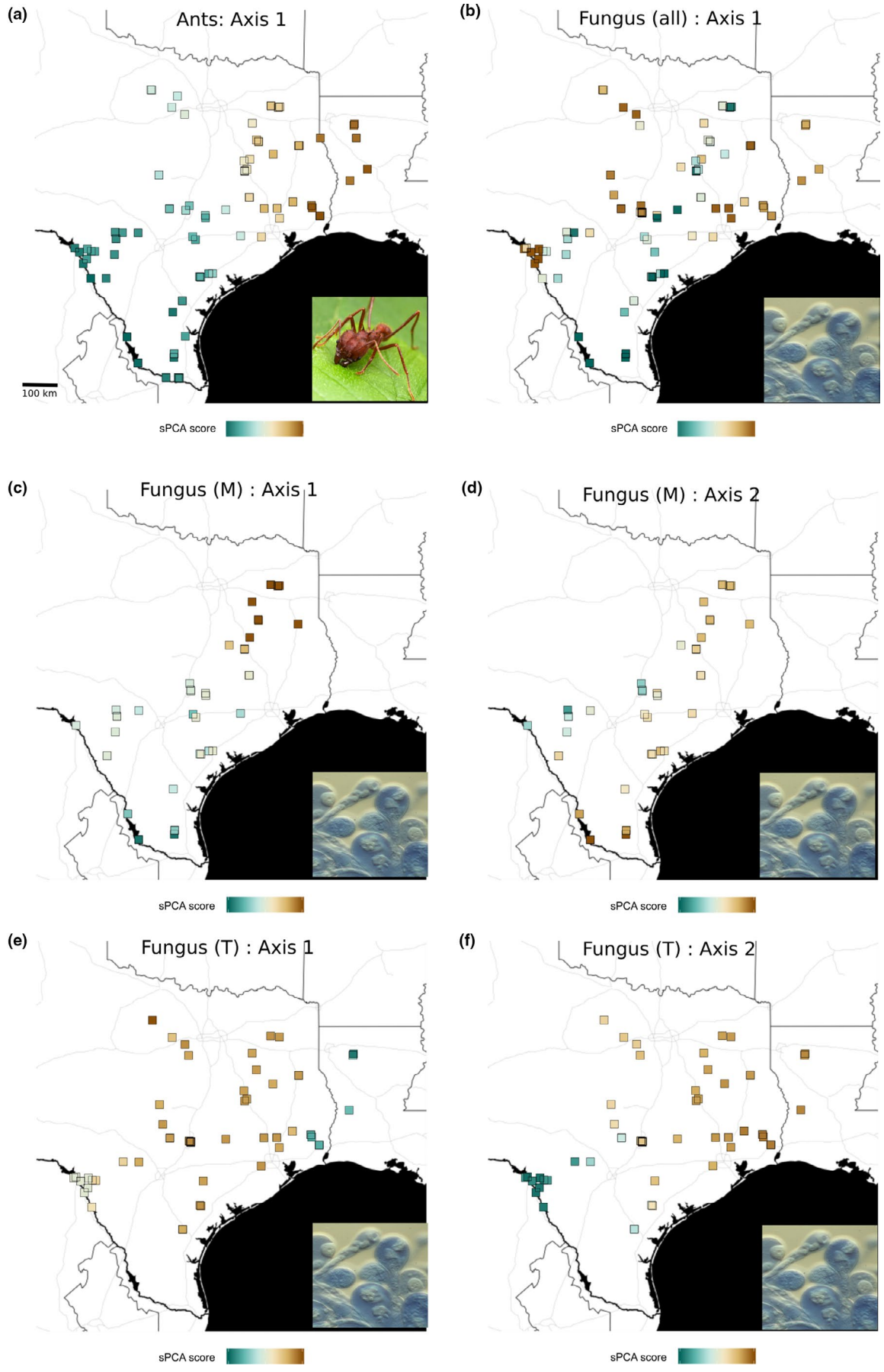
(a)
Ants: Axis 1
100 km
sPCA score
(b)
Fungus (all) : Axis 1
sPCA score
(c)
Fungus (M) : Axis 1
sPCA score
(d)
Fungus (M) : Axis 2
sPCA score
(e)
Fungus (T) : Axis 1
sPCA score
(f)
Fungus (T) : Axis 2
sPCA score

FIGURE 1 Global scores of the spatial principal component analyses (sPCA) for (a) the leafcutter ant *Atta texana* and (b–f) *Leucocoprinus gongylophorus* fungal cultivars collected from the same nests. The fungi can be grouped into two distinct types (Mueller, Mikheyev, Hong, et al., 2011; Mueller, Mikheyev, Solomon, et al., 2011), called M- and T-fungi, and three analyses are therefore presented here for *L. gongylophorus*: the first axis of the sPCA when analysing all fungal samples in the complete data set combining M- and T-fungi (b); the first axis (c) and second axis (d) when analysing only M-type fungi; and the first axis (e) and second axis (f) when analysing only T-type fungi. Inset in (a) is an *A. texana* worker (photo courtesy of Alex Wild), and insets in (b–f) are gongylidia (hyphal-tip swellings) of *L. gongylophorus* cultivar (photo by Ulrich Mueller)

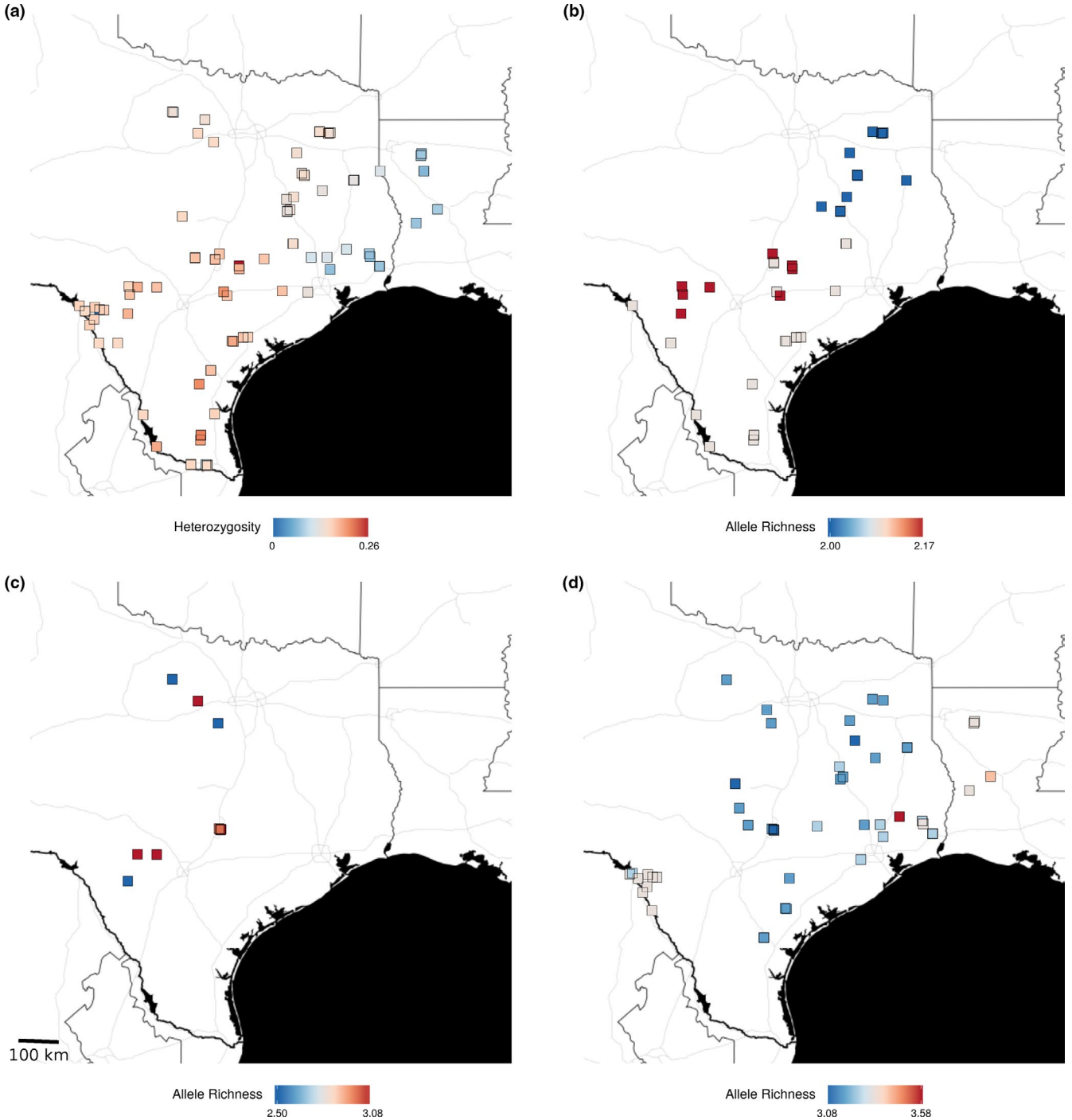

FIGURE 2 Genetic diversity in (a) the leafcutter ant *Atta texana*, and (b) putative triploid, (c) putative tetraploid and (d) putative pentaploid *Leucocoprinus gongylophorus* fungal cultivars. Putative triploid cultivars are exclusively M-fungi (n = 48), putative tetraploid cultivars are T-fungi (n = 7) or admixed genotypes (n = 3), and putative pentaploid cultivars are T-fungi (n = 56) or C-fungi (n = 3). The placement into the two dominant fungal groups (M- and T-fungi) and the two rare groups (admixed, C-fungi) is taken from Mueller, Mikheyev, Solomon, et al. (2011)

differentiation in *A. texana* (Table 1). A one degree increase in temperature was equivalent to 60.2 km (mean of two MCMC runs) of genetic differentiation due to IBD among nests, while every 1 cm increase in precipitation was equivalent to 20.6 km of genetic differentiation due to IBD.

Genetic differentiation between ants associated with either M- versus T-fungi was relatively large, comparable to 719 km of IBD. Relative to the abiotic factors, this is equal to an 11.9°C difference in temperature (719 km/60.2°C/km), or a 34.9 cm difference in precipitation (719 km/20.6 cm/km) (Figure 3). Fungal genotype-cluster thus had a larger effect than temperature, which differs by 7.1°C average January temperature (interquartile range = 2.7°C) from the coldest to the warmest locations, but less than that of precipitation, which differs by 220.2 cm annual rainfall (interquartile range = 152.9 cm) between the driest and wettest locations. Our BEDASSLE models also produced highly variable *F*-statistics across ant populations (range = 3.96e−4 to 0.528, *SD* = 0.133), with high values resulting from either high inbreeding or poor model fit. However, there was no association between *F*-values and fungal type (linear model: $t = 0.26$, $p = 0.80$).

A caveat in BEDASSLE analysis is that correlations among ecological factors can confound the interpretation of the effect sizes. Hotter areas in our data set tended to be dryer (Spearman $r = -0.48$, $p < 0.001$, Figure S8a), and thus the effect sizes for precipitation and temperature (Table 1) are not completely independent. Comparing nests with T- or M-fungi, nests with T-fungi were more probably collected in wetter areas than nests with M-fungi, although the correlation was low (Mann-Whitney *U*: $r = 0.29$, $p = 0.05$, Figure S8b). This correlation between precipitation and fungus type is not surprising because only T-fungi have been found so far in Louisiana and far-east Texas, the wettest part of the range of *A. texana* (Figure S1), and because only M-fungi have been found so far in southernmost Texas, the hottest part of the range with far lower precipitation than in Louisiana (Figure S1; Mueller, Mikheyev, Hong, et al., 2011; Mueller, Mikheyev, Solomon, et al., 2011). In contrast, no significant difference was found between fungus types with respect to temperature (Mann–Whitney *U*: $r = 0.13$, $p = 0.85$, Figure S8c). Differences in precipitation between nests cultivating M- and T-fungi thus might make a small contribution to the observed effects of fungal type on ant differentiation (Table 1).

TABLE 1 Median (95% credible interval) posterior effect sizes of geographical distance (α_D) and ecological factors (α_E) on genetic differentiation in the ant *Atta texana*. Rows in the same cell correspond to two independent MCMC runs

Source	*α (95% credible interval)*	α_E/α_D
Geographical distance	1.34e−06 (8.17e−07, 1.89e−06)	
	1.52e−06 (9.14e−07, 2.18e−06)	
Temperature (per °C)	7.78e−05 (5.85e−09, 2.99e−04)	59.1 (4.33e−03, 211)
	8.86e−05 (3.00e−08, 3.78e−04)	61.4 (1.75e−02, 248)
Precipitation (per cm annual rainfall)	2.70e−05 (1.62e−05, 3.88e−05)	20.3 (15.3, 26.8)
	3.14e−05 (1.79e−05, 1.14e−04)	20.9 (15.9, 27.0)
Fungal genotype cluster (M- or T-fungi)	8.64e−04 (3.52e−04, 1.62e−03)	673 (271, 1,210)
	1.12e−03 (4.60e−04, 1.95e−03)	766 (373, 1,240)

4 | DISCUSSION

The ecological and evolutionary processes driving and limiting range expansions have been debated since Darwin suggested that both abiotic and biotic factors can limit species boundaries (Darwin, 1859; Schoville et al., 2012; Sexton et al., 2009). The role of mutualisms in facilitating or inhibiting range expansions, however, has received far less attention than other species interactions, such as competition and predation (Gilman et al., 2010; Lavergne et al., 2010; Urban, 2011). We found that the range expansion sometime during the last 11,000 years by the northernmost leafcutter ant *A. texana* across south-central USA (Texas and Louisiana) has left a strikingly different genetic footprint on each partner in this obligate ant–fungus mutualism, indicating that the evolutionary processes accompanying expansions may be markedly different in these obligately interdependent species. Second, we observed that genetic differentiation among the ant hosts depends upon the genotype of their symbiotic fungal partner (M-fungi or T-fungi), and that the strength of this effect was of the same order of magnitude over local geographical scales as two abiotic environmental factors, temperature and

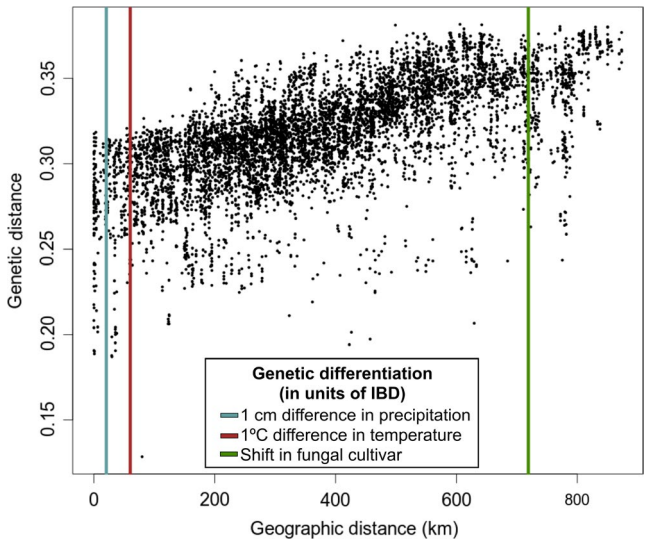

FIGURE 3 Isolation-by-distance (IBD) and isolation-by-ecology in the leafcutter ant *Atta texana*. Genetic distance is in units of X (i.e., method 2 in the dist.genpop function of the ADEGENET R package). Coloured bars indicate the impact of ecological differences relative to IBD (see Table 1 for additional information)

precipitation. Our results (Figures 1a and 2a) support theory predicting that (a) range expansion can leave an indelible genetic signature; and (b) both symbiotic associations and environmental factors impact genetic differentiation of a host. These observations have important implications for our understanding of how symbioses affect range expansions, because discordance in the evolutionary forces acting on codependent partners has the potential to affect adaptation and colonization of new environments (Bronstein, 1989; Dixon et al., 2015; Hume et al., 2016).

4.1 | Magnitude of effects of symbiont type, temperature and precipitation on genetic differentiation in *A. texana*

4.1.1 | Symbiont type

Isolation-by-environment occurs when genetic differences among populations increase along an environmental gradient independent of their geographical distance (Wang & Bradburd, 2014). We found that temperature, precipitation and fungal genotype-cluster were all correlated with genetic differentiation across the range of *A. texana*, suggesting that both abiotic factors and ant–fungus interactions influenced evolution during range expansion. Genetic differences between *A. texana* associated with M- or T-fungi of *L. gongylophorus* were relatively large (Figure 3), equivalent to the amount of IBD of ant populations 719 km apart (i.e., almost the entire known species range of *A. texana*, spanning in total about 850 km south to north and about 900 km west to east). Three hypotheses can explain these results.

First, genetic interactions between *A. texana* and *L. gongylophorus* could affect survival (e.g., nutrition) and reproduction [e.g., fungus-dependent mate choice; see Mehdiabadi, Mueller, Brady, Himler, and Schultz (2012) for a discussion], resulting in correlations between genetic variation in ant and fungal genotypes because specific ant–fungus combinations are selectively favoured. Genetic interactions (i.e., epistasis) are traditionally studied between nuclear genes, but epistasis can also occur between genomes (Gilbert, 2002; Wade, 2007; Wolf, 2000), such as nuclear–mitochondrial (Ballard & Melvin, 2010; Dowling, Friberg, & Lindell, 2008), nuclear–chloroplast (Wolf, 2000) or host–symbiont interactions (Heath, 2010; Mueller et al., 2005; Wade, 2007). In leafcutter ants, novel ant–fungus combinations might arise by mutation and hybridization in the fungi, or by cultivar switching of ant colonies between M- and T-fungi. We expect beneficial, co-adapted genome–genome combinations will be more likely to copropagate than mismatched (i.e., selectively inferior) combinations, thus facilitating colonization of suboptimal habitat at the edge of the species' range. Genome-by-genome fitness effects of mutualisms have been tested in controlled common-garden experiments, for example in endophytic or rhizobial mutualists of plants (Gilbert et al., 2010; Heath, 2010), whereas our survey suggests that intergenomic epistasis for mutualisms could operate under natural conditions across the range of a host species.

Although less plausible than intergenomic epistasis, there are two alternative explanations for fungus-associated genetic differentiation. First, genetic differentiation between ants associated with either M- or T-fungi could be due to a spurious correlation with another factor, such as if ants with different fungal types evolved in allopatry. Ant nests cultivating either M- and T-fungi, however, are sympatric across much of the range, occurring as close as 50 m apart in some instances, less than the typical diameter of 100–150 m of a mature nest's foraging territory (Mueller, Mikheyev, Solomon, et al., 2011; Phillips, Zhang, & Mueller, 2017). With no obvious physical or environmental barriers preventing cultivar switching between nests and with ample opportunity for gene flow between ants with different fungal types, genetic differentiation of ants with M- and T-fungi due to allopatry is unlikely.

Second, the association of genetic variation between ants and fungus type (M- or T-fungi) could be due to a demographic process unrelated to ant-by-fungal interactions per se, such as population growth of specific ant lineages cultivating either M- or T-fungi. Because cultivars are vertically transmitted, fungal strains could "hitchhike" with a successful ant lineage much like loci in linkage disequilibrium with selected genes during a selective sweep (Vitti, Grossman, & Sabeti, 2013). The most likely scenario for this would be a "double invasion" of *A. texana* expansion into Texas, one invasion by ants bearing the T-fungus and a second bearing the M-fungus. Because gene flow among ant lineages would erode genetic differences between M- and T-cultivating ant lineages, the second invasion would have to occur quickly relative to the rate of interbreeding between ant lineages. However, given the slow dispersal rate of *A. texana* (see below) and sympatry between M- and T-cultivating ants across most of the range of *A. texana* (Mueller, Mikheyev, Solomon, et al., 2011), this scenario seems very unlikely. Moreover, the lack of association between fungal type and *F*-values in our BEDASSLE models also suggests that a purely demographic explanation is unlikely. Models reconstructing different possible demographic histories (Schraiber & Akey, 2015) are needed to formally test this hypothesis.

4.1.2 | Temperature and precipitation

Temperature and precipitation were also associated with genetic differentiation in *A. texana*, independent of IBD in the ants. Genetic differentiation increases more rapidly with temperature, but the absolute potential for differentiation was greater along the east–west precipitation cline because of greater rainfall differences (i.e., annual precipitation increases 210% from the western to the eastern range limit of *A. texana*, while annual temperature increases 42% from the northern to the southern range limit; Figure S1). Wang and Bradburd (2014) identified several processes that generate genome-wide associations between genetic differentiation and environment across the landscape: (a) local adaptation and selection against maladapted immigrants, assuming local selection is sufficiently strong (Aeschbacher & Bürger, 2014; Aeschbacher, Selby, Willis, & Coop,

2017); (b) biased dispersal of genotypes to a preferred habitat, leading to a correlation between genotype and habitat regardless of whether selection acts once migrants arrive; and (c) IBD, which is controlled in the BEDASSLE analysis.

Biased dispersal is unlikely to account for our results because the spatial scale of our sampling over the environmental gradient far exceeds the dispersal distance of female leafcutter ants during the mating flight. In other words, while queens can choose specific microhabitats for nest building (e.g., shaded areas under trees), they would need to fly much further than they can to sample habitats that differ significantly in mean annual temperature and precipitation. To illustrate this, the invasive leafcutter ant *Acromyrmex octospinosus* expanded across the Caribbean island of Guadeloupe at about ~0.5 km/year, generating IBD at geographical scales as small as 1,000 km^2 (Mikheyev, 2008), <1% of the area examined here for *A. texana*. Dispersal distance of *A. texana* females in the field is not known, but average dispersal is estimated to be no more than 10 km per mating flight (Moser, 1967) or much less (Mueller, Mikheyev, Solomon, et al., 2011; Phillips et al., 2017). Isolation-by-distance can also lead to spurious correlations between genetic differentiation and environmental factors, but this was explicitly controlled for in our BEDASSLE analysis. Local adaptation and strong selection against maladapted immigrants thus remains a viable explanation for the genome-wide differentiation observed across the clines in temperature and precipitation.

Temperature and precipitation are environmental factors that have wide-ranging effects on physiological function of insects, and leafcutter ants have evolved adaptations to cope with cold, heat and drought (Bollazzi, Kronenbitter, & Roces, 2008; Bollazzi & Roces, 2002, 2010a, 2010b; Ruchty, Roces, & Kleineidam, 2010). *Atta texana* exhibits behavioural adaptations to provide environments suitable to maintain the productivity of fungal cultivars, for example by building garden chambers at soil depths that meet an acceptable range of temperature and humidity, or by moving fungal gardens to depths with optimal environmental conditions (Mueller, Mikheyev, Hong, et al., 2011). The observation that fungal cultivars from colder locales exhibit greater cold tolerance suggest that environmental gradients in temperature are steep enough to have led to a response to selection (Mueller, Mikheyev, Hong, et al., 2011), at least in the fungal symbionts.

An important caveat is that the observed genome-wide differentiation can be caused by other, unknown factors correlated with the environmental gradients. Although BEDASSLE corrects for genetic differences between populations due to geographical distance, we cannot rule out that unmeasured variables other than temperature and precipitation might be ultimately responsible to the observed patterns. One such factor could be the south-to-northeast direction of the range expansion, and therefore the axis of genetic drift, which is not completely orthogonal to the gradients in temperature (north/south) and precipitation (east/west) (Figure S1). Second, correlations among environmental factors are not uncommon and can add uncertainty as to which factor is driving genetic divergence. On the other hand, the correlation between temperature and precipitation in our study is weak (Figure S8a), because temperature and precipitation clines are almost completely orthogonal to each other across the range of *A. texana* (Figure S1). Laboratory experiments testing the performance of ants under cold and desiccation stress, as well as genomic analyses of selection, are required to further evaluate the different adaptive hypotheses.

4.2 | Spatial associations between genetic variation in ants and their fungal cultivars

4.2.1 | Population genetic structure in *A. texana*

We found (a) a broad cline in genetic differentiation (Figure 1a) and (b) a reduction in heterozygosity (Figure 2a) across the entire range of *A. texana*, from the Rio Grande at the USA–Mexico border to northeast Texas and Louisiana. These observations are consistent with a northeastward expansion of *A. texana* following glacial retreat in the Pleistocene, accompanied by limited gene flow across the range as new habitat was colonized by the ants. The continuous and graded cline in genetic differentiation is probably due to the lack of prominent physical barriers to dispersal across the range of *A. texana* (i.e., there are no mountain ranges or large river basins that prohibit the movement of female reproductives during dispersal flights). Instead, gene flow is probably restricted by the short dispersal distance of female reproductives during their annual mating flight (<10 km, or far less; Moser, 1967; Mueller, Mikheyev, Solomon, et al., 2011; Phillips et al., 2017), generating strong signals of IBD (Figure S4) and clinal population structure (Figure 1a).

The observed decline in heterozygosity in *A. texana* towards the range limit (Figure 2a) is expected if genetic variation existing in ancestral populations was eroded by drift as founding queens dispersed along the front of the expanding population (Eckert et al., 2008; Excoffier et al., 2009; Sexton et al., 2009). This cline in population structure and loss of genetic diversity across the range might have important evolutionary consequences because (a) smaller effective population sizes at the wave front increase drift, weakening the efficacy of selection; (b) deleterious mutations can "surf" to high frequency along the wave front due to genetic drift; and (c) immigration of alleles from the centre of the range can constrain the speed of adaptation to conditions at the range edge (Excoffier & Ray, 2008; Kirkpatrick & Barton, 1997; Klopfstein, Currat, & Excoffier, 2005; Mayr, 1963; Nei, Maruyama, & Chakraborty, 1975; Peischl, Dupanloup, Kirkpatrick, & Excoffier, 2013; Sexton et al., 2014,2009). These factors are probably compounded in mutualisms if both partners in the symbiosis must adapt independently to conditions at the wave front to survive, and when a symbiont propagates asexually, as is the case for the largely asexual fungi cultivated by *A. texana* (Marti et al., 2015; Mueller, Mikheyev, Solomon, et al., 2011; Mueller et al., 2010). Genetic drift operating at the expanding range front might also reduce the number of possible combinations of ant and fungal genomes coexisting in a population at the range edge, limiting the opportunity for advantageous genome–genome interactions (i.e., coadaptations) that facilitate adaptation.

4.2.2 | Population-genetic structure in the fungal cultivar compared to ant host

The striking contrast in the spatial distribution of genetic variation of *L. gongylophorus* fungi compared to the ant hosts suggests that the evolutionary forces operating on each partner in this mutualism can be discordant. In contrast to the smooth cline in population structure (Figure 1a) and the spatially contiguous corridors of shared genetic diversity in the ant host (Figures S5a and S5b), population structure in the fungi is patchily distributed across the range (Figure 1b) (Mueller, Mikheyev, Solomon, et al., 2011), and there was no consistent reduction in genetic diversity of fungal symbionts in the direction of the host's population expansion (Figure 2b–d).

One possible explanation for this mismatch is that the differentiation between M- and T-fungi pre-dated the migration of *A. texana* across Texas into Louisiana (rather than differentiation arising after this range expansion), and that both types were propagated by the ants as the population expanded from southern Texas northeastward across Texas to Louisiana. This hypothesis is partially supported by our independent analyses of M- and T-fungi, which revealed a cline in population structure within M-fungi that matches the cline found in the ants (compare Figure 1a with c and d), consistent with the hypothesis that M-fungi comigrated with the ants during the range expansion.

In contrast, the biogeographical patterns in the T-fungi are more discordant with the cline observed in the ant hosts (compare Figure 1a with e and f), suggesting that evolution proceeded differently in T-fungi than in M-fungi. The monolithic bloc of genetically similar T-fungi dominating the central swathe of the range (Figure 2e) could suggest that selection may have favoured these T-fungi over other cultivars and that these fungi spread recently between nests across the central range. If so, such a selective sweep of novel superior T-fungi originating in central Texas must have been recent, which would then also explain why the T-fungi in Louisiana are more genetically similar to T-fungi in southwest Texas (Figure 1e). On the other hand, the second sPCA of T-fungi (Figure 1f) shows a cline in population structure from southwest Texas to Louisiana, suggesting that a gradual range expansion may have occurred at some point in the history of the T-fungi. As a third hypothetical possibility, Louisiana might have been colonized by a long-distance dispersal event of T-fungi from southeastern Texas to generate the observed genetic similarity between T-fungi in southwest Texas and Louisiana, but such events are rare in *L. gongylophorus* and are difficult to reconcile with the observed west-to-east cline in population structure in the T-fungi (Figure 1f). Genotyping-by-sequencing analyses of T-fungi may be able to distinguish between the above hypothetical processes that are insufficiently resolved by our microsatellite marker analyses.

5 | CONCLUSION

The observed differences between *A. texana* and *L. gongylophorus* in the importance of adaptation, genetic drift and gene flow have implications for evolution during the northward range expansion of leafcutter ants since the end of the last glaciation 11,000 years ago. Average temperature in January at the range front in northern Texas (−3 to 3°C), for example, is currently about 10°C lower than in the south (7–10°C) (Mueller, Mikheyev, Hong, et al., 2011), and both M- and T-fungi cultivated by *A. texana* in northern populations are more cold-tolerant than fungi from the corresponding two groups cultivated in southern populations (see Figure S6 in Mueller, Mikheyev, Hong, et al., 2011). If north–south gene flow in *L. gongylophorus* has occurred, it has not precluded local adaptation of the fungus to temperature. Whether local adaptation at the range front has similarly occurred in the ant hosts has yet to be tested experimentally, but the limited opportunity for dispersal by the ants does make it unlikely that gene flow precludes range-limit adaptation in the ants. Genetic drift through successive founder events during the range expansion of *A. texana*, on the other hand, reduces the number of possible combinations of interacting alleles between ants and their fungal cultivars, possibly limiting the opportunity for beneficial intergenomic synergisms that facilitate adaptation at the range front. Whether range expansion of leafcutter ants is impacted by interspecific interactions therefore depends on complex interactions between selection, gene flow and drift acting in parallel in both hosts and symbionts to either maintain or erode adaptive intergenomic synergisms.

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AUTHOR CONTRIBUTIONS

C.C.S., J.N.W., U.G.M., F.R., M.B., K.K. and J.N.S. planned sampling and coordinated research. C.C.S. and J.N.W. designed the sequencing strategy and conducted population-genomic analyses. C.C.S. archived data. A.S.M. assembled the draft genome of *Atta texana*. C.C.S., U.G.M. and J.N.W. wrote the manuscript. U.G.M., K.K., M.B., F.R. and J.N.S. procured funding. All authors revised the manuscript and approved the final manuscript.

DATA ACCESSIBILITY

RAD-sequence data are available in NCBI BioProject PRJNA395768. Sampling metadata for *Atta texana* collections, SNP, and microsatellite data are available in the Appendix S1. The draft genome of *A. texana* is available at NCBI under accession QEPB00000000.

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