

Evolution of cold-tolerant fungal symbionts permits winter fungiculture by leafcutter ants at the northern frontier of a tropical ant–fungus symbiosis

Ulrich G. Mueller^{a,b,1}, Alexander S. Mikheyev^{a,c}, Eunki Hong^a, Ruchira Sen^a, Dan L. Warren^a, Scott E. Solomon^{a,d}, Heather D. Ishak^a, Mike Cooper^a, Jessica L. Miller^a, Kimberly A. Shaffer^{a,e}, and Thomas E. Juenger^{a,b}

^aSection of Integrative Biology, ^bInstitute for Cellular and Molecular Biology, University of Texas, Austin, TX 78712; ^cOkinawa Institute of Science and Technology, Onna-son, Kunigami, Okinawa 904-2234, Japan; ^dDepartment of Ecology and Evolutionary Biology, Rice University, Houston, TX 77005; and ^eSchool of Life Sciences, Arizona State University, Tempe, AZ 85287

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The obligate mutualism between leafcutter ants and their *Attomyces* fungi originated 8 to 12 million years ago in the tropics, but extends today also into temperate regions in South and North America. The northernmost leafcutter ant *Atta texana* sustains fungiculture during winter temperatures that would harm the cold-sensitive *Attomyces* cultivars of tropical leafcutter ants. Cold-tolerance of *Attomyces* cultivars increases with winter harshness along a south-to-north temperature gradient across the range of *A. texana*, indicating selection for cold-tolerant *Attomyces* variants along the temperature cline. Ecological niche modeling corroborates winter temperature as a key range-limiting factor impeding northward expansion of *A. texana*. The northernmost *A. texana* populations are able to sustain fungiculture throughout winter because of their cold-adapted fungi and because of seasonal, vertical garden relocation (maintaining gardens deep in the ground in winter to protect them from extreme cold, then moving gardens to warmer, shallow depths in spring). Although the origin of leafcutter fungiculture was an evolutionary breakthrough that revolutionized the food niche of tropical fungus-growing ants, the original adaptations of this host-microbe symbiosis to tropical temperatures and the dependence on cold-sensitive fungal symbionts eventually constrained expansion into temperate habitats. Evolution of cold-tolerant fungi within the symbiosis relaxed constraints on winter fungiculture at the northern frontier of the leafcutter ant distribution, thereby expanding the ecological niche of an obligate host-microbe symbiosis.

ecological gradient | selection gradient | range limit | frontier mutualism

Mutualistic symbioses between microbial symbionts and eukaryotic hosts generated some of the most remarkable evolutionary transitions and biological diversifications (1–3). The success of such host–microbe mutualisms derives partly from innovations inherent in host-symbiont synergisms, but also from a level of adaptability to environmental stress that is inaccessible for nonsymbiotic organisms. Under environmental stress, host–microbe mutualisms can respond facultatively by symbiont reassociation (substituting, purging, or acquiring symbionts) or by modulating the reciprocal physiological effects that partners have on each other, thereby broadening the ecological conditions under which a host can exist (4–8). In addition to such plastic responses, host–microbe associations can respond evolutionarily to environmental stress whenever selection acts on one or both partners, particularly in obligate symbioses. Stress-mediated evolution occurring within an obligate symbiosis is more difficult to document than facultative symbiont reassociation, but adaptive evolution within an obligate symbiosis can be inferred experimentally (9) or by correlating variation in a selected trait with variation of selection intensity that changes systematically along an ecological cline (e.g., steep climate gradient) (10). Using a leafcutter ant–fungus symbiosis that ranges across a steep south-to-north temperature cline in the southern United States, we provide here evidence for

adaptive evolution of the fungal symbiont that occurred within the host–microbe symbiosis under cold-temperature stress at the northernmost frontier of the leafcutter distribution.

Leafcutter ants depend on the cultivation of *Attomyces* fungi for food, and the fungi have strict humidity and temperature demands (11–14). The vast majority of leafcutter species occur in the tropics, generally in low- to midelevation rainforest, where temperature and humidity vary only moderately throughout the year (11, 15). Growth rates of tropical *Attomyces* fungi are optimized around 25 °C; growth stagnates below 20 °C, and prolonged exposure to temperatures below 10 °C can be lethal to tropical *Attomyces* (12–14). Tropical leafcutter ants therefore construct fungal chambers that maintain gardens within a temperature window of 20 to 30 °C, typical temperatures for tropical rainforest soils (15–19). Moreover, leafcutter ants possess an antennal sensitivity to temperature gradients that is far more acute than in any other insect (20), and this sensitivity enables leafcutter workers to rapidly assess temperature gradients. Within minutes after gardens are displaced experimentally to unfavorable temperatures, leafcutter workers begin to relocate gardens to favorable temperatures (18).

Despite the narrow temperature window required by tropical *Attomyces*, several leafcutter ant species have dispersed with their *Attomyces* cultivars across steep climate gradients (clines) into subtropical and temperate habitat at the extreme southern range in Argentina and the extreme northern range in the United States (Fig. 1 *A* and *C*). By correlating variation in heritable cold-tolerance with variation of selection intensity changing systematically along a temperature cline, these leafcutter mutualisms provide test cases for analysis of symbiont evolution within a host–microbe symbiosis.

Attomyces fungi are obligately dependent on leafcutter ants (21–24) and they are clonally propagated by the ants within nests and from maternal to offspring nests, but *Attomyces* strains are occasionally also transferred between nests of sympatric leafcutter ant species (21, 25, 26). Because a local community of tropical leafcutter ants shares a corresponding local community of cultivar lineages (23, 25), *Attomyces* cultivars in the tropics evolve within the comparatively warm microhabitats occupied by the diverse leafcutter ant species.

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¹To whom correspondence should be addressed. E-mail: umueller@mail.utexas.edu.

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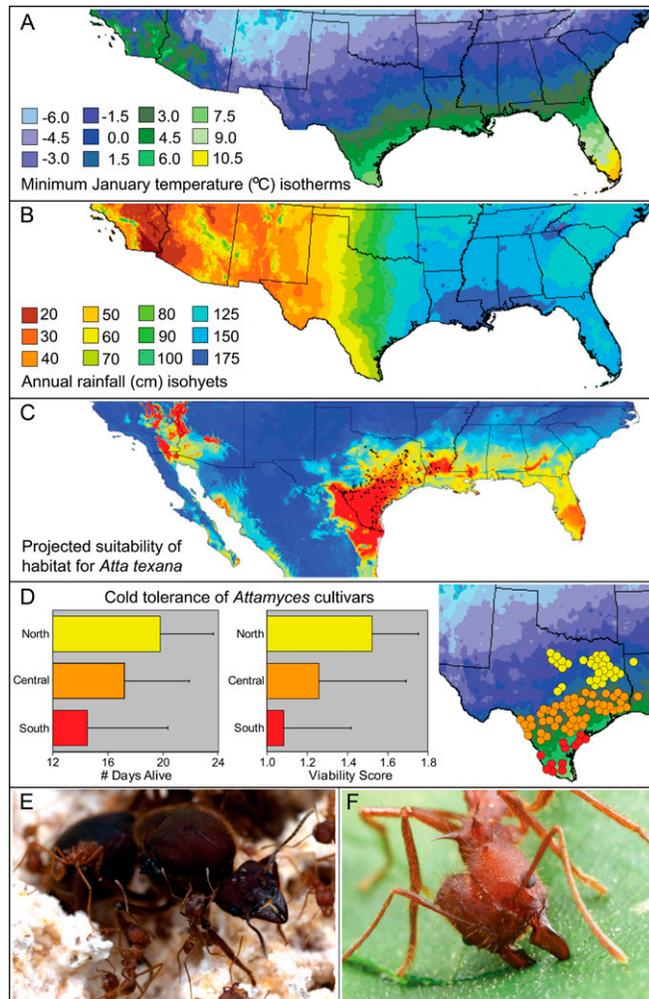


Fig. 1. Selection for cold-tolerance of *Attamyces* fungi across a latitudinal temperature cline. (A) Isotherm map of average minimum air temperature (in degree centigrade) in January for the southern United States. (B) Isohyet map of average annual precipitation (in centimeter rainfall). (C) Habitat suitability for *A. texana* projected by ecological niche modeling. Warmer colors indicate higher estimated suitability; black dots indicate confirmed occurrence localities of *A. texana*. (D) Clinal variation in cold-tolerance of *Attamyces* cultivated by *A. texana* across its range. Error bars show one SD. Cold-tolerance was measured by the number of days alive (survivorship) and by the growth vigor of *Attamyces* growing *in vitro* under increasingly lower temperatures (12 to 5 °C). *Attamyces* accessions are grouped into southern (red), central (burnt orange), and northern (yellow) representatives and mapped onto the January minimum-temperature isotherm map. As predicted by selection along the temperature cline, cold-tolerance of *Attamyces* increases toward the north (viability $P = 0.0011$; survivorship $P = 0.0015$). *Attamyces* symbionts in the northern range of *A. texana* are more cold-tolerant and thus better adapted to colder winter temperatures. A parallel study testing for drought-tolerance did not find a significant association between desiccation-resistance of *Attamyces* and rainfall patterns along the longitudinal moisture gradient shown in B. (E) Queen and workers of *A. texana* on their garden. (F) Worker of *A. texana* cutting a leaf as substrate for fungiculture. Isotherm and isohyet maps redrawn from maps of the National Oceanic and Atmospheric Administration-Southern Regional Climate Center (www.srcc.lsu.edu/climateNormals/). Photos copyright Alex Wild, with permission.

In contrast to tropical populations, *Attamyces* cultivars at the northern range limit are subject to different selection pressures, for two reasons. First, unlike tropical populations, the northernmost populations experience seasonally low temperature extremes (Fig. 1A and Table S1). Second, the northernmost leafcutter populations do not exist sympatrically with any other leafcutter species,

so the northernmost *Attamyces* cultivars cannot be exchanged between leafcutter species. For example, the Texas leafcutter ant *Atta texana* is the only leafcutter species within its range in the United States (Fig. 1C and *SI Text: Study System and Field Methods*), and *Attamyces* cultivars therefore evolve here within the background of a single, soil-nesting ant host. We capitalized on these features to test whether selection on *A. texana* cultivars generates local adaptation along the steep temperature and precipitation clines across the range of *A. texana* (Fig. 1A and B).

Leafcutter ants can protect their gardens against some environmental fluctuations (18, 19), but the northernmost *A. texana* populations experience harsh winters in which soils can freeze for prolonged times to significant depths (Table S1). *A. texana* ants can prevent garden desiccation by foraging for groundwater in their nests' deep tunnel systems [reaching as deep as 32 m (27)] and by supplying gardens with moisture during fungiculture [e.g., manuring of gardens with droplets of liquid feces (11, 28, 29)]. In contrast to such control of garden moisture through fecal manuring, temperate leafcutter ants are unable to maintain garden temperatures in winter at the warmth required for the survival of tropical *Attamyces*. At the northern range limit of the leafcutter distribution, the warmest soil temperatures in winter (around 15 °C) occur at depths below 10 to 15 m, whereas more shallow depths, where fungal gardens are maintained, are significantly colder (5–15 °C) (Table S1 and *SI Text: Study System and Field Methods*). Consequently, fungiculture in the northernmost leafcutter populations must operate throughout winter at temperatures that would critically compromise growth and survivorship of tropical *Attamyces* (i.e., the most favorable winter temperatures in nests of the northernmost *Atta* populations would compromise survival of tropical *Attamyces*). Because the ants can regulate garden moisture (by foraging for groundwater), but the ants have only limited control over winter temperature [by relocating gardens vertically from the coldest, shallow layers (around 5 °C) to deeper layers (around 10–15 °C)], we expected that selection for *Attamyces* cold-tolerance along the latitudinal temperature cline (Fig. 1A) is stronger than selection for desiccation-resistance along the longitudinal precipitation cline (ranging from wetter, eastern habitat to drier, western habitat across the range of *A. texana*) (Fig. 1B).

To assess *Attamyces* adaptations, we collected gardens from nests throughout the United States range of *A. texana* (*SI Text: Study System and Field Methods*) and tested live *Attamyces* isolates (accessions) from these gardens under standardized stress tests for cold-tolerance ($n = 100$ *Attamyces* accessions) and desiccation-resistance ($n = 78$) (*SI Text: Cold-Tolerance and Desiccation-Resistance Stress Tests*). Cold-tolerance was quantified by measuring survivorship and viability of *Attamyces* isolates exposed to gradually decreasing temperatures (12 to 5 °C) (Figs. S1 and S2), simulating the temperature decline that shallow gardens experience in winter in northern populations. A second experiment simulated gradual desiccation that gardens may experience in drying soil in summer.

Results and Discussion

A linear regression analysis of the performance of 100 *Attamyces* accessions in stress tests identified a significant negative relationship between minimum January temperature at the accession collection site and cold-tolerance for survivorship ($P = 0.0008$) and viability measures ($P = 0.0007$) (Fig. S3). Cold-tolerant *Attamyces* accessions tended to occur at colder northern sites, and cold-susceptible *Attamyces* tended to occur at warmer southern sites. In addition, we observed a strong relationship between geographic region of the *Attamyces* accessions and cold-tolerance for survivorship ($P = 0.0015$) and viability ($P = 0.0011$) (Fig. 1D). Similar analyses found no relationship between *Attamyces* desiccation-resistance and rainfall characteristics of the

collection sites (e.g., annual rainfall, rainfall of driest month in July) (all $P > 0.5$ for survivorship and viability) (Figs. S4 and S5).

To correlate genotypic diversity with the observed phenotypic diversity in fungal stress tolerance, we genotyped each *Attamyces* accession with a panel of 12 microsatellite loci (30), grouped accessions into 36 unique haplotypes (31), and grouped haplotypes conservatively into 23 clones (haplotypes that differed by only a single microsatellite marker) (SI Text: *Microsatellite DNA Fingerprinting of Attamyces Fungi*). We found significant diversity of haplotypes and clones at most collection sites. Most clones were collected from several nests at distant locations (31); sample sizes averaged 4.35 accessions per clone, with a maximum of 15 accessions per clone and a minimum of a single accession per clone. Variance component analyses detected significant among-clone genetic variability for cold-tolerance (viability: $P < 0.0001$; survivorship: $P < 0.0001$), but not for desiccation-resistance (viability: $P > 0.20$; survivorship: $P > 0.50$) (SI Text: *Cold-Tolerance and Desiccation-Resistance Stress Tests*). We identified substantial broad-sense heritability in cold-tolerance (viability: $H^2 = 0.43$; survivorship: $H^2 = 0.46$), revealing considerable genetic differentiation in this important ecological trait among the clonal genomes. A hierarchical clustering analysis of the microsatellite marker profiles had previously identified two main clonal groups of *Attamyces* fungi cultivated by *A. texana* (31). The geographic distributions of these two clonal clusters broadly overlap, but with some differentiation along the south-north and east-west axes (Fig. S6). The basis of the phylogeographical structuring of *Attamyces* of *A. texana* is unknown, but could relate to historical vicariance and dispersal patterns of the host, or to range expansion of *Attamyces* through between-colony transfer from southern sources. We found no significant differences between these two *Attamyces* groups in average cold-tolerance and average desiccation-resistance phenotypes (all $P > 0.50$) (SI Text: *Cold-Tolerance and Desiccation-Resistance Stress Tests*), but both *Attamyces* groups showed significant increases in cold-tolerance between southern and northern *Attamyces* populations (Fig. S6).

In sum, across the temperature gradient from southern to northern Texas (Fig. 1A), *Attamyces* from sites with warmer winter temperatures were on average more cold-susceptible, and *Attamyces* from sites with colder winter temperatures were on average more cold-tolerant (Fig. 1D and Figs. S3 and S6). These differences have a genetic basis and support the hypothesis of local adaptation for cold-tolerance in northern sites. There was no trend in desiccation-resistance across the east-west rainfall gradient (Fig. 1B and Figs. S4 and S5). The patterns are consistent with our expectation that selection for cold-tolerance of *Attamyces* should be stronger than selection for desiccation-resistance across the range of *A. texana* (see above).

A. texana colonies cultivate gardens in deeper chambers during winter, but use more superficial chambers in spring as surface-soil temperatures increase and become favorable for fungiculture and brood rearing (Fig. S7). Seasonal, vertical relocation of gardens has been previously hypothesized for *A. texana* (27), but we report here the dependency of this vertical garden movement on latitude (Fig. S7). In southern latitudes, *A. texana* is able to maintain gardens throughout winter at shallow depths, whereas the ants collapse shallow gardens in winter at the northern limit of *A. texana* and restrict winter fungiculture to the somewhat warmer soil layers below 3 m. The relocation behavior improves growth conditions for gardens at northern latitudes, but still exposes the cultivated fungi to significantly colder temperatures (around 10–15 °C) than the warm soil temperatures experienced by tropical leafcutter fungi (20–30 °C).

Ecological niche modeling in Maxent (32) implicated the average temperature of the coldest quarter (i.e., winter) as the most significant abiotic factor limiting the northern extent of *A. texana* (Fig. 1D and SI Text: *Ecological Niche Modeling of the Leafcutter Ant A. texana*). The importance of cold winter temperature as

a key ecological parameter selecting on the ant–fungus mutualism corroborates the conclusions of our analyses of clinal variation in *Attamyces* cold-tolerance (Fig. 1D) and the facultative behavioral responses of the ants to maintain gardens at deeper, warmer soil layers during winter (Fig. S7). Because our niche-model construction focused on northern range limits, it is possible that other factors limit the eastward expansion of *A. texana* (e.g., shallow water table across the Mississippi valley). However, because suitable nesting habitat (e.g., sandy soils) and foraging substrate for leafcutter ants occur abundantly outside the current range of *A. texana* in the United States, *A. texana* may eventually expand across the southeast United States under the milder winters predicted by global climate change (SI Text: *Ecological Niche Modeling of the Leafcutter Ant A. texana* and Figs. S8 and S9).

Conclusion

Experiments with human-cultivated crops have shown that there is great potential for yield increase that remains unrealized because of suboptimal crop adaptation to cultivation environments (33, 34); moreover, traditional efforts to improve crop tolerance to drought, salinity, and low-temperature through breeding have had only moderate success because of the genetic complexity of stress responses (34, 35). Selection on fungal symbionts within the leafcutter ant–fungus mutualism of *A. texana* appears to have overcome some of the complexities of stress responses and generated fungal variants that are adapted to local temperature conditions. Cold-tolerant strains presumably increase garden productivity and colony fitness in the northernmost *A. texana* populations, possibly through adequate garden yields even at lower temperatures, or possibly through an extension of the annual growth season (e.g., earlier garden reinvigoration in spring, later garden dormancy in fall). Future studies could evaluate such cultivar-dependent garden productivity in live leafcutter nests maintained under simulated winter condition in laboratory experiments. Such experiments may also reveal geographic variation in cold-adaptation of the ants, and may determine which of the two symbiotic partners is more cold-sensitive and thus more influential in limiting the northern range of this ant–fungus symbiosis. Studies of leafcutter ants along temperature gradients at the southern end of the leafcutter distribution in Argentina, or across elevation gradients in the Andes or in Central America, present additional opportunities to test for evolved stress responses in the fungal symbionts and their ant hosts, as well as for the role of such adaptations in past and future range expansions of leafcutter species (36–38).

Materials and Methods

Live *Attamyces* fungal cultivars were collected from gardens excavated from nests throughout the range of the northernmost leafcutter ant *A. texana* (Fig. 1C and SI Text: *Study System and Field Methods*) (39), spanning a steep latitudinal winter-temperature cline (Fig. 1A) and a steep longitudinal rainfall cline (Fig. 1B). Axenic (pure) *Attamyces* accessions were isolated (40–42), genotyped with a panel of 12 polymorphic microsatellite markers (30, 31, 43) (SI Text: *Microsatellite DNA Fingerprinting of Attamyces Fungi*), and tested for cold-tolerance and desiccation-resistance in standardized laboratory stress tests (SI Text: *Cold-Tolerance and Desiccation-Resistance Stress Tests*). *Attamyces* cold-tolerance was quantified in a common-garden experiment by measuring survivorship (number of days remaining alive when exposed to cold) and viability (growth vigor after revival from cold) of *Attamyces* isolates maintained on potato-dextrose medium under gradually decreasing temperatures (from 12 to 5 °C). This temperature regime simulates the gradual cooling that gardens experience in winter at a shallow depth in the ground (Table S1 and SI Text: *Study System and Field Methods*). Survivorship and viability measures of cold-tolerance are correlated, but they estimate different fitness components; survivorship measures the ability to withstand cold temperature for prolonged time, viability measures the ability to reactivate quickly and produce vigorous growth when revived from cold temperature. The desiccation-resistance stress test followed the basic common-garden design of the cold-tolerance stress test, but simulated gradual desiccation that a shallow garden may experience in summer when precipitation is at a seasonal low and soil moisture decreases under the intense summer heat. All stress responses were scored blind with respect to

accession genotype and collection locality. Regression and covariance analyses explored the relationships between measured stress responses, genotype, and climate conditions at the sites of origin of each *Attomyces* accession (*SI Text: Cold-Tolerance and Desiccation-Resistance Stress Tests*). Such quantitative-genetic analyses of phenotypes measured in common-garden experiments permit inference of adaptive, genetic differentiation along latitudinal clines (10). Ecological niche models built in Maxent (version 3.3.2) (32) concentrated on the environmental factors determining the northern range limit of the *A. texana*–*Attomyces* symbiosis (*SI Text: Ecological Niche Modeling of the Leafcutter Ant A. texana*).

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Supporting Information

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Study System and Field Methods

Study System of *Atta texana* Leafcutter Ants. *Atta texana* is a soil-nesting leafcutter ant and the northernmost species of its genus. Its two closest relatives (1), *Atta mexicana* (from Mexico and adjoining Central American countries) and *Atta insularis* (from Cuba), are also North American denizens, suggesting a likely North American origin of this clade. All three *Atta* species cultivate *Attamyces* fungi, a name given by Kreisel (2) to the anamorphic fungus cultivated by *Atta insularis*. Hundreds of cultivar fungi genotyped so far from *A. texana* were all *Attamyces* (3). The *Attamyces* of North American leafcutter species originally derived from tropical *Attamyces* lineages cultivated by tropical leafcutter species (4–7). Because *Attamyces* fungi do not appear to exist independently of leafcutter ants (ref. 6 and references therein), *Attamyces* evolution is closely coupled with the biology of the leafcutter ant hosts. For example, *Attamyces* lineages were vectored by dispersing leafcutter queens during their postglacial range expansion northward into the current range of *A. texana* in the southern United States. Where *A. texana* existed during the last Pleistocene glaciation is unclear; refugia in Mexico or perhaps southernmost Texas would seem to be the most likely possibilities because the entire southern United States was significantly colder at that time. The northward expansion of *A. texana* from these putative southern refugia can be dated only broadly (i.e., northward expansion during the past 10,000–15,000 y). *A. texana* was established in central and east Texas at the time when European settlers arrived (8, 9), whereas the presence of *A. texana* in Louisiana at that time is not documented, but likely.

Local adaptation by *Attamyces* fungi to local climatic conditions is plausible because all *Attamyces* fungi studied to date have strict humidity and temperature demands. To buffer their gardens from environmental changes, leafcutter ants evolved a series of behavioral adaptation to adjust temperature, humidity, and gas exchange of their nests. For example, leafcutter ants manipulate nest architecture to regulate aeration, seal nest entrances to reduce water loss, choose leaves of different moisture content to regulate water influx to their gardens, or move gardens to deeper soil layers if moisture declines in superficial layers (10–16). In the tropics, surface soil moisture and water tables can vary between wet and dry seasons, whereas soil temperatures vary little between tropical seasons. Specifically, subsurface temperatures of soil inhabited by *Atta* in Panamanian rainforest remain stable around 25 °C throughout the tropical year (17). Tropical *Attamyces* cultivars therefore are grown by the ants at near constant 100% humidity and within a temperature range typical for tropical soils (broadly 20–30 °C) (10–12, 17–21). In contrast, subtropical and temperate leafcutter ants have to cope with drastic seasonal temperature changes, and temperature of the inhabited soil is expected to constrain the distribution of leafcutter ant species at these latitudes.

Because temperatures have not been measured directly inside any *A. texana* nest, latitudinal trends in nest temperature across the *A. texana* range have to be understood from latitudinal trends in soil and air temperatures (Table S1). In general, surface soil temperatures follow the seasonal air temperatures. Consequently, surface soil temperatures are variable between seasons, but seasonal temperature variation decreases with depth until temperatures stabilize throughout the year, typically at a depth between 5 and 10 m. For the northernmost *A. texana* populations, therefore, the warmest soil temperatures in winter (around 15 °C) occur at depths below 10 m, whereas shallower depths (where the ants maintain gardens) are significantly colder than 15 °C.

The average number of frost-free days is 160 to 220 d for the northernmost *A. texana* populations (average first freeze is in October, last freeze in March), but frosts are exceedingly rare for the southern *A. texana* populations in the United States (22). Extreme frost penetration is about 25-cm depth for northern *A. texana* populations, about 10 cm for midlatitudinal populations, and zero for southern *A. texana* populations (23) (www.ngs.noaa.gov/PUBS_LIB/GeodeticBMs/). For the northernmost *A. texana* populations, soil temperatures at depths of shallow gardens (50–80 cm) rarely exceed 20 °C, even in summer; for the southern *A. texana* populations, soil temperatures at the same depths measure 20 to 30 °C throughout the year (24) (www.wcc.nrcs.usda.gov/nwcc/site?sitenum=2016&state=tx), which is comparable to soil temperatures in the tropics (see above). Ground temperatures are stable year-round below 10 to 15 m, and they can be estimated from the temperature of ground water, which measures around 15 °C for the northernmost *A. texana* populations, but around 20 to 26 °C for the southern populations (Table S1). At depths between 0.5 and 3 m (i.e., depths of spring and summer gardens), temperatures vary most extremely between winter and summer in northern populations, as explained above.

The environmental gradient spanned by *A. texana* across its 850-km latitudinal range (latitude N 25.8° to N 33.2°) can also be gauged by the number of plant temperature-hardiness zones across this range. *A. texana* ranges across 5 of the 15 temperature-hardiness zones recognized for crops in the continental United States (25), from zone 9b for southern populations to zone 7b for the northernmost *A. texana* populations. This substantial environmental gradient is expected to impact fungal growth and garden productivity in *A. texana* nests, generating potential for selection for cold-tolerant *Attamyces* genotypes in northern ranges. Selection for cold-tolerance may be particularly strong in newly established, incipient nests, which are most vulnerable to freezing in winter because of their shallow gardens at 30- to 45-cm depth.

A. texana forages throughout winter even in the northernmost populations. Although night temperatures may be below freezing for weeks in winter in the northern range of *A. texana*, on many sunny winter days the early-afternoon temperatures are sufficiently warm to permit the ants to forage for at least a few hours. One of the authors (U.G.M.) has observed such winter foraging in the northernmost *A. texana* populations (e.g., freezing night temperatures falling below –10 °C, but early afternoon temperatures rising to 10 to 15 °C to permit foraging). Foraging activity is presumably slower in winter in the northern than in the southern populations, but *A. texana* does not cease foraging completely in winter in northern populations. Fungal gardens therefore receive regular additions of some fresh substrate for fungal growth throughout winter, even in the northern populations.

Methods: Collection of *A. texana* and Isolation of *Attamyces* Cultivars.

Locality information. Information on the occurrence of nests of *A. texana* leafcutter ants was compiled between 2003 and 2008 to accumulate a comprehensive list of localities for collection of *Attamyces* material across Texas, Louisiana, and northern Mexico. Garden collections were needed for isolation of live *Attamyces* for the cold-tolerance and desiccation-resistance stress tests (*SI Text: Cold-Tolerance and Desiccation-Resistance Stress Tests*), for preservation of garden for population-genetic analyses of *Attamyces* (*SI Text: Microsatellite DNA Fingerprinting of *Attamyces* Fungi*), and for ecological niche modeling (*SI Text: Ecological Niche Modeling of the Leafcutter Ant *Atta texana**).

Locality information of *A. texana* was obtained by (i) examining material in museum collections (Entomology Collection, Brackenridge Field Laboratory, Austin, TX; Insect Collection, Texas A&M University, College Station, TX; Museum of Texas Tech University, Lubbock, TX; Louisiana State Arthropod Museum, Baton Rouge, LA; National Museum of Natural History, Washington, DC; Museum of Comparative Zoology, Harvard University, Cambridge, MA; Los Angeles County Museum of Natural History, Los Angeles, CA; Bohart Museum of Entomology, University of California at Davis, CA; California Academy of Sciences Collection, San Francisco, CA; American Museum of Natural History, New York, NY); (ii) extracting information from the literature (refs. 26–31 and references therein); (iii) surveying roadsides by car until suitable habitat was located, then inquiring with local residents about the location of *A. texana* nests; and (iv) networking with naturalists, nature centers, State Park rangers, extension agents, pest-control businesses, and farmers. Because we have found that even experienced naturalists can confuse harvester ants with leafcutter ants (both have conspicuous mounds, both construct foraging trails and forage on plant material), we included in our dataset only locality information that we could verify by examining museum specimens or by visiting locations to confirm the presence of *A. texana*.

Particular effort was spent to locate nests and collect garden material at the limits of the reported distribution of *A. texana* (26–29), including the westernmost populations (Del Rio, Val Verde County, TX), the northernmost populations (Fort Belknap, Young County, TX; Ogburn, Wood County, TX; Minden, Webster Parish, LA), and the easternmost populations (Catahoula Parish, LA; Pineville, Rapides Parish, LA; Oberlin, Allen Parish, LA). We concentrated on the northernmost populations to elucidate the ecology and evolution of *A. texana* and its cultivated fungi under the environmental conditions at the northern limit of the entire leafcutter distribution. The southern populations of *A. texana* in the United States along the lower Rio Grande River (Cameron, Hidalgo, Starr, Zapata, and Webb Counties, TX) were less extensively surveyed; however, gardens from populations near Salineno (Starr County) and Raymondsville (Cameron County) were collected as the southernmost representatives for our population-genetic analyses and common-garden stress tests of *Attamyces*. We could not confirm the presence of *A. texana* reported for northeast Texas in Foard, Knox, Denton, and Grayson Counties in north Texas (26), nor in Bowie, Red River, and Cass Counties (29, 31), despite considerable effort to find nests in these counties using the strategies mentioned above. Such unconfirmed county records were not included in our database used for ecological niche-modeling (*SI Text: Ecological Niche Modeling of the Leafcutter Ant *Atta texana**). Our final dataset included 402 confirmed locality records of *A. texana* (indicated as black dots in Fig. 1C).

Collection of garden material. Gardens of *A. texana* were collected by digging into the center of leafcutter mounds with a shovel to the depth of the topmost gardens. Because *A. texana* cultivates a monoculture of the same fungal strain throughout its hundreds of gardens (32), a fragment from a single garden was sufficient to obtain the resident *Attamyces* strain cultivated by a particular nest. Between late November 2006 and mid-May 2007, live garden fragments were collected to permit isolation of live *Attamyces* accessions ($n = 108$) for cold-tolerance and desiccation-resistance stress tests. To facilitate *Attamyces* isolation, excavations aimed to access gardens from the side (rather than from above) to prevent dirt from falling onto and contaminating gardens. Uncontaminated garden fragments were collected into sterile 5-dram snap-cap vials (taking three to five duplicate vials as backups per garden) by carefully separating a clean fragment from a garden with forceps, then carefully placing the fragment with attending ants into the vial without compressing the fragment (compression or injury of a garden fragment generally

results in eventual destruction of the fragment by contaminant fungi). Vials were filled to near completion with garden fragments to minimize jostling of the garden during transport to the laboratory. For the subsequent *Attamyces* isolations, healthy garden fragments with attending ants could be kept at room temperature in the snap-cap vials for several weeks without opening for aeration.

Nests were chosen for excavation principally because of ease of access (e.g., permission by landowner; location along roadside or on public land), rather than ease of excavation in sand versus alluvial clay. In some cases, no clean garden could be collected for *Attamyces* isolation because too much soil collapsed onto the garden and compressed it; in such cases, a garden sample could still be ethanol-preserved for genotyping, but isolation of a live *Attamyces* culture was not attempted. Two excavation attempts in alluvial clay soil failed because no garden could be found within the top 2 m, and one attempt in sandy soil failed because the garden could not be accessed between the roots of a large oak tree. Except for these three failed excavation attempts, the genotyped *Attamyces* accessions represent an unbiased sample of the nests chosen between November 2006 and May 2007 for *Attamyces* isolation.

Isolation of *Attamyces* accessions. Cultivars were isolated from garden fragments within a week of excavation, but at least 3 d after collection to allow the attending ants to clean the garden fragments. Isolation methods followed a standard isolation protocol for attine fungi [potato dextrose agar (PDA) medium without antibacterial supplements, as described in refs. 33 and 34]. Isolations that failed initially were repeated until a pure *Attamyces* isolate could be obtained. Live *Attamyces* were obtained for each nest from which healthy garden could be collected for the purpose of isolation; the *Attamyces* accessions included in the stress tests therefore represent an unbiased sample from the nests chosen for isolation (i.e., our collection of *Attamyces* accessions was not biased by viability differences between *Attamyces* genotypes on PDA medium). Gardens for *Attamyces* isolation were collected from *A. texana* between late November 2006 and mid-May 2007. Isolations from these gardens yielded a total of 108 live, axenic *Attamyces* accessions (each from a separate nest of *A. texana*). This set of *Attamyces* accessions formed the core material for the subsequent cold-tolerance and desiccation-resistance assays.

Sample sizes. Of 108 *Attamyces* accessions isolated from gardens of *A. texana* between November 2006 and May 2007, three accessions were lost because culture plates became contaminated before the start of the stress tests; these three lost accessions had been collected in south Texas. Of the remaining 105 *Attamyces* accessions, exactly 100 isolates were unambiguously assigned through microsatellite-marker genotyping to one of two main *Attamyces* subgroups [so-called M-group and T-group *Attamyces* (35), see details in *SI Text: Microsatellite DNA Fingerprinting of *Attamyces Fungi**], whereas five *Attamyces* accessions were identified as “admixed” (possibly hybrid) genotypes. Because of the unusual genetic makeup of these five admixed accessions, and to simplify secondary analyses comparing stress-tolerances between T-group and M-group accessions, these admixed accessions were not included in the stress analyses. The total number of live *Attamyces* accessions available for testing therefore was exactly 100.

Cold-Tolerance and Desiccation-Resistance Stress Tests

Methods. Cold-tolerance stress test. *Attamyces* cold-tolerance was quantified in a common-garden experiment by measuring viability and survivorship of *Attamyces* accessions growing under gradually decreasing temperatures (from 12 to 5 °C). This temperature regime simulates the kind of gradual cooling that gardens experience during fall and winter if grown at a shallow depth in the ground. Six weeks before the start of the assay, *Attamyces* isolates were subcultured onto the center of new PDA plates (2% agar), wrapped with parafilm, and grown at room temperature to attain a healthy colony (about 1–2 cm diameter). These plates (termed

“refrigeration plates”) were designated to support growth of *Attamyces* during the cold-tolerance assay. To minimize variation in *Attamyces* phenotypes because of growth condition, PDA plates were poured very carefully to standardize volume/plate and evenness of PDA medium. To minimize between-plate variation, about 300 plates were first poured, and from these a set of the most standardized plates was chosen for the experiment. Plates were randomly assigned to the different *Attamyces* accessions. On Day 0 of the experiment, a box with about 35 of the refrigeration plates was moved into a refrigerating incubator (set to 12 °C) to start the temperature regime of decreasingly colder temperatures. Temperatures were lowered by 2 °C every other day for three 2-d cycles (12, 10, 8, 6 °C), then for one 2-d cycle by 1 to 5 °C, followed by eight 2-d cycles at constant temperature of 5 °C. The entire temperature regime lasted therefore 22 d: 12 °C (until day 2), 10 °C (day 4), 8 °C (day 6), 6 °C (day 8), 5 °C (day 10), 5 °C (day 12), . . . 5 °C (day 22) (Fig. S1A). At the end of each 2-d cycle, before the temperature was lowered in the refrigerating incubator, the box with refrigeration plates was moved into a laminar flow hood for subculturing of *Attamyces* accessions onto a corresponding set of “recovery plates.” Recovery plates were always kept at room temperature, and the refrigeration plates were parafilm-wrapped after subculturing and returned to the incubator (now set to the next-lower temperature for the next refrigeration cycle). Subculturing of a set of 35 isolates took about 1.5 h, during which the refrigeration plates were temporarily exposed to room temperature. Subculturing involved the cutting of a lengthy 3-mm × 10-mm strip (Fig. S1B) radially into the mycelium on the refrigeration plate, to ensure that both older and younger mycelium was subcultured (sometimes the youngest mycelium did not recover, but older mycelium did). Each isolate was subcultured onto its own recovery plate, which was then kept parafilm-wrapped at room temperature to permit recovery of mycelium and evaluate survivorship and viability at recovery. Survivorship was measured as the number of days that an *Attamyces* isolate remained alive during the refrigeration regime and exhibited growth on the recovery plate after subculturing; for example, the *Attamyces* accession on the plate in the bottom left corner of Fig. S1B remained alive for 22 d (= 11 2-d subculturing cycles), whereas the *Attamyces* accession at the top left remained alive for only 12 d (= six 2-d subculturing cycles). Viability was scored on the third day after each subculturing by examining the subcultured agar plug under a stereomicroscope, using the following viability scale: viability score 0 = no growth; 1 = one to five hyphae sprouting from the agar plug; 3 = six or more sprouting hyphae. An overall viability score was calculated for a particular *Attamyces* accession by summing each of the 11 individual scores obtained from the 11 subculturing cycles. Viability scores therefore could attain a maximal value of 22 (11 records of maximum viability 2 for a particular *Attamyces* accession). Although these measures of survivorship and viability are correlated, both measures estimate different fitness components, as shown in Fig. S1B: the *Attamyces* on the bottom-left and the bottom-right plates were both alive for 22 d (same survivorship score), but the *Attamyces* accession on the bottom left received a higher viability score because it exhibited more vigorous growth at recovery during the last few days of the 5 °C phase compared with the *Attamyces* accession on the bottom right.

Because of the large number of *Attamyces* accessions tested in the cold-tolerance assay ($n = 100$), samples were randomly assigned to three batches, which were tested in successive test series in the summer of 2007. Within each batch of plates, the stacking arrangement and the position of stacks was changed every 2 d within the plastic box used to house the plates in the refrigerator; such rearrangement rotated plates regularly between bottom, middle, or top of a stack of plates in the refrigerator box and aimed to randomize any minor temperature differences in different positions in a box. Temperature in the

refrigerator was monitored to maintain the desired temperature at the shelf level of the refrigeration box, and temperature fluctuations deviated from the desired temperature by no more than ± 0.2 °C. All scoring of *Attamyces* survivorship and viability was conducted blind by E.H. without knowledge of the collecting locations and the genotypes of the *Attamyces* accessions tested.

Repeatability of the cold-tolerance assay. To elucidate the repeatability of the survivorship and viability measures in the cold-tolerance stress tests, we repeated in the summer of 2008 the same cold-tolerance assay for a subsample of 35 *Attamyces* accessions assayed first in the summer of 2007. The repeat assay assumed that maintenance in the laboratory at room temperature of 20 to 23 °C for over a year, involving two cycles of subculturing onto new PDA plates, did not result in evolutionary change adapting *Attamyces* to the warm laboratory conditions (i.e., we assumed that any such evolutionary change in vitro did not completely erase initial genetic differences underlying the cold-tolerance phenotypes of the tested accessions). The 35 accessions retested were a subsample of the *Attamyces* that were available as live accessions in June 2008 (a number of accessions had been lost between the summer of 2007 and the summer of 2008), and they were chosen from among these live accessions because they exhibited typical, staphylae-bearing growth of *Attamyces*. Staphylae are aggregations of hyphal-tip swellings known only from *Attamyces* fungi and close relatives (6, 11), and some *Attamyces* accessions lose the tendency to form staphylae after about 1- to 2-y growth on PDA medium in the laboratory. Accessions that had lost the tendency to grow staphylae by the summer of 2008 therefore were excluded from the repeat assay, and the 35 *Attamyces* chosen for the repeat assay were all competent to produce staphylae (as was the entire set of *Attamyces* accessions tested in the initial assay). As before, survivorship and viability was scored blind by E.H. without knowledge of *Attamyces* identity and the scores in the initial assay in 2007. Survivorship (Pearson's $r = 0.569$, $P = 0.0004$) and viability ($r = 0.392$, $P = 0.019$) were significantly correlated between the initial assay in 2007 and the repeat assay in 2008 (Fig. S2). Two conclusions emerge: First, survivorship and viability are repeatable measures, but the survivorship measure showed greater repeatability than the viability measure (Fig. S2). Second, both survivorship and viability measured an intrinsic, genetic property of *Attamyces* that persisted for a year in the laboratory and through two subculturing cycles (through two clonal “generations”).

Fig. S2 shows that the length of the assay (22 d) bounds the scores of survivorship, and perhaps also the scores of viability (2.0 maximum viability score) (see also Figs. S3–S5). This means that a longer assay over more than 22 d likely would have generated greater variation in cold-tolerance measures among the longest-surviving and most-viable phenotypes. Future studies therefore can improve on our cold-tolerance assay by observing *Attamyces* responses for at least 30 d, perhaps running the assay until the great majority of *Attamyces* tested show reduced survivorship and viability (this is the experimental design that we chose for the desiccation assay; see below). Second, it should be possible to improve reliability of the cold-tolerance measures by testing multiple replicates within single individuals (within each accession). Because of the time-intensive experimental procedure, because only about 35 accessions could be processed at the same time (leading to potential between-batch variation and random assignment of individuals to different batches), and because of the large number of individuals available to us, we opted against replication within each accession, and rather relied on the strength of our large sample size of 100 *Attamyces* accessions (i.e., we maximized coverage of identical *Attamyces* genotypes cultivated in different nests of *A. texana*).

Desiccation-resistance stress test. The desiccation-resistance assay followed the basic experimental steps of the cold-tolerance assay, but simulated gradual desiccation that a shallow garden may

experience in summer when precipitation is at a seasonal low and soil moisture decreases under the intense summer sun. Desiccation was simulated by slowly drying culture plates in a desiccator and regularly testing for survivorship and viability of *Attamyces* cultivars on the drying “desiccation plates.” By the start of the experiment in August 2009, 30 *Attamyces* accessions from the original set of isolates had been lost, and only 78 *Attamyces* accessions remained for testing. *Attamyces* accessions were first subcultured onto the center of new PDA plates (2% agar), parafilm-sealed, and maintained for 2 mo to permit mycelial growth for subculturing during the desiccation assay. PDA plates were standardized and randomly assigned to *Attamyces* accessions, as described above in the cold-tolerance assay. At the start of the desiccation assay, the desiccation plates with live *Attamyces* were unsealed and moved into a sterilized desiccator kept at room temperature (average humidity $48.5 \pm 3.6\%$; average temperature 24.8 ± 0.5 °C). The stacking arrangement and position of stacks were changed every 2 d within the desiccator; such rearrangement rotated plates regularly between bottom, middle, or top of a stack of plates and aimed to randomize any minor humidity differences in different positions in the desiccator. Every second day, the desiccation plates were moved into a laminar flow hood for subculturing onto corresponding “recovery plates.” Viability of the subcultured *Attamyces* growing on the recovery plate was scored on the second day after subculturing, using the same scale as in the cold-tolerance assay: viability score 0 = no growth; 1 = one to five hyphae sprouting from the subcultured agar plug; 2 = six or more sprouting hyphae. The 2-d subculturing cycle was continued for 60 d (30 cycles) until the drying agar medium became brittle and only two *Attamyces* remained alive. An overall viability score was calculated for a particular *Attamyces* accession by summing each of the 30 individual scores obtained from the corresponding 30 subculturing cycles. Viability scores therefore could attain a maximal value of 60 (30 records of maximum viability 2 for a particular *Attamyces* accession). Survivorship was measured as the number of days that an *Attamyces* isolate remained alive during desiccation and exhibited growth on the recovery plate after subculturing. Desiccation plates were given random codes by U.G.M., and survivorship and viability were scored blindly by R.S. without knowledge of the collecting locations and genotypes of the *Attamyces* accessions tested.

Statistical analyses. Because *Attamyces* fungi are cultivated as monocultures within *Atta* nests and are propagated asexually by the ants (32, 35), our field collections from nests therefore represent a sampling of the diversity of *Attamyces* “genotypes” occurring across the range of *A. texana* in Texas and Louisiana. We used simple linear regression analyses in JMP version 8 (SAS Institute Inc.) to explore the relationships between measured stress tolerances and climate conditions at the sites of origin of each accession. Climatic information (temperatures and rainfall data) was obtained from the National Oceanic and Atmospheric Administration (NOAA) Southern Regional Climate Center (<http://www.srcc.lsu.edu/climateNormals>), which disseminates climatic information specifically for the southern United States but obtains this information from the NOAA’s Environmental Satellite, Data, and Information Service (<http://cdo.ncdc.noaa.gov/cgi-bin/climatenormals/climatenormals.pl>). Climate information was used from the respective weather station nearest the collecting site of an accession. We also explored geographic patterns of fungal tolerance by collapsing accessions into three regions (northern, central, southern) or (eastern, central, western) based on natural breaks in the geographic distribution of our collections. These regions were tested for effects on tolerance characters using fixed effect ANOVAs in JMP.

We explored genetic diversity and clonal relationships among the field collections by screening each accession with a panel of 12 microsatellite loci (3, 32) (*SI Text: Microsatellite DNA Finger-*

printing of Attamyces Fungi). At each locus, we scored fragment lengths as alleles and sorted alleles into unique haplotypes (a unique combination of alleles across the 12 loci). Unique haplotypes represent clonal lineages resulting from asexual reproduction (32). We conservatively pooled haplotypes that differed by only a single microsatellite marker and called these pooled groupings “*Attamyces* clones.” We explored the genetic basis of the tolerance phenotypes using linear mixed models including a fixed “batch” effect and a random clone effect with Proc Mixed in SAS (36). This analysis partitioned the variability in cold-tolerance and desiccation-resistance into an among-clone component and a residual variance. The variance components associated with the random effects were estimated using restricted maximum likelihood and assessments of significance were based on likelihood ratio tests with one-sided *P* values because of our prior expectation that northern populations are more cold-tolerant. We estimated the “broad-sense” heritability (H^2) of the abiotic stress-tolerance traits by computing the ratio of V_C/V_P , where V_C equals the among-clone variance component and V_P equals the total phenotype variance (V_C + the residual variance) in models without fixed batch effects. This ratio provides a useful metric for describing the degree of genetic determination or the clonal repeatability of the measured phenotypes. It is important to note, however, that the causes of among-clone genetic variability in *Attamyces* are likely to be complex and may consist of both additive and nonadditive genetic effects, as well as differences resulting from the multinucleate (polyploid-like) nature of the fungi. We explored the relationship between clone mean phenotype values and the average climate of clonal collection sites using linear regression in JMP 8.

Results. Cold-tolerance stress test. There was a significant relationship between cold-tolerance of *Attamyces* and the average January low temperature (JanTemp) at the nest sites from which *Attamyces* accessions had been collected; that is, cold-tolerant *Attamyces* tended to occur at colder sites, and cold-susceptible *Attamyces* tended to occur at warmer sites (Fig. S3, Upper graphs). This relationship was statistically significant for both survivorship ($F = 12.09$, $df = 1/98$, $P = 0.0008$) and viability ($F = 12.23$, $df = 1/98$, $P = 0.0007$). Because JanTemp correlates with latitude (Fig. 1A), cold-tolerance also increases significantly with latitude for both the survivorship ($F = 14.73$, $df = 1/98$, $P = 0.0002$) and the viability measure of cold-tolerance ($F = 13.51$, $df = 1/98$, $P = 0.0004$). When pooling genotypes into clones (no more than one allele difference within each clone), we observed a significant relationship between the average JanTemp of a clone’s collection sites and the clone mean cold-tolerance phenotype (viability: $F = 3.06$, $df = 1/21$, one-sided $P = 0.047$; days alive: $F = 4.15$, $df = 1/21$, one-sided $P = 0.027$) (Fig. S3, Lower graphs). This result further supports a genetic matching of the cold-tolerance phenotype with the putative climatic selective regime.

Desiccation-resistance stress test. *Attamyces* desiccation-resistance did not correlate significantly with annual rainfall (Fig. S4) nor with July rainfall (Fig. S5). July rainfall was used in addition to annual rainfall because it is a measure of summer drought-stress (July is the driest month across the range of *A. texana* and, together with August, also one of the two hottest months).

Annual rainfall. At the level of all *Attamyces* accessions tested ($n = 78$ accessions), there was no significant relationship between desiccation-resistance and annual rainfall for both the viability measure of desiccation-resistance ($F = 0.81$, $df = 1/76$, $P = 0.71$) and the survivorship measure ($F = 0.005$, $df = 1/76$, $P = 0.95$) (Fig. S4, Upper graphs). At the clone level ($n = 22$ clones tested), there was no significant relationship between the clone mean desiccation-tolerance phenotype and the average annual rainfall across the sites at which a particular clone had been collected (viability: $F = 0.03$, $df = 1/20$, $P = 0.86$; days alive: $F = 0.09$, $df = 1/20$, $P = 0.76$) (Fig. S4, Lower graphs).

July rainfall. At the accession level ($n = 78$ accessions), there was no significant correlation between desiccation-resistance and July rainfall for both the viability measure of desiccation-resistance ($F = 0.02$, $df = 1/76$, $P = 0.90$) and the survivorship measure ($F = 0.13$, $df = 1/76$, $P = 0.72$) (Fig. S5, Upper graphs). At the clone level ($n = 22$ clones), there was no significant relationship between the clone mean desiccation-resistance phenotype and the average July rainfall across the sites at which a particular clone had been collected (viability: $F = 0.10$, $df = 1/20$, $P = 0.75$; days alive: $F = 0.47$, $df = 1/20$, $P = 0.50$) (Fig. S5, Lower graphs).

Sensitivity Analysis. The variance component analysis used to estimate broad-sense heritability is based on restricted maximum likelihood methods as implemented in Proc Mixed in SAS (see above). Maximum likelihood methods are robust to imbalance in experimental design and as such provide variance component estimates and significance tests that should be valid with varying numbers of *Attamyces* clones or varying replication within clones. Nevertheless, as we collected clones blind in the field (without prior knowledge of *Attamyces* genotypes), different clones were collected at different frequencies, resulting in an imbalance in our experimental design: samples sizes averaged 4.35 independent isolates (accessions) per clone, with a maximum of 15 isolates per clone and a minimum of a single isolate per clone. To explore the possible biases of the imbalanced sampling across clones, we culled the unreplicated clones (four cases of singletons) and repeated the analyses; we observed no major change for either statistical significance (viability: $P < 0.0001$; survivorship: $P < 0.001$) or the pattern of broad-sense heritability (viability: $H^2 = 0.45$; survivorship: $H^2 = 0.48$) for cold-tolerance phenotypes. We also completed an analysis where we dropped the most highly replicated clone, and observed only a minor reduction in broad-sense heritability (viability: $H^2 = 0.37$, $P < 0.004$; survivorship: $H^2 = 0.40$, $P < 0.0001$). Moreover, we conducted a bootstrap resampling analysis to explore the dependence of heritability estimates on the inclusion of specific clones and on the clone-frequency distribution using the software H2boot [available at darkwing.uoregon.edu/~pphil/software.html (37)] and 10,000 bootstrap samples. The bootstrap analyses yielded very similar estimates of broad-sense heritability for cold-tolerance (viability: $H^2 = 0.30 \pm 0.12$ SE, $P = 0.0012$; survivorship: $H^2 = 0.41 \pm 0.11$ SE, $P = 0.0002$). In sum, sensitivity analyses indicate that the broad-sense heritability estimates are robust and unbiased by the unequal sampling across *Attamyces* clones.

Microsatellite DNA Fingerprinting of *Attamyces* Fungi

Methods. To explore the genetic basis of cold tolerance and desiccation-resistance of *Attamyces* along the environmental gradients in Texas and Louisiana, we profiled *Attamyces* accessions with a panel of 12 polymorphic microsatellite markers (3). An extensive survey had previously established that *A. texana* cultivates a monoculture of the same fungal strain throughout all gardens of a single nest (32). Because of this monoculture, it is sufficient to genotype a fragment from a single garden to profile the resident *Attamyces* strain cultivated by a specific nest. *Attamyces* fungi were preserved in 100% ethanol at the time of collection and genotyped using standard methods (3, 32, 35). Microsatellite marker sizes were scored using GeneMarker v1.5 (Softgenetics). We scored fragment lengths as alleles and sorted alleles into unique haplotypes (a unique combination of alleles at multiple loci). Unique haplotypes likely represent clonal lineages resulting from asexual reproduction. We conservatively pooled haplotypes that differed by only a single microsatellite marker and called these pooled groupings *Attamyces* clones.

Results. Because *Attamyces* fungi are multinucleate (yielding up to five alleles per locus per individual (3, 32, 35), screening of 12 loci yielded information on the presence or absence of 91 variable markers across all samples. *Attamyces* accessions grouped into 36 unique haplotypes, and haplotypes could be grouped conservatively into 23 clones (haplotypes that differed by only a single microsatellite marker). We found significant diversity of haplotypes and clones at most collection sites. Many clones were collected from several nests; the most common clone was collected from 15 nests. The grouping into haplotypes and into clone lineages permitted exploration of the genetic basis of the tolerance phenotypes with linear mixed models, as described above (SI Text: Cold-Tolerance and Desiccation-Resistance Stress Tests).

A full account of the population-genetic patterns of *Attamyces* cultivated by *A. texana* and other North American leafcutter ants is provided by Mueller et al. (35). This comprehensive analysis of the population genetics and biogeography North American *Attamyces* clustered the clonal *Attamyces* lineages cultivated by *A. texana* into two main subgroups (so-called T-group and M-group cultivars) (35). The cold-tolerance phenotypes are analyzed separately for T-group and M-group *Attamyces* in Fig. S6; both groups show the significant increase in cold-tolerance phenotype with latitude that is also apparent in the global analysis where T-group and M-group *Attamyces* are pooled (compare Fig. S6 with Fig. 1D). Separate quantitative-genetic analyses for T-group *Attamyces* (19 clones) and M-group *Attamyces* (4 clones) revealed significant heritability in both groups for cold-tolerance phenotypes (viability: M-group, $H^2 = 0.45$, $P < 0.0001$; T-group, $H^2 = 0.52$, $P < 0.0007$) (survivorship: M-group, $H^2 = 0.50$, $P < 0.001$; T-group, $H^2 = 0.51$, $P < 0.001$).

Ecological Niche Modeling of the Leafcutter Ant *Atta texana*

Methods: Ecological Niche Modeling. Ecological niche models were built using Maxent (version 3.3.2) (38). Maxent uses environmental data from known species localities and estimates of the background distribution of environmental variables, and attempts to construct an estimate of the species' environmental niche that explains the distribution of known occurrence points given the available habitat. Because Maxent attempts to build a model that distinguishes presence from pseudoabsence localities (localities in the study area for which no presence records are known), predictions can be affected by the distribution of occurrence points and by the study area from which pseudoabsence data are drawn during model construction; model calibrations using an appropriately defined study area therefore are expected to make more accurate predictions (39). For this study, the initial study area was selected by creating circular buffer zones around known occurrences of *A. texana* with a radius of 1 arc-minute (Fig. S8A). To concentrate on the environmental factors determining the northern limit of the species distribution, we trimmed the study area to the south, east, and west of the known distribution of the species. The resulting study area is shown in gray in Fig. S8A. Because pseudoabsence data are primarily drawn in this analysis from areas within the known range and to the north of the known range of *A. texana*, we expect that models built using this study area to be more informative about the factors limiting the northern range of the species than about the factors limiting the ranges to the east, west, or south (see also ref. 39).

Models were constructed using a regularization multiplier of 2.0, and 20% of occurrences were randomly withheld from model construction to be used for model validation. Models were built using the 19 BIOCLIM layers available from Worldclim (40), a set of environmental data commonly used in environmental niche modeling that contains various measurements of temperature, precipitation, and seasonality. Model fit is measured using the area under the receiver-operating characteristic curve (AUC). AUC values range from 1, where models perfectly distinguish presences from pseudoabsences, to 0.5, where models are effective

tively no better than random. The AUC value for the initial model built using the reduced study area and all 19 BIOCLIM variables was 0.731 on training occurrences and 0.706 on test occurrences. AUC values are useful in many cases, but they can be strongly affected by the study area used in model evaluation; more narrowly defined study areas tend to contain a higher proportion of habitat that is similar to training points, resulting in an average increase in the prediction of habitat suitability at background points. This increase in suitability results in lower AUC values when contrasted with models projected onto a broader, more environmentally heterogeneous region, regardless of overall model quality. When this model was evaluated within the study area using all available occurrence points (training and test), it produced an AUC of 0.735, but when projected over the broader geographic area, including the southern United States and northern Mexico (Fig. S8B), it produced an AUC value of 0.843, indicating a good fit of the model to the data.

Because environmental niche modeling can be negatively affected by spatial correlations between environmental variables (particularly when the intent is to determine which variables are most important in limiting species distributions), we measured the Pearson correlation coefficient (r) between all 19 BIOCLIM variables within the study area, and constructed a new model using a reduced set of variables. Variables were selected via a simple heuristic: (i) starting with the most important variable in the initial model (as measured by Maxent's "percent contribution" score), eliminate all other variables correlated with the focal variable such that $|r| > 0.7$; (ii) move to the highest-ranked variable that has not yet been eliminated; and (iii) repeat the procedure until no pair of variables with $|r| > 0.7$ remains. This procedure yielded a set of eight variables: mean diurnal range, isothermality, mean temperature of wettest quarter, mean temperature of driest quarter, mean temperature of warmest quarter, mean temperature of coldest quarter, precipitation seasonality, and precipitation of warmest quarter. The model constructed with this reduced set of variables is expected to have a lower AUC value within the study area because of the reduced possibility of exactly fitting (or overfitting) the model to the input data, but this effect was not strong (AUC of 0.716 for the reduced set of 8 variables compared with 0.735 for the full set of 19 variables), whereas the fit over the broader geographic space was considerably improved (AUC of 0.907 for the reduced set of 8 variables compared with 0.843 for the full set of 19 variables). The projected model is shown in Fig. S8C.

In addition to estimating habitat suitability, Maxent produces heuristic estimates of the relative contribution of environmental

variables to determining the species' distribution by tracking the increase in the regularized training gain of the model as each variable's weight in the model is increased or decreased. The variable showing the strongest contribution to the model, and therefore the variable most likely to limit the northern extent of the species range, was the average temperature of the coldest quarter (accounting for 38% of the reduced model). This variable also had the highest contribution to the model built using all 19 layers. Second in importance was the average temperature of the warmest quarter (25%), followed by the mean temperature of the driest quarter (15.1%) and the mean diurnal temperature range (8.5%). No other variable accounted for more than 5% of the model.

Interestingly, the projected model indicates suitable habitat in areas outside of *A. texana*'s known range in four disjunct areas: western Mexico, Baja California, southwestern Arizona and southeastern California, and southern Georgia and Florida. The predicted areas of suitable habitat in California, Arizona, and Baja California closely correspond to the known distribution of the leafcutter ant *Acromyrmex versicolor* (*Acromyrmex* is the sister genus of *Atta*) (Fig. S9). Because *Ac. versicolor* cultivates *Attamyces* closely related to the *Attamyces* of *A. texana* (35), this correspondence indicates that at least some of the environmental factors limiting the distribution of *A. texana* may be conserved in other leafcutter ants in North America. The predicted suitable habitat in southern Georgia and Florida may indicate the potential for further eastward expansion of *A. texana*. However, given that model construction in this study focused on northern range limits, it is possible that other factors limit the species' eastward expansion (e.g., shallow water table across the Mississippi valley, which prevents *A. texana* from digging deep nests needed to escape cold winter temperatures).

The leafcutter ant *A. texana* may present a unique opportunity to test models of range expansion under ongoing global warming, for three reasons. First, as the northernmost representative of its group, the northward range expansion of *A. texana* is not impeded by competition with other leafcutter species occupying overlapping niche space. Second, as a generalist herbivore, *A. texana* is not limited by plant availability in more northern habitat. Third, because of its dependency on *Attamyces* that recently evolved cold-adapted traits, further evolution of cold-adaptation may be limited by the lack of requisite genetic variation in *Attamyces*, making it more likely that range shifts are dictated by ecological responses to global warming rather than by evolutionary responses. Such a constellation of characters may make the ant–fungus mutualism of *A. texana* particularly responsive to global temperature changes.

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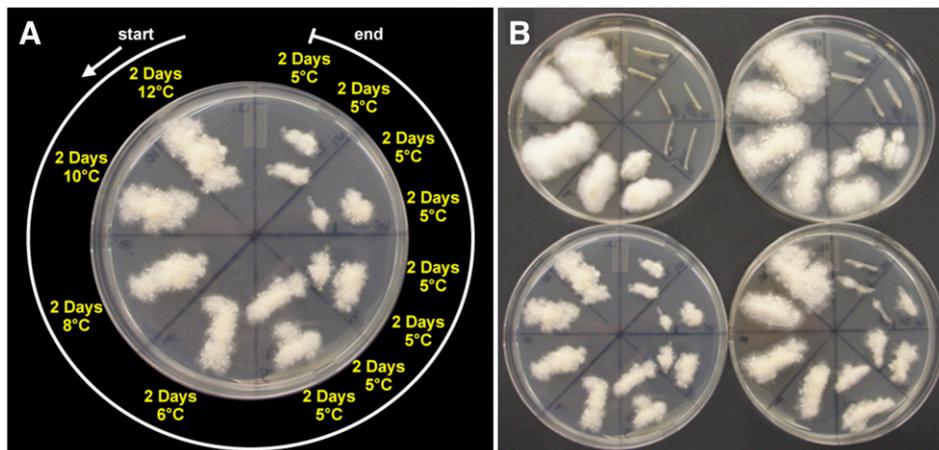


Fig. S1. (A) Recovery plate in the cold-tolerance assay of *Attamyces*. After 2 d of refrigeration at 12 °C, an *Attamyces* strain was subcultured onto the 11 o’clock position of this recovery plate, and again after 2 d more (now at 10 °C) onto the 9 o’clock position, then continuing counterclockwise through the eleven 2-d subculturing cycles as the refrigeration was lowered to 5 °C. The particular *Attamyces* accession survived for the duration of the entire assay. (B) Variation in survivorship and in viability of four *Attamyces* accessions. See text for explanation of differences between the stress responses of these four accessions.

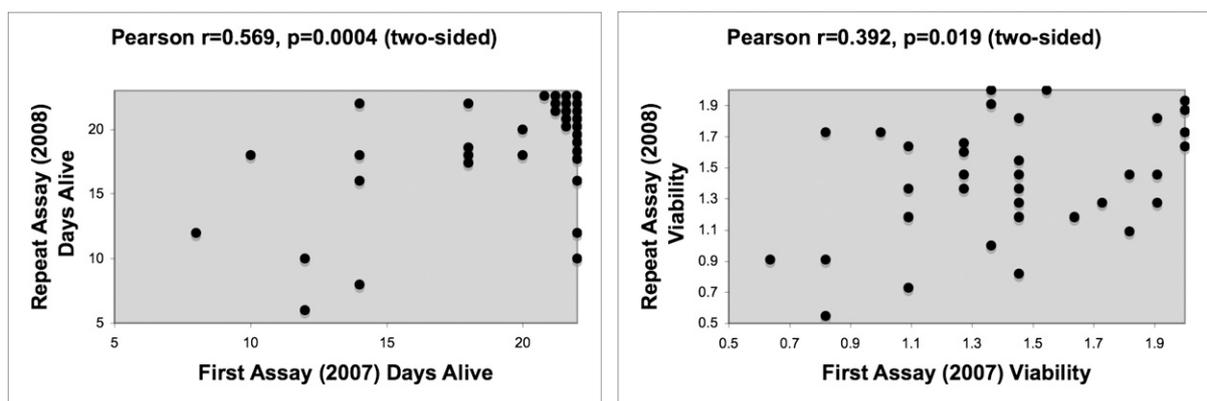


Fig. S2. Repeatability of survivorship and viability as two measures of cold-tolerance. Thirty-five *Attamyces* strains were assayed in the summer of 2007 and again in the summer of 2008 to evaluate whether survivorship and viability measure an intrinsic property that persists through two subculturing cycles and a year of growth on laboratory medium. Correlations were calculated as the Pearson product-moment correlation.

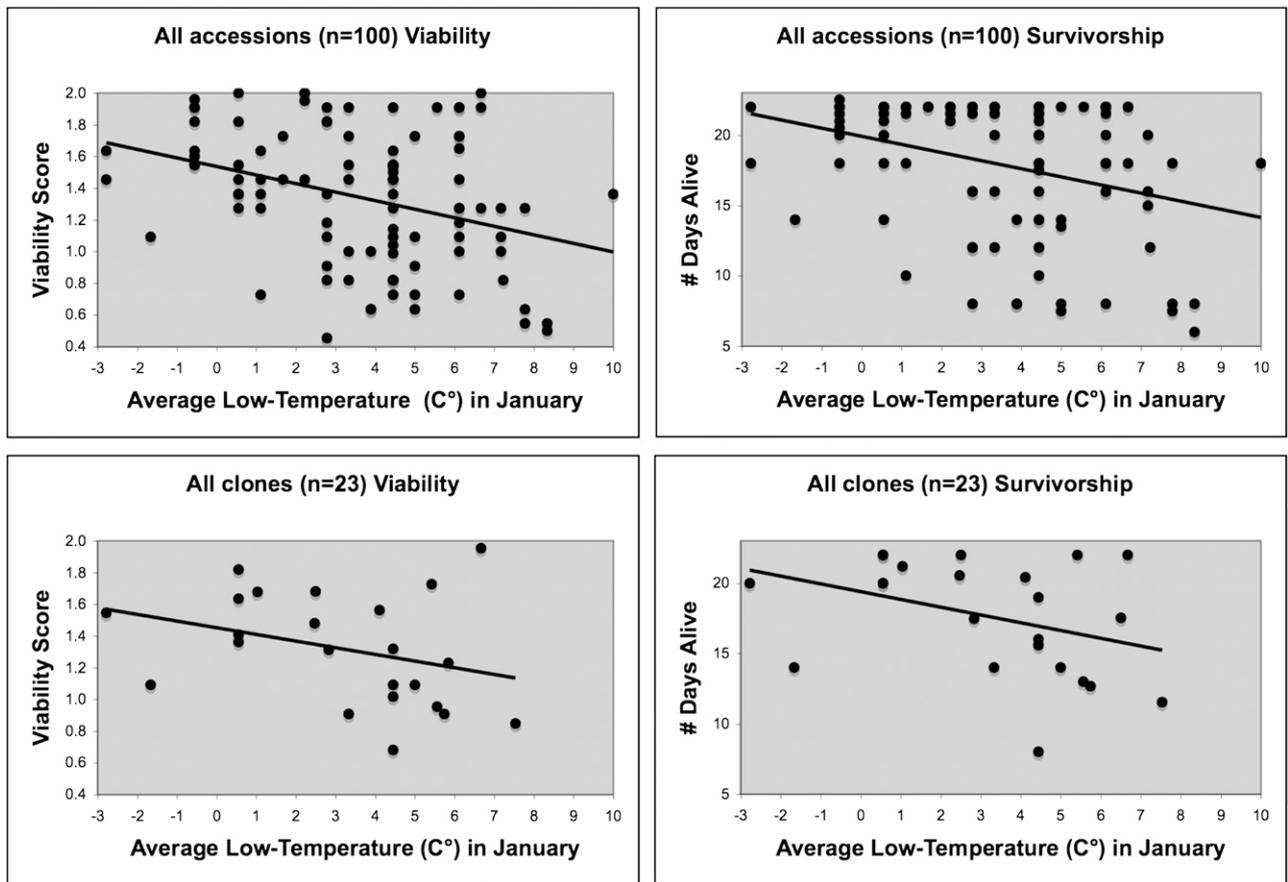


Fig. S3. Cold-tolerance of *Attomyces* cultivated by *A. texana* ants along a latitudinal temperature gradient across the range of *A. texana*, analyzed for all *Attomyces* accessions tested ($n = 100$; *Upper* graphs) and for the *Attomyces* clones into which these accessions could be grouped ($n = 23$; members of a clone are haplotypes that differed from each other by no more than one microsatellite marker; *Lower* graphs). Cold-tolerance decreases significantly with increasingly warmer average low-temperature in January (JanTemp) at the nest sites from which *Attomyces* had been collected; that is, *Attomyces* with greater cold-tolerance tended to be collected from sites with colder January temperatures. For all *Attomyces* accessions ($n = 100$, *Upper* graphs), this relationship was statistically significant for both the viability measure of cold-tolerance ($F = 12.23$, $df = 1/98$, one-sided $P = 0.0007$) and the survivorship measure ($F = 12.09$, $df = 1/98$, one-sided $P = 0.0008$). For *Attomyces* clones ($n = 23$, *Lower* graphs), the relationships between the average JanTemp of a clone collection sites and the clone mean cold-tolerance phenotype were also significant (viability: $F = 3.06$, $df = 1/21$, one-sided $P = 0.047$; days alive: $F = 4.15$, $df = 1/21$, one-sided $P = 0.027$). These results support a genetic matching of the cold-tolerance phenotype with the putative climatic selective regime.

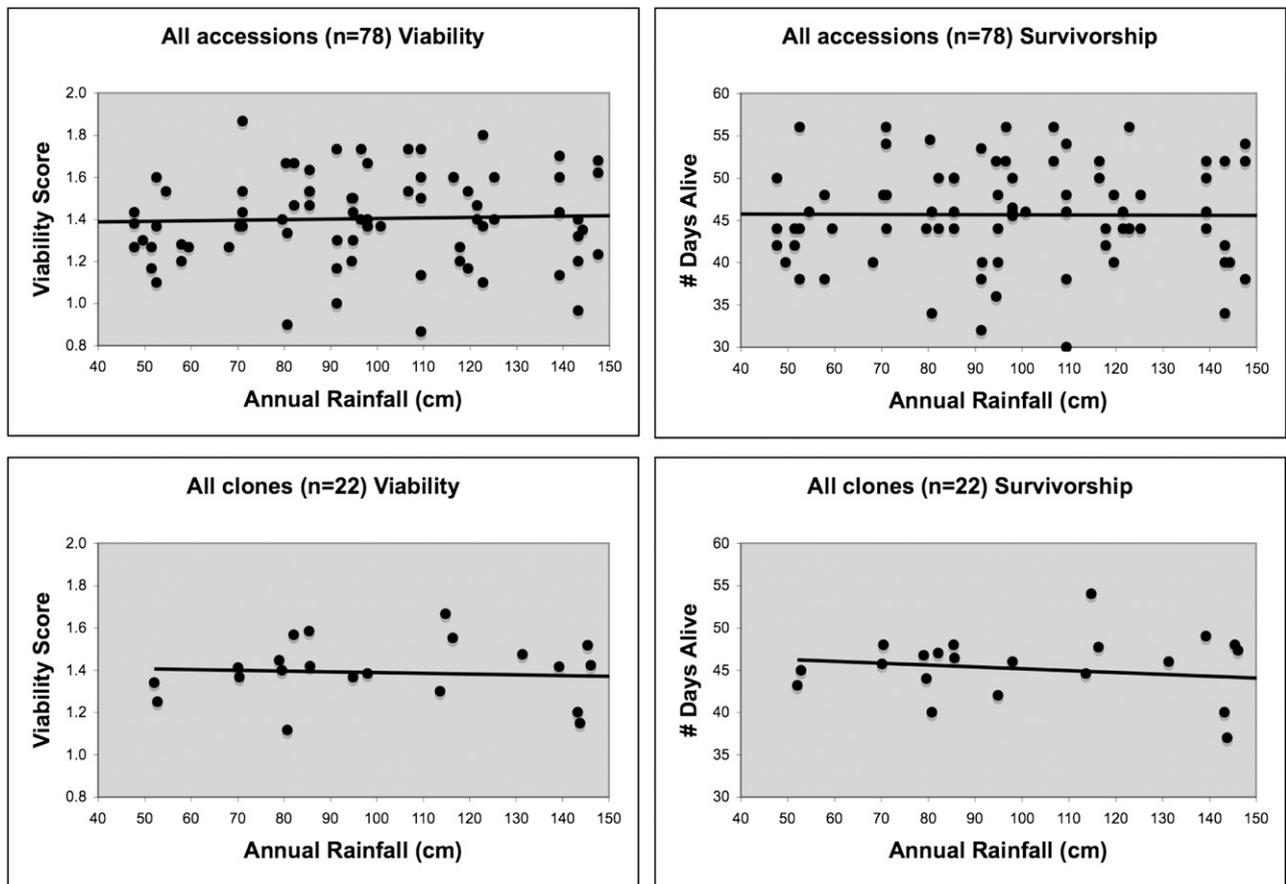


Fig. S4. Desiccation-resistance of *Attomyces* cultivated by *A. texana* ants along a longitudinal precipitation gradient (annual rainfall, 30-y average) across the range of *A. texana*, analyzed for all *Attomyces* accessions tested ($n = 78$; Upper graphs) and for the *Attomyces* clones into which these accessions could be grouped ($n = 22$; members of a clone are haplotypes that differed from each other by no more than one microsatellite marker; Lower graphs). Desiccation-resistance does not change significantly with annual rainfall at the nest sites from which *Attomyces* had been collected. For all *Attomyces* accessions ($n = 78$, Upper graphs), this relationship was not statistically significant for both the viability measure of desiccation-resistance ($F = 0.81$, $df = 1/76$, $P = 0.71$) and the survivorship measure ($F = 0.005$, $df = 1/76$, $P = 0.95$). Likewise for *Attomyces* clones ($n = 22$, Lower graphs), the relationship between the average annual rainfall of a clone's collection sites and the clone mean desiccation-resistance phenotype were not statistically significant (viability: $F = 0.03$, $df = 1/20$, $P = 0.86$; days alive: $F = 0.09$, $df = 1/20$, $P = 0.76$).

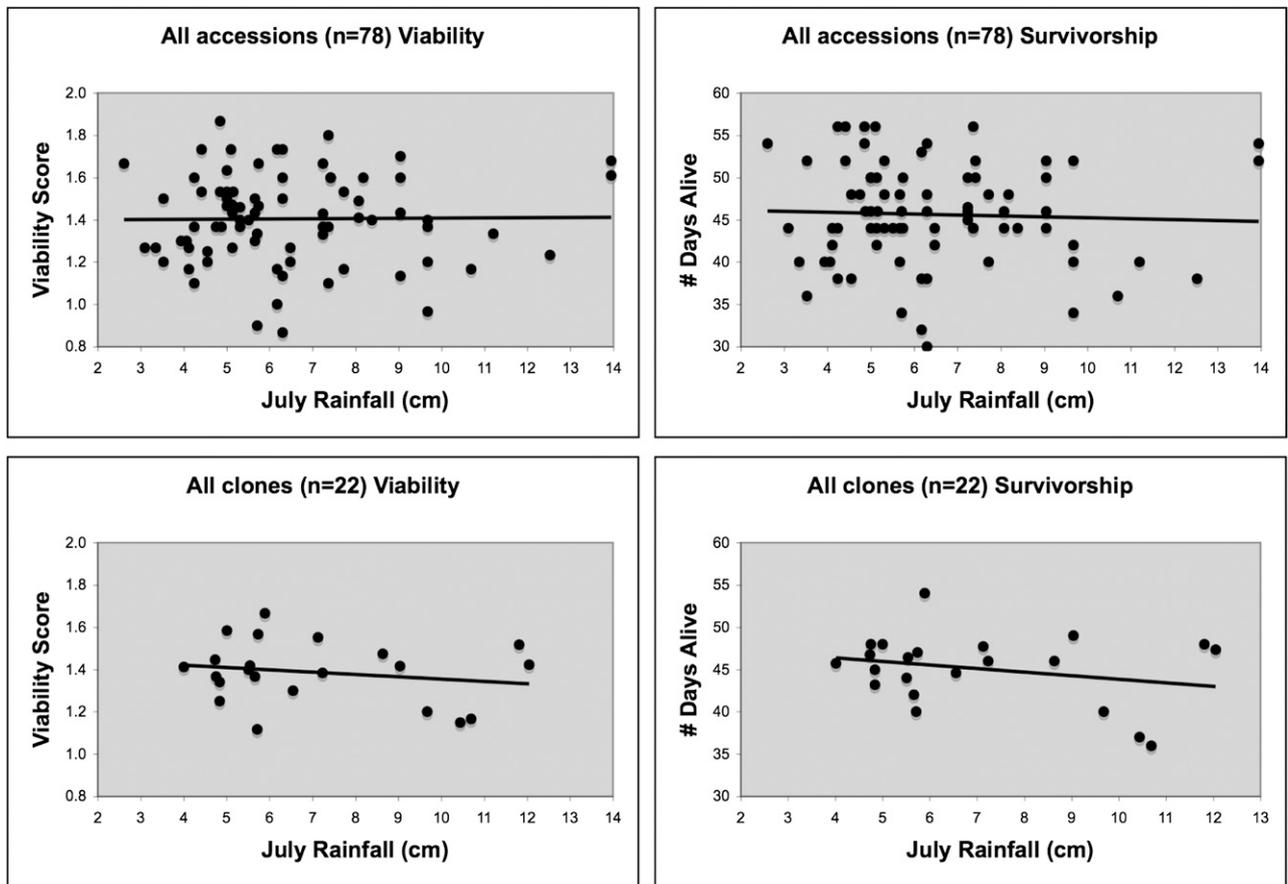


Fig. S5. Desiccation-resistance of *Attomyces* cultivated by *A. texana* ants along a longitudinal precipitation gradient (July rainfall, 30-y average) across the range of *A. texana*, analyzed for all *Attomyces* accessions tested ($n = 78$; Upper graphs) and for the *Attomyces* clones into which these accessions could be grouped ($n = 22$; members of a clone are haplotypes that differed from each other by no more than one microsatellite marker; Lower graphs). Desiccation-resistance does not change significantly with July rainfall at the nest sites from which *Attomyces* had been collected. For all *Attomyces* accessions ($n = 78$, Upper graphs), this relationship was not statistically significant for both the viability measure of desiccation-resistance ($F = 0.02$, $df = 1/76$, $P = 0.90$) and the survivorship measure ($F = 0.94$, $df = 1/76$, $P = 0.72$). Likewise for *Attomyces* clones ($n = 22$, Lower graphs), the relationship between the average July rainfall of a clone's collection sites and the clone mean desiccation-resistance phenotype were not statistically significant (viability: $F = 0.10$, $df = 1/20$, $P = 0.75$; days alive: $F = 0.47$, $df = 1/20$, $P = 0.50$).

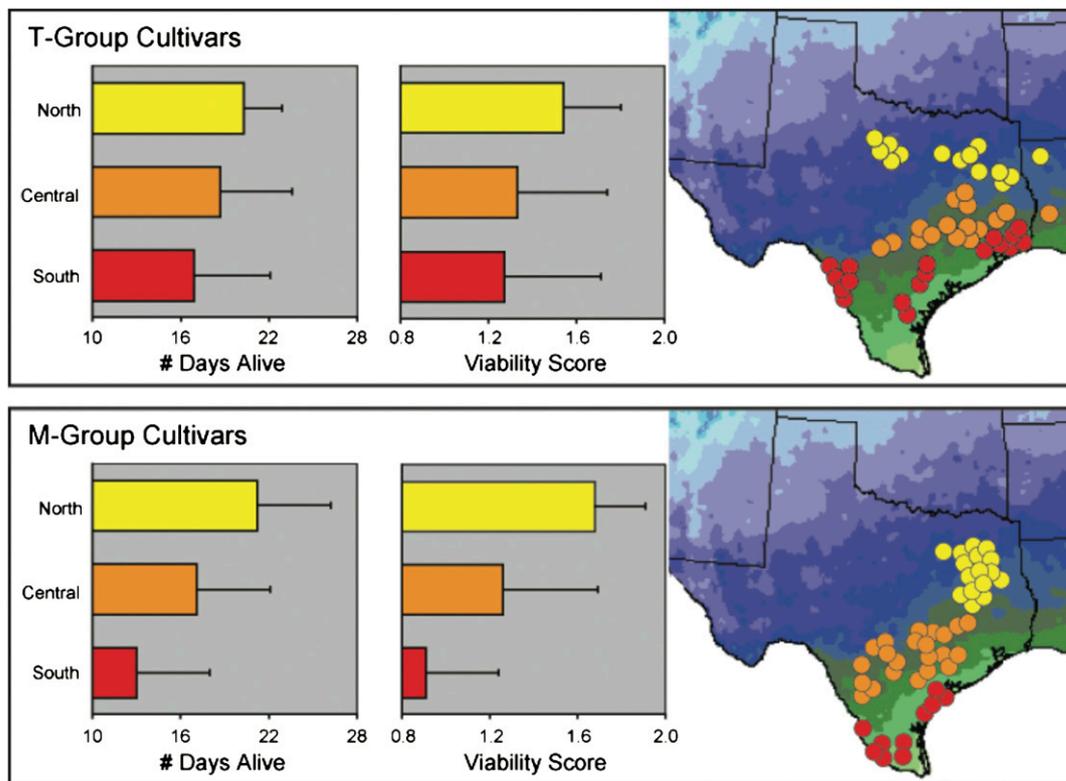


Fig. 56. Clinal variation in cold-tolerance of T-group *Attamyces* fungi (Upper) and M-group *Attamyces* fungi (Lower) cultivated by the leafcutter *A. texana* across its range. Cold-tolerance was measured by the number of days alive and by the growth vigor of *Attamyces* accessions growing in vitro under increasingly lower temperatures (12 to 5 °C). T-group and M-group *Attamyces* are stratified across their ranges into Southern (red), Central (orange), and Northern (yellow) representatives and mapped onto a January minimum-temperature isotherm map on the right. *Attamyces* cold-tolerance increases significantly along the temperature cline across the range of *A. texana* (survivorship T-fungi $r = 0.297$, $P = 0.0164$; survivorship M-fungi $r = 0.597$, $P = 0.0000038$; viability T-fungi $r = 0.261$, $P = 0.0308$; viability M-fungi $r = 0.621$, $P = 0.0000012$; all tests are based on Pearson product-moment correlations). Within both groups of *Attamyces* cultivars, representatives from the northernmost range of *A. texana* are more cold-tolerant and adapted to the colder, local winter temperatures.

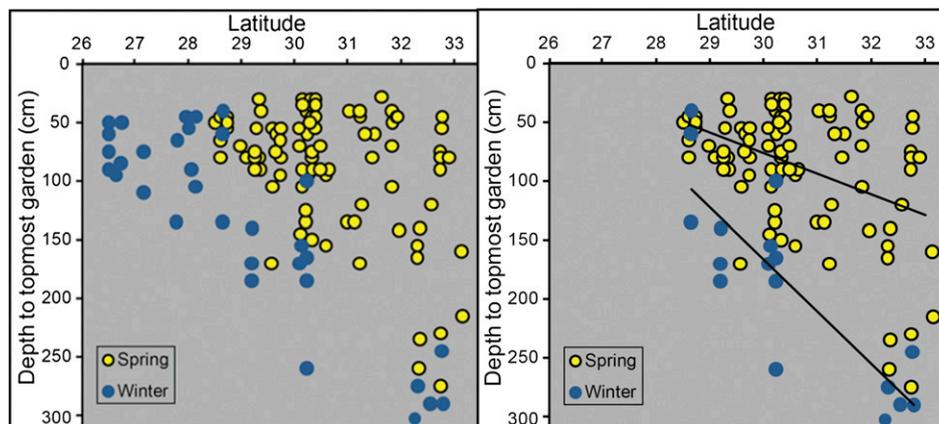


Fig. 57. Clinal variation in garden-relocation behavior by *A. texana* leafcutter ants. Depth of topmost garden changes between winter and spring along a latitudinal cline across the range of *A. texana*. In northern latitudes, cold temperatures force colonies to abandon shallow garden chambers in winter and restrict fungiculture to the warmest soil layers below 3 m, but colonies reactivate gardens in shallow chambers in spring as the surface soil warms. In southern latitudes, surface soils remain warm throughout the year (Table S1) and ant colonies maintain shallow gardens throughout the subtropical winter. (Left) Complete dataset. (Right) Dataset pruned to include only observation from latitudes for which depths of both spring gardens and winter gardens were known; this pruned dataset was used to statistically test for changes in depths as a function of latitude: the average depth to the topmost garden changes as a function of latitude for both winter gardens ($F = 18.22$, $df = 1/15$, $P < 0.001$) and for spring gardens ($F = 39.47$, $df = 1/91$, $P < 0.001$), but as latitude increases, winter gardens are located increasingly deeper than spring gardens (comparison of regression slopes: $F = 10.04$, $df = 1/106$, $P = 0.002$). The left graph could suggest that spring gardens do not occur at latitudes south of 28.5° North; however, the apparent absence of spring gardens at lower latitudes is a sampling artifact: at latitudes below 28.5° North, nests were excavated only in winter, but not in spring. In southern latitudes, *A. texana* maintains shallow gardens throughout winter (expected soil temperatures at those depths are ~20–35 °C) (Table S1), whereas *A. texana* in the northernmost latitudes abandon shallow chambers in winter (projected soil temperatures at those depths are ~5–15 °C) and the ants restrict fungiculture to below 3-m depth in winter.

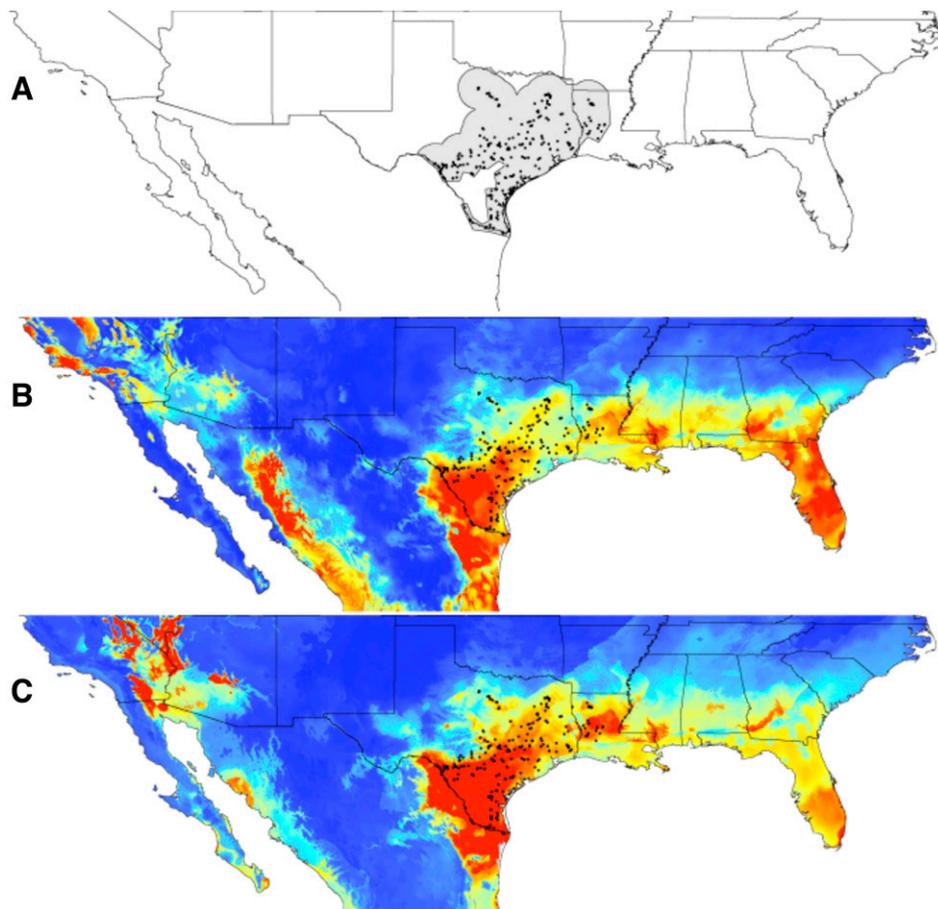


Fig. S8. Environmental niche modeling. (A) Study area used as environmental background for model construction. The initial study area (shaded gray) was selected by (i) creating circular buffer zones around known occurrences of *A. texana* with a radius of one arc minute, and (ii) trimming the study area to the south, east, and west of the known distribution of the species to concentrate on the environmental factors determining the northern limit of *A. texana*. The range of *A. texana* extends a little into Mexico south of the United States border (*SI Text: Study System and Field Methods*) and *A. texana* is replaced in Mexico by the closely related species *A. mexicana*. (B) Projected suitability of habitat for *A. texana* using all 19 environmental (Bioclim) variables. Warmer colors indicate higher estimated suitability. (C) Projected suitability of habitat for *A. texana* using the reduced set of eight environmental variables. Warmer colors indicate higher estimated suitability. Fig. S8C is identical to Fig. 1C, but is shown here again to permit direct comparison with Fig. S8B.

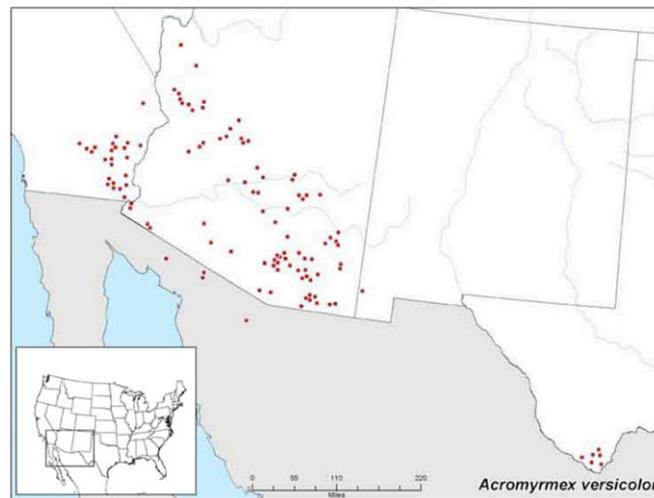


Fig. S9. Distribution in Arizona and California of the desert leafcutter ant *Acromyrmex versicolor*, the northernmost representative of the leafcutter genus *Acromyrmex* (sister genus of *Atta*). South of Arizona and California, the range of *Ac. versicolor* extends through the Chihuahuan and Sonoran deserts [the southmost collection locality that we are aware of is Mesquite (Mezquitilito, latitude 26° 08' N) in northern Sinaloa; not shown on the map]. The collection locations on the map were compiled as described in ref. 35. The ants of California, Arizona, and New Mexico have been extensively surveyed, and it is unlikely that additional *Ac. versicolor* populations currently exist in the United States that are significantly outside the range delimited by the collection records shown on the map. The known distribution of *Ac. versicolor* matches suitable habitat predicted for *A. texana* outside the known range of *A. texana* (compare Fig. S9 with Fig. S8C). This finding indicates that some of the environmental factors limiting the distribution of *A. texana* may be conserved in other leafcutter ant species in North America. Figure drawn by Damon Broglie and Christian Rabeling.

Table S1. Temperature parameters expected for soil inhabited by the leafcutter ant *Atta texana* for northern, central, and southern populations

| Temperature parameter | Northern <i>A. texana</i> populations | Central <i>A. texana</i> populations | Southern <i>A. texana</i> populations | Reference |
|---|---|--|---|---|
| Average low temperature of air in January | −3 to 3 °C | 0 to 6 °C | 7 to 10 °C | Values are for the collections in the present study; see Methods in SI Text: Cold-Tolerance and Desiccation-Resistance Stress Tests |
| Average high temperature of air in July | 32 to 38 °C | 33 to 37 °C | 32 to 38 °C | Values are for the collections in the present study; see Methods in SI Text: Cold-Tolerance and Desiccation-Resistance Stress Tests |
| Average snowfall | 3–6 cm | 0–4 cm | 0 cm | www.wcc.nrcs.usda.gov/climate/ |
| Number of frost-free days (last spring frost to first fall frost) | 160–220 d | 210–270 d | 270–330 d | http://cdo.ncdc.noaa.gov/climate normals/clim20supp1/states/TX.pdf (22) |
| Extreme-frost penetration into soil (frost line) | ~25 cm | ~10 cm | ~0 cm | (23) |
| Soil temperature at depth 40–80 cm in January | ~5 to 15 °C | ~10 to 20 °C | ~20 to 25 °C | www.wcc.nrcs.usda.gov/climate/clim-reports.html (24) |
| Soil temperature at depth 40–80 cm in July | ~15 to 25 °C | ~20 to 30 °C | ~20 to 30 °C | www.wcc.nrcs.usda.gov/climate/clim-reports.html (24) |
| Temperature of ground water (= temperature below 10–15 m) | ~15 to 19 °C | ~18 to 22 °C | ~20 to 26 °C | http://water.usgs.gov/ogw/gwrp/ |

Populations are grouped as shown in the map of Fig. 1D.