

# 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<sup>a,b,1</sup>, Alexander S. Mikheyev<sup>a,c</sup>, Eunki Hong<sup>a</sup>, Ruchira Sen<sup>a</sup>, Dan L. Warren<sup>a</sup>, Scott E. Solomon<sup>a,d</sup>, Heather D. Ishak<sup>a</sup>, Mike Cooper<sup>a</sup>, Jessica L. Miller<sup>a</sup>, Kimberly A. Shaffer<sup>a,e</sup>, and Thomas E. Juenger<sup>a,b</sup>

<sup>a</sup>Section of Integrative Biology, <sup>b</sup>Institute for Cellular and Molecular Biology, University of Texas, Austin, TX 78712; <sup>c</sup>Okinawa Institute of Science and Technology, Onna-son, Kunigami, Okinawa 904-2234, Japan; <sup>d</sup>Department of Ecology and Evolutionary Biology, Rice University, Houston, TX 77005; and <sup>e</sup>School of Life Sciences, Arizona State University, Tempe, AZ 85287

Edited by Nancy A. Moran, Yale University, West Haven CT, and approved January 26, 2011 (received for review October 20, 2010)

**The obligate mutualism between leafcutter ants and their *Attamyces* 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 *Attamyces* cultivars of tropical leafcutter ants. Cold-tolerance of *Attamyces* cultivars increases with winter harshness along a south-to-north temperature gradient across the range of *A. texana*, indicating selection for cold-tolerant *Attamyces* 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

**M**utualistic 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 *Attamyces* 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 *Attamyces* fungi are optimized around 25 °C; growth stagnates below 20 °C, and prolonged exposure to temperatures below 10 °C can be lethal to tropical *Attamyces* (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 *Attamyces*, several leafcutter ant species have dispersed with their *Attamyces* 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.

*Attamyces* 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 *Attamyces* 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), *Attamyces* cultivars in the tropics evolve within the comparatively warm microhabitats occupied by the diverse leafcutter ant species.

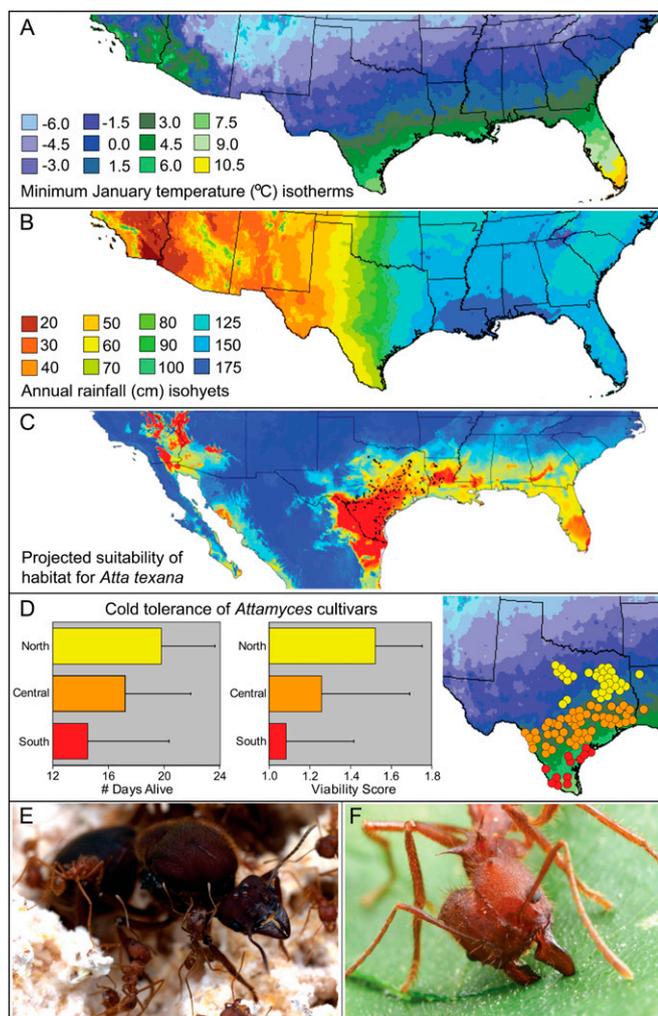
Author contributions: U.G.M. designed research; U.G.M., A.S.M., E.H., R.S., D.L.W., S.E.S., H.D.I., M.C., J.L.M., and K.A.S. performed research; U.G.M., A.S.M., D.L.W., and T.E.J. contributed new reagents/analytic tools; U.G.M., A.S.M., R.S., D.L.W., S.E.S., H.D.I., M.C., and T.E.J. analyzed data; and U.G.M., A.S.M., R.S., D.L.W., and T.E.J. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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

This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1015806108/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1015806108/-DCSupplemental).



**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/](http://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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