Local adaptation of an introduced transgenic insect fungal pathogen due to new beneficial mutations

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Genetically modified *Metarhizium* spp represent a major new arsenal for combating insect pests and insect-borne diseases. However, for these tools to be used safely and effectively, we need a much better understanding of their evolutionary potential and invasion ecology. In order to model natural as well as anthropogenic dispersal scenarios, we investigated evolutionary processes in a green fluorescent protein tagged strain of *Metarhizium robertsii* following transfer from a semitropical to a temperate soil community. Adaptive changes occurred over four years despite recurrent genetic bottlenecks and lack of recombination with locally well adapted strains. By coupling microarray-based functional analysis with DNA hybridizations we determined that expression of cell wall and stress response genes evolved at an accelerated rate in multiple replicates, whereas virulence determinants, transposons, and chromosome structure were unaltered. The mutable genes were enriched for TATA boxes possibly because they are larger mutational targets. In further field trials, we showed that the new mutations increased the fitness of *M. robertsii* in the new range by enhancing saprophytic associations, and these benefits were maintained in subsequent years. Consistent with selection being habitat rather than host specific, populations of an avirulent mutant cycled with seasons similarly to the wild type, whereas a mutant unable to adhere to plant roots showed a linear decrease in population. Our results provide a mechanistic basis for understanding post-release adaptations, show that agents can be selected that lack gene flow and virulence evolution, and describe a means of genetically containing transgenic strains by disrupting the *Mad2* gene.

adaptive evolution | biocontrol | transgenic microbes | entomopathogenic fungi | soil fungi

Predicting the consequences of different types of human intervention has become an increasingly important challenge in light of habitat fragmentation, climate change, invasive species, and genetically modified introductions (1). Genetically modified (GM) *Metarhizium* strains represent a major new arsenal for combating agricultural pests, insect vectors of disease, or malaria parasites within mosquitoes (2, 3). However, the current predictive data base for risk assessment of genetically engineered microbes is very small. There is little information available on survival of individual genotypes (clones) of microbes in nature, nor is there experimentally derived information on gene transfer risks between strains. Lack of knowledge about the fate of genotypes at the population and ecosystem levels creates an inherent uncertainty about the efficacy, survivability, and environmental risk posed by any biocontrol agent. Even for classical biocontrol agents to be used safely and effectively there is a need to fill a vast gap in our knowledge regarding the potential consequences of their evolving (4). Some of the outstanding evolutionary questions address fundamental yet poorly understood issues, including: What roles do different kinds of mutants play in adaptation? When organisms adapt to new environments, do they do so because of changes in few genes or many? Are the same genes involved in independent cases of adaptation to the same environment? To date these questions have been addressed by long term culture of model organisms under conditions in which they were not originally suited (5). This approach has provided an unprecedented view of natural selection in action, but might involve unnaturally harsh selective pressures and the relevance to natural environments is unclear (5). Although there are also many examples of rapid adaptation of parasites in natural populations (6), it has been difficult to disentangle the effects of new mutations from preexisting genetic variation, and the beneficial mutations are rarely identified.

Metarhizium spp. are typically mitosporic, haploid soil fungi. Soil fungi have a central role in ecosystem functioning as pathogens, decomposers, and plant symbionts, but their natural selection and invasion ecology has received scant attention (7–9). It can however be reasonably assumed that genotypic plasticity and natural or anthropogenic dispersal of fungi has contributed to their abundance and diversity (10). *Metarhizium* spp may exemplify these processes, as they are very common (sometimes exceeding 10^6 propagules per gram of soil) (11), and contain numerous intraspecific variants that range from small areas to continents, and exhibit different insect host preferences (12). Recent studies have shown that some species, including *Metarhizium robertsii* (formerly known as *M. anisopliae* var. *anisopliae*) also colonize the rhizosphere (the layer of soil influenced by root metabolism) of specific plant types (13–15). This result was unexpected, and given the abundance of *Metarhizium* could have profound implications for plant biology. Potentially, a better understanding of these interactions could lead to the engineering of *Metarhizium* strains into comprehensive symbionts for plant growth enhancement (16).

In spite of risk assessment concerns, in practice many deliberately introduced pest pathogens and plant symbiotic fungi are reported to have had little invasive success, and this has been attributed to the absence of genetic variation and a failure to recombine with well adapted local populations (9). However, it is also possible that establishment and spread of inconspicuous introductions could have been missed because they were not tagged with a marker. In order to model natural or human dispersal scenarios beginning with a clonal founding population, we set out to identify evolutionary processes in a green fluorescent protein (GFP) expressing *M. robertsii* strain Mr2575 (Mr2575-GFP) (originally isolated in humid semitropical South Carolina), after its introduction into a temperate grassland habitat in Maryland. Mr2575 is the type strain of *M. robertsii* (17), and is frequently employed as a model for studies on host pathogen interactions and genetic engineering (18).

Results

Metarhizium robertsii Mr2575-GFP Persisted in the New Range. An assumption about genetically modified organisms is that they will be eliminated from the environment due to the metabolic burden of expressing a foreign gene (19). Following the initial application of Mr2575-GFP, there was a 94% decline in population size, measured as colony forming units (CFUs) in soil samples, but several intermittent recoveries occurred over four years (Fig. 1). These population cycles coincided with temperature extremes. Although Mr2575 belongs to a genotypic class mostly found in semitropical parts of the United States (12), which usually have milder winters and longer summers than Maryland, temperatures >32 °C repressed population size more than temperatures <0 °C (Mann-Whitney test, $p < 0.001$). The more days in a particular month with temperatures above 32 °C, the lower the CFU counts for that month ($p < 0.001$). While the introduced Mr2575-GFP initially suppressed the native *Metarhizium* strains, suggesting a maximum carrying capacity for the soil, the native populations had recovered four months post application, and the introduced strain comprised ~30% of the total population throughout the rest of the study. Subsequently, native and Mr2575-GFP strains cycled in tandem (Fig. 1), indicating that their population sizes are controlled by the same biotic and abiotic factors.

Metarhizium robertsii Mr2575-GFP Exhibits Adaptive Evolution in Gene Expression. Measuring changes in gene expression with microarrays provides a very sensitive assay for mutant accumulation as mutations in many different loci can affect abundance of any one transcript and a single mutation in a regulatory locus can affect dozens of genes (20). We used cDNA microarrays composed of genes differentially expressed in soil or insect hosts (21), to inventory the accumulation of mutations in five Mr2575-GFP isolates (Re01-Re05) collected after four years in separate turf plots; i.e., in replicate populations. We found that 3.6% of the arrayed genes had significant statistical differences (≥1.5 fold change, Student's *T*-test, $p < 0.05$) in expression across isolates, with 1.3% showing differences higher than twofold relative to the introduced strain. The average percentage of mutated genes per isolate was 0.72%. Significantly, genes coding for traits with obvious ecosystem effects such as the secreted products and pathogenicity determinants (including 45 genes for proteases), that are listed in refs. 21–23 were unaltered in regulation (Fig. 2*A*), in spite of approximately 60% of the arrayed genome encoding secreted products (22). Genes for most metabolic pathways were also highly conserved whereas >12% of genes encoding cell wall proteins, cell polarity, and stress response proteins were mutable;

a highly significant overrepresentation in each case (Fisher's exact test, $p < 0.0005$) (Fig. 2*A*). We used quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (24) to validate the microarray results for five cell wall proteins, one stress response protein, one hypothetical protein, and one transporter protein in Re01-Re05, and five additional isolates (Re06-Re10) (Tables S1 and S2). The same cell wall and stress response genes showed a ≥1.5-fold change ($p < 0.01$) in expression in at least 4 of the 10 isolates, whereas the transporter and hypothetical genes were each altered in just two of the isolates (Table S2). We next investigated whether promoter properties might influence the evolvability of gene expression. In studies with model systems, the minority of genes with TATA boxes were more likely than TATA-less genes to accumulate gene expression mutations owing to their larger cis- and trans-mutational target sizes (25). Consistent with this, mutable genes in the recovered isolates are significantly enriched for TATA boxes (Fisher's exact test, $p = 0.02$). Thus, while 49% of genes encoding cell wall proteins harbor a TATA box, 47% of these had significantly ($p < 0.05$) altered gene expression in one or more isolates, as compared to only 11% of TATA-less genes (Fig. 2*B*). TATA boxes do not increase the likelihood that mutations will be adaptive as ~70% of genes encoding secreted proteases have TATA boxes, but none were altered in regulation, suggesting that mutations in these genes had been selected against.

Re04 and Re05 have several parallel mutations in cell wall and stress response genes. To determine if the microarray results are predictive of phenotypic differences, we conducted a cell wall rigidity assay using Congo red which interferes with the construction of the cell wall. Both Re04 and Re05 exhibited increased tolerance to Congo red as shown by more rapid colony growth relative to the introduced strain (Fig. 3 *A* and *B*), indicative of more rigid and impermeable cell walls (26).

Field Evolved Isolates Rescued Four Years After Release of Mr2575-GFP Are Better Adapted to the New Range. A state of local adaptation can fluctuate in time as well as space (4). To determine if the accumulated mutations have fitness benefits that were maintained in subsequent years, Re04 and Re05 were reintroduced to the field and their survival compared with their parent strain Mr2575-GFP (Fig. 3*C*). In the first month following application (March 10 to April 10, 2009), Mr2575-GFP declined significantly more than Re04 and Re05, whereas throughout summer (June to August), Re05 declined the most. We hypothesized that Re05 may show less heat tolerance as a result of reduced expression of heat-shock protein HSP70 (Fig. S1*A*). As predicted, Re05 showed a significantly reduced germination rate following heat-shock (6 h
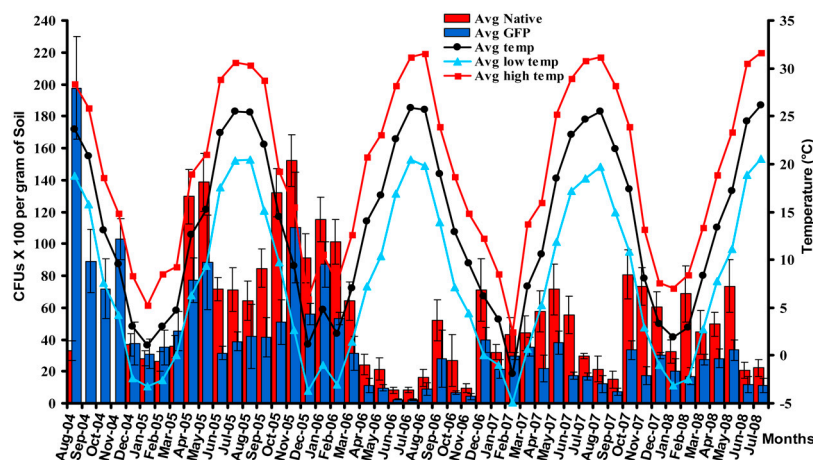

Fig. 1. *Metarhizium robertsii* Mr2575-GFP in turf plots cycled with seasonal changes. Soil samples were collected over four years from turf grass plots. The seasonal changes in population counts of the introduced strain were also shown by the native *Metarhizium* strains, indicating that they were controlled by the same biotic and abiotic factors. Error bars indicate SD, $n = 15$.
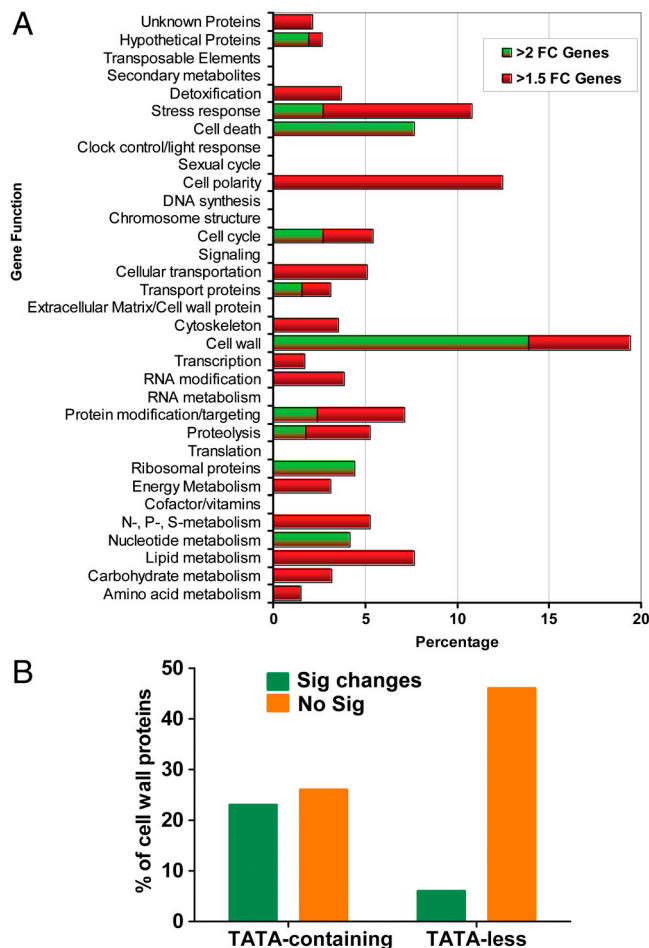
A

B

Fig. 2. Microarray analysis of five isolates recovered four years after field application compared with the original introduced strain Mr2575-GFP. (*A*) Percentage of genes in each functional group with a significant >1.5-fold change in gene expression in at least one of the five isolates. The cell wall, cell polarity, and stress response categories contained the highest proportions of genes with >1.5 fold changes in expression. (*B*) Cell wall genes with significantly ($p < 0.05$) altered expression (Sig changes) are enriched for TATA boxes compared to genes with unaltered expression (No sig). FC, fold change.

at 42 °C) compared to other isolates (Student's *T*-test, $p < 0.03$) whereas growth rates under optimal conditions were the same (Fig. S1 *B and C*). A possible explanation for the altered expression of HSP70 is that the responsible mutation is pleiotropic and beneficial effects offset deleterious effects. During the cooler fall season, Re05 recovered more rapidly than the other isolates, and for the rest of the year trended with Re04. Both Re04 and Re05 ended the year at significantly higher levels than Mr2575-GFP ($p = 0.002$). Bioassays showed no significant differences between Re04, Re05, and Mr2575-GFP in their virulence against *Galleria mellonella* caterpillars (Log-rank test, $p = 0.42$) (Fig. S1*D*). Unaltered virulence against caterpillars is consistent with the better fitness of field evolved isolates not being dependent on insects although it cannot exclude a change in virulence against one or more native species of field insect.

Populations of *M. robertsii* Are Principally Maintained by Rhizospheric Associations. To determine whether populations of *Metarhizium* are principally maintained by rhizospheric associations or in insects, we compared survival of Mr2575-GFP vs. two mutant strains, Δ*Mcl1*-RFP [expressing red fluorescent protein (RFP); disrupted in an immune evasion gene and with low infectivity to insects] (27) and Δ*Mad2*-GFP (expressing GFP; disrupted in a gene required for adhesion to roots) (28) (Fig. S2). *Mcl1* and

Mad2 are specifically up-regulated by insect blood and root exudates, respectively, and lack pleiotropic effects in a panel of tests (27, 28). Scarab beetle larvae (*Phyllophaga* spp.), comprised approximately 80% of insects infected by green fluorescent Mr2575-GFP and Δ*Mad2*-GFP in the field plots, with the balance being made up largely of other beetles (*SI Text*). We also identified ants and hemipteran bugs infected with GFP-expressing Mr2575-GFP and Δ*Mad2*-GFP during the first month postrelease, when spore levels were very high in the application zones. Throughout the course of the study we did not find any insects infected with RFP-expressing fungus, confirming that Δ*Mcl1*-RFP is avirulent. The Δ*Mcl1*-RFP population cycled with seasons in the same manner as Mr2575-GFP (Spearman correlation coefficient $\rho = 0.99$, $p = 1.6 \times 10^{-7}$), albeit CFU counts were lower (Fig. 4). Between October 2007 and March 2008, Δ*Mad2*-GFP declined in parallel with Mr2575-GFP ($\rho = 0.90$, $p < 0.001$), but correlation was less between March and May 2008 ($\rho = 0.8$, $p = 0.1$) and between June 2008 to June 2009 ($\rho = 0.58$, $p = 0.2$) because Δ*Mad2*-GFP exhibited a continuing significant (Mann-Whitney test, $p < 0.01$) linear decrease in population levels without intermittent increases. In contrast to the wild type and Δ*Mcl1*-RFP, Δ*Mad2*-GFP was not detected in the field eighteen months post release.

M. robertsii Mr2575 Is Exclusively Clonal. Mutation is the ultimate source of genetic variation, but genetic and fitness changes could also result from recombination, whereas local adaptation will be favored by low gene flow (4). To assay for recombination, we released a double-labeled strain Mr2575-GFP/RFP in which *gfp* and *rfp* are on two different chromosomes. The strain stability and possible dissemination of genetic material through hybridization was investigated by examining whether Mr2575-GFP/RFP retained both marker elements in their original state. During two years of continuous screening ($n = 126,820$ colonies), we did not find any colonies with only GFP or RFP (Table S3). We also released Mr2575-GFP and Mr2575-RFP together to measure intrastrain recombination events and found that within one month, 1.2% of CFUs were unstable monokaryon diploids (Table S3). In the laboratory, these diploids gave rise to parasexual segregants during the first parameiosis (Fig. S3). Although parasexuality can enhance adaptation (29), we did not detect segregants in the field, suggesting that they have reduced fitness.

Insects are hot spots for recombination events for some *Metarhizium* strains (30). To confirm that Mr2575 has a very low capacity for gene introgression, we looked for recombinants in insects coinfected with different strains labeled with either GFP or RFP (Table S4). We found that twenty-four percent of spores harvested from insects infected with Mr2575-GFP and Mr2575-RFP were unstable diploids (Table S5, Fig. 5). However, no recombinants were observed when Mr2575-RFP and *M. robertsii* strain Mr23-GFP [from North Carolina, and in the same genotypic class as Mr2575 (12)] attacked the same insect, and several other combinations of strains were also incompatible (Table S5). Changes in genome structure and population genetic variability in fungi are often attributed to transposable elements (31). However, the distribution of transposons in Mr2575 and Mr23 are very similar (Fig. 6), suggesting that chromosomal changes and recombination events have not played a part in adaptation because Mr2575 and Mr23 were somatically compatible. Analysis of the complete genome of Mr23 revealed that it is probably exclusively asexual and clonal (23), and the current study provides experimental evidence for clonality. Of the ten selected Mr2575 transposable elements, five could not be detected and three were in single copy in *M. anisopliae* strain Ma549, suggesting these transposons had been active in the common ancestor of Mr23 and Mr2575 after it diverged from *M. anisopliae*. Isolates recovered from the field four years after application displayed no distribution pattern variation in these transposons (Fig. S4). These results suggest that new beneficial mutations are the only cause of fitness
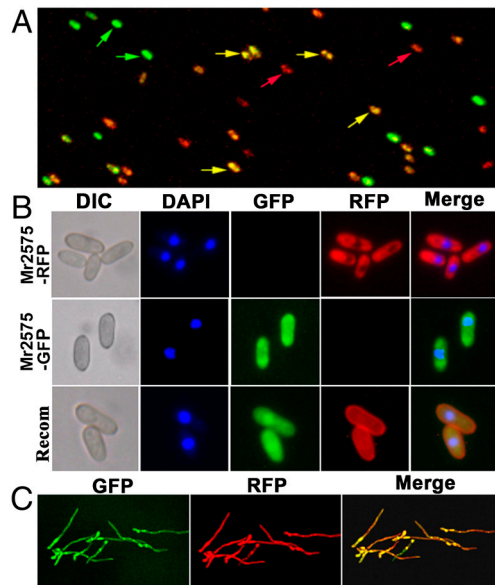
Fig. 3. The evolved isolates Re04 and Re05 possessed more rigid and impermeable cell walls than the parent strain (Mr2575-GFP), and were better adapted to field conditions. To determine susceptibility of Mr2575-GFP, Re04, and Re05 to Congo red, 5 μL of each spore suspension (range 5×10 conidia/mL to 5×10^4 conidia/mL) was spotted onto PDA (*A*) or PDA complemented with Congo red (300 μg/mL) (*B*). The sensitivity to Congo Red (CR) is determined by comparing the extent of colony formation. To measure population levels, Re04, Re05 and the introduced strain Mr2575-GFP were collected from turf plots and CFUs were counted (*C*). Error bars indicate SD, $n = 15$.

increases in Re04 and Re05, and that there is little risk of introgression of new mutations or transgenes into native strains of fungi.

Discussion

Most of the existing data on how fungi adapt to their environments has been obtained from highly artificial experiments (32). The present work on *M. robertsii* is of interest both fundamentally and practically with respect to several understudied areas of fungal evolution and invasion biology, and provides a potential model for assessing fungal evolution in natural communities. We show that contrary to expectations (4, 9), adaptive changes in a clonal lineage can occur within a few years, even in the absence of pre-existing genetic variation and recombination, and that they occur despite recurrent genetic bottlenecks.

Biocontrol agents are usually selected from regions of the native range that environmentally match the release area reason-

ably well, but the importance of finer scale adaptation of biocontrol agents is unclear (4). We reasoned that dispersal ∼450 miles north of its origin would not subject Mr2575 to harsh selective pressures, and it would model common usages of biocontrol agents, as well as many natural dispersal scenarios. The recurring drops in population levels caused by thermal stresses are consistent with laboratory determinations of permissive temperatures for sporulation, germination, growth, and infection processes (33). However, many grasses go semidormant in temperatures >32 °C, with reduced photosynthetic rates, root formation, and the death of older roots (34). Reduced productivity of plants could detrimentally impact population structures of insects and *M. robertsii*. In contrast to Δ*Mad2*, the Δ*Mcl1* mutant cycles with the wild type suggesting that population levels depend on temperature-dependent interactions with plants rather than insects.

Fig. 4. Rhizosphere competence plays an important role in maintaining *Metarhizium*'s population. Mr2575-GFP, the avirulent mutant Δ*Mcl1*-RFP and rhizosphere incompetent mutant Δ*Mad2*-GFP were recovered from turf plots. Different letters indicate a significant difference (*p* < 0.05) in counts of CFUs. Error bars indicate SD, $n = 15$.

Fig. 5. Intrastrain recombinants are unstable diploids. (*A*) Confocal fluorescent image of conidia harvested from cadavers of *Galleria mellonella* coinfected by Mr2575-GFP and Mr2575-RFP. Green and red arrows indicate conidia expressing GFP or RFP only. Yellow conidia are recombinants expressing both GFP and RFP. (*B*) Parental strains (Mr2575-GFP and Mr2575-RFP) and their recombinants are uninucleate, confirming that recombinants are diploid. DNA was stained with DAPI (blue, 4′,6-diamidino-2-phenylindole). (*C*) Recombinant mycelia expressing both GFP and RFP appear yellow when GFP and RFP fields are merged. Scale bar indicates 10 μm.

There is considerable controversy regarding the proportion of expression divergence in model systems that is attributable to natural selection rather than genetic drift (35). However, several
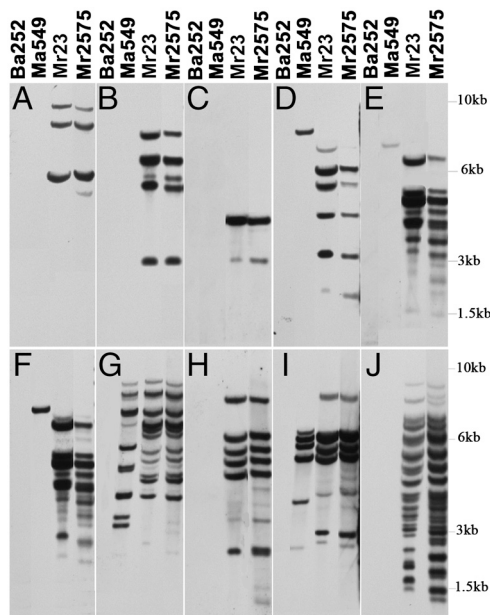

Fig. 6. Genetic relatedness revealed by transposable elements in Southern blot analysis. *Beauveria bassiana* Bb252, *Metarhizium anisopliae* Ma549, *Metarhizium robertsii* Mr23 and Mr2575 genomic DNA were hybridized with DIG-11-dUTP-labeled probes corresponding to the complete ORF sequences of Mr2575 transposable elements: transposase CN808808 (*A*), transposase AJ272685 (*B*), pol polyprotein AJ274203 (*C*), gag-like polyprotein AJ274338 (*D*), polyprotein AJ272783 (*E*) polyprotein AJ274240 (*F*), reverse transcriptase CN809546 (*G*), transposase-like protein AJ273429 (*H*), transposase CN808708 (*I*), restless-like transposase AJ274202 824*J*). Molecular size markers are indicated on the right.

lines of evidence suggest that the regulatory mutations in *M. robertsii* cell wall and stress response genes are the result of positive selection. First, parallel mutations in replicate populations is consistent with directional (positive) selection (36). Second, stress responses, cell polarity, and the cell wall, are functionally linked. The cell wall determines shape and environmental signals redirect cell wall deposition effecting cell polarity during stress responses (26). Third, these genes are likely to have a direct impact on fitness of individual conidia and germtubes. Stress responses are an essential component of survival (26), while the cell wall is the interphase with the environment, and plays a vital role in morphology, adhesion, resource acquisition, and resistance to toxins. The fact that the survival of diversified field isolates was increased suggests that microarrays can identify the mechanistic basis for adaptation to the Maryland field site.

In the absence of similar field-based evolution studies with other fungi we cannot say whether the mutation rate or functional categories of genes undergoing adaptive regulatory evolution in *M. robertsii* are typical. The genetic variation we observed suggests divergence is habitat rather than host specific, and this is consistent with the broad host range *M. robertsii* being commonest in grasslands, whereas several other broad host range *Metarhizium* species prefer woodlands (13). However, in light of extensive empirical support for the Red Queen hypothesis (37), the absence of changes in pathogenicity-related genes is not a result we would have predicted. TATA boxes are present in many genes encoding secreted products that have been implicated in pathogenicity. The failure to detect mutations in these genes suggests that mutations have been selected against. It is possible that purifying selection was intense because the Mr2575 phenotype is already well adapted for pathogenicity to multiple hosts. Mr2575 was isolated from the pecan weevil in South Carolina but principally infected scarab beetle larvae in Maryland. The ability to attack multiple host species may require conservation of a generic pathogenicity "tool kit," and heterogeneous hosts could also constrain the ability to coevolve with just one host. We suggest that as hosts were heterogeneous *M. robertsii* instead locally adapted to more consistent components of the environment, such as grass roots. In spite of purifying selection of pathogenicity genes, the ability of the avirulent Δ*Mcl1*-RFP to cycle with the seasons in a similar manner to Mr2575-GFP suggests that insects were not the major constraint on Mr2575-GFP populations. Although it has wild-type infectivity to insects (28), the Δ*Mad2*-GFP mutant showed a linear decrease in population confirming that the ability to adhere to root surfaces plays an important part in maintaining population size and seasonal cycling, irrespective of the presence of insects. *Metarhizium* is thus in the fortuitous position of being able to maintain populations on plant roots while the additional lifestyle option of entomopathogenicity enables it to build up population levels above the carrying capacity of the rhizosphere, and possibly also escape competition from other microbes. There is not enough experimental data to conclude whether or not adaptation in a new environment is an important part of efficacious biological control (4). However, even in the absence of changes to virulence, if local adaption of Re04 and Re05 increases population size then this by itself may enhance biocontrol.

Little is known about the extended periods many plant, animal, and insect pathogens survive in soil in the absence of their hosts (38). As plant roots stabilize Mr2575-GFP populations it is feasible they play a similar role with other fungal pathogens. If so, this information would be useful for being able to predict and control outbreaks of commercially important pathogens. The data thus far from our field trials suggest that at least some *Metarhizium* strains pose little risk from gene flow or from evolution of host virulence in a new habitat. This knowledge alleviates science-based risk assessment concerns that are a potential obstacle to application of transgenic *Metarhizium* technologies. However, surveying native tropic interactions of biocontrol agents is

clearly crucial in order to fully understand patterns of establishment, and also to identify sets of genes that can be used for monitoring adaptation. Furthermore, with knowledge of such genes, the precision and malleability of molecular techniques could allow the design of multiple pathogens with different strategies to be used for different ecosystems. While root colonization increases the potential of an introduced biocontrol agent to replicate and persist in the environment providing long term control, it will also increase the difficulty of eliminating an agent following unanticipated environmental effects. Fortunately, the ability to selectively block root colonization by knocking out the *Mad2* gene provides an easy means of genetically containing the persistence of introduced or transgenic strains, if so required by regulatory agencies. This containment strategy could be immediately useful for transgenic strains which are not designed to recycle; e.g., *Metarhizium* strains expressing arthropod toxins to control vector borne diseases (2).

Materials and Methods

A detailed description of the methods is available in *SI Text*. Briefly, we used two strains of *Metarhizium robertsii* (Mr2575 and Mr23), two strains of *M. anisopliae* (Ma549 and Ma1080), and one strain of *Beauveria bassiana* (Bb252) (Table S4). Plasmid construction and fungal genetic transformation were performed as described in *SI Text*. Fungal release and field sample collection were performed as previously described (14); additional details are available in *SI Text*. We investigated genetic recombination by coinfecting insects and coinoculation of GFP and RFP-expressing strains in the field.

Southern blot, real-time PCR, heat tolerance, cell wall rigidity assays, insect bioassay, and other procedures were performed as described in *SI Text*.

Microarray Analysis. We used cDNA microarrays for array based mutation accumulation assays. The cDNA microarrays and RNA hybridizations were performed as described (22, 39). A loop design was used to compare gene differential expression between the original introduced strain (designated as Mr2575-GFP) and five field strains (designated as Re01, Re02, Re03, Re04, and Re05), and three microarray slides were used per comparison. Competitive hybridization of the second biological replicate was performed using a reverse dye-assignment to eliminate bias from dye incorporation. Microarray dataset and methods are available at the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo) under accession number GSE30175.

Statistical Analysis. Comparisons of population differences were analyzed using Wilcoxon-Mann-Whitney tests. Survival curves for insects were analyzed using the Log-rank (Mantel-Cox) test. The correlation between changes in gene expression and TATA boxes was analyzed by Fisher's exact test. Correlations in population sizes were analyzed using Spearman correlation analysis. Pair-wise comparisons of mRNA expression levels in qRT-PCR were analyzed using Student's T-test. Except for microarray data, all statistics were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, www.graphpad.com). P-values of less than 0.05 were considered statistically significant.

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