Supporting Information

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SI Text

Description of Fungal Strains and Culture Conditions. Two strains of *Metarhizium robertsii* (Mr2575 and Mr23), two strains of *M. anisopliae* (Ma549 and Ma1080) and one strain of *Beauveria bassiana* (Bb252) (Table S3) were obtained from the U.S. Department of Agriculture Entomopathogenic Fungus Collection (ARSEF) in Ithaca, NY.

M. robertsii strain Mr2575 was originally isolated from a pecan weevil in South Carolina. We also employed Mr2575 gene disruption mutants Δ*Mcl1* (disrupted in an immune evasion gene) and Δ*Mad2* (disrupted in a gene required for adhesion to roots) (1, 2). *Mcl1* is expressed exclusively in response to insect hemolymph, and it has no pleiotropic effects, whereas Δ*Mcl1* is rapidly attacked by hemocytes and shows low virulence to insects (1). Unlike the wild-type (WT) Mr2575, at 10^6 conidia/ml dosages Δ*Mcl1* was not pathogenic against scarab beetle larvae harvested from the prospective field plots and thus is unlikely to cause mortality under field conditions, where concentrations seldom exceed 10^6 conidia/gm of soil. Thus a reduction in persistence in the field will be due to failure of Δ*Mcl1* to cycle in insect populations. In contrast, disruption of *Mad*2 (for *Metarhizium* adhesin-like protein 2) has no effect on virulence but greatly reduces the ability of *Metarhizium* to adhere to and colonize roots (2).

Fungal strains were maintained at 27 °C on Potato Dextrose Agar (PDA). Genomic DNA was prepared from mycelia cultured for 36 hours in Sabouraud Dextrose Broth (SDB) as described (3).

Plasmid Construction and Fungal Transformation. The red fluorescent protein (RFP) gene was amplified from pMT-mRFP1 (4) with a *Bam*HI-containing primer (5′-CGC*GGATCC*GCAATTCATGGCCTCCTCCGAGGAC-3′) and an *Eco*RI-containing primer (5′-CG*GAATTC*GGTACCTTAGGCGCCGGTGGAG-3′) using AccuPrime Taq DNA Polymerase High Fidelity (Invitrogen). The digested product was cloned into *Bam*HI and *Eco*RI sites of pBarGPE1 (5) to generate pBarRFP. pBarRFP was digested with *Nde*I, blunted with T4 DNA polymerase, and the RFP cassette was excised with *Spe*I. pFBenGFP (6) was digested with *Eco*RI, blunted with T4 DNA polymerase, and the GFP cassette was excised with *Xba*I to generate linear plasmid pBen. The RFP cassette was ligated with pBen to generate pBenRFP.

Metarhizium robertsii strains Mr2575 and Mr23, *M. anisopliae* Ma549, *M. anisopliae* Ma1080, and *B. bassiana* strain Bb252 were transformed with *Agrobacterium tumefaciens* AGL-1 containing a binary vector pFBarGFP harboring a phosphinothricin acetyltransferase (bar) selective marker (7) to produce GFP-tagged transgenic strains Mr2575-GFP, Mr23-GFP, Ma549-GFP, Ma1080-GFP, and Bb252-GFP. Mr2575 and Mr2575-GFP were transformed with pBenRFP to produce Mr2575-RFP and Mr2575-GFP/RFP. The two fungal knockout mutants Δ*Mad2* and Δ*Mcl1* were transformed with pFBenGFP and pBenRFP harboring a benomyl resistance gene, benA3 from *A. nidulans*, respectively, to produce GFP or RFP-tagged transgenic strains Δ*Mad2*-GFP and Δ*Mcl1*-RFP. The verified transgenic isolates chosen for field trial and coinfection had parent-type growth rate, colony morphology, level of conidial production, and relative virulence as determined by standard laboratory protocols (8).

Fungal Release and Samples Collection. The field site was located at the University of Maryland, Paint Branch Turf Grass Research Facility at 395 Greenmeade Drive, MD. The soil is a Monmouth fine sandy loam. A block design was used to set up five 1 m^2 turf grass plots per treatment. The treatments were applications of 0 (control) and 10^9 spores/m^2 of: (*i*) Mr2575-GFP (pathogenic and root exudate competent); (*ii*) Δ*Mcl1*-RFP (to test the hypothesis that populations are maintained by cycling through insects); and (*iii*) Δ*Mad2*-GFP (to test the hypothesis that populations are maintained on roots). Conidia were applied with a hydraulic sprayer followed by irrigation to carry spores into the ground. During the application, the paths between plots were covered with plastic sheeting to prevent contamination. Transfer of the fungus by mechanical means was minimized by field test design and field test protocol that will include the buffer zones and tool and footwear disinfestations as described before (10).

Population levels of *Metarhizium* per gram of soil were determined on Rose-Bengal Selective Medium (RBSM) (10). At the end of each month, three soil samples were taken using a cork borer from a depth of 5 cm from each plot. Soil samples (1 g) were vortexed in 0.05% Tween 80 solution, serial dilutions were made, and 100 μL portions of each dilution were spread on each of five plates of RBSM. The red (RFP) or green (GFP) fluorescent introduced strains and recombinants were distinguished from indigenous strains of *Metarhizium* spp. by fluorescent microscopy. Total colony forming units (CFUs) of *Metarhizium* and the subset of these expressing GFP or RFP were counted and visually compared to the original strain to identify any noticeable changes in growth rate, colony morphology, and level of conidial production. Samples were also collected from untreated control turf plots to titer the native *Metarhizium* CFUs in the absence of recombinants.

The effects of temperature were plotted against CFUs to measure any influence that temperature may have on fungal survival (Fig. 1). All weather data were obtained from the National Oceanic and Atmospheric Administration (NOAA). To determine whether field recovered isolates could become more or less adapted to the field conditions than the introduced isolate, two field recovered isolates (Re04 and Re05) and the initial strain were reintroduced to the field. The experimental set up was randomized block design with five replications per isolate. The fungal culture, release, and soil sample collection, and strain identification were as described above.

Genetic Recombination of *Metarhizium robertsii* Mr2575 Revealed by Coinfecting Insects and by Coinoculation of GFP and RFP-Expressing Strains in the Field. We looked for genetic recombination *in insecta* by coinoculating larvae of *Galleria mellonella* with conidia of Mr2575-RFP and either Mr2575-GFP, Mr23-GFP, Ma549-GFP, Ma1080-GFP, or Bb252-GFP. Larvae were immersed in a suspension containing 1×10^7 conidia/mL of Mr2575-RFP and a second strain, and individually transferred to clean plastic cups. Mortality was checked twice daily and dead insects were transferred to sterile petri dishes containing a sterile moistened cotton ball to promote external conidiation. Counts of GFP and/or RFP fluorescent conidia harvested from cadavers were made using a Leica SP5 X confocal system. Spores were also plated onto PDA-dodine/cyclohexamide selective medium (PDADC) (11) to isolate recombinants and examine their genetic stability.

To determine the extent to which genetic exchange occurs in the field, turf plots were treated with (*i*) Mr2575-GFP/RFP which contains unlinked *gfp* and *rfp* genes, or (*ii*) Mr2575-GFP plus Mr2575-RFP.

Microarray Analysis. We used cDNA microarrays (12), for array based mutation accumulation assays. Gene expression levels of five isolates recovered from turf plots 4 y after initial application

were directly compared with the introduced strain (which was assayed contemporaneously after having been kept frozen during the intervening period of time) by cohybridizing cDNA's obtained six hours after transfer from SDB to 2% *Manduca sexta* cuticle. Cuticle triggers substantial transcriptional activity with broad interstrain variation in gene expression (12, 13). Single spore isolations were transferred to RBSM selective media twice to eliminate transitional variations due to physiological adaptation to field conditions. The RNA was extracted using a QIAGEN RNeasy Plant Mini kit and treated with DNase I.

The cDNA microarrays and RNA hybridizations were performed as described (12, 14). 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). Three microarray slides were used per comparison (cDNAs are replicated in triplicate on each slide). Competitive hybridization of the second biological replicate was performed using a reverse dye-assignment to eliminate bias from dye incorporation.

To obtain differential signal expression, the TM4 microarray suite (http://www.tm4.org) was used to process data. All microarrays underwent Locally Weighted Scatterplot Smoothing (LOWESS) normalization and flip-dye analysis for the Cy3 (green) and Cy5 (red) dyes. In-slide-replicate analysis was implemented on all normalized arrays, and the MeV (http://www.tm4.org/mev/) was used to perform t-tests. Resultant volcano plots were obtained, as well as significance values of differential gene expression, using a p-value of 0.05 as the cut-off. All genes with significant differential gene expression were categorized by fold change (15). Genes with a fold change of >1.5 with significant ($p < 0.05$) differential gene expression were analyzed and sorted by function.

Real-Time Quantitative PCR. SYBR dye technology (Applied Biosystems, CA) and quantitative real-time PCR (qRT-PCR) analysis were used to verify the validity of microarray results as described before (16). Primers corresponding to eight genes randomly chosen from microarray results were designed using Primer3 (v.0.4.0) software (http://frodo.wi.mit.edu/). Primers from *gpd* and *tef* house-keeping genes were tested as internal controls.

Heat Tolerance and Cell Wall Rigidity Assays. To test heat tolerance, 40 µL fresh spore suspensions (10^7 spores/mL) of the initial introduced strain Mr2575-GFP and the two recovered isolates (Re04 and Re05) were incubated at 42 °C for 6 h, and then transferred into 2 mL of SDB broth medium and incubated at 27 °C. Germination rates were recorded at 4 h intervals.

To test the effect on cell wall rigidity of mutations in cell wall genes, 10^7 conidia/mL spore suspensions of Mr2575-GFP and the two recovered isolates (Re04 and Re05) were used to make dilution series. Aliquots (5 µL) were spotted onto PDA or PDA complemented with Congo red (CR, 80–500 µg/mL) and the sensitivity to CR was determined by comparing the extent of colony formation (17).

Insect Bioassay. Newly emerged fifth-instar larvae of *Galleria mellonella* were used for bioassays by immersing them in spore suspensions containing 10^7 conidia/mL for 12 s. Insects were maintained at 27 °C and >95% relative humidity for 24 h and subsequently at 80% relative humidity. Each treatment had three replicates with 10 insects each, and the experiments were repeated twice. Mortality was recorded every 12 h.

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Fig. S1. qRT-PCR analysis of *hsp70* expression, effect of heat stress on conidia germination and bioassay analysis. (*A*) Real-time RT-PCR analysis of *hsp70* expression in Mr2575-GFP, Re04 and Re05. Two house-keeping genes *gpd*, glyceraldehyde 3-phosphate dehydrogenase (AY461523) and *tef*, translation elongation factor 1-α (AY445082) were used as references. Values represent the average fold change for each recovered isolate relative to mRNA levels in the introduced strain Mr2575-GFP. (*B*) Effect of heat shock on conidial germination rate of Mr2575-GFP (solid square), Re04 (solid triangle), and Re05 (solid diamond). Conidia pretreated with heat shock (42 °C for 6 h), and the germination rate was determined in SDB at 27 °C. (*C*) Controls (no heat shock). Germination rate of Mr2575-GFP, Re04 and Re05 was determined in SDB at 27 °C. (*D*) Kinetics of insect survivorship in bioassays. *Galleria mellonella* larvae were infected by topical application of 1×10^7 conidia/mL of Mr2575-GFP (solid diamond) and the two recovered field evolved isolates Re04 (solid square) and Re05 (solid triangle). Control insects (black solid circle) were dipped in 0.01% Tween 80. There was no significant difference between strains in their virulence to *G. mellonella*.

Fig. S2. Fluorescent images of transgenic *Metarhizium robertsii* strains tagged with GFP or RFP. Imaging conidia (*A*, *B*), germlings (*C*, *D*) and PDA-grown colonies (*E*, *F*) of GFP-tagged strain (left boxes) and RFP-tagged strain (right boxes).

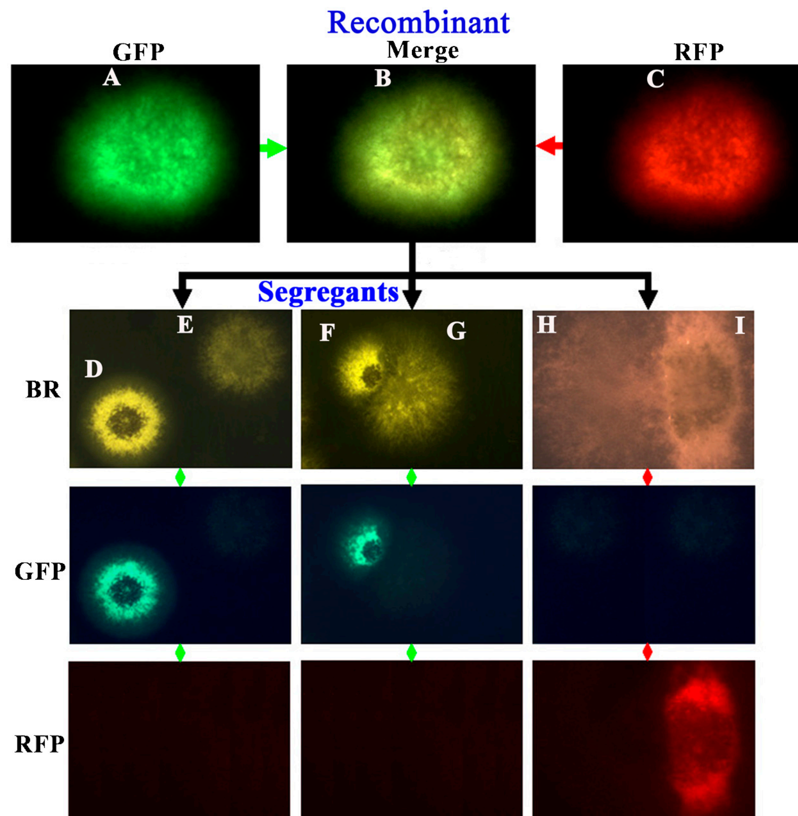

Fig. S3. The diploid recombinants were highly unstable. The diploid recombinants resulting from Mr2575-GFP × Mr2575-RFP crosses were highly unstable giving rise to parasexual segregants that were nonfluorescent or had lost either gfp or rfp. The recombinants expressing both green (GFP) and red (RFP) fluorescence proteins in GFP (*A*) and RFP (*C*) fields, respectively, and appear yellow (*B*) when the pictures are merged. (*D*) and (*F*) are GFP-expressing segregants; (*I*) is an RFP-expressing segregant; (*E*), (*G*), and (*H*) are nonfluorescing segregants. Only 0.134% of segregants still carried both GFP and RFP markers during the first parameiosis, and subsequent subcultures revealed no segregants expressing both GFP and RFP.

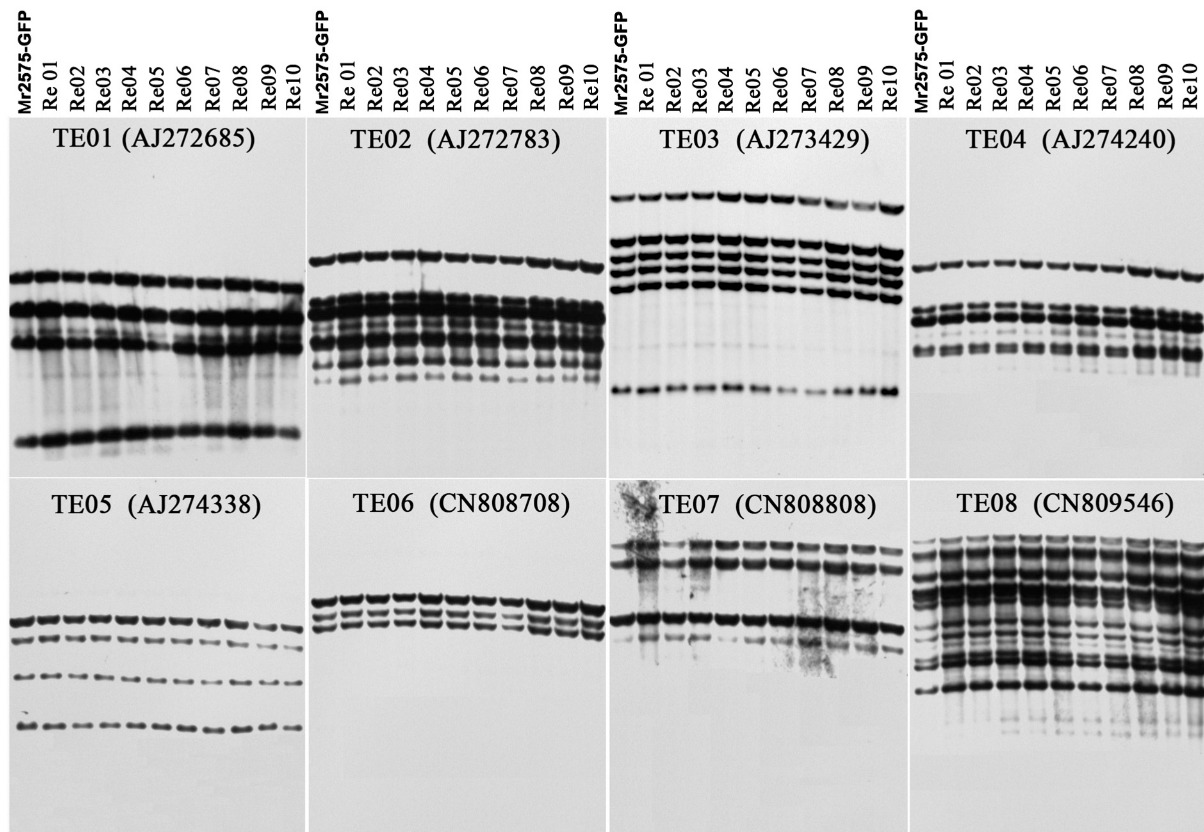

Fig. S4. Southern blot analysis of ten isolates recovered 4 y after being applied to turf plots show no chromosomal changes. Eight transposable elements (TE01-TE08, GenBank# J272685, AJ272783, AJ273429, AJ274240, AJ274338, CN808708, CN808808, and CN809546) were used as probes to detect jumping events in chromosome structure. Genomic DNA samples (10 ug) were digested overnight at 37 °C using 100 units of *Eco*RI, *Eco*RV, *Bam*HI, *Bgl*II, or *Xba*I (New England Biolabs). The digested DNA samples were electrophoresed in 0.8% TAE agarose gels at 22 V, and blotted onto Hybond N filters (Amersham). Probe preparation, hybridization and immunological detection were as previously described (3).

Table S1. Primers used in this study

No	Genes	Primer sequence (5′ to 3′ end)
1	CN809178	Forward: AGCCTCACCCTTCTTACGG Reverse: GCTTCTCCAACAATTCCACTG
2	AJ273870	Forward: GCGTTCAGCCATTATCCAG Reverse: CCAGAAAAGTTACCACAGGGA
3	AJ272799	Forward: GGTGTCTATTCTGGTGGTCTCA Reverse: CCAAACAGGGTGAAGAAAACT
4	AJ272811	Forward: CAGGGTCAGATGACGAACG Reverse: TGAAAACGACGAGTAGAATGC
5	CN809258	Forward: AGGAGATGGGTTCTGTGGG Reverse: GAGGGGCATTGGTTTTGTA
6	AJ273011	Forward: CTGCGGAAATGCTCTTGAC Reverse: TCGGTGCCGAAATACTTGA
7	CN808187	Forward: CCGGTTTCAACGGAGATG Reverse: CGAGACTTTGGGCAATGGT
8	CN808112	Forward: AATCCACCCTTCACCTCGTT Reverse: GGGAATGCCTTCTTTATCCTG
9	HSP70	Forward: CCGCACTCGCATCACAA Reverse: CCTGGACATCGGCATCG
10	gpd	Forward: CGCATCGTCTTCCGCAAC Reverse: TGGGAACACGCATGGACA
11	tef	Forward: AGGACGACAAGACTCACATC Reverse: GTTCAGCGGCTTCCTTCTC

Table S2. RNA intensities from eight selected genes with significant changes in gene expression (according to microarray analysis), were examined using qRT-PCR

Genes	Mr2575	Re01	Re02	Re03	Re04	Re05	Re06	Re07	Re08	Re09	Re10
AJ273870	1.00	1.09 ± 0.11 *	2.08 ± 0.16	2.22 ± 0.04	−1.18 ± 0.01	3.03 ±0.23	−1.28 ± 0.03	1.07 ± 0.09	2.29 ± 0.23	1.02 ± 0.13	1.57 ± 0.04
CN809178	1.00	1.17 ± 0.12	1.89 ± 0.17	1.99 ± 0.03	2.16 ± 0.01	2.28 ± 0.13	−1.26 ± 0.03	1.21 ± 0.11	1.71 ± 0.25	−1.11 ± 0.11	2.27 ± 0.03
AJ272799	1.00	1.62 ± 0.11	2.88 ± 0.16	2.10 ± 0.03	1.15 ± 0.01	2.32 ± 0.12	1.56 ± 0.02	1.46 ± 0.07	−1.29 ± 0.11	−1.27 ± 0.10	2.28 ± 0.03
AJ272811	1.00	−1.06 ± 0.09	1.18 ± 0.12	−1.28 ± 0.02	1.06 ± 0.01	3.16 ± 0.22	1.99 ± 0.02	−1.27 ± 0.07	−1.12 ± 0.13	−1.10 ± 0.11	−1.21 ± 0.05
CN809258	1.00	1.41 ± 0.11	1.21 ± 0.12	1.16 ± 0.03	1.82 ± 0.01	2.01 ± 0.02	1.04 ± 0.04	1.38 ± 0.11	1.20 ± 0.09	−1.12 ± 0.11	−1.35 ± 0.04
CN808112	1.00	1.14 ± 0.11	1.78 ± 0.15	−1.12 ± 0.02	2.05 ± 0.08	1.07 ± 0.02	1.81 ± 0.04	1.25 ± 0.11	2.19 ± 0.23	−1.28 ± 0.10	1.01 ± 0.06
AJ273011	1.00	−1.68 ± 0.11	−1.89 ± 0.16	1.21 ± 0.03	−1.20 ± 0.01	−2.28 ± 0.11	−1.46 ± 0.03	−1.29 ± 0.07	−2.07 ± 0.08	−1.45 ± 0.09	−1.17 ± 0.05
CN808187	1.00	−1.62 ± 0.08	−1.13 ± 0.11	1.22 ± 0.03	1.34 ± 0.02	−2.14 ± 0.06	−1.54 ± 0.02	−1.34 ± 0.06	−1.98 ± 0.08	−2.19 ± 0.07	−2.16 ± 0.04

House-keeping genes g*pd*, glyceraldehyde 3-phosphate dehydrogenase (Genbank #AY461523) and *tef*, translation elongation factor 1-α (Genbank #AY445082) were used as references. RNA extracted from ten recovered isolates (Re01-Re10) was compared to the isogenic input strain (Mr2575)

*RT-PCRs (±S.E. ΔCt) were calculated from three biological replicates. Values represent the average fold change for each recovered isolate relative to mRNA levels in the input strain.

Table S3. Recombinants recovered from fields treated with Mr2575-GFPxMr2575-RFP or Mr2575-GFP/RFP

Treatments	Isolates assayed	No. of recombinants	Percentage of recombinants
Mr2575-GFP×Mr2575-RFP	8,091	97	1.2%*
Mr2575-GFP/RFP	126,820	0	0

Over a four year period, all isolates from fields treated with Mr2575-GFP/RFP expressed both gfp and rfp.

*Recombinants expressing both gfp and rfp fluorescence recovered from fields treated with Mr2575-GFP and Mr2575-RFP in the first month post application.

Table S4. Fungal strains used in this study, and their origin

Strains	Species	Host origin/substrate	Geographic origin and date
Mr2575	*Metarhizium robertsii*	*Curculio caryae*, Coleoptera: Curculionidae	South Carolina ,USA, 1988
Mr23	*Metarhizium robertsii*	*Conoderus* sp., Coleoptera: Elateridae	North Carolina, USA, 1961
Ma549	*Metarhizium anisopliae* sensu lato	Metabiol.	Brazil, 1980
Ma1080	*Metarhizium* anisopliae sensu stricto	*Helicoverpa zea*, Lepidoptera: Noctuidae	Gainesville, Florida, USA, 1983
Bb252	*Beauveria bassiana* (Balsamo-Crivelli) Vuillemin	*Leptinotarsadecemlineata*,Coleoptera: Chrysomelidae	Orono, Maine, USA, 1978

Table S5. Recombinants recovered from insects coinfected with different strains of *M. robertsii*, *M. anisopliae*, or *B. bassiana*

Crosses	Conidia analyzed	Recombinant conidia *	Percentage ofrecombinants (%)
Mr2575-RFP × Mr2575-GFP	2,634	624	23.7
Mr2575-RFP × Mr23-GFP	>100,000	0	0
Mr2575-RFP × Ma549-GFP	>100,000	0	0
Mr2575-RFP × Ma1080-GFP	>100,000	0	0
Mr2575-RFP × Bb252-GFP	>100,000	0	0

*Recombinant conidia express both fluorescent GFP and RFP.