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Comparative Genomics Using Microarrays Reveals Divergence and Loss of Virulence-Associated Genes in Host-Specific Strains of the Insect Pathogen *Metarhizium anisopliae*▽†

Sibao Wang, Andreas Leclerque, Monica Pava-Ripoll, Weiguo Fang, and Raymond J. St. Leger*

Department of Entomology, University of Maryland, College Park, Maryland 20742

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Many strains of *Metarhizium anisopliae* have broad host ranges, but others are specialists and adapted to particular hosts. Patterns of gene duplication, divergence, and deletion in three generalist and three specialist strains were investigated by heterologous hybridization of genomic DNA to genes from the generalist strain Ma2575. As expected, major life processes are highly conserved, presumably due to purifying selection. However, up to 7% of Ma2575 genes were highly divergent or absent in specialist strains. Many of these sequences are conserved in other fungal species, suggesting that there has been rapid evolution and loss in specialist *Metarhizium* genomes. Some poorly hybridizing genes in specialists were functionally coordinated, indicative of reductive evolution. These included several involved in toxin biosynthesis and sugar metabolism in root exudates, suggesting that specialists are losing genes required to live in alternative hosts or as saprophytes. Several components of mobile genetic elements were also highly divergent or lost in specialists. Exceptionally, the genome of the specialist cricket pathogen Ma443 contained extra insertion elements that might play a role in generating evolutionary novelty. This study throws light on the abundance of orphans in genomes, as 15% of orphan sequences were found to be rapidly evolving in the Ma2575 lineage.

It is difficult to trace and reconstruct the evolutionary processes of diversification and radiation of species. In particular, genes that contribute to ecological diversification and the nature of the evolutionary forces acting during this process are poorly understood, partly because genes directly involved in ecological attributes are hard to identify (13). This is not the case with fungi, as they have genes encoding secreted products with specific environmental adaptations, e.g., scavenging nutrients and penetrating host barriers. During its pathogenic life cycle the ubiquitous insect pathogen *Metarhizium anisopliae* secretes a formidable array of hydrolytic enzymes, antimicrobial compounds, and toxins. These properties, plus its experimental tractability, have made *M. anisopliae* a common research subject and model system for studying pathogenicity and for developing useful products for medicine, agriculture, and biotechnology (33).

The phylogeny of the *Metarhizium* genus has been well characterized (12). It is a largely clonal organism (4), containing subtypes with wide host ranges (e.g., *M. anisopliae* var. *anisopliae* Ma2575) and subtypes that, like *M. anisopliae* var. *acridum* Ma324 (used for locust control), show specificity for certain locusts, beetles, crickets, homopterans, etc., and are unable to infect other insects (5). While some specialized lineages, such as *M. anisopliae* var. *acridum*, are phylogenetically distant from generalist strains, implying evolutionarily conserved host use patterns, closely related strains can also differ greatly in host range and requirements for germination (16, 40, 42). Evidence that most specialists arose from generalists includes the following: (i) the vast majority of isolates found in nature belong to the genetically very diverse *M. anisopliae* var. *anisopliae* and typically demonstrate wide host ranges; (ii) specialist strains are scattered among generalists in phylogenies and have independently adapted to different insects; (iii) specialization is associated with conditions that are assumed to be derived, including reduced diet breadth (2, 35, 40). Specialist and generalist strains are often closely linked in phylogenies, indicating that there are genetic mechanisms allowing rapid adaptation (40).

We are using genetic variation to explore the evolutionary history and pathogenic adaptations of *M. anisopliae*. The goal is to provide a detailed molecular classification of multiple strains and address the origins of intraspecific differences (gene loss/gain/divergence or modulation of gene expression). Correlation of strain differences with adaptations to specific hosts will identify the underlying regulatory, metabolic, and biosynthetic differences that define host preferences. To initiate this study, we used expressed sequence tag (EST) approaches to compare gene expression patterns between Ma2575 and Ma324 (17). These are two of the most distantly related strains and essentially span the range of variation within *M. anisopliae* (12, 40). About 60% of the ESTs expressed by Ma2575 during growth on insect cuticle encode secreted enzymes and toxins. We speculated that the large number and diversity of these effectors may be the key to Ma2575's ability to infect a wide variety of insects. In contrast, Ma324 ESTs revealed fewer hydrolytic enzymes and very few toxins. This relates to life-styles. Strain Ma2575 kills hosts quickly via toxins and grows saprophytically in the cadaver. In contrast, Ma324 causes a systemic infection of host tissues before the host dies. This study showed that ESTs allow dif-

* Corresponding author. Mailing address: Department of Entomology, 4112 Plant Science Building, University of Maryland, College Park, MD 20742-4454. Phone: (301) 405-5402. Fax: (301) 314-9290. E-mail: stleger@umd.edu.

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ferent pathogenic strategies to be understood from a broad perspective.

Patterns of gene duplication, divergence, and deletion can be specifically determined by heterologous hybridization of total genomic DNA to microarrays (11, 20, 27). Heterologous hybridization has provided a fast and powerful tool facilitating the merging of functional genomics with physiology, ecology, and evolution (7, 31, 38) in species of yeast (22, 27), fish (9, 24), mammals (23, 25), and plants (1, 15). We have already verified that an array of Ma2575 ESTs can be used for heterologous hybridization with cDNAs. Thus, Ma2575 arrays were used to probe the causes of sectorization (production of nonsporulating cultures) in two commercial strains of *M. anisopliae* var. *anisopliae*. Probes from both strains cross-reacted strongly with the arrays, although with different expression profiles (46). We also used Ma2575 arrays to identify hundreds of genes differentially regulated by Ma324 in response to host or nonhost cuticles (45). Although only 8% of paralogous Ma2575 genes have greater than 80% identity, we expected cross-hybridization would potentially overestimate the overlap in genes expressed by different strains. However, individual genes within gene families were distinguished, revealing processes unique to Ma324 (45). In this study we exploit the fact that heterologous cDNA can provide information on physiological processes to allow us to gain a mechanistic perspective on the different life-styles that exist in insect-fungus interactions.

MATERIALS AND METHODS

Fungal strains and growth conditions. This study employed six strains of *M. anisopliae* that represent the range of phylogenetic variation and evolutionary distance within the species and that differ in host ranges and in responses to chemical and physical stimuli (40). All fungal strains were obtained from the U.S. Department of Agriculture Entomopathogenic Fungus Collection in Ithaca, NY. The three generalist strains are all *M. anisopliae* var. *anisopliae*. Strain numbers are Ma2575, Ma549, and Ma820. Besides their original hosts, they can at least infect caterpillars (*Manduca sexta*) and crickets (*Acheta domestica*) in the lab and usually more insects, for example, Ma2575 was isolated from the pecan weevil but also infects locusts (10). Generalist strains can germinate in many nutrients and produce appressoria against a hard hydrophobic surface (plastic petri dish) in yeast extract medium (40). The three specialized strains infect only a few species. They show little or no germination in yeast extract or glucose medium, e.g., *M. anisopliae* var. *acridum* Ma324 (specific for acridids), *M. anisopliae* var. *majus* Ma297 (specific for scarab beetles), and *M. anisopliae* var. *anisopliae* Ma443 (specific for gryllids) (40). Fungal strains were routinely grown at 27°C on potato dextrose agar. For preparation of genomic DNA, fungal spores were cultured in Sabouraud dextrose broth at 27°C.

Genomic DNA preparation and construction of microarray. Fungal mycelia from 48-h Sabouraud dextrose broth cultures were collected by filtration and washed with sterile distilled water three times. The high-molecular-mass total genomic DNA of each strain was prepared as previously described (47). The construction of the cDNA microarrays used in this study has been previously described (46). This array harbors PCR-amplified fragments from the unique cDNA clones from *M. anisopliae* var. *anisopliae* Ma2575 and a few genes from *M. anisopliae* var. *acridum* Ma324 absent from the libraries of Ma2575. In total, 1,748 amplified clones were printed in triplicates on the slides. Additional background control was provided by 8 randomly distributed spots of 3× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Printing, hybridization, and scanning of slides was as described before (18).

DNA labeling and microarray hybridization. For DNA labeling experiments, 2 μg of total genomic DNA was fragmented by restriction endonuclease digestion with RsaI and MseI (New England Biolabs). The digested genomic DNAs were labeled by random priming with the BioPrime DNA labeling system (Invitrogen) according to the manufacturer's instructions. The aminoallyl-labeled genomic DNAs were concentrated with Microcon YM-30 filters (Millipore) and labeled with green Cy3 dUTP-tagged or red Cy5 dUTP-tagged nucleotides as described previously (27).

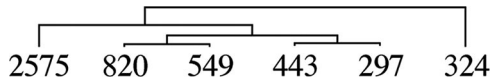

FIG. 1. A hierarchical tree constructed for three generalist strains (*Metarhizium anisopliae* var. *anisopliae* Ma2575, Ma549, and Ma820) and three specialized strains (*M. anisopliae* var. *acridum* Ma324, *M. anisopliae* var. *majus* Ma297, and *M. anisopliae* var. *anisopliae* Ma443), based on the microarray results.

Three microarray slides were used per comparison (cDNAs were replicated in triplicate on each slide). Each strain's DNA was cohybridized with Ma2575 DNA in dye-swapping replicate experiments and the relative hybridization efficiencies (fluorescence ratios) of their DNA for Ma2575 genes were compared. We used standard microarray hybridization conditions for this work (46) that provides a stringency at which 90% matching over 60 bases should suffice to form a stable hybrid (48).

CGH data analysis. The intensity of the spots on the cDNA slides was calculated using the Spotfinder version 2.2.4 program from the Microarray software suite TM4 from JCVI (http://www.tm4.org/spotfinder.html) and applying local background subtraction for each spot. The spotted microarray data preprocessing platform Ginkgo was used for comparative genomic hybridization (CGH) analysis (http://pfgrc.jcvi.org/index.php/bioinformatics/ginkgo.html). Intensity-dependent variation was normalized using the histogram mode centering algorithm with means and standard deviations as parameters. A dye consistency check was performed after normalization to eliminate systematic errors. This flip-dye analysis used a standard deviation criterion of threefold to filter spots with inconsistent values. Consolidation of the triplicate intensity values of channels A and B (1,756 unigenes, with channel A referring to the reference Ma2575) was also performed using the in-slide replicate algorithm of this platform.

Southern blot analysis. Genomic DNA samples (10 μg) were digested overnight at 37°C using 100 units of EcoRI, EcoRV, BamHI, BglII, or XbaI (New England Biolabs). Using standard protocols, digested samples were electrophoresed in 0.8% Tris-acetate-EDTA–agarose gels and blotted onto Hybond N filters (Amersham). Probes were amplified from cDNA clones and digoxigenin (DIG) labeled using the random primed DNA labeling kit (Roche). The genes analyzed and their primers are listed in Table S1 in the supplemental material. Following hybridization (42°C for 4 h) membranes were washed twice in 2× SSC containing 0.1% sodium dodecyl sulfate (room temperature) and twice in 0.5% SSC containing 0.1% sodium dodecyl sulfate (65°C). Genomic DNA hybridized with probe was immunologically detected by anti-DIG antibody conjugated with alkaline phosphatase (Roche), according to the manufacturer's instructions.

Microarray data accession number. The microarray data obtained in this study have been deposited in the NCBI Gene Expression Omnibus database under accession number GSE14846.

RESULTS

CGH. Fragmented genomic DNA from six *M. anisopliae* strains (Ma297, Ma324, Ma443, Ma549, Ma820, and Ma2575) was cohybridized with DNA from strain Ma2575 to microarrays of Ma2575 ESTs. The resulting hybridization patterns were used to organize the strains into a hierarchical tree that placed *M. anisopliae* var. *acridum* strain Ma324 into a relative outgroup position (Fig. 1). This is consistent with phylogenies of *Metarhizium* spp. (12). Normalized average intensity ratios (I_B/I_A, with I_A for channel A referring to the reference strain Ma2575) for the different strain combinations in three replicates varied from 0.94 (Ma2575 versus Ma2575) to 0.41 (Ma2575 versus Ma324) (Table 1) in replicate dye-swap array experiments. The 0.94 value indicates the limit of detection of significant differences in hybridization with three replicate slides.

To investigate the relationship between the strength of hybridization of homologous gene pairs and their degree of nucleotide identity, we used 30 corresponding cDNA sequences from *M. anisopliae* strains Ma324 and Ma2575 identified by

TABLE 1. Normalized average I_B/I_A ratios for the different strain combinations[a]

Cohybridization	Avg in-slide I_B/I_A ratio
Ma2575 vs Ma2575	0.94
Ma820 vs Ma2575	0.69
Ma549 vs Ma2575	0.72
Ma443 vs Ma2575	0.62
Ma297 vs Ma2575	0.55
Ma324 vs Ma2575	0.41

[a] The I_A (from channel A) refers to the signal for the reference strain, *Metarhizium anisopliae* var. *anisopliae* Ma2575, and I_B is the normalized signal value of the indicated test strain (Ma820, Ma549, Ma443, Ma297, or Ma324).

Freimoser et al. (17) to plot the percent nucleotide identity against $\log_2$ ratios calculated from cohybridization data (Fig. 2). The percentage of nucleotide sequence identity ranged from 87.7% to 99.5%. Linear regression analysis was employed to predict the best-fit line, demonstrating a moderately good correlation ($r^2 = 0.66$; $P < 0.0001$) between nucleotide sequence identity and the $\log_2$ ratio. It is likely that the correlation was not closer to 1, because hybridization is also influenced by the GC/AT ratio and the distribution of highly conserved regions in sequences (48).

Categorization of conserved and divergent genes. Greater than 90% of all the arrayed genes showed decreasing efficiency of hybridization with increasing distance of the two strains as determined by the normalized intensity ratio averages (Table 1). However, individual genes evolve at different rates. Using the cohybridization of strains Ma324 and Ma2575 as a point of reference, we arbitrarily divided genes into three groups according to their normalized intensity ratios. The high-similarity group 1, with I_B/I_A ratios of ≥ 0.5, corresponds to $\geq 95\%$ nucleotide sequence similarity; the moderate-similarity group 2 with I_B/I_A ratios of 0.25 to 0.5 corresponds to 60 to 95% similarity; the group 3 sequences with I_B/I_A ratios of <0.25 possess low homologies or are absent in Ma324.

Previously, ESTs of *M. anisopliae* were subdivided into broad functional categories (17). Functional categories where >90% of the genes fall into group 1, i.e., have high nucleotide identity, include amino acid metabolism, nucleotide metabolism, cell cycle/division and growth, N, P, and S metabolism, translation and posttranslational modification, regulatory function, DNA replication, recombination and repair, energy metabolism, transcription, and RNA processing and degradation. Thus, as expected, major life processes are highly conserved, presumably due to purifying selection.

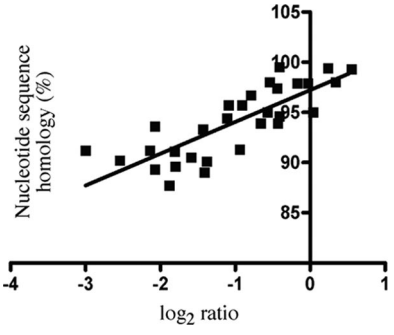

FIG. 2. Standard curve used to determine the relationship between nucleotide sequence similarity for genes of *Metarhizium anisopliae* strains Ma2575 and Ma324 and normalized fluorescence ratios. Sequences for 30 *M. anisopliae* genes of known similarity were included (17). The means of the $\log_2$ ratio values for each gene were plotted against the percent similarity.

TABLE 2. Functional categories of *M. anisopliae* var. *anisopliae* Ma2575 genes predicted to be of low nucleotide sequence similarity or absent in *M. anisopliae* var. *acridum* Ma324

Functional category	No. of genes	% of total
Hypothetical proteins or orphans	55	46.61
Cell metabolism	19	16.10
Lipid metabolism	7	5.93
Carbohydrate metabolism	7	5.93
Amino acid metabolism	3	2.54
Nucleotide metabolism	2	1.69
Cell structure and function	13	11.02
Cell wall protein	7	5.93
Transport proteins	3	2.54
Signaling	3	2.54
Transposable elements	10	8.47
Stress response and defense	10	8.47
Stress response	4	3.39
Detoxification	3	2.54
Secondary metabolites	3	2.54
RNA metabolism	4	3.39
Protein metabolism	4	3.39
Ribosomal proteins	1	0.85
Proteolysis	2	1.69
Protein modification/targeting	1	0.85
Energy metabolism	3	2.54
Total	118	100.00

A total of 118 (6.7%) of Ma2575 genes were predicted to be highly divergent or absent ($I_B/I_A < 0.25$) in Ma324, indicative of functional differences (Table 2; Fig. 3). The absence of homologous sequences in strain Ma324 was confirmed by Southern hybridization analysis with probes comprising the complete *M. anisopliae* strain Ma2575 open reading frame (ORF) of 26 genes (Fig. 4, 5, and 6). The genes were chosen as a representative sampling of transposable elements, cell metabolism, stress response and defense, cell structure, and signaling functions. In every case, the Southern blotting data validated the results from microarrays.

Thirty-five out of 237 Ma2575 orphan sequences (i.e., with no homologs in databases) only hybridized to Ma2575 DNA. Low-stringency Southern hybridization confirmed the absence of CN808668 in the genome of Ma324 (Fig. 5E). Several other sequences with homologs in phylogenetically distant organisms but none in published fungal genomes hybridized only to Ma2575 DNA, e.g., EST CN809303, which is similar ($E = 2 \times 10^{-7}$) to a bacterium (*Rubrobacter xylanophilus*) short chain dehydrogenase/reductase. Its absence in Ma324 and Ma443 was confirmed by Southern analysis (Fig. 5A). However, se-

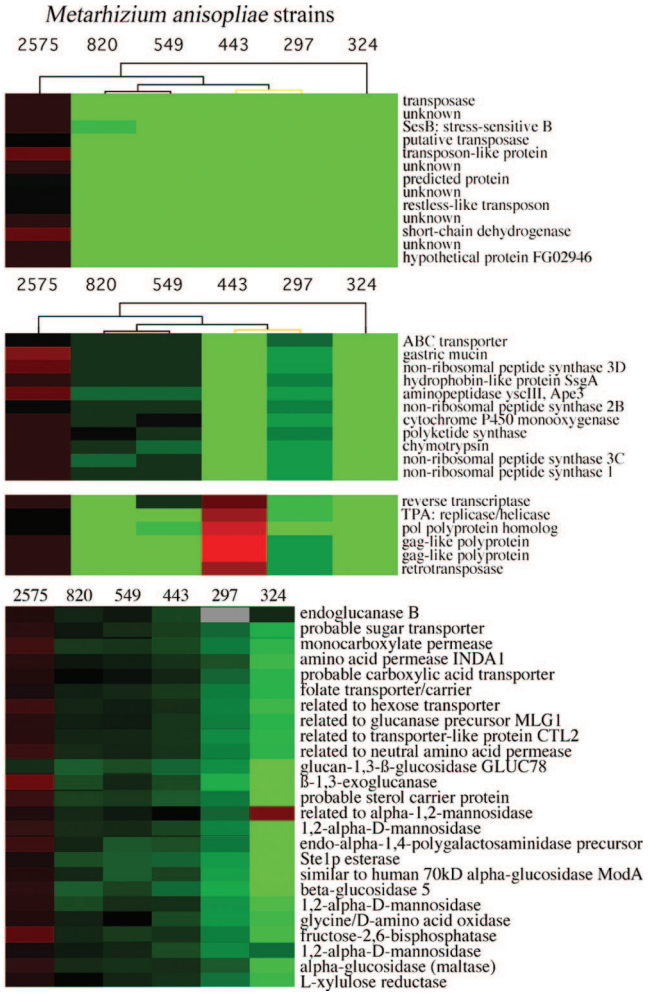

FIG. 3. Gene content of *Metarhizium* strains. Cluster analysis results are shown for portions of microarray cohybridizing DNA from five strains with Ma2575 DNA. Some sequences are absent or have diverged beyond hybridization, but Ma443 contains more copies of some insertional elements while others (bottom) are missing.

quences from Ma2575 that are highly conserved in other fungal species could also show rapid evolution and loss in *Metarhizium* genomes. Thus, the strain Ma2575 EST AJ273858, which is similar (4×10^{-42}) to the ankyrin repeat protein (stress response protein) of *Aspergillus fumigatus*, was highly divergent in most strains. Low-stringency Southern hybridization confirmed the absence of this sequence in Ma443 and Ma324 (Fig. 5B).

Ten genes encoding proteins involve in the stress response and defense were found to hybridize poorly with *M. anisopliae* var. *acridum* Ma324 genomic DNA. Four of these had high similarity to genes encoding proteins involved in the stress response. Aside from the ankyrin repeat protein (AJ273858), these included CN809313, similar (6×10^{-55}) to the extracellular cell wall glucanase Crf1/allergen Asp F9 of *Penicillium marneffei* (XP_002146076); CN808429, similar (3×10^{-49}) to the integral membrane protein Mpv17 (ethanol metabolism) of *Aspergillus fumigatus*; and CN808677, similar (2×10^{-10}) to the Wsc4p cell wall integrity and stress response component of

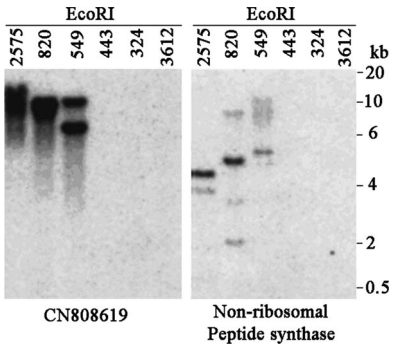

FIG. 4. Southern hybridization analysis of *Metarhizium anisopliae* var. *anisopliae* (strains Ma2575, Ma820, Ma549, and Ma443) and *M. anisopliae* var. *acridum* (strains Ma324 and Ma3162) DNA with DIG-11-dUTP-labeled probes corresponding to the ORF sequences of the *M. anisopliae* Ma2575 polyketide synthase (CN808619) and peptide synthase genes. Genomic DNA from each strain was digested with EcoRI. Molecular size markers are indicated on the right. Washes were performed at reduced stringency (65°C in 0.5× SSC).

Saccharomyces cerevisiae. Of the others, three were involved in detoxification, including AJ273280, which is similar (1×10^{-14}) to an ABC transporter of *Emericella nidulans*, which is involved in contact-dependent secretion, virulence, and resistance to antifungal compounds (43), CN808854, similar (8×10^{-121}) to an *Aspergillus fumigatus* flavin-binding monooxygenase-like protein, and CN808382, similar (1×10^{-161}) to the pyridine nucleotide-disulfide oxidoreductase of *Aspergillus clavatus*. The latter two were confirmed to be absent in Ma324 and Ma443 genomic DNA (Fig. 5).

Nineteen genes encoding proteins involved in lipid, carbohydrate, and amino acid metabolism were also divergent. Southern analysis confirmed that both Ma324 and Ma443 lacked genes encoding extracellular 3-ketosteroid 1-dehydrogenase (CN809570), squalene-hopene cyclase (CN808855), and cytochrome P450 monooxygenase (CN808662), all of which are involved in lipid metabolism, and carbohydrate glycosyl hydrolase (CN808813) and short chain dehydrogenase CN809303 (involved in carbohydrate metabolism) (Fig. 5A). Except for CN809303, the Ma2575 genes were very similar to sequences in *Aspergillus fumigatus* (Table 3). The EST AJ274133, which encodes an esterase STE1 that increases virulence when expressed in *Beauveria bassiana* (34), was also absent in Ma324 (Fig. 5A).

Other poorly conserved genes in Ma324 (e.g., those with I_B/I_A ratios of <0.25) have previously been associated with *M. anisopliae* Ma2575 virulence. These include a set of functionally coordinated genes comprising four nonribosomal peptide synthases and a polyketide synthase (CN808619) likely involved in toxin generation (17, 45), which were divergent or missing in Ma443 and Ma324 as confirmed by Southern blot analysis. We confirmed that the absence of these sequences was a characteristic of *M. anisopliae* var. *acridum* by including genomic DNA from *M. anisopliae* var. *acridum* strain 3612 in the Southern blot assays (Fig. 4). The cytochrome P450 (CN809217) was very similar ($E = 6 \times 10^{-70}$) to an enzyme involved in secondary metabolite biosynthesis in *Fusarium sporotrichioides* (6). Other key divergent or missing genes in specialists included a chymotrypsin (AJ273081) produced by

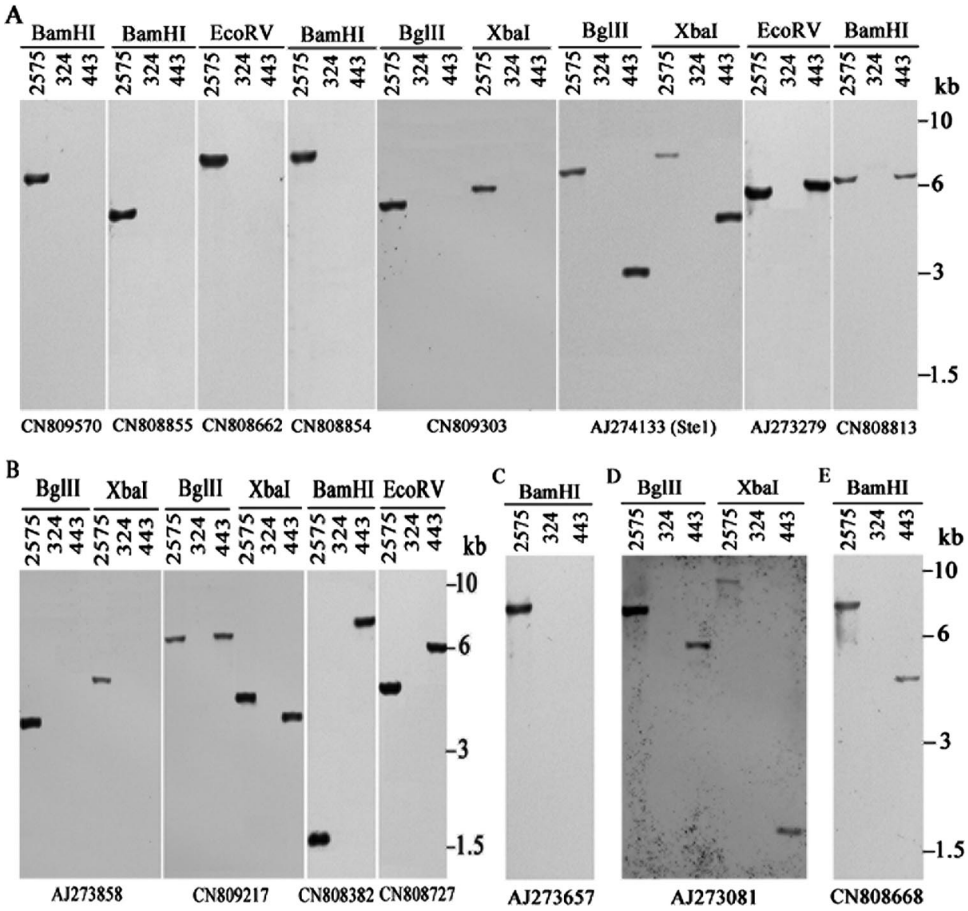

FIG. 5. Southern hybridization analysis of *Metarhizium anisopliae* var. *anisopliae* Ma2575, *M. anisopliae* var. *acridum* Ma324, and *M. anisopliae* var. *anisopliae* Ma443 DNA with DIG-11-dUTP-labeled probes corresponding to the complete ORF sequences of the Ma2575 genes involved in cell metabolism (CN809570, CN808855, CN808662, CN808854, CN809303, AJ274133 [Ste1], AJ273279, and CN808813) (A); stress response and defense (AJ273858, CN809217, CN808382, and CN808727) (B); cell structure and signaling function (AJ273657) (C); proteolysis (AJ273081) (D); and an orphan gene (CN808668) (E). The ORFs were amplified from the unique cDNA with the primer set M13F/M13R. Each blot contains BamHI-, BglII-, EcoRV-, or XbaI-digested genomic DNA from Ma2575, Ma324, and Ma443. Washes were performed at low stringency (65°C in 0.5× SSC). Molecular size markers are indicated on the right.

Ma2575 during penetration of host cuticle (36) and absent from published fungal genomes (21). Southern blot analysis confirmed its absence in both Ma443 and Ma324 (Fig. 5D), suggesting that this gene is highly expendable and multiple lineages have independently lost it. An aflatoxin biosynthesis ketoreductase (CN808727) involved in the biosynthesis of aflatoxins by *Aspergillus parasiticus* (44) was absent in Ma324 (Fig. 5). EST AJ274066, a Ma2575 homolog (7.9×10^{-58}) of yeast aminopeptidase yscIII, had diverged beyond hybridization in Ma443 but was present in Ma324. The yeast enzyme is a vacuolar Y-aminopeptidase with a viable null mutant that cannot hydrolyze Lys-Ala (19).

A Ma2575 hydrophobin (AJ274156) involved in cell wall structure and nonspecific adhesion to hydrophobic cuticle surfaces (40) showed only 38.9% sequence identity to its counterpart in Ma324 (BQ143508) according to CLUSTAL W alignment analysis. Several other cell wall proteins were also highly divergent, including glycophosphatidylinositol-anchored cell wall beta-1,3-endoglucanase (AJ273279), which is important for cell wall stability (32), mixed-linked glucanase precursor MLG1 (CN808527), cell wall synthesis protein (CN808518), cell wall protein (CN808796), putative endochitinase CHI2 (CN808888), and an extensin-like protein (CN808213).

Ten of the transposable elements in Ma2575, including Restless-like transposase (AJ274202), polyprotein (AJ272783 and AJ274240), transposases (AJ274329, AJ272685, AJ273458, CN808708, and CN808808), reverse transcriptase (CN809546), and Gag-like polyprotein (AJ274338), either lack homologs in the other strains or they are highly divergent (Fig. 6). All were absent in Ma324. However, a transposase (CN808708) and Gag-like polyprotein (AJ274338) that each hybridized to four bands with Ma2575 DNA were highly redundant in Ma443 (Fig. 6A). These findings are in agreement with the array data and suggest that these sequences had multiplied in the Ma443 genome.

Thirty Ma324 sequences were also arrayed with the Ma2575 sequences and employed in dye swap experiments. These sequences included CN808542, similar (1×10^{-33}) to an endoglucanase B from *Aspergillus kawachi*, and

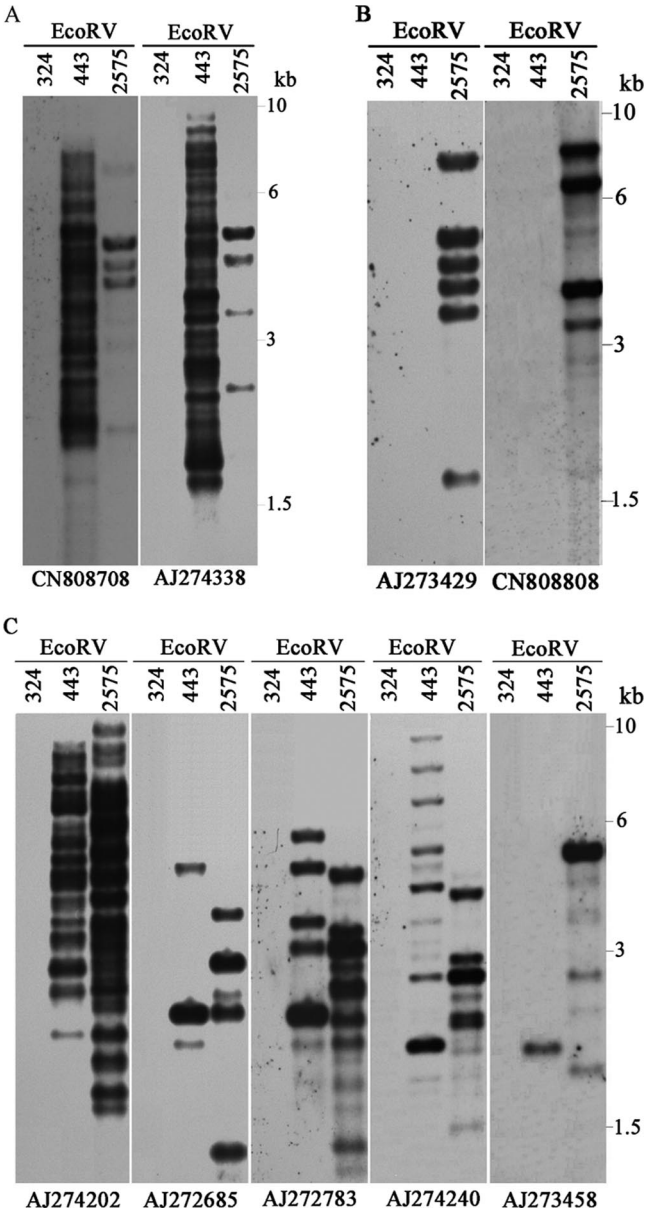

FIG. 6. Southern hybridization analysis of *Metarhizium anisopliae* var. *anisopliae* Ma2575, *M. anisopliae* var. *acridum* Ma324, and *M. anisopliae* var. *anisopliae* Ma443 DNA with DIG-11-dUTP-labeled probes corresponding to the complete ORF sequences of the Ma2575 transposable elements. (A) Hybridization with probes of transposase (CN808708) and Gag-like polyprotein (AJ274338), which were absent in Ma324 but highly redundant in Ma443. (B) Hybridization with probes of transposase-like protein (AJ273429) and transposase (CN808808), which were absent in both Ma324 and Ma443. (C) Hybridization with probes of Restless-like transposase (AJ274202), putative transposases (AJ272685 and AJ273458), and polyproteins (AJ272783 and AJ274240), which were absent in Ma324. The blot contains Ma324, Ma443, and Ma2575 genomic DNA digested with EcoRV. Washes were conducted at low stringency (65°C in 0.5× SSC). Molecular size markers are indicated on the right.

CN809639, related (3×10^{-43}) to α-1,2-mannosidase. The endoglucanase B was conserved in all strains, while the α-1,2-mannosidase hybridized to Ma324 genomic DNA to a much greater extent.

DISCUSSION

The central strategy of this work was to examine pathogen genome evolution and host range usage by confining the comparisons within a single species while exploring adaptive radiation within this species as far as possible. *M. anisopliae* is a particularly good model system for studying evolutionary processes because it consists of strains that in terms of developmental processes are almost indistinguishable from each other but that differ dramatically in host range and possibly saprophytic competence. Given that specialization has occurred many times in *M. anisopliae*, this organism provides an unusual opportunity to study a species containing a large number of independently evolved models of adaptation and response. These comparisons provide a novel perspective on the evolution and strategies of highly specialized fungi. As a radiating species, its natural molecular variation offers the chance of finding processes of both adaptive change and phylogenetic differentiation still in operation, even in intermediate states.

We used PCR-amplified cDNA, as their longer sequences are better than short oligonucleotides for heterologous gene expression studies. The Ma2575 sequences we arrayed are full length, or nearly so, and most are 600 to 800 bp long. For genes encoding secreted products (hydrophobins, subtilisins, chitinases, etc.), nucleotide identities between orthologs in Ma324 and Ma2575 range from 93% to 98%, with the most closely matching sequences at functional domains (3, 17, 37). The findings presented in this study reinforced these data, showing that ~6.7% of Ma2575 genes were absent or highly divergent in Ma324. We may have underestimated the level of functionally divergent genes in different lineages, because our ability to detect divergence or inactivation of genes relies on sequence divergence or deletion of the locus preventing hybridization with the probe on the microarray. However, mitigating this consideration is that a small amount of sequence divergence may change hybridization intensity. Also, for genes facilitating an opportunistic life-style, we are confident that the arrays included loci required for living in multiple habitats. That is because the libraries were created from a strain with a broad host range and the arrayed genes included those expressed when the organism lives saprophytically (46). It is likely, given the reduced diet breadth of specialized lineages (40), that these genes will be represented in any strains undergoing degradation. Thus, our experiments have a high probability of providing a picture of the features characterizing specialized and generalized fungal pathogen populations.

The evidence for divergence of genes in Ma324 suggests a potential role for specific gene loss in the emergence of some of the differences between strains in diet breadth and host range. Although Ma324 is the most divergent strain from Ma2575, other specialists also show reduced diet breadth and loss of toxins (41). Degenerative changes occur in bacteria that are obligate pathogens because some genes are no longer needed or possibly because specialization reduces effective population size, which increases fixation of deleterious mutations (28). However, gene loss has also been proposed as an important force driving the evolution of recently evolved novel lineages (29).

The most obvious categories of genes expected to undergo degradation or deletion in a specialized pathogen will be those

TABLE 3. *M. anisopliae* var. *anisopliae* Ma2575 genes predicted to be of low similarity (subgroup 3) in the genome of *M. anisopliae* var. *acridum* Ma324 based on CGH analysis

Category and gene identifier[a]	Putative or known function	Best match	Closest relationship	E-value
Transposable elements				
CN808708	Transposase	AAB63315	*Botryotinia fuckeliana*	7.0E-146
AJ274338	Gag-like polyprotein	CAA96388	*Fusarium poae*	6.0E-07
AJ274202	Restless-like transposase	AAK16925	*Nectria hematococca*	200E-13
AJ272685	Transposase	AAV28708	*Fusarium oxysporum*	5.0E-13
AJ273458	Transposase	AAV28708	*Fusarium oxysporum*	2.0E-59
CN809546	Reverse transcriptase	ABC24970	*Monascus pilosus*	4.0E-34
AJ272783	Polyprotein	CAB91877	*Phaeosphaeria nodorum*	5.0E-165
AJ273429	Transposase	AAV28708	*Fusarium oxysporum*	6.0E-33
AJ274240	Polyprotein	CAB91877	*Phaeosphaeria nodorum*	1.0E-22
CN808808	Transposase-like protein	BAA32244	*Fusarium oxysporum*	7.0E-39
Stress response and defense				
Stress response				
AJ273858	Ankyrin repeat protein	EDP48589	*Aspergillus fumigatus*	4.0E-42
CN809313	Ice nucleation protein	AAQ14297	*Gibberella acuminata*	4.0E-78
CN808429	Integral membrane protein, Mpv17/PMP22 family	XP_748225	*Aspergillus fumigatus*	3.0E-49
CN808677	Cell wall integrity and stress response component 4; Wsc4p	NP_011835	*Saccharomyces cerevisiae*	2.0E-10
Detoxification				
CN808854	Flavin-binding monooxygenase-like protein	XP747888	*Aspergillus fumigatus*	8.0E-121
CN808382	Pyridine nucleotide-disulfide oxidoreductase	XP001272526	*Aspergillus clavatuse*	1.0E-161
AJ273280	ABC transporter	AAF29805	*Emericella nidulans*	1.0E-14
Secondary metabolites				
CN809217	Cytochrome P450	AAK33073	*Fusarium sporotrichioides*	3.0E-138
CN808619	Polyketide synthase	AAR90254	*Botryotinia fuckeliana*	1.0E-66
CN808727	Aflatoxin biosynthesis ketoreductase NOR-1	Q00278	*Aspergillus parasiticus*	1.0E-24
Cell structure and function				
Cell wall protein				
CN808527	Related to mixed-linked glucanase precursor MLG1	CAB88654	*Neurospora crassa*	1.0E-37
CN808796	Cell wall protein	BAD01559	*Aspergillus kawachii*	8.0E-13
CN808518	Cell wall synthesis protein	BAC82548	*Penicillium chrysogenum*	1.0E-57
CN808888	Putative endochitinase CHI2	CAC07216	*Metarhizium anisopliae*	1.0E-137
AJ273279	GPI-anchored cell wall beta-1,3-endoglucanase EglC	XP001939526	*Pyrenophora tritici-repentis*	3.0E-70
CN808213	Extensin-like; with SH3 Src homology domain	NP594446	*Schizosaccharomyces pombe*	1.0E-15
AJ274156	Hydrophobin-like protein ssgA precursor	AAA33418	*Metarhizium anisopliae*	3.0E-50
Transport proteins				
CN809357	Transporter, putative	CAE47906	*Aspergillus fumigatus*	2.0E-32
CN808929	Related to NCE102 protein, nonclassical export membrane protein	CAD37009	*Neurospora crassa*	9.0E-25
CN808769	Adaptin-ear-binding coat-associated protein	DAA01434	*Mus musculus*	5.0E-16
Signaling				
AJ273356	Putative mitogen-activated protein kinase kinase 2	CAC07966	*Leishmania mexicana*	2.0E-17
AJ273657	SRPK1-like kinase	NP_013943	*Saccharomyces cerevisiae*	7.0E-12
CN808754	Serine kinase SRPK2	AAC05299	*Homo sapiens*	4.0E-21
Cell metabolism				
Lipid metabolism				
AJ274133	Esterase; STE1	CAB63910	*Metarhizium anisopliae*	3.9E-99
CN808855	Squalene-hopene cyclase	XP751356	*Aspergillus fumigatus*	1.0E-74
CN808662	Cytochrome P450 monooxygenase	AAF26280	*Aspergillus parasiticus*	8.0E-09
CN808333	Putative P450 monooxygenase	AAO73449	*Fusarium sporotrichioides*	1.0E-173
CN809570	Putative 3-ketosteroid-delta-1-dehydrogenase	NP_822771	*Streptomyces avermitilis*	3.0E-15
CN809534	Phosphatidylinositol phospholipase	CAB92911	*Candida albicans*	8.0E-23
CN808853	Lanosterol synthase-related protein	XP_326612	*Fusarium oxysporum*	2.0E-22

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TABLE 3—*Continued*

Category and gene identifier[a]	Putative or known function	Best match	Closest relationship	E-value
Carbohydrate metabolism				
CN808775	Homocitrate synthase, mitochondrial precursor	Q12726	*Yarrowia lipolytica*	2.0E-76
CN808813	Glycosyl hydrolase	XP001273136	*Aspergillus clavatus*	1.0E-70
CN809153	Probable homoaconitase precursor	CAD71225	*Neurospora crassa*	3.0E-84
CN809303	Short chain dehydrogenase/reductase family protein-like	BAD09200	*Oryza sativa*	2.0E-09
CN809316	*n*-Alkane-inducible cytochrome P450 gene (ALK1) essential for *n*-decane assimilation	BAA31433	*Yarrowia lipolytica*	2.0E-22
CN808940	CGI-49 protein, saccharopine dehydrogenase	AAD34044	*Homo sapiens*	1.0E-15
CN808435	Glucosidase	AAO34674	*Gibberella zeae*	7.0E-17
Amino acid metabolism				
AJ274200	Proline oxidase	CAC18796	*Emericella nidulans*	2.0E-42
CN808802	Glycine/D-amino acid oxidases	ZP_00096788	*Novosphingobium aromaticivorans*	4.0E-14
CN808928	Glutamine synthetase (glutamate-ammonia ligase)	EAA69962	*Gibberella zeae* PH-1	1.0E-119
Nucleotide metabolism				
CN809258	Similar to rRNA intron-encoded homing endonuclease	XP_372959	*Homo sapiens*	1.0E-17
CN808663	Adenine phosphoribosyltransferase	AAA68956	*Mastomys hildebrantii*	4.0E-06
Energy metabolism				
CN808777	Putative oxidoreductase	AAL58884	*Aspergillus nidulans*	4.0E-32
CN809440	Cytochrome p450 (E-class), putative	CAF32039	*Aspergillus fumigatus*	5.0E-07
AJ274160	Probable short chain dehydrogenase	AAG05038	*Pseudomonas aeruginosa*	1.0E-13
Protein metabolism				
Ribosomal protein				
CN809545	40S ribosomal protein S0	Q01291	*Neurospora crassa*	1.0E-110
Proteolysis				
AJ273081	Chymotrypsin	CAB44651	*Metarhizium anisopliae*	2.0E-51
CN809169	Glutathione *S*-transferase I	NP_588298	*Schizosaccharomyces pombe*	2.0E-42
Protein modification/ targeting				
CN809424	Transferase family protein	XP_001266554	*Neosartorya fischeri*	2.0E-39
RNA metabolism				
CN809525	Probable transcription factor	T37601	*Schizosaccharomyces pombe*	7.0E-09
CN809374	Flavocytochrome b_{558} (NADPH oxidase gp91^{phox})	BAA95154	*Tursiops truncatus*	1.0E-07
CN808682	TPR (transcriptional repressor)-containing protein Mql1	AAK58576	*Ustilago maydis*	1.0E-107
CN809621	Antisilencing protein, causes depression of silent loci when overexpressed; Asf1p	NP_012420	*Saccharomyces cerevisiae*	4.0E-42
Hypothetical proteins or orphans				
CN808665	Hypothetical protein FG02705	EAA67953	*Gibberella zeae PH-1*	5.0E-17
CN808811	Hypothetical protein FG02893	EAA72393	*Gibberella zeae PH-1*	2.0E-64
CN808833	Hypothetical protein MG04736	EAA50977	*Magnaporthe grisea*	6.0E-53
CN809268	Hypothetical protein FG10959	EAA74243	*Gibberella zeae* PH-1	2.0E-07
CN809246	Hypothetical protein FG11155	EAA75365	*Gibberella zeae* PH-1	1.0E-19
CN809389	Predicted protein	EAA51141	*Magnaporthe grisea*	3.0E-72
AJ274045	Hypothetical protein FG06966	EAA76426	*Gibberella zeae* PH-1	1.0E-43
CN809166	Hypothetical protein MG03337	EAA51742	*Magnaporthe grisea*	2.0E-22
CN808764	Hypothetical protein FG09539	EAA76655	*Gibberella zeae* PH-1	9.0E-12
CN808135	Hypothetical protein MG09337.4	EAA51320	*Magnaporthe grisea*	2.0E-58
AJ273482	Predicted protein	EAA68277	*Gibberella zeae* PH-1	4.0E-16
AJ272778	Hypothetical protein AN2582.2	EAA64687	*Aspergillus nidulans*	5.0E-22
CN809260	Hypothetical protein	XP384749	*Gibberella zeae*	2.0E-11
CN808381	Hypothetical protein MG06755.4	EAA55098	*Magnaporthe grisea*	6.0E-16

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TABLE 3—*Continued*

Category and gene identifier[a]	Putative or known function	Best match	Closest relationship	E-value
CN809340	Hypothetical protein FG02946	EAA71001	*Gibberella zeae* PH-1	3.0E-29
CN809111	Putative membrane protein	CAB65565	*Streptomyces coelicolor*	1.0E-09
CN809278	Hypothetical protein FG00492	EAA68724	*Gibberella zeae* PH-1	4.0E-12
CN808208	Hypothetical protein FG09877	EAA70103	*Gibberella zeae* PH-1	3.0E-07
CN809490	Hypothetical protein AN9016.2	EAA64348	*Aspergillus nidulans*	2.0E-73
CN809544	Hypothetical protein AN3935.2	EAA59244	*Aspergillus nidulans*	4.0E-09
CN809615	Orphan			
AJ274328	Orphan			
CN808763	Orphan			
CN808668	Orphan			
CN808749	Orphan			
CN808778	Orphan			
CN808897	Orphan			
CN808997	Orphan			
CN809648	Orphan			
CN809229	Orphan			
AJ272938	Orphan			
CN808540	Orphan			
CN808793	Orphan			
CN809674	Orphan			
CN809028	Orphan			
CN808529	Orphan			
AJ273884	Orphan			
CN809072	Orphan			
CN809454	Orphan			
CN808989	Orphan			
CN808807	Orphan			
CN809563	Orphan			
CN809452	Orphan			
CN808412	Orphan			
CN808880	Orphan			
CN809004	Orphan			
AJ273764	Orphan			
AJ272837	Orphan			
AJ272822	Orphan			
AJ274006	Orphan			
AJ273965	Orphan			
CN809633	Orphan			
CN808693	Orphan			
CN809144	Orphan			
AJ273455	Orphan			

[a] Gene identifiers refer to the accession number of the EST in the NCBI database.

required primarily to live in alternative hosts or as a saprophyte. One of the most intensely studied virulence attributes of *M. anisopliae* is the ability to secrete destruxins encoded by peptidyl synthases (33). The selective divergence or loss of toxin-encoding genes in the specialists suggests they confer considerable selectable functions for Ma2575 but either provide no benefits to Ma324 or are detrimental. It is probably significant that specialist strains kill their host slowly (8). Presumably, strains that are not specifically adapted to subvert/avoid/overcome the immune response of a particular insect are best served by achieving a rapid kill with toxins, whereas an adapted strain may optimize utilization of host nutrients and production of infectious propagules by growing within the living host. Production and transmission of propagules only occurs after host death, so if hosts are in the growing phase during infection, the reduced virulence resulting from loss of toxins may also allow for increased reproduction per host by allowing the pathogen to exploit the extra host tissue generated by the additional host growth (14). Consistent with this, genetic engineering to increase the speed of kill by *M. anisopliae* resulted in reduced sporulation on cadavers (30). Active toxins may therefore have placed a specific pathogen at a selective disadvantage that could drive inactivation of the gene. Loss-of-function mutations are presumably a one-way street and will be deleterious to a specialized strain if it returns to its ancestral habitat. It could also constrain opportunistic host switching.

From the likely function of genes that have been degraded (or silenced), we can assess their conceivable effects on host specialization, virulence, and/or expendability for virulence. However, for a given gene, this is only a hypothesis and needs separate experimental support. A direct approach is through examination of genotype-phenotype correlations. Consistent with our array results, Ma2575 has several peptide synthases and produces destruxins A, B, and E, while strain Ma297 only produces destruxin A (2). Likewise, destruxins do not play a role in the pathogenesis of Ma324 (35). Specificity, or lack of it, has always been an issue with *M. anisopliae* (26). Instances where generalist strains are closely related to specialists suggest that host range can be substantially altered in a short period of evolutionary time (40). It is possible therefore that

some imported specialized biocontrol agents may have the potential to switch hosts in response to selection pressures in new habitats. What we lack is a means to predict when such evolution is, and is not, expected. An understanding of genetic changes that have enabled evolution of major host range changes in the past could help predict future evolutionary changes. For example, genomic degradation could reasonably be expected to reduce specialists future adaptive options. Confirmation of gene loss would therefore impact risk assessment issues.

This study throws light on the "mysterious abundance" of orphans in genomes (39). Only 35 out of 237 (15%) arrayed "*Metarhizium*-specific sequences" were in the highly divergent group 3. This suggests that 85% arose de novo in a common ancestor of Ma2575 and Ma324, presumably after it had already diverged from other pyrenomycetous fungi, given that those other fungi lack the sequences. Heterologous probes from different strains showed reduced signal strength for the same 35 orphans as Ma324 probes, suggesting that these have lost sequence similarities because of rapid evolution in Ma2575. They are unlikely to be pseudogenes, as they were identified from cDNAs, confirming active expression of mRNA (45).

Several Ma2575 retrotransposon genes were not detected in other strains, indicating that since their divergence Ma2575 may have acquired different mobile genetic elements. Conversely, there was an expansion in the number of a subset of insertion elements in Ma443 that hybridized to respective sequences from Ma2575. This has obvious implications for strain stability that are of importance when considering the commercial development of a strain and the possibility of alterations in virulence and host range.

This study shows that comparing multiple strains will provide an excellent framework for the analysis of pathogenesis and host specificity. Genetic variation is a powerful tool to study adaptation, and we expect future studies to address a number of basic yet poorly understood questions that span much of molecular evolution, including the following: what roles do changes in gene complement or expression profiles play in generating intraspecific differences? How do these differences correlate with metabolic and biosynthetic adaptations to specific hosts? What are the relative rates of different kinds of mutations and do these vary between strains? What are the mechanisms by which novel pathogens emerge with either wide or narrow host ranges? Are the same genes involved in the evolution and maintenance of specialization in different strains? What variables drive the functional divergence of gene variants between strains, e.g., does genic novelty correlate with life-style or lineage?

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