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Evolutionary Dynamics of Mating-Type Loci of *Mycosphaerella* spp. Occurring on Banana▽†

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The devastating Sigatoka disease complex of banana is primarily caused by three closely related heterothallic fungi belonging to the genus *Mycosphaerella*: *M. fijiensis*, *M. musicola*, and *M. eumusae*. Previous phylogenetic work showing common ancestry led us to analyze the mating-type loci of these *Mycosphaerella* species occurring on banana. We reasoned that this might provide better insight into the evolutionary history of these species. PCR and chromosome-walking approaches were used to clone the mating-type loci of *M. musicola* and *M. eumusae*. Sequences were compared to the published mating-type loci of *M. fijiensis* and other *Mycosphaerella* spp., and a novel organization of the MAT loci was found. The mating-type loci of the examined *Mycosphaerella* species are expanded, containing two additional *Mycosphaerella*-specific genes in a unique genomic organization. The proteins encoded by these novel genes show a higher interspecies than intraspecies homology. Moreover, *M. fijiensis*, *M. musicola*, and *M. eumusae* contain two additional mating-type-like loci, containing parts of both *MAT1-1-1* and *MAT1-2-1*. The data indicate that *M. fijiensis*, *M. musicola*, and *M. eumusae* share an ancestor in which a fusion event occurred between *MAT1-1-1* and *MAT1-2-1* sequences and in which additional genes became incorporated into the idiomorph. The new genes incorporated have since then evolved independently in the MAT1-1 and MAT1-2 loci. Thus, these data are an example of the evolutionary dynamics of fungal MAT loci in general and show the great flexibility of the MAT loci of *Mycosphaerella* species in particular.

In fungi belonging to the Pezizomycotina, sexual development is controlled by a single mating-type locus (*MAT*). In all heterothallic filamentous ascomycetes studied to date, the mating-type locus contains one of two forms of dissimilar sequences (known as the idiomorph) occupying the same chromosomal position in the genome (23). By convention, mating-type idiomorphs of complementary isolates are termed *MAT1-1* and *MAT1-2* (32). In contrast, homothallic fungi contain both mating-type genes (either linked or unlinked) in a single genome and even some homothallic *Neurospora* species exist that contain only a *MAT1-1* homologue (20). Until now, the idiomorphs of all heterothallic members of the *Dothideomycetes* have been characterized by the presence of a single gene. MAT1-1 isolates contain a single gene encoding a protein with an alpha domain (*MAT1-1-1*), and MAT1-2 isolates a single gene encoding a protein containing a high-mobility group (HMG) domain (*MAT1-2-1*) (32). Homothallic members of the *Dothideomycetes* can carry both mating-type genes in their haploid genome, linked or unlinked (37). *MAT1-1-1* and *MAT1-2-1* encode transcription factors controlling the signal transduction pathway involved in mating identity and development of the sexual cycle (7, 24, 35).

After wheat, rice, and corn, bananas (*Musa* spp.) are the fourth-most-important staple food crop. Banana production can be severely impaired by a variety of diseases. Currently, the most serious and economically important leaf spot diseases of bananas are caused by different species of the fungal genus *Mycosphaerella*. The Sigatoka leaf spot disease complex of bananas involves three closely related fungi, *Mycosphaerella fijiensis* (anamorph *Pseudocercospora fijiensis*), causing the black Sigatoka disease; *M. musicola* (anamorph *Pseudocercospora musae*), responsible for yellow Sigatoka disease; and *M. eumusae* (anamorph *Pseudocercospora eumusae*), causing eumusae leaf spot disease (2, 10, 18). Of these, *M. fijiensis* is currently regarded as the most important pathogen, causing premature ripening of the fruits and yield losses of up to 50% (21).

The chronology of the disease record around the world suggests that, as for their host genus *Musa*, Southeast Asia is the center of origin for all three pathogens. Yellow Sigatoka was first described on banana in Java in 1902 and was found worldwide throughout the whole banana production area during the 1940s. After its discovery on the Fiji islands in the early 1960s, *M. fijiensis* spread rapidly across all continents, thereby replacing *M. musicola* as the main disease agent. The third species, *M. eumusae*, was recognized as a new constituent of the Sigatoka disease complex in the mid-1990s and is currently still restricted to Southeast Asia and parts of Africa (5, 10, 18, 26). A recent study of the phylogeny of *Mycosphaerella* species occurring on banana indicated that 20 species of *Mycosphaerella* or its anamorphs can occur on banana. Several of these species are able to coinfect a single leaf or even lesion. This study also showed that the three major pathogens represent a monophyletic clade and share common ancestry (3). We rea-

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TABLE 1. List of species and isolates used in this study

Species	Accession no. for isolate[a]	Mating type	Origin
Mycosphaerella eumusae	CIRAD670/CBS111438	MAT1-1	Vietnam
	CIRAD485/CBS121381	MAT1-2	Thailand
Mycosphaerella fijiensis	CIRAD86/CBS120258	MAT1-1	Cameroon
	CIRAD251/CBS121358	MAT1-2	Costa Rica
Mycosphaerella musicola	UQ2003/CBS121374	MAT1-1	Australia
	CIRAD90/CBS121368	MAT1-2	Colombia

[a] Accession numbers are from the Centre de Coopération Internationale en Recherche Agronomique pour le Dévelopment (CIRAD), Montpellier, France, or the University of Queensland (UQ), Australia, and Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

soned that analyzing the structure and organization of the mating-type loci of the primary *Mycosphaerella* species occurring on banana might provide better insight into the evolutionary history of these species and possibly even into the history of the disease. Therefore, we identified and cloned the mating-type loci of these species and compared them to mating-type loci of other *Mycosphaerella* spp.

MATERIALS AND METHODS

Isolates. A list of the *Mycosphaerella* strains isolated from banana and used in this study is provided in Table 1. Isolates were maintained in a 10% glycerol solution at −80°C, and all are deposited at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

Isolation and characterization of mating-type loci of *Mycosphaerella* spp. Previously published degenerate primers were used to amplify a conserved region within *MAT1-1-1* and *MAT1-2-1* from different *Mycosphaerella* species occurring on banana. PCR amplification and sequencing conditions were as previously described (17). Sequence fragments were assembled using SeqMan (Lasergene package; DNAstar, Madison, WI). Nested primer sets were designed based upon contigs corresponding to *MAT1-1-1* and *MAT 1-2-1* sequences. These primer sets were used in combination with primers provided with a DNA-walking SpeedUp kit (Seegene, Inc., Rockville, MD) to amplify fragments adjacent to the initially cloned fragments. Amplified fragments were purified using GFX PCR DNA and a gel band purification kit (GE Healthcare, Piscataway, NJ) and cloned in a pGEM-T vector system I (Promega, Madison, WI). The identities of the cloned fragments were confirmed by sequencing, and subsequent genome-walking steps were performed to obtain the complete idiomorph.

DNA and amino acid sequence comparisons and bioinformatics. Sequence data obtained from genome walking were assembled and edited using the SeqMan and EditSeq programs from the Lasergene 7.2.1 package (DNAstar, Madison, WI). Consensus sequence files were exported to the Vector NTI 10.1 software package (Invitrogen, United States) for further analysis and the creation of graphical maps. Sequence data were analyzed using the basic local alignment search tools (BLAST) at NCBI (http://www.ncbi.nlm.nih.gov) (1). Open reading frames (ORFs) and intron positions were predicted by comparing the sequence data with known *MAT* sequences from other filamentous fungi, as well as by means of the FGENESH gene prediction module from the MOLQUEST software package (Softberry, Inc., Mount Kisco, NY) (28) with the *Stagonospora nodorum* data set as reference. Genomic comparisons to the recently released genomic sequences of the *M. graminicola* isolate IPO323 and the *M. fijiensis* isolate CIRAD86 were performed at the respective genome portal sites at the JGI-DOE (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html and http://genome.jgi-psf.org/Mycfi1/Mycfi1.home.html). Phylogenetic analyses were performed using the Phylogeny.fr platform (www.phylogeny.fr) and comprised the following steps. Sequences were aligned with T-Coffee (version 6.85), and gaps and poorly aligned regions were removed with Gblocks (version 0.91b), with a minimum length of block after gap cleaning of 10, no gap positions allowed in the final alignment, and a maximum of 8 contiguous nonconserved positions. The phylogenetic tree was reconstructed based upon the maximum likelihood method implemented in the PhyML program using the WAG amino acid substitution model and the NNI tree building algorithm, and the reliability of the branches was assessed by 500 bootstrap replicates (12).

DNA and RNA manipulations. Basic DNA and RNA manipulations were performed based on standard procedures (29). *Escherichia coli* strain JM109 (Promega) was used for propagation of constructs. Genomic DNA from axenic cultures grown on agar plates was extracted using a commercial DNA isolation kit (MoBio Laboratories, Carlsbad, CA) according to the vendor's instructions. Total RNA from *M. fijiensis* isolates (Table 1) grown for 7 days at 25°C in liquid potato dextrose broth was isolated using Trizol reagent (Life Technologies). Reverse transcription-PCR (RT-PCR) was performed on cDNA made using a Superscript III first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instructions. Primers used for RT-PCR were designed to distinguish between genomic DNA and cDNA (Table 2).

Nucleotide sequence accession numbers. GenBank accession numbers for the sequences reported are as follows: *M. eumusae* MAT1-1, GU046393; *M. eumusae* MAT1-2, GU046394; *M. musicola* MAT1-1, GU057991; and *M. musicola* MAT1-2, GU057992.

RESULTS

Cloning and characterization of mating-type loci. PCR amplification of genomic DNA from *M. musicola* and *M. eumusae* MAT1-1 and MAT1-2 isolates using degenerate primers yielded fragments with homology at the nucleotide level to the mating-type genes of other *Dothideomycetes*. Several subsequent chromosome-walking steps were performed in both the downstream and upstream direction to obtain the full sequence of the mating-type genes, as well as the whole idiomorph.

The predicted gene structures of the cloned *MAT1-1-1* and *MAT1-2-1* genes of *M. eumusae* and *M. musicola* were compared to the published gene structures of the *M. fijiensis* *MAT1-1-1* and *MAT1-2-1* genes (GenBank accession numbers DQ787015 and DQ787016, respectively) (6). The predicted

TABLE 2. Primers used for RT-PCR and expected sizes of amplicons from the *Mycosphaerella fijiensis* target genes

Primer	Sequence	Target gene	Expected size of amplicon from:	
			Genomic DNA	cDNA
Mat1F	CATGAGCACGCTGCAG CAAG	*MAT1-1-1*	702	547
Mat1R	GTAGCAGTGGTTGACCA GGTCAT			
Mat2F	GGCGCTCCGGCAAAT CTTC	*MAT1-2-1*	720	616
Mat2R	CTTCTCGGATGGCTTG CGTG			
ORF1F	CTATCCAGCAAGGC CCAG	*MATORF1*	441	382
ORF1R	TTCTGCTGCATCTCC TCCA			
ORF2F	CTCACGCATGACACCT CCGA	*MATORF2*	733	683
ORF2R	GCGRTTCTGCGTAGTCA CATC			

MAT1-1-1 genes of the three pathogens were remarkably similar; all encode a protein of 388 amino acids, and in all three species, the ORF was interrupted by three introns of almost identical size (51, 55, and 49 nucleotides in the *M. fijiensis* and *M. musicola MAT-1-1* and 51, 50, and 49 nucleotides in the *M. eumusae MAT1-1-1*). The first two introns were located in the α-domain comprising areas of *MAT1-1-1* at exactly the same positions as described for other *MAT1-1-1* genes. The predicted third intron was located downstream from the α-domain. This third intron was absent from the *MAT1-1-1* gene from *Mycosphaerella graminicola* and *Septoria passerinii*, but its presence and location were identical for the third intron recently described within the *MAT1-1-1* gene of several *Cercospora* species and *Passalora fulva* (17, 30). BLASTP searches of the predicted MAT1-1-1 of *M. eumusae* and *M. musicola* showed that both proteins exhibited the highest similarity to MAT1-1-1 of *M. fijiensis*, with 94% and 95% identity, respectively. The second-best hits were obtained with MAT1-1-1 of *Mycosphaerella pini* and *P. fulva* (69% identity). The predicted MAT1-1-1 sequences of *M. eumusae* and *M. musicola* were 94% identical.

The published size of *M. fijiensis MAT1-2-1*, as well as the published sizes of the introns (33 and 50 nucleotides), deviated from the predicted gene models in *M. eumusae* and *M. musicola*. This led us to reevaluate the published model of the *M. fijiensis MAT1-2-1* using the same prediction tools used for *M. eumusae* and *M. musicola*, resulting in a model corresponding to the other two banana pathogens. The predicted proteins varied in size, being 411 (*M. musicola*), 423 (*M. eumusae*), and 433 amino acids (*M. fijiensis*) in length, but the sizes and positions of the two predicted introns were identical (54 and 50 nucleotides). The positions of the predicted introns corresponded to the positions of these introns in *MAT1-2-1* of other *Mycosphaerella* species. BLASTP searches showed that the MAT1-2-1 proteins of *M. eumusae* and *M. musicola* have the highest similarity to the *M. fijiensis* MAT1-2-1 (89% and 87% identity, respectively). The second-best hits were obtained with the MAT1-2-1 proteins of several *Cercospora* species (~60% identity). The predicted amino acid sequences of MAT1-2-1 of *M. eumusae* and *M. musicola* were 90% identical.

Approximately 12 kb of *M. musicola* genomic DNA from MAT1-1 and MAT1-2 isolates was obtained by genome walking and subsequently sequenced. BLAST2 analyses (using the BLASTN algorithm with standard settings) of these sequences indicated that in the MAT1-1 and MAT1-2 isolates, 3.9 kb of *MAT1-1* and 4.3 kb of *MAT1-2* were dissimilar and therefore by definition belonged to the idiomorph. BLASTP and BLASTX analyses of the genomic region upstream of the idiomorph revealed the presence of a DNA lyase gene with highest homology to the *M. fijiensis* DNA lyase, both at the nucleotide (76% identity over 1,083 nucleotides) and the protein level (67% identity over 312 amino acids). The DNA lyase gene is commonly found upstream from the mating-type loci in other ascomycetes (8, 33, 36). Besides the DNA lyase gene, no other genes could be identified upstream from the mating-type loci (Fig. 1). Analyses of the genomic region downstream from the idiomorph revealed the presence of two putative genes. BLAST comparisons of these gene models with the publicly available genomic sequences of *M. fijiensis* and *M. graminicola* indicated that this downstream region in *M. musicola* was highly syntenous to the corresponding genomic region in *M. fijiensis* and not to that in *M. graminicola* (Fig. 1). The two predicted genes were highly homologous to *M. fijiensis* gene models encoding Mycfi1-29759 (88% identity at the nucleotide and 88% identity at the amino acid level) and Mycf1-77284 (85% identity at the nucleotide and 88% identity at the amino acid level).

The organization of MAT1-1 and MAT1-2 in isolates of *M. eumusae* was different (Fig. 1). Approximately 12 kb of genomic DNA containing the mating-type loci from MAT1-1 and MAT1-2 isolates of *M. eumusae* was sequenced. BLAST2 analyses indicated that in the MAT1-1 and MAT1-2 isolates, at least 5.1 kb of *MAT1-1* and 6.1 kb of *MAT1-2* sequences were dissimilar and thus belonged to the idiomorph. However, the mating-type loci of *M. eumusae* are likely to be even larger, as attempts to clone the upstream boundaries of the *M. eumusae* idiomorph (putatively containing the DNA lyase) by extended genome walking were not successful. A part of the *M. eumusae* DNA lyase gene was amplified using degenerate primers, and this fragment was extended by genome walking to a 3.7-kb fragment that was identical in the MAT1-1 and MAT1-2 isolates and, thus, did not belong to the idiomorph. Additionally, an attempt to bridge the *M. eumusae* idiomorph to the flanking regions by long-range PCR between the idiomorph sequence and the DNA lyase gene was not successful. BLASTX comparisons of the *M. eumusae* region neighboring the DNA lyase against the *M. fijiensis* genome revealed the presence of a gene highly homologous to a predicted *M. fijiensis* gene model (Mycf1-29705 protein) encoding an acyl-coenzyme A transferase/carnitine dehydratase (79% identity on the amino acid and 81% identity on the nucleotide level). Interestingly, in *M. fijiensis*, this gene was located on the same scaffold as the mating-type locus but separated by ~39 kb from the *MAT1-1-1* gene.

Similar to the situation in *M. musicola*, the genomic region downstream from the idiomorph of *M. eumusae* was highly syntenous with the corresponding genomic region in *M. fijiensis*. This region contained two predicted genes highly homologous to *M. fijiensis* gene models for Mycf1-29759 (88% identity on the nucleotide and 90% identity on the amino acid level) and Mycf1-77284 (84% identity on the nucleotide and 82% identity on the amino acid level).

A BLAST2 pairwise alignment of the *MAT1-1* and *MAT1-2* idiomorphs, extended with sequences flanking the idiomorphs, revealed the presence of two inverted regions within the idiomorphs. These inversions were present in *M. fijiensis* (as described before [6]), *M. musicola*, and *M. eumusae* but were clearly absent from the *M. graminicola* idiomorph (Fig. 2). The inverted regions present within the *M. musicola* idiomorphs were homologous to the inverted regions present within the *M. fijiensis* idiomorphs. The same pairwise alignment of the *MAT1-1* and *MAT1-2* idiomorphs of *M. eumusae* revealed a more complicated pattern that included three inverted regions. Two of these inversions shared high similarity with the inversions observed in *M. musicola* and *M. fijiensis*, whereas the other (an ~1.3-kb inversion) was restricted to *M. eumusae* (Fig. 2).

The mating-type loci of members of the Sigatoka disease complex contain additional genes. The inverted regions observed within the *M. fijiensis* idiomorph were analyzed in more

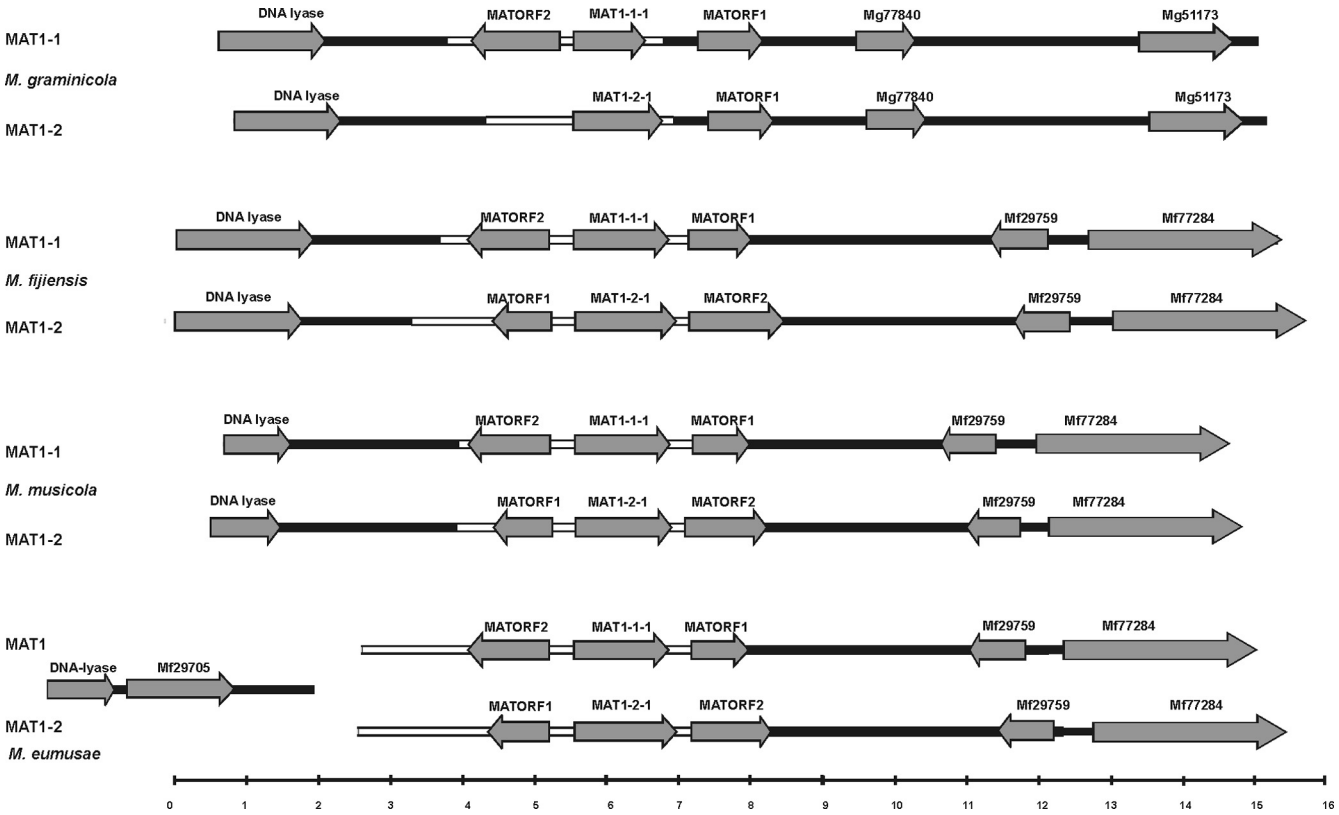

FIG. 1. Organization of mating-type loci of *Mycosphaerella graminicola*, *M. fijiensis*, *M. musicola*, and *M. eumusae*. Dissimilar sequences (idiomorphs) are indicated by white boxes, and identical sequences by black boxes. Predicted gene models are indicated by arrows. The scale at the bottom shows size in kilobases.

detail by using BLASTX analysis and the FGENESH gene prediction module. These analyses predicted the presence of two putative genes, designated *MATORF1* and *MATORF2*, respectively. The *M. fijiensis MATORF1* is located downstream on the same strand as *MAT1-1-1*, whereas *MATORF2* is found upstream from the *MAT1-1-1* gene on the opposite DNA strand in a head-to-head orientation. The *MATORF1* and *MATORF2* homologs present within the *M. fijiensis* MAT1-2 idiomorph are present in an inverted order; *MATORF1* is located upstream from *MAT1-2-1* and *MATORF2* downstream from *MAT1-2-1* (Fig. 1), thus giving rise to the inverted regions observed in the pairwise alignments.

Analysis of the mating-type loci of *M. musicola* and *M. eumusae* and the published idiomorph of *M. graminicola* indicated the presence of orthologs of these new genes in all of these species, although not in all mating types (Fig. 1 and Table 3). Furthermore, within the available idiomorph sequences of the related *S. passerinii* (GenBank accession numbers AF483193 and AF483194), partial *MATORF1* and *MATORF2* genes could be distinguished (16). The idiomorphs of *M. graminicola* and *S. passerinii* are very similar, with *MATORF2* lacking in both *MAT1-2* idiomorphs and *MATORF1* located outside the idiomorphs (Fig. 1).

A directed BLASTX search identified only a single sequence with homology to each ORF. The single hit with *MATORF2* (e-value, 5e-21), as well as the single hit with *MATORF1* (e-value, 6e-7), corresponded to recently published unknown genes (GenBank accession numbers DQ659350 and DQ659351, respectively) found within the idiomorph of *Passalora fulva*, a species that also belongs to the *Mycosphaerellaceae* (30).

A phylogenetic comparison of the deduced proteins encoded by the *MATORF1* and *MATORF2* genes revealed that the interspecies homology between the three banana pathogens was greater than the intraspecies homology. Thus, MATORF1 from an *M. fijiensis MAT1-1* isolate exhibited higher homology to MATORF1 from an *M. eumusae* or *M. musicola MAT1-1* isolate (88% and 84% amino acid identities, respectively) than to MATORF1 from an *M. fijiensis MAT1-2* isolate (64% identity). The same phenomenon was observed for MATORF2. This contrasted with the situation in *M. graminicola* and *S. passerinii*, where the intraspecies homology was clearly the highest (Fig. 3; also see Table S1 in the supplemental material).

***MATORF1* and *MATORF2* are expressed in a mating-type-dependent fashion.** RT-PCR experiments were performed on *M. fijiensis* isolates of opposite mating type to determine whether *MATORF1* and *MATORF2* are expressed and thus encode functional genes. Attempts to cross the complementary isolates CIRAD251 and CIRAD86 were unsuccessful. Therefore, the expression could only be analyzed under conditions nonconducive for mating. No fragments indicative of expression of *MAT1-1-1* or *MAT1-2-1* were observed. However, RT-PCR performed on RNA isolated from the *MAT1-2* isolate CIRAD251 yielded a fragment corresponding to the expected

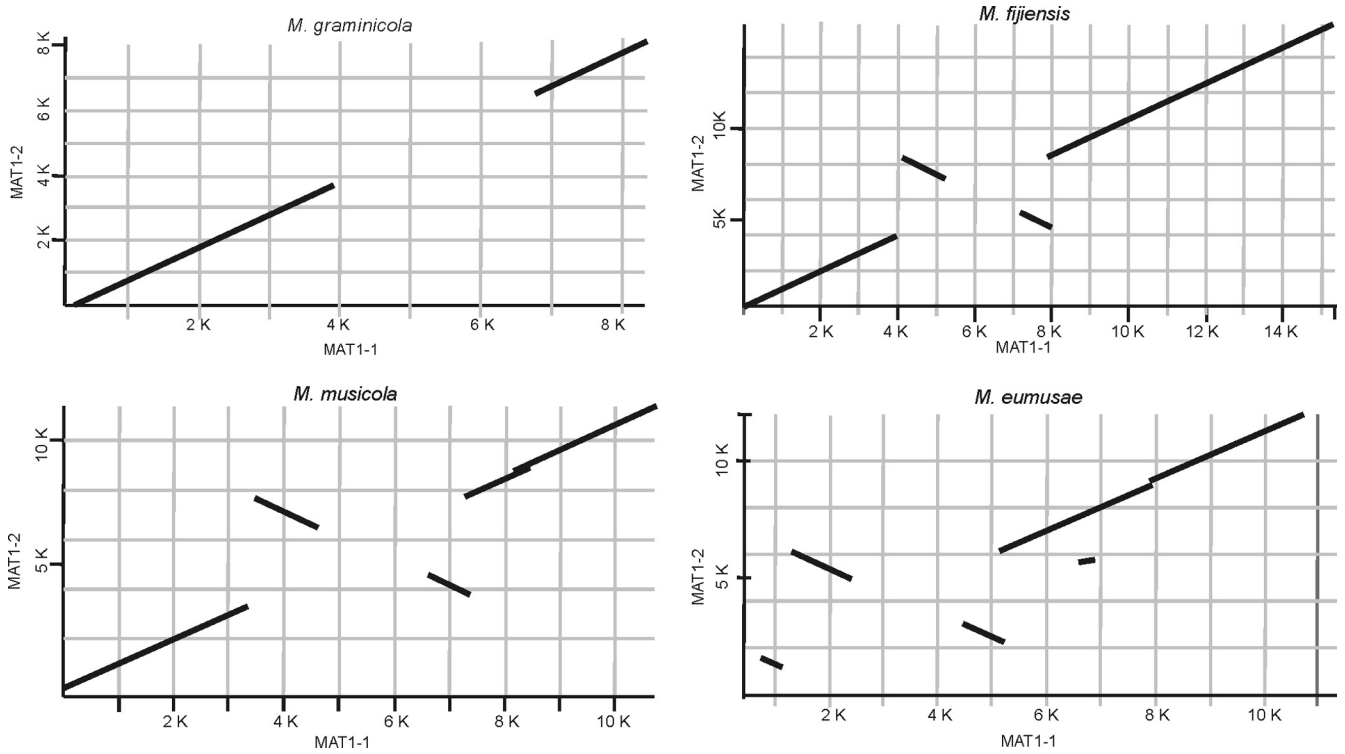

FIG. 2. Pairwise comparison of mating-type loci of *Mycosphaerella graminicola*, *M. fijiensis*, *M. musicola*, and *M. eumusae*. *MAT1-1* and *MAT1-2* and flanking regions of *M. graminicola*, *M. fijiensis*, *M. musicola*, and *M. eumusae* were analyzed by BLAST2. Plots show the presence of inverted regions with high levels of identity within the idiomorphs of the banana pathogens. The MAT1-1 sequences are plotted on the *x* axis, and the MAT1-2 sequences are plotted on the *y* axis. Numbers indicate the size of the analyzed fragments in kilobases.

size of an expressed *MATORF1* protein. Similarly, a fragment with the expected size of an expressed *MATORF2* protein was detected in RNA isolated from the *MAT1-1* isolate CIRAD86 (Fig. 4). These fragments were cloned, and sequence analysis confirmed their proper identities, as well as the predicted intron boundaries. This RT-PCR experiment was performed on two independently generated RNA samples. In both experiments, the expression of *MATORF1* was restricted to the *MAT1-2* isolate, and the expression of *MATORF2* was limited to the *MAT1-1* isolate.

Members of the Sigatoka disease complex contain additional loci of fused mating-type-like genes. The RT-PCR experiments did not reveal any expression of the *M. fijiensis* mating-type genes *MAT1-1-1* and *MAT1-2-1*, but these experiments did yield a surprising result. Negative control reactions performed on genomic DNA of CIRAD86 (*MAT1-1*) using *MAT1-2-1*-specific primers sometimes yielded a fragment. Similarly, PCR using *MAT1-1-1*-specific primers irregularly produced an amplicon in *MAT1-2* isolates. This was investigated in more detail.

Primers Mat2F and Mat2R were used to amplify part of *MAT1-2-1* from a *MAT1-2* isolate. This sequence was compared to the genome sequence of the *M. fijiensis MAT1-1* isolate CIRAD86 by BLAST analysis. Surprisingly, this *MAT1-2-1*-specific sequence gave a significant hit with sequences on scaffold 15 of the genome sequence of this *MAT1-1* isolate

TABLE 3. Comparison of the organization of the newly identified *MATORF1* and *MATORF2* genes in both idiomorphs of *Mycosphaerella graminicola*, *M. fijiensis*, *M. eumusae*, and *M. musicola*

Species	*MATORF1*[a]			*MATORF2*[a]		
	Gene size (nt)	Intron size(s) (nt)	Protein size (aa)	Gene size (nt)	Intron size(s) (nt)	Protein size (aa)
M. eumusae MAT1-1	791	59	243	1,135	49	361
M. eumusae MAT1-2	818	59	252	1,145	50	364
M. fijiensis MAT1-1	791	59	243	1,136	50	361
M. fijiensis MAT1-2	818	59	252	1,315	50, 77	395
M. graminicola MAT1-1	901	53, 53	264	1,226	56, 49, 57	354
M. graminicola MAT1-2	910	53, 53	267			
M. musicola MAT1-1	791	59	243	1,135	49	361
M. musicola MAT1-2	818	59	252	1,145	50	364

[a] nt, nucleotides; aa, amino acids.

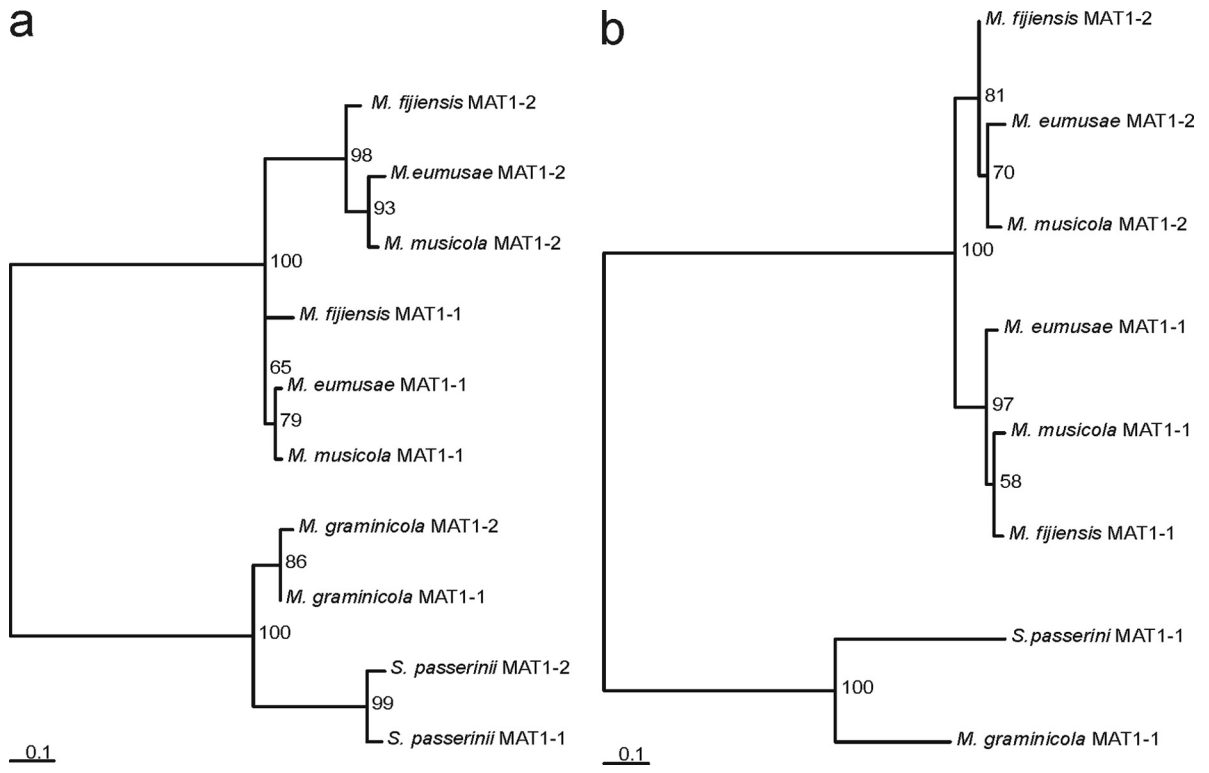

FIG. 3. Phylogram of the predicted amino acid sequences of MATORF1 and MATORF2. The phylograms are based upon the maximum likelihood method implemented in the PhyML program. Analyses were performed for MATORF1 (a) and MATORF2 (b) from both idiomorphs of *Mycosphaerella eumusae*, *M. fijiensis*, *M. musicola*, *M. graminicola*, and *Septoria passerinii*. Bootstrap support values from 500 replicates are shown as percentages at the nodes. Scale bars indicate the number of substitutions per site.

(>90% identity in 336 bp). Additionally, Mat1F and Mat1R were used to amplify part of *MAT1-1-1* from a *MAT1-1* isolate and the sequence was compared to the *M. fijiensis* genome sequence. As expected, this BLAST analysis gave a hit with the *M. fijiensis MAT1-1-1* locus on scaffold 2, but it also gave a significant hit with sequences on scaffold 10 (>90% identity in 452 bp).

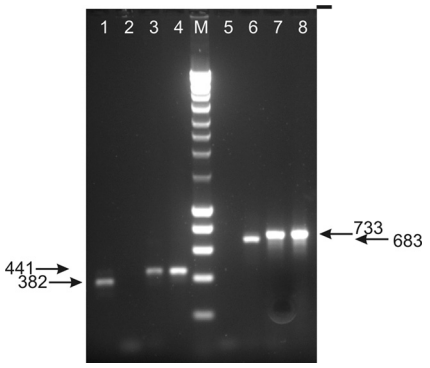

FIG. 4. RT-PCR analysis of *MATORF1* and *MATORF2* expression in *Mycosphaerella fijiensis*. The isolates used were the *MAT1-1* isolate CIRAD86 and the *M. fijiensis MAT1-2* isolate CIRAD251. PCR with primer combinations ORF1F/ORF1R (lanes 1 to 4) and ORF2F/ORF2R (lanes 5 to 8) was performed on cDNA derived from CIRAD251 (lanes 1 and 5), cDNA derived from CIRAD86 (lanes 2 and 6), genomic DNA from CIRAD251 (lanes 3 and 7), and genomic DNA from CIRAD86 (lanes 4 and 8). The lane labeled M contains a DNA marker. The numbers to left and right show nucleotide sizes of amplified fragments.

The *M. fijiensis* genome sequences surrounding these areas of scaffold 10 and scaffold 15 were analyzed in more detail. This revealed the presence of two genomic regions sharing extensive homology (>90% identity on nucleotide level) with parts of both the *MAT1-1-1* and *MAT1-2-1* genes of *M. fijiensis*. These two fusion loci, composed of both *MAT1-1-1* and *MAT1-2-1* sequences, were designated *MAT1/2A* and *MAT1/2B*. Both *MAT1/2A* and *MAT1/2B* were unique in their composition and showed a distinct pattern of *MAT1-1-1* and *MAT1-2-1* sequences. There was no sequence similarity between the two fused mating-type loci (Fig. 5). Neither was there any sequence similarity between the sequences flanking the two fusion loci. No clear gene models could be annotated for either fusion region. A partial α-domain was only distinguishable in *MAT1/2A*, whereas no HMG domain region was detectable in either *MAT1/2A* or *MAT1/2B*.

To answer the question of whether the presence of these loci was restricted to MAT1-1 isolates of *M. fijiensis* or whether they would also be present in *M. fijiensis* MAT1-2 isolates, primers were designed based upon the sequences of the predicted *MAT1/2A* and *MAT1/2B* fusion loci (Fig. 5). Furthermore, these primers were also used on MAT1-1 and MAT1-2 strains from the other two main constituents of the Sigatoka disease complex, *M. musicola* and *M. eumusae*. This led to the amplification of fragments corresponding to the *MAT1/2A* and *MAT1/2B* fusion loci from both MAT1-1 and MAT1-2 isolates

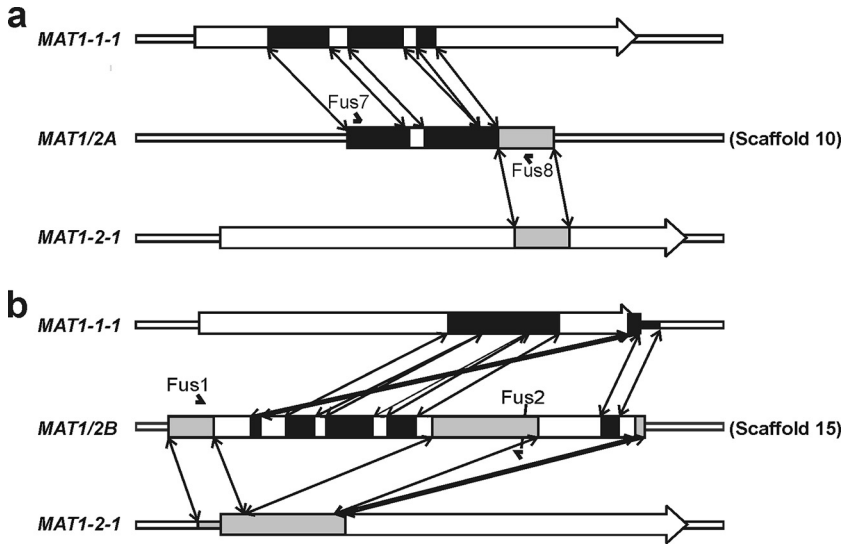

FIG. 5. Schematic overview of the organization and composition of the *Mycosphaerella fijiensis MAT1/2A* and *MAT1/2B* fusion loci. Indicated are the areas sharing >90% nucleotide identity between the *Mycosphaerella fijiensis MAT1/2A* (a) and *MAT1/2B* (b) fusion loci and the *M. fijiensis* mating-type genes *MAT1-1-1* and *MAT1-2-1*. *MAT1-1-1* sequences and their corresponding positions in *MAT1/2A* and *MAT1/2B* are marked in black. *MAT1-2-1* sequences and corresponding locations in *MAT1/2A* and *MAT1/2B* are marked in gray. Double-ended arrows mark the positions of corresponding homologous stretches. The positions and names of the primers used to amplify the fusion loci from *M. eumusae* and *M. musicola* are marked with carets.

from all three banana pathogens. In all three species, the *MAT1/2A* and *MAT1/2B* fusion loci found in MAT1-1 and MAT1-2 isolates were identical. The organization of both the *MAT1/2A* and the *MAT1/2B* fusion loci was highly conserved between the three *Mycosphaerella* species examined (see Fig. S1 in the supplemental material). Finally, the released genome sequence of the related *M. graminicola* was searched for the presence of these fusion loci. Neither the *MAT1/2A* nor the *MAT1/2B* fusion locus was found within the *M. graminicola* genome.

DISCUSSION

Expansion of the mating-type locus. Since the initial characterization of the idiomorphs of *Cochliobolus heterostrophus* (31), mating-type loci have been characterized for many other *Dothideomycetes*. Until now, a single gene per idiomorph has been found in all species examined (11). This is in contrast to the situation in *Sordariomycetes* and *Leotiomycetes*, where the mating-type loci often contain additional genes besides the *MAT1-1-1* and *MAT1-2-1* genes (11). However, our results clearly show that the mating-type loci of the three main constituents of the Sigatoka disease complex and other *Mycosphaerella* species deviate from the typical dothideomycetous pattern. First, the idiomorphs show an expansion in size compared to the idiomorphs of other *Dothideomycetes* or even other *Mycosphaerellaceae* (4, 16, 30, 31, 33). The idiomorph of *M. graminicola* is 2.8 kb, whereas the sizes of the *M. fijiensis* idiomorphs are 3.9 kb and 4.4 kb and the sizes of the *M. musicola* idiomorphs are 3.9 and 4.3 kb (Fig. 1). The failed attempts to obtain the 5′ flanking region of the *M. eumusae* MAT1-1 and MAT1-2 loci by chromosome walking indicate that the sizes of these idiomorphs are at least 5.1 and 6.1 kb, respectively. Second, in comparison to the other *Dothideomycetes*, the *Mycosphaerella* idiomorphs contain additional genes (*MATORF1* and *MATORF2*) and exhibit a novel organization (Fig. 1). Homologs of *MATORF2* were also identified in the published *MAT1-1* idiomorphs of *M. graminicola* and *S. passerinii*, previously thought to adhere to the one gene per idiomorph rule (16, 33). Moreover, in these species, a homolog of *MATORF1* was found outside the *MAT1-1* and *MAT1-2* idiomorphs.

The pattern of an expanded mating-type locus concomitant with the incorporation of flanking regions and genes inside the mating-type loci has also been described for *Coccidioides immitis*, *C. posadasii*, and, most strikingly, *Cryptococcus neoformans* (15, 19). It is suggested that the expansion of mating-type loci by the incorporation of adjacent genes could represent a step in the evolution toward sex chromosomes (14, 19). In *C. neoformans*, the idiomorph is greatly expanded (>100 kb) and contains extensive rearrangements of genes between the mating types. For instance, the *C. neoformans RP041*α and *RP041***a** genes are highly similar (97%) but are organized in opposite directions. This is strikingly similar to the observed similarity and inversion of *MATORF1* and *MATORF2* in the mating-type loci of *M. eumusae*, *M. fijiensis*, and *M. musicola*.

Presence and expression of *MATORF1* and *MATORF2*. The newly identified *MATORF1* and *MATORF2* are found only in the *Mycosphaerellaceae*. BLAST analyses indicated the presence of a homolog only in *Passalora fulva*, which also belongs to the *Mycosphaerellaceae*. A reexamination of the published mating-type loci of *S. passerinii* and *M. graminicola*, both members of the *Mycosphaerellaceae* (34), also revealed the presence of these ORFs (Fig. 1). Furthermore, additional studies aimed at the characterization of mating-type loci of the homothallic *M. musae* revealed the presence of *MATORF2* in close association with a mating-type gene (unpublished data). Analysis of the proteins encoded by *MATORF1* and *MATORF2* indicated

the presence of several conserved protein motifs with no known counterparts present in the databases (data not shown). These motifs might be used to design specific primers that can be used to test whether these genes are indeed specific for the *Mycosphaerellaceae* and potentially might yield a *Mycosphaerellaceae*-specific barcode.

The results of the RT-PCR experiments showed that both *MATORF1* and *MATORF2* of *M. fijiensis* are expressed and thus are not pseudogenes. A striking feature is the observed mating-type-dependent expression of these genes: *MATORF1* is expressed in the *MAT1-2* isolate and *MATORF2* in the *MAT1-1* isolate. This mating-type-dependent expression was observed under conditions nonconducive for mating. It would be interesting to see if and how the expression of *MATORF1* and *MATORF2* is regulated during mating. Such studies could also validate the predicted gene models for *MAT1-1-1* and *MAT1-2-1*. Unfortunately, several attempts to cross complementary isolates *in vitro* proved unsuccessful. The mating-type-dependent expression can be well explained by the genomic organization within the idiomorphs and the assumptions that *MATORF2* and *MAT1-1-1* are under the control of a bidirectional promoter and that a similar-acting bidirectional promoter region is controlling *MATORF1* and *MAT1-2-1* (Fig. 1). To address this properly, promoter studies need to be performed. It is not clear whether the location of *MATORF1* and *MATORF2* near the mating-type genes is meaningful in relation to a function in sexual development or mating. Currently, experiments are under way to generate *MATORF1*/*MATORF2* knockout mutants to assess their function.

In contrast to the situation in *M. graminicola* and *S. passerinii*, the interspecies homology of both MATORF1 and MATORF2 in the three banana pathogens examined was higher than the intraspecies homology. This looks like an example of *trans*-specific polymorphism (polymorphisms that are maintained in populations through speciation events) and confirms the common evolutionary history for *M. eumusae*, *M. fijiensis*, and *M. musicola* (3). *trans*-specific polymorphisms are distinguished by alleles from a species being less related to alternate alleles from the same species than to alleles from other species (22, 25). This is regularly found to occur on self-incompatibility loci (such as mating-type loci) and has recently been shown for the pheromone receptors pr-MatA1 and pr-MatA2 in *Microbotryum* spp. (13). *trans*-specific polymorphisms are considered to be maintained primarily by balancing selection (25). We hypothesize that after the incorporation of *MATORF1* and *MATORF2* into the mating-type region of an ancestor species, balancing selection exerted strong pressure, as mating can only occur between individuals with different idiomorphs. Consequently, after speciation, the intraspecies homology is lower than the interspecies homology.

Members of the Sigatoka disease complex contain additional loci of fused mating-type-like genes. The amplification of fragments from an *M. fijiensis* MAT1 isolate using *MAT1-2-1*-specific primers, as well as from an *M. fijiensis* MAT2 isolate using *MAT1-1-1*-specific primers, revealed the presence of two loci containing both *MAT1-1-1* and *MAT1-2-1* sequences. Homologs of these loci were also present in *M. eumusae* and *M. musicola*. Furthermore, preliminary results of PCR amplification with a specific primer set for the fused mating-type loci reveal that these loci are also present in other closely related *Pseudocercospora* spp. from banana, *viz.*, *P. longispora*, *P. indonesiana*, and *P. assamensis*, which, based on organismal gene phylogeny, display the same pattern of evolutionary history (3). Attempts to amplify the fused mating-type loci from genomic DNA isolated from other *Mycosphaerella* species occurring on banana, e.g., the homothallic species *M. musae* and *M. thailandica* and the heterothallic *M. citri* (data not shown), or an *in silico* analysis of the publicly available genome of the related *M. graminicola* were unsuccessful. These results suggest that the presence of these loci is restricted to a monophyletic clade of *Mycosphaerella* spp. occurring on banana. This clade contains the three major banana pathogens and the three above-mentioned *Pseudocercospora* spp. It is doubtful that *MAT1/2A* and *MAT1/2B* are true genes since no clear gene models could be predicted. However, if the fusion loci comprise functional genes, they will probably not function as mating-type genes, as no HMG box domains and only a partial α-domain (in *MAT1/2A*) were present.

The presence of additional MAT loci has been described in the homothallic euascomycete *Neosartorya fischeri*, where a *MAT2* region, including flanking nonfunctional DNA lyase (*APN1*) and cytoskeleton assembly control (*SLA2*) sequences, became incorporated into a genome already containing a functional *MAT1* region, thereby resulting in a homothallic mating system (27). However, the apparent MAT1/MAT2 recombinations that resulted in two distinct shuffled MAT1/2 fusion loci in the banana pathogens are novel findings. An explanation for the observed presence and structure of the *MAT1/2* fusion loci could be the occurrence of unequal crossover events within the idiomorph of a heterothallic ancestor. These crossover events might have occurred within the inverted regions present within the idiomorphs, and thus, within *MATORF1* and *MATORF2*.

Evolution of the *Mycosphaerella* MAT loci. Taken as a whole, these data suggest great evolutionary dynamics acting at the MAT loci of *Mycosphaerella* species. This evolutionary flexibility might underlie the huge diversity of this fungal genus that comprises several thousands of species (9).

Based upon the results obtained, we propose the following working model for the evolution of the *Mycosphaerella* MAT loci. In a hypothetical *Mycosphaerella* ancestor, both *MATORF1* and *MATORF2* were present adjacent to the idiomorphs. In a MAT1-2 ancestor of the *M. graminicola*/*S. passerinii* lineage, *MATORF2* was deleted. This deletion resulted in sequence divergence between the MAT1-1 and MAT1-2 idiomorphs, and thus, *MATORF2* became incorporated into the MAT1-1 idiomorph.

In the "banana" *Mycosphaerella* lineage, an inversion of MAT1-1 or MAT1-2 plus flanking genes occurred. This expanded the area of nonhomology between the MAT1-1 and MAT1-2 strains. Consequently, the former non-MAT genes *MATORF1* and *MATORF2* became incorporated into the idiomorphs, and both genes have since undergone independent evolution in the MAT1-1 and MAT1-2 loci. A subsequent inversion of *MAT1-1-1* or *MAT1-2-1* would then result in the MAT organization observed in the banana pathogens. In the "banana" lineage, multiple additional recombination events resulted in the occurrence of the two additional nonfunctional fused mating-type loci. Finally, this common ancestor gradually split into *M. fijiensis*, *M. eumusae*, and *M. musicola*, which have undergone further minor modifications at the MAT locus

(the extension in size in *M. eumusae*). Currently, the organization of MAT loci of additional *Mycosphaerella* species is being characterized to test this scenario of MAT locus evolution.

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