Mating Type Sequences in Asexually Reproducing Fusarium Species

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To assess the potential for mating in several Fusarium species with no known sexual stage, we developed degenerate and semidegenerate oligonucleotide primers to identify conserved mating type (MAT) sequences in these fungi. The putative α and high-mobility-group (HMG) box sequences from Fusarium avenaceum, F. culmorum, F. poae, and F. semitectum were compared to similar sequences that were described previously for other members of the genus. The DNA sequences of the regions flanking the amplified MAT regions were obtained by inverse PCR. These data were used to develop diagnostic primers suitable for the clear amplification of conserved mating type sequences from any member of the genus Fusarium. By using these diagnostic primers, we identified mating types of 122 strains belonging to 22 species of Fusarium. The α box and the HMG box from the mating type genes were transcribed in F. avenaceum, F. culmorum, F. poae, and F. semitectum. The novelty of the PCR-based mating type identification system that we developed is that this method can be used on a wide range of Fusarium species, which have proven or expected teleomorphs in different ascomycetous genera, including Calonectria, Gibberella, and Nectria.

The genus Fusarium contains filamentous ascomycete fungi with a worldwide distribution. Fusarium species can parasitize cultivated plants (1) and/or produce mycotoxins that pose serious hazards to human and animal health (9, 18). Species of Fusarium can grow successfully on a variety of substrates, can tolerate diverse environmental conditions, and have high levels of intraspecific genetic and genotypic diversity (for examples, see references 8, 12, 17, and 26). Neither the origins of this diversity nor the mechanisms that maintain it are well understood.

Meiotic recombination can generate and maintain genotypic variation and result in the reassembly of genes that govern traits such as virulence or toxin production (7). The sexual spores (ascospores) produced by some Fusarium species such as virulence or toxin production (7). The sexual variation and result in the reassortment of genes that govern horizontal resistance could be more effective against pathogens comprising genetically diverse populations as a result of mating and meiotic recombination (16).

The known teleomorphs of Fusarium species belong to the genera Calonectria, Gibberella, and Nectria (5). In heterothallic species, e.g., Gibberella fujikuroi, mating type is controlled by a single locus with two idiomorphic alleles, termed MAT-1 and MAT-2. These alleles contain a conserved α box domain and a high-mobility-group (HMG) box domain, respectively. Strains of Gibberella zeae (anamorph of Fusarium graminearum), a homothallic species, carry both the MAT-1 and MAT-2 idiomorphs, closely linked together (27). Strains of Fusarium oxysporum, a species complex with no known sexual stage, also contain transcribed MAT alleles (4, 13, 27). However, the MAT genes have not been studied in other mitotic holomorph species within the genus Fusarium.

PCR amplification of MAT sequences from various Fusarium species belonging to the G. fujikuroi species complex has been utilized to standardize the mating type terminology for mating populations of this species complex (13) and to develop assays for identifying the presence of the MAT allele without sexual crosses (22, 25). However, the primers used in these previous studies were inadequate for the rest of the genus (13, 22), probably due to sequence divergence that may occur even in conserved MAT sequences of these fungi. The aims of the present study were (i) to demonstrate whether mating type sequences can be found in Fusarium species with no known sexual stage, (ii) to develop a PCR-based technique for the rapid identification of mating types in a wide range of Fusarium species with proven or expected teleomorphs, (iii) to demonstrate the transcription of mating type genes in selected “asexual” Fusarium species during their vegetative growth.

(A preliminary version of this work was presented at the 6th European Conference of Fungal Genetics [A. Moretti, Z. Ker-
Fusarium acuminatum

Fusarium camptoceras

Fusarium chlamydosporum

Fusarium compactum

Fusarium graminearum

Fusarium poae

Fusarium semi-tectum

Fusarium sporotrichioides

Gibberella moniliformis

G. fujikuroi

Common DNA and RNA manipulation techniques

Molecular techniques.

C. For genomic DNA extractions, DNA and RNA were isolated by electrophoresis in agarose gels, stained with ethidium bromide, and visualized with UV light.

RT-PCR experiments. Total RNAs were extracted from the mycelia grown on carrot agar plates by use of the TRI reagent (Sigma, St. Louis, Mo.) according to the manufacturer’s instructions. The first-strand cDNA reaction was performed by using Tag polymerase (Stratagene) according to the manufacturer’s instructions. Amplifications were performed with the same program as described above, except that the number of cycles was increased to 35, and the elongation time was expanded to 30 s.

Nucleotide sequence accession numbers. The sequences of the amplified regions of F.avenaceum, F. culmorum, F. poae, and F. semitectum were deposited in the EMBL database under accession numbers AJ353625 to AJ353632.

RESULTS

Cloning of MAT-1 genes from Fusarium species with no known sexual stage. Regions flanking the α or HMG boxes were amplified by inverse PCR from F.avenaceum, F. culmorum, F. poae, and F. semitectum, and the resulting fragments were cloned and sequenced. Based on these sequences, new PCR primer pairs were designed for border regions of the MAT-1 and MAT-2 genes, and the DNA fragments generated by these primer pairs were cloned and sequenced.

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sequences available for the MAT idiomorphs of *F. oxysporum*, *G. fujikuroi*, and *G. zeae* (27). The MAT-1-1 gene identified in *F. avenaceum* ITEM 859 was 1,218 bp long and encoded a putative protein with an α-box motif. The sequences of MAT-1-1 genes from *F. culmorum* strain 19A1, *F. poae* TAPO21, and *F. semitectum* ITEM 3192 were 1,085, 1,203, and 1,129 bp long, respectively, and encoded putative proteins with conserved α-box domains. All of these MAT-1-1 gene sequences contained introns at conserved positions (20). No in-frame stop codons were found in these sequences.

The MAT-2 gene from *F. avenaceum* ITEM 858 was 860 bp long and encoded a putative protein with an HMG box domain. Similar sequences from *F. culmorum* 11F1, *F. poae* TAPO34, and *F. semitectum* ITEM 3390 were 865, 859, and 856 bp long, respectively, and encoded proteins with conserved HMG domains. These MAT-2 gene sequences also contained introns at conserved positions (20). In-frame stop codons were not found in these sequences. Sequence similarities ranged from 49 to 99%, but the percentages of similarity between the MAT-specific box sequences were always higher than the values obtained from comparisons of entire MAT gene sequences. The Treebase database accession number for these comparisons is SN 1779.

## TABLE 1. Primers used for amplification of entire MAT-1-1 and MAT-2 genes from *F. avenaceum*, *F. culmorum*, *F. poae*, and *F. semitectum*

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5′-3′)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVE-1-F</td>
<td>TCTTTAACCTCTCCTCTCTGCCC</td>
<td><em>F. avenaceum</em> MAT-1-1 forward</td>
</tr>
<tr>
<td>AVE-1-R</td>
<td>ACCCTTGGACAAACGAGGCTT</td>
<td><em>F. avenaceum</em> MAT-1-1 reverse</td>
</tr>
<tr>
<td>AVE-2-F</td>
<td>CACCCCAACAAAACCTCCCGGATT</td>
<td><em>F. avenaceum</em> MAT-2 forward</td>
</tr>
<tr>
<td>AVE-2-R</td>
<td>CAATGGGAGTGGAGCAGCTT</td>
<td><em>F. avenaceum</em> MAT-2 reverse</td>
</tr>
<tr>
<td>CUL-1-F</td>
<td>AATTCTACCTCTGCTGCTT</td>
<td><em>F. culmorum</em> MAT-1-1 forward</td>
</tr>
<tr>
<td>CUL-1-R</td>
<td>ATTTCGACGCCCTAGATCTCATT</td>
<td><em>F. culmorum</em> MAT-1-1 reverse</td>
</tr>
<tr>
<td>CUL-2-F</td>
<td>TTCAGAACGCCAGGACCAG</td>
<td><em>F. culmorum</em> MAT-2 forward</td>
</tr>
<tr>
<td>CUL-2-R</td>
<td>GAGCGGGACGTTTGTGCCTACTTA</td>
<td><em>F. culmorum</em> MAT-2 reverse</td>
</tr>
<tr>
<td>POA-1-F</td>
<td>CACCCCAACAAAACCATTCCTTC</td>
<td><em>F. poae</em> MAT-1-1 forward</td>
</tr>
<tr>
<td>POA-1-R</td>
<td>CAGTCAAACCTCCAATCAACC</td>
<td><em>F. poae</em> MAT-1-1 reverse</td>
</tr>
<tr>
<td>POA-2-R</td>
<td>AGTCGAGGAGGTCGTCAATCAAT</td>
<td><em>F. poae</em> MAT-2 forward</td>
</tr>
<tr>
<td>POA-2-F</td>
<td>ACGTACCATCTGACACTTGCTCG</td>
<td><em>F. poae</em> MAT-2 reverse</td>
</tr>
<tr>
<td>SEM-1-F</td>
<td>GCTTCACCTCTCTGCTTCTTC</td>
<td><em>F. semitectum</em> MAT-1-1 forward</td>
</tr>
<tr>
<td>SEM-1-R</td>
<td>TCTCTTTCTCTCATCGGCTT</td>
<td><em>F. semitectum</em> MAT-1-1 reverse</td>
</tr>
<tr>
<td>SEM-2-F</td>
<td>TTCAGAACGCCAGGACCAG</td>
<td><em>F. semitectum</em> MAT-2 forward</td>
</tr>
<tr>
<td>SEM-2-R</td>
<td>GAGCGGGACGTTTGTGCCTACTTC</td>
<td><em>F. semitectum</em> MAT-2 reverse</td>
</tr>
</tbody>
</table>

## TABLE 2. Similarity of MAT sequences of *Fusarium* species with no known sexual stage to MAT sequences described previously for other members of the genera *Fusarium* and *Gibberella*.

<table>
<thead>
<tr>
<th>MAT sequence</th>
<th>Accession no.</th>
<th>% Similarity for entire gene</th>
<th>% similarity for conserved boxes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. avenaceum</em> MAT-1-1</td>
<td>AJ535625</td>
<td>63.1/64.9</td>
<td>51.1/75.7</td>
</tr>
<tr>
<td><em>F. avenaceum</em> MAT-1-1</td>
<td>AJ535626</td>
<td>48.7/58.9</td>
<td>52.7/75.7</td>
</tr>
<tr>
<td><em>F. poae</em> MAT-1-1</td>
<td>AJ535627</td>
<td>52.5/66.7</td>
<td>55.3/76.6</td>
</tr>
<tr>
<td><em>F. semitectum</em> MAT-1-1</td>
<td>AJ535628</td>
<td>49/68.3</td>
<td>60.5/75.2</td>
</tr>
<tr>
<td><em>F. avenaceum</em> MAT-2</td>
<td>AJ535629</td>
<td>61.7/73.8</td>
<td>67.6/71.6</td>
</tr>
<tr>
<td><em>F. culmorum</em> MAT-2</td>
<td>AJ535630</td>
<td>52.8/71.6</td>
<td>58.8/68.4</td>
</tr>
<tr>
<td><em>F. poae</em> MAT-2</td>
<td>AJ535631</td>
<td>57.7/73.8</td>
<td>59.7/69.5</td>
</tr>
<tr>
<td><em>F. semitectum</em> MAT-2</td>
<td>AJ535632</td>
<td>61.4/74.2</td>
<td>62.6/69.5</td>
</tr>
</tbody>
</table>

### Diagnostic PCR for mating type in *Fusarium* species.

We designed new degenerate oligonucleotide primers, namely fusALPHAfor (CGCCCTCTKAAYGCYTACAT), fusALPHArev (GGARTARACTTGGTACGGGCGGT), fusHMGfor (CGACCTCCCAAYGCYTACAT), and fusHMGrev (TGGGCGGTACGGGCGGTACGG), and designed appropriate PCR conditions. The positions of these primers in the α and HMG box sequences of *F. avenaceum* (AJ535625 and AJ535629) are nucleotides 282 to 302, 456 to 479, 540 to 559, and 775 to 795, respectively. The sizes of the MAT-1-1 and MAT-2-specific fragments amplified from different species of *Fusarium* were 200 and 260 bp, respectively (Fig. 1).

The usability of the diagnostic PCR method for mating type identification was tested on 122 *Fusarium* strains representing species with no known sexual stage. (A) Amplification of MAT-1-specific α box by using the fusALPHAfor and fusALPHArev primers. (B) Amplification of MAT-2-specific HMG box by using the fusHMGfor and fusHMGrev primers. Lanes 1 and 10, 100-bp DNA ladder; lanes 2 to 9, *F. avenaceum* ITEM 859 (MAT-1) and ITEM 858 (MAT-2), *F. culmorum* 19A1 (MAT-1) and 11F1 (MAT-2), *F. poae* TAPO21 (MAT-1) and TAPO34 (MAT-2), and *F. semitectum* ITEM 3192 (MAT-1) and 3390 (MAT-2), respectively. Lanes 11 to 13, *F. graminearum* FGSC 7600 (MAT-1) and FGSC 7603 (MAT-2) and *F. graminearum* 24F1 (MAT1/2), respectively (used as controls).
22 species from 9 sections. Both MAT-1 and MAT-2 individuals were identified among strains of *F. acuminatum* subsp. *acuminatum*, *F. acuminatum* subsp. *armeniacum*, *F.avenaceum*, *F. cerealis*, *F. chlamydosporum*, *F. compactum*, *F. culmorum*, *F. equiseti*, *F. poae*, *F. scripi*, *F. semitectum*, *F. solani*, *F. sporotrichioides*, *F. torulosum*, and *F. tricinctum*. Only the MAT-2 mating type was found among strains of *F. camptoceras*, *F. decemcellulare*, *F. longipes*, *F. merismoides*, and *F. tudmidum*, but the number of isolates we could obtain for this assay was limited. The only strain for which both MAT-specific amplicons were identified belonged to *G. zeae*, a true homothallic member of the genus Gibberella that is known to harbor both MAT-1 and MAT-2 idiromorphs (27). The two opposing mating types of the reference strains of *F. verticillioides* were also clearly identified by using the semidegenerate primers developed in this work. The mating types of all of the strains are indicated in Materials and Methods.

**Transcription of MAT genes in Fusarium species with no known sexual stage.** RT-PCR experiments primed with α box- or HMG box-specific primers, respectively, were performed to examine the expression of *MAT* genes in *F.avenaceum*, *F.culmorum*, *F.poae*, and *F.semitectum*. Electrophoretic separation of the RT-PCR products resulted in the appearance of one characteristic band at the appropriate size, i.e., an ~150-bp and an ~200-bp fragment in all samples (Fig. 2). The size differences observed between the amplicons obtained by RT-PCR and fragments generated from the genomic DNAs were due to the presence of an intron in the genomic copies of these *MAT* boxes. Northern blot analyses of these RT-PCR products, with the appropriate cloned MAT-1-1 or MAT-2 gene as a probe, confirmed the identities of the fragments. Thus, both the MAT-1-1 and MAT-2 genes were transcribed in all asexual *Fusarium* species involved in this experiment.

**DISCUSSION**

For the present study, we developed a robust PCR-based method suitable for the identification of mating type in several *Fusarium* species with no known sexual stage. To achieve this, we designed PCR primers on the basis of known mating type sequences as well as conserved α and HMG box sequences of four asexual *Fusarium* species. All *Fusarium* species involved in this work were found to contain one or the other mating type idiromorph, with the exception of *F. graminearum*, which was used as a control. The MAT-1- and MAT-2-specific fragments that were amplified from these fungi showed substantial sequence similarities to conserved motifs of the MAT-1-1 and MAT-2 genes from *F. oxysporum*, *G. fujikuroi*, and *G. zeae*, suggesting that these partial sequences represent the mating type idiromorphs in these fungi. Degenerate MAT-specific primers designed by Arie et al. (3) or the *G. fujikuroi*-specific MAT primers developed in previous studies (13, 22) were unsuitable for generating unambiguous PCR fragments in such diverse *Fusarium* species (representatives of nine sections) due to sequence differences within the conserved MAT regions of these fungi.

Our findings clearly show that conserved MAT-specific sequences are present and expressed in *Fusarium* species with no known sexual stage. Since the strains of *F. acuminatum*, *F.avenaceum*, *F. campioceras*, *F. cerealis*, *F. chlamydosporum*, *F. compactum*, *F. culmorum*, *F. equiseti*, *F. longipes*, *F. merismoides*, *F. poae*, *F. semitectum*, *F. scripi*, *F. sporotrichioides*, *F. torulosum*, *F. tricinctum*, and *F. tudmidum* all contained only a single MAT allele, presumably they are capable of heterothallic, but not homothallic, mating. These results are consistent with the hypothesis (24) that these fungi may have a cryptic sexual cycle, even though sexual structures have not been identified in field collections and there are no reports of successful forced parings among them in laboratory experiments.

Leslie and Klein (15) explained the absence of sexual reproduction in local populations of the *G. fujikuroi* species complex by the presence of mutations that concomitantly resulted in female sterility with an increased vegetative propagation capability. Selection for an increased number of asexual propagules can result in a selective accumulation of female sterile strains, which could become prevalent even in large geographic areas. Under such conditions, mating is limited by the absence of normal female fertile partners. The *Fusarium* species that we examined seem to have functional mating type genes, are aggressive pathogens, and can colonize a wide range of decaying substrates. Populations of these fungi could easily be dominated by successful female sterile clone lineages that produce more asexual propagules and are therefore not under significant immediate selection pressure to participate in sexual reproduction. The female fertile strains could be such a small minority (<10% in some natural populations [15]) that they are likely to be infrequent, especially under epidemic conditions. Thus, their sexual structures may not be observed in nature simply because of their rarity. Alternatively, the purportedly asexual species also may require environmental conditions for sexual reproduction that are uncommon when disease epidemics occur or that are difficult or unusual conditions to mimic in the laboratory.

The PCR method that we developed for the mating type assessment of these *Fusarium* species facilitates the recognition of potentially compatible strains that could be used in crossing experiments to obtain teleomorphic structures. This approach could increase our knowledge of reproductive strategies in these fungi and allow a realistic evaluation of the potential for generating strains with new pathotypes and/or...
altered mycotoxin-producing abilities and could be used to assess disease control strategies that presume that limited genotypic variation and rearrangement occur within the pathogen population.

ACKNOWLEDGMENTS

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