Genomic and Population Analyses of the Mating Type Loci in *Coccidioides* Species Reveal Evidence for Sexual Reproduction and Gene Acquisition

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*Coccidioides* species, the fungi responsible for the valley fever disease, are known to reproduce asexually through the production of arthroconidia that are the infectious propagules. The possible role of sexual reproduction in the survival and dispersal of these pathogens is unexplored. To determine the potential for mating of *Coccidioides*, we analyzed genome sequences and identified mating type loci characteristic of heterothallic ascomycetes. *Coccidioides* strains contain either a *MAT1-1* or a *MAT1-2* idiomorph, which is 8.1 or 9 kb in length, respectively, the longest reported for any ascomycete species. These idiomorphs contain four or five genes, respectively, more than are present in the *MAT* loci of most ascomycetes. Along with their cDNA structures, we determined that all genes in the *MAT* loci are transcribed. Two genes frequently found in common sequences flanking *MAT* idiomorphs, *APN2* and *COX13*, are within the *MAT* loci in *Coccidioides*, but the *MAT1-1* and *MAT1-2* copies have diverged dramatically from each other. Data indicate that the acquisition of these genes in the *MAT* loci occurred prior to the separation of *Coccidioides* from *Uncinocarpus reesii*. An analysis of 436 *Coccidioides* isolates from patients and the environment indicates that in both *Coccidioides immitis* and *C. posadasii*, there is a 1:1 distribution of *MAT* loci, as would be expected for sexually reproducing species. In addition, an analysis of isolates obtained from 11 soil samples demonstrated that at three sampling sites, strains of both mating types were present, indicating that compatible strains were in close proximity in the environment.

*Coccidioides* is known to reproduce asexually in the soil by the segmentation of hyphae to produce arthroconidia, the infectious propagules, and by the internal cleavage of host-borne spherules to produce endospores, which spread the infection. Population genetics studies suggest that *Coccidioides* is not restricted to asexual reproduction but rather that recombination occurs (5, 20). The potential for sexual reproduction is important when considering the epidemiology of the disease: ascospores and conidia likely differ in their survival potential in the soil and may play different roles as reservoirs for the disease.

One criterion that is necessary but not sufficient for fungal sexual reproduction is the presence of mating type genes, which have been identified in a number of ascomycete fungi (reviewed in references 7, 8, and 21). For mating to occur, the mating type loci of compatible strains must contain a high-mobility group (HMG) domain gene in one strain and an α box protein gene in the other, or in the case of homothallic species, both genes must be present in the same strain (7, 8). Some species have additional genes at the mating type loci that may contribute to sexual reproduction, although for a number of fungi, only the single genes are necessary.

Among the *Eurotiomycetes*, which include *Coccidioides*, mating type loci in only two species, *Emericella nidulans*, which is self-fertile (10), and *Aspergillus fumigatus*, which like *Coccidioides* has no known sexual cycle (29), have been identified...
previously, *E. nidulans*, like other homothallic species, contains genes indicative of both ascomycete mating types in the same genome. The distribution of mating type genes in *A. fumigatus* is typical of that in heterothallic ascomycetes, with each strain containing one of two idiomorphs, nonhomologous regions of DNA at a chromosomal position flanked by common sequences, at the mating type locus (26). In the *A. fumigatus* genome, a gene encoding a MAT1-2 HMG domain protein was identified, and this identification was followed by a survey of other strains, which revealed the presence of the alternate mating type gene, encoding a MAT1-1 α box protein, in ~50% of isolates. These data, taken together with the results of population analyses suggesting that recombination in *A. fumigatus* occurs and the results of expression analyses indicating pheromone-associated gene transcription, were used to propose that *A. fumigatus* has a recent evolutionary history of a sexual cycle and may still be capable of sexual reproduction (29).

To investigate the potential for sexual reproduction in *Coccidioides*, we analyzed genome sequences for potential mating type loci and identified idiomorphs indicative of a typical heterothallic ascomycete. MAT1-1 and MAT1-2 idiomorphs of *C. immitis* and *C. posadasii* show sequence conservation between the two species. The idiomorphs in *Coccidioides* are the largest reported to date for an ascomycete, with the MAT1-1 idiomorph being 8 kb and the MAT1-2 idiomorph being 9 kb in length. The MAT1-1 idiomorph contains four open reading frames (ORFs), and the MAT1-2 idiomorph has five ORFs, more than the number observed in any other member of the *Pezizomycotina*. Interestingly, amino acid identity and nucleotide sequence comparisons between species show that both loci contain a cytochrome c oxidase subunit VIa gene (COX13) and a DNA lyase gene (APN2) that have limited similarity to their counterparts in the other locus. An analysis of 436 isolates of *C. immitis* and *C. posadasii* revealed that each isolate contained either a MAT1-1 locus or a MAT1-2 locus and that the loci were distributed in a 1:1 ratio in both species. Further, we showed that all the genes in the MAT loci were expressed as determined by either serial analysis of gene expression (SAGE) or reverse transcriptase PCR (RT-PCR), supporting the idea that the genes are functional. A comparison of the *Coccidioides* MAT loci to those of related fungi is also presented.

**MATERIALS AND METHODS**

**Strains.** *Coccidioides* strains from a variety of sources were analyzed for the presence of mating type loci. Details of the 436 strains are listed in Table S1 in the supplemental material. A total of 20 *C. immitis* strains representing samples from the full geographic range of the species were analyzed. These included 16 strains from the Roche Molecular Systems Culture Collection, along with strains RS and HS384, whose genomes have been sequenced previously. Two additional *C. immitis* isolates were obtained from patients in Arizona hospitals. The *C. posadasii* strains analyzed included a collection of 190 human clinical isolates from the University of Arizona Medical Center collected from 1976 to 1999. Another 81 human clinical samples were isolated from patients at the Southern Arizona Veterans Administration Health Care System and other Arizona hospitals. DNA corresponding to a group of 72 isolates collected at Arizona hospitals between December 2004 and January 2006 was received from Kelsea Jewell of the Arizona Department of Health Services. Eleven isolates from nonhuman mammals were received from the University of Arizona Veterinary Diagnostic Laboratory. These included six feline isolates, three canine isolates, and individual isolates from a llama and a rock hyrax. A set of 62 environmental isolates from 11 soil sites in the Tucson area were also analyzed. The isolation of these strains is described below. For the details of the strains, see Table 3 and Table S1 in the supplemental material.

**Fungal spore isolation from soil.** Fungal spores were extracted from soil by using a modified protocol from Omieczynski and Swatek (28). Briefly, 5 g of soil was mixed with 25 ml of 30% NaCl and 0.01% Tween 80 (Sigma-Aldrich, St. Louis, MO). This soil slurry was shaken thoroughly to mix and allowed to settle for 30 min. Two 10-ml aliquots of the supernatant were incubated with penicillin G (20,000 U/liter; Research Products International Corp., Mt. Prospect, IL) and streptomycin (300 mg/liter; Sigma-Aldrich) for 30 min at room temperature. The volume of each aliquot was brought to 40 ml with distilled water, and the aliquots were centrifuged at 5,000 × g for 40 min at 4°C. Pellets were rinsed twice with 25 ml of H2O and resuspended in 0.5 ml of phosphate-buffered saline, pH 7.4, with penicillin G (5,000 U/liter) and streptomycin (30 mg/liter). The two aliquots were combined, and 0.25 ml was injected intraperitoneally into two to four female BALB/c mice (6 to 8 weeks old). After 14 days (unless the mice were clinically moribund prior to 14 days), mice were sacrificed and the lungs, spleen, and any gross abdominal lesions were removed. Organs were macerated with phosphate-buffered saline and plated onto 2% glucose-yeast extract (GYE) agar plates with streptomycin (200 mg/liter). Any fungal colony that developed within 2 weeks was subcultured on fresh 2% GYE medium, and DNA was extracted from the mycelium as described below. All strains that were collected are independent isolates, although in several cases multiple isolates were collected from the same mouse (see Table 3). In most cases, only a single isolate was taken from any one organ (lung, spleen, or abdominal tissue). Positive *Coccidioides* identification was performed by PCR amplification with *Coccidioides*-specific primers (16) followed by species determination by using microsatellite primers as described by Fisher et al. (22).

**Nucleic acid isolations.** DNA was isolated from mycelia grown on 2% GYE agar plates at 28°C for approximately 2 weeks as described previously (18). For RNA isolation from mycelia, 5 × 10⁷ arthroconidia were inoculated into 100 ml of 2% GYE and shaken at 180 rpm for 48 h at 28°C. Total RNA was extracted as described by Mandel et al. (25). Poly(A) RNA was isolated from 300 μg of total RNA by using the PolyATtract mRNA Isolation System III according to the instructions of the manufacturer (Promega, Madison, WI). The quantification of nuclear acids was performed using a NanoDrop system (NanoDrop Technologies, Wilmington, DE).

**Oligonucleotide primer design.** Oligonucleotide primer sequences were designed based either on sequences conserved among the locus of *C. immitis* strain HS338.4, the sequence of which was recently released, and the corresponding loci from *C. posadasii* strain C735 and *C. immitis* strain RS or on conserved regions from putative ORFs within the loci. All primers used in this report are listed in Table 1.

**Detection of mating types of patient and soil isolates.** To determine the mating types of *Coccidioides* strains, approximately 50 ng of genomic DNA was used for PCR with three sets of oligonucleotide primers: OAM1039 and OAM1040, which amplify a 483-bp fragment adjacent to the mating type locus in both *MAT1-1* and *MAT1-2* strains; OAM1048 and OAM1049, which amplify an open reading frame from the α box gene of the *MAT1-1* idiomorph; and OAM1050 and OAM1051, which amplify a fragment of 950 bp from the HMG domain gene, present in the *MAT1-2* idiomorph. PCR reactions were performed with GoTaq Green Master Mix (Promega) under the following conditions: 2 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; and 5 min at 72°C. Samples were visualized on agarose gels.

**Isolation of the full-length *MAT1-1* locus from *C. posadasii* strain RMSCC 1040.** The *MAT1-1* locus was amplified as an 8-kb fragment with primers OAM1039 and OAM1049 (Table 1) and in two overlapping sections of 4 kb each with primers OAM1039 and OAM1052 (left half) and OAM1053 and OAM1049 (right half). The left and right ends of the mating type locus are defined according to the orientation of the *MAT1-2* locus in the *C. immitis* RS genome (Broad Institute). The 3' end of the locus was isolated as a 1.6-kb fragment with primers OAM1048 and OAM1044. PCR amplifications were performed with AccuPrime Taq DNA polymerase (Invitrogen, San Diego, CA) in buffer II by following the manufacturer’s specifications. PCR fragments were cloned into pGEM-T Easy (Promega). Single clones of the full locus and the left half were obtained and sequenced. Several clones of the right half and the 1.6-kb 3' end were obtained, and two of each type were sequenced.

**Transcription analysis.** To determine the expression of putative genes in the *MAT* idiomorphs, primers corresponding to both sides of predicted introns were designed for use in RT-PCR. For the *MAT1-2* idiomorph, oligonucleotide primers OAM1071 and OAM1072 correspond to either side of the third intron of the putative cytochrome c oxidase subunit VIa gene, OAM1073 and OAM1074 correspond to either side of the fifth intron of the putative DNA lyase gene, OAM1075 and OAM1076 correspond to either side of the sixth intron of a
TABLE 1. Oligonucleotide primers used in this study

| Primer          | Sequence                                      |
|-----------------|-----------------------------------------------|
| OAM1039         | AGCGAGAACACATCAAGGAGGTTGCC                    |
| OAM1040         | GCAGATACATCAACAAACTATATGTAGT                 |
| OAM1044         | CGTGGGCGGCACTCCCTGCTG                       |
| OAM1048         | CCCCTTGGCCCATGGTAGTTG                        |
| OAM1049         | TATCCGTGCTAATCCCTGCTG                       |
| OAM1050         | ATTGGTGAGGTTGCGGAAATGC                      |
| OAM1051         | GATATTATATCCTACTCATGAGG                     |
| OAM1052         | CCCCTGCGCGCAGTGATGTG                        |
| OAM1053         | CCACCGGCCTATTTCTGGTCTTG                     |
| OAM1056         | CAGGAGGGCTTCTTCAACAGCC                     |
| OAM1057         | CGGAAACGCTTCTATGAGG                        |
| OAM1067         | GATGGGACACACAGGAGGTG                       |
| OAM1068         | AGTTCGCCGCTATGGTGTG                        |
| OAM1071         | ATGCCACATTAGAAGACAGGGG                     |
| OAM1072         | CCTTTGTAGTGTAGTCTGCTGTG                     |
| OAM1073         | CGGAGTCTATATCCAGAGGGGC                     |
| OAM1074         | CCAAGGTGGGTCATATTACGAGG                     |
| OAM1075         | GGAAGAATCCATATGTCGAAAGAA                   |
| OAM1076         | GTATATCATGGCCGGTGCTGTAG                     |
| OAM1077         | GCATCAACCATGCGAGGACT                      |
| OAM1079         | GCCCTTCATGCGGTTGGAATT                      |
| OAM1080         | TCAATATTGTGTCACATGG                         |
| OAM1081         | CGGTCTGACAGTCCAAAGTACAGG                   |
| OAM1082         | GGTGAATTAGGTCATCTTATGAGG                   |
| OAM1084         | CGCAAAGAATGCTCCATCTATGAGG                   |
| OAM1085         | CTGCATTCTGCTGCTGTG                         |
| OAM1086         | TTTGCAAGTTTAGCTCGGACT                      |
| OAM1087         | CTCTGGTCTGCTGCTGCTGT                       |
| OAM1102         | CAAATGTGGTTCCCCAAGGTG                      |
| OAM1103         | ACAGGCCGCGAATAACTGAGAG                     |

hypothetical gene, and OAM1048 and OAM1087 correspond to either side of the single predicted intron of the MAT1-1-1 a box gene (see Fig. 3). For the MAT1-2 idiomorph, primers OAM1102 and OAM1103 correspond to either side of the second intron of the predicted ORF of the MAT1-2-2 gene, CIMG_00407.2 (Broad Institute).

Poly(A) RNA was isolated from mycelial cultures of C. posadasii strains Silveira and RMSCC 1040 and C. immitis strain RMSCC 3703 as described above. First-strand cDNA was synthesized with poly(A) RNA and oligo-(dT)12–18 by using the SuperScript Reverse Transcriptase III system according to manufacturer's specifications and by using buffer II and 3-min extension steps. Amplified fragments were cloned into pGEM-T Easy and sequenced.

**SAGE data.** We constructed LongSAGE libraries of C. posadasii strain Silveira (15, 32) from RNA extracted from mycelia grown in liquid at 37°C and from spherules grown in vitro for 24, 48, 72, 96, and 120 h at 39°C. Approximately 100,000 SAGE tags from each library have been sequenced (M. A. Mandel, E. M. Kellner, L. Li, J. N. Galgiani, and M. J. Orbach, unpublished results). We analyzed the expression of the five mating type genes within the MAT1-2 idiomorph in C. posadasii strain Silveira by identifying the numbers of SAGE tags corresponding to the genes. Data for the expression of actin (Broad Institute annotation code, CIMG_02791.2) are provided for comparison.

**Sequence analysis.** A combination of BLASTN (1) and NUCmer (from the MUMmer package) (9) was used to define the boundaries of the mating type idiomorphs by the alignment of genome sequences of Coccidioides. Genome sequences used were those of RS and H538.4 strains of C. immitis, downloaded from The Broad Institute website (http://www.broad.mit.edu/annotation /gi), and the C735 strain of C. posadasii, downloaded from The Institute for Genomic Research website (http://www.tigr.org/tdb/e2k1/c735/). Structures of predicted genes within each idiomorph were defined manually with the Artemis genome viewer and editor (3) by using a combination of evidence obtained from expressed sequence tag (EST) analyses, RT-PCR products, gene prediction programs, and analyses of homology to related genes. Nomenclature assigned to genes identified within the idiomorphs followed the conventions proposed by Turgeon and Yoder (33). If genes were homologous to previously described MAT genes, they took similar allelic designations. For example, the a box gene was named MAT1-1-1, and the HMG domain gene was designated MAT1-2-1. The genes for which no homologous MAT genes had been described previously were named beginning with the designation MAT1-1-5 in the MAT1-1 locus and MAT1-2-3 in the MAT1-2 locus.

Alignments of multiple nucleotide sequences corresponding to the predicted COX13 and APN2 coding regions were generated using ClustalW and were used to produce neighbor-joining phylogenetic trees with version 3.1 of the MEGA software package (22). The model of substitution selected in the phylogenetic analysis was the LogDet method of Hillis et al. (16a), which does not assume that all sequences within the matrix have evolved the same pattern of nucleotide substitutions. Support for each resulting topology was evaluated with 1,000 bootstrap replicates.

For comparative analyses, the genomes of Uncinocarpus reesi strain 1704 (Broad Institute) and Histoaspasma capulatum class NAMl strain WU24 (Broad Institute) were analyzed. Genes from several fungi were used for alignments of the COX13 and APN2 sequences. These genes were manually annotated from the publicly available genome sequences of Aspergillus nidulans, A. fumigatus, A. oryzae, A. terreus, Podospora anserina, Chaetomium globosum, Fusarium graminearum, Magnaporthe grisea, and Neurospora crassa.

**Nucleotide sequence accession numbers.** The consensus sequence of each C. immitis strain RSCC 1040 idiomorph was submitted to GenBank with the accession numbers EF512013-2016. Consensus genome sequences of the cytochrome c oxidase subunit IVA gene, the DNA lysis gene, the hypothetical protein gene, and the a box gene were submitted to GenBank with the accession numbers EF512009 to EF512012.

**RESULTS**

**Identification of mating type idiomorphs in Coccidioides.** In the genome sequences of C. immitis strain RS and C. posadasii strain C735, a MAT1-2 ORF with similarity to the HMG domain mating type gene that defines the MAT1-2 loci of other ascomycetes (33) was identified. At the same chromosomal position in C. immitis strain H538.4 (Broad Institute), a region of nonhomology to strains RS and C735 is present (Fig. 1A). This region contains a MAT1-1-1 a box ORF that encodes the protein characteristic of the MAT1-1 locus proteins of ascomycetes. Analyses of the sequences surrounding these genes defined regions of 9 kb in strains RS and C735 and 8.1 kb in strain H538.4 with no significant DNA homology to one another. These regions are characteristic of hetertrophic allici mating type idiomorphs and are not present at other positions within the alternate strains. The sequences flanking these idiomorphs are highly conserved in both C. immitis strains, with an average of >99% nucleotide identity over 200-kb windows on
either side of the loci. Interestingly, the left flanks (defined as the region to the left of the mating type idiomorph on super-contig 1 of the RS genome) show two distinct components (Fig. 1B). Distal to the idiomorphs, sequence identity averages more than 99%, which is typical of the levels of identity between the genome sequences of different strains of \textit{C. immitis}. However, the most proximal 2.5 kb of these flanking regions displays a marked reduction in identity, down to 97.5%, between the RS and H538.4 strains of \textit{C. immitis}. The regions flanking the right side of the MAT idiomorphs show no such effect and correspond to an abrupt increase to 99.8% identity immediately beyond the boundary of the idiomorphs.

The mating type loci of the 15 \textit{Coccidioides} strains whose genomes are being sequenced were determined by performing PCR with three pairs of primers. One set amplifies DNA sequences present in the flanking regions common to both mating type idiomorphs, and the other sets amplify a part of the HMG domain protein gene characteristic of MAT1-2 loci and a part of the α box protein gene characteristic of MAT1-1 loci. For all 15 strains, amplification occurred with the pair of primers that recognizes the common sequences in the flanking regions. Each strain also exhibited amplification with either the primers that identify the MAT1-1 locus or those that identify the MAT1-2 locus, with none producing products with both sets of primers (Fig. 2). This result supports the mating type locus structure defined by \textit{C. immitis} strains RS and H538.4, which is characteristic of most heterothallic ascomycetes, in which only one of the two alternate mating type idiomorphs is present in each strain. Of the four \textit{C. immitis} strains screened, two (H538.4 and RMSCC 3703) contained the α box sequences and seven (C735, RMSCC 3700, RMSCC 1038, Silveira, RMSCC 1037, CPA0020, and CPA0066) contained the HMG domain sequences.

The \textit{C. posadasii} MAT1-1 idiomorph was defined by sequencing overlapping PCR fragments of the locus of \textit{C. posadasii} strain RMSCC 1040. Comparison of these sequences with the MAT1-2 region of C735 defined a region of nonhomology similar to that in \textit{C. immitis}, spanning 9 kb in C735 and 8.1 kb in RMSCC 1040. Both strains are from the Arizona set of \textit{C. posadasii} isolates (reference 11 and B. M. Barker and M. J. Orbach, unpublished results).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1}
\caption{Mating type idiomorphs of \textit{Coccidioides} spp. (A) Schematic representation of the two mating type idiomorphs of \textit{Coccidioides} are shown in the boxed regions. The horizontal lines to the left and right of the idiomorphs indicate the common flanking sequences. The 8,071-bp MAT1-1 idiomorph contains four predicted genes (gray arrows with white dots), and the 9,046-bp MAT1-2 idiomorph contains five predicted genes (white arrows with black dots). There are COX13 and APN2 genes in both idiomorphs; the levels of amino acid identity between the products of these idiomorph genes in \textit{C. immitis} are indicated in brackets. (B) Sequence identity of the idiomorph-flanking regions of \textit{C. immitis} strains RS and H538.4. The flanking regions 10 kb on either side of the idiomorphs are represented by horizontal lines. Notice the reduced identity in the proximal region of the left flank.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Determination of mating types of \textit{Coccidioides} spp. PCR amplification of \textit{C. immitis} strains RMSCC 3703 and RMSCC 2394 and \textit{C. posadasii} strains RMSCC 1040, RMSCC 3488, RMSCC 1037, and Silveira with primers common to both mating types in the region to the left of the loci (+) and with idiomorph-specific primers (arrowheads below the MAT1-1-1 and MAT1-2-1 genes) revealed that strains have either the MAT1-1 (1-1) or MAT1-2 (1-2) mating type.}
\end{figure}
Sequence analysis of the corresponding idiomorphs in *C. immitis* and *C. posadasii* revealed nucleotide identity of 98.1% for MAT1-1 (*C. immitis* H538.4 and *C. posadasii* RMSCC 1040) and 98.4% for MAT1-2 (*C. immitis* RS and *C. posadasii* C735). These levels are typical of genome-wide levels of similarity between the two species. For example, the average identity between RS and C735 for all predicted genes is 98.7%.

**Annotation of predicted genes in MAT1-1 and MAT1-2 idiomorphs.** The genes in the MAT1-2 idiomorph had previously been partially identified by the Broad Institute's annotation of the *C. immitis* RS genome. To refine the annotation for this idiomorph, we used a combination of methods but primarily sequence alignments with genomic sequences from other fungi and ESTs from RS (available from the Broad Institute website, http://www.broad.mit.edu/annotation/fungi). Based on these results, we predict that there are five genes in the MAT1-2 idiomorph. The first gene is a single-exon ORF that encodes a protein similar to mannosyl transferases and has corresponding ESTs G896P517FN22.T0 and G896P517RN22.T0, which are two sequences from opposite ends of a single cDNA clone (not annotated by the Broad Institute). The second gene (Broad Institute annotation code, CIMG_00404.2) has five exons and encodes a cytochrome c oxidase subunit VIa-like protein similar to COX13 in *Saccharomyces cerevisiae*. ESTs from RS are available and help define the 5’ and 3’ untranslated regions. The third gene (CIMG_00405.2) encodes a DNA lyase-like protein similar to APN2 in *S. cerevisiae*, and the available ESTs define two splice forms. The longer splice form, which appears to be conserved in other species, has six coding exons. The alternative splice form encodes a much shorter sequence with no significant similarity to those of proteins in *S. cerevisiae* and *H. capsulatum*.

The fourth gene (CIMG_00406.2) has the same intron number and position as its MAT1-2 counterpart, but the two proteins are only 53.7% identical at the amino acid level. The APN2 RT-PCR product from RMSCC 3703 exhibited an unspliced 57-bp second intron, which would add 19 amino acids to the encoded protein, without a change of reading frame. The third gene of the idiomorph contains seven exons that were confirmed by RT-PCR, but the predicted protein sequence has no significant similarity to those of proteins in GenBank and is therefore of unknown function. The RT-PCR product sequence from RMSCC 1040 exhibits an alternative splice form that truncates the second exon, thus changing the reading frame and producing a substantially different protein sequence. The final, two-exon gene is the α box protein gene common to all MAT1-1 mating type loci (Fig. 3).

Based on proposed mating type gene-naming conventions (33), we designated the HMG domain gene MAT1-1-1 and the α box protein gene MAT1-1-1. In the MAT1-1 idiomorph, the other genes were designated MAT1-1-5 for the cytochrome c oxidase subunit VIa gene, MAT1-1-6 for the DNA lyase gene, and MAT1-1-7 for the gene of unknown function. For the MAT1-2 idiomorph, besides the HMG box gene, the others were designated MAT1-2-3 for the mannosyl transferase-like gene and MAT1-2-4 for the gene of unknown function that has similarity to the *A. fumigatus* and *N. fischeri* genes. We used MAT1-2-5 for the cytochrome c oxidase subunit VIa gene and MAT1-2-6 for the DNA lyase gene based on similarity to the designations for the MAT1-1 locus.

**Phylogeny of COX13 and APN2 genes.** Since MAT1-1 and MAT1-2 idiomorphs both contain COX13 and APN2 gene homologs that have diverged substantially from each other, we were interested in examining the phylogeny of these two genes. For this analysis, we included *U. reesii*, a nonpathogenic relative of *Coccidioides*. The genome sequence for one strain of *U. reesii* (strain 1704; Broad Institute) is available, and BLAST analysis shows this strain to have a MAT1-1 mating type, based on the presence of a MAT1-1 α box gene and the absence of a MAT1-2 HMG domain gene. Furthermore, in *U. reesii*, all genes of the *Coccidioides MAT1-1* idiomorph are present in identical order. For each COX13 and APN2 gene, we took predicted coding sequences from four strains of *Coccidioides* and 12 other fungal genomes, including that of *U. reesii*, and generated multiple sequence alignments and neighbor-joining phylogenetic trees (Fig. 4). These trees clearly show that the COX13 and APN2 genes present in the MAT1-1 strains of both species of *Coccidioides* are more closely related to the homologs found in a MAT1-1 strain of *U. reesii* than to those in the MAT1-2 strains of *Coccidioides*. However, the APN2 genes present in MAT1-2 idiomorphs of *Coccidioides* still group within the *Onygenales* and not with more distantly related fungi.

**Transcription analyses of the MAT1-1 loci in *Coccidioides* species.** To determine whether the putative ORFs in the MAT1-1 loci were expressed, RNA was isolated from mycelial cultures of *C. immitis* (RMSCC 3703) and *C. posadasii*
and cDNA was synthesized using specific primers that amplify sequences across predicted introns. For all four predicted ORFs, amplification from cDNA yielded DNA fragments smaller than those amplified from genomic DNA, indicating that a spliced transcript of each gene was produced (Fig. 3).

We have previously prepared SAGE libraries from mycelial RNA and from spherule RNA from *C. posadasii* strain Silveira at different developmental stages (M. A. Mandel, E. M. Kellner, L. Li, J. N. Galgiani, and M. J. Orbach, unpublished results, and http://www.broad.mit.edu/annotation /genome/coccidioides_immitis). We used our SAGE data to analyze the transcript levels of the five predicted ORFs in the MAT1-2 idiomorph in *C. posadasii*. As shown in Table 2, the *COX13* and the *APN2* genes are the most highly expressed at all levels of differentiation. The expression of the HMG box gene was detected at low levels at all stages except in 72-h spherules, where it was not detected. The mannosyl transferase-like gene and the gene of unknown function were expressed only at very low levels in a few developmental stages.

**Distribution of mating type loci in Coccidioides.** To analyze the distribution of the two mating types, 436 isolates of *Coccidioides* from patients living throughout the geographic range of the pathogen and from soil samples in Arizona were sur-
veyed using the three sets of primers described above. Only one mating locus was present in each Coccidioides isolate, and the distribution was approximately 1:1. Although the number of C. immitis strains analyzed (n = 20) was limited, there was a 1:1 ratio (10:10) of mating types. The C. immitis isolates included 16 strains that have previously been subjected to detailed microsatellite analyses and represent the full population range of the species (11, 12a). Additionally, two strains whose genomes have been sequenced (RS and H538.4) and two other isolates from clinical strain collections were included.

The majority of the isolates analyzed (n = 406) were C. posadasii strains from Arizona. This group included a combination of strains from patients, nonhuman mammals, and soil. There were 10 strains of C. posadasii from places other than Arizona. Among strains from human patients in Arizona (n = 334), the MAT1-1/MAT1-2 ratio was 170:164, and among the 10 C. posadasii strains from outside Arizona, the ratio was 5:5. When the strains were analyzed based on the type of infection, the ratios of mating types were similar (Fig. 5A). The distribution of idiomorphs among isolates from patients with lung-associated infections was 111:106; among those from patients with systemic infection, the ratio was 37:38. For those isolates for which the site of infection was undefined, which were likely

![FIG. 4. Phylogenetic trees of the COX13 and APN2 genes. Neighbor-joining trees for the COX13 (A) and APN2 (B) coding sequences found in each Coccidioides idiomorph indicate that the incorporation of these two genes into the mating type loci likely occurred in an ancestor common to both Uncinocarpus and Coccidioides. The numbers at the branches are percentages indicating the frequency at which each branch was obtained in the bootstrap analysis. CI, C. immitis; CP, C. posadasii.](image)

**TABLE 2. Expression of C. posadasii strain Silveira MAT1-2 genes**

| Gene name | Gene product or function | Spherules grown for indicated time (h) |
|-----------|--------------------------|----------------------------------------|
|           |                          | Mycelia | 24 | 48 | 72 | 96 | 120 |  |
| MAT1-2-3  | Mannosyl transferase     |         | 2  | 0  | 0  | 1  | 2  |  |
| MAT1-2-5  | Cytochrome c oxidase     |         | 126 | 69 | 70 | 56 | 78 | 30 |
| MAT1-2-6  | DNA lyase                |         | 16 | 11 | 6  | 7  | 14 | 25 |
| MAT1-2-7  | HMG box                  |         | 2  | 1  | 1  | 0  | 4  | 3  |
| MAT1-2-4  | Hypothetical protein     |         | 0  | 0  | 0  | 0  | 2  | 1  |
| actin     | Actin                    |         | 86 | 198| 34 | 67 | 48 | 27 |

* a SAGE data from mycelia and spherules at different developmental stages are expressed as numbers of tags per 100,000.

* b Expression data corresponding to actin are shown for comparison purposes.

![FIG. 5. Distribution of C. posadasii mating type idiomorphs among human isolates. (A) C. posadasii isolates from human patients sorted by date of collection. If the date was not available, strains were grouped as “unknown.” A trend toward greater numbers of MAT1-1 idiomorphs among the 2002 to 2005 data set was found; however, this difference was not significant (P = 0.08). (B) C. posadasii isolates from human patients sorted by infection type. If infection data were not available, strains were grouped as “undefined infection.”](image)
associated with a mixture of pulmonary and systemic infections, the \textit{MAT1-1/MAT1-2} ratio was 26:25.

A limited set of isolates (\(n = 11\)) was obtained from non-human mammals in Arizona, including domestic cats (six isolates), dogs (three isolates), and a llama and a rock hyrax (one isolate each). All of these isolates were identified as \textit{C. posadasii} by microsatellite analysis (Barker and Orbach, unpublished), and they had a 7:4 ratio of \textit{MAT1-1} to \textit{MAT1-2} genes; this divergence from 1:1 is most likely the result of the small sample size, although the distribution of alleles among cat isolates (\(n = 6\)) was 3:3.

A total of 62 \textit{C. posadasii} environmental strains, which were collected from mice inoculated with soil extracts from 11 sites in the Tucson area, were analyzed (Table 3). The sites include six individual locations and five sites along a 10-m transect. Strains were collected from lungs, spleens, or abdomens of

| Soil sample and aliquot* | Mouse no. | No. of isolatesb | MAT allelec | Organ(s)d | Isolate identification(s) |
|-------------------------|-----------|------------------|-------------|-----------|--------------------------|
| 407                     | a         | 1                | 2           | \textit{MAT1-1} | L, S | CPA0001, CPA0002 |
|                         |           | 2                | 2           | \textit{MAT1-1} | L, S | CPA0003, CPA0004 |
|                         | b         | 3                | 3           | \textit{MAT1-1} | A, L, S | CPA0005, CPA0006, CPA0007 |
|                         |           | 4                | 3           | \textit{MAT1-1} | A, L, S | CPA0008, CPA0009, CPA010 |
| 409                     | a         | 1                | 3           | \textit{MAT1-1} | A, L, S | CPA0016, CPA0017, CPA0018 |
|                         |           | 2                | 3           | \textit{MAT1-2} | A, L, S | CPA0013, CPA0014, CPA0015 |
| 448                     | a         | 1                | 2*          | \textit{MAT1-2} | L | CPA0019 |
| 485                     | a         | 1                | 2           | \textit{MAT1-2} | L, S | CPA0020, CPA0021 |
|                         |           | 2                | 1           | \textit{MAT1-2} | S | CPA0025 |
|                         | b         | 3                | 2           | \textit{MAT1-2} | L, S | CPA0022, CPA0024 |
|                         |           | 4                | 2           | \textit{MAT1-2} | L, S | CPA0026, CPA0027 |
|                         |           | 5                | 2           | \textit{MAT1-2} | L, S | CPA0028, CPA0029 |
| 500                     | a         | 1                | 6*          | \textit{MAT1-2} | L | CPA0030 |
| 573                     | a         | 1                | 2           | \textit{MAT1-1} | L, S | CPA0032, CPA0034 |
|                         | b         | 2                | 2           | \textit{MAT1-1} | L, S | CPA0031, CPA0033 |
|                         |           | 3                | 2           | \textit{MAT1-1} | L, S | CPA0035, CPA0036 |
| 574                     | a         | 1                | 3           | \textit{MAT1-1} | A, L, S | CPA0037, CPA0038, CPA0039 |
|                         | b         | 2                | 2           | \textit{MAT1-1} | L, S | CPA0040, CPA0041 |
|                         |           | 3                | 2           | \textit{MAT1-1} | L, S | CPA0042, CPA0043 |
|                         |           | 4                | 2           | \textit{MAT1-1} | L, S | CPA0044, CPA0045 |
|                         |           | 5                | 2           | \textit{MAT1-1} | L, S | CPA0046, CPA0047 |
| 578                     | a         | 1                | 2           | \textit{MAT1-2} | L | CPA0050 |
|                         | b         | 2                | 2           | \textit{MAT1-1} | A, L, S | CPA0048, CPA0049, CPA0051 |
|                         |           | 3                | 1           | \textit{MAT1-1} | L | CPA0053 |
| 580                     | a         | 1                | 3           | \textit{MAT1-1} | A, L, S | CPA0054, CPA0056, CPA0058 |
|                         | b         | 2                | 2           | \textit{MAT1-2} | L, S | CPA0055, CPA0057 |
|                         |           | 3                | 3           | \textit{MAT1-1} | A, L, S | CPA0059, CPA0060, CPA0061 |
|                         |           | 4                | 2           | \textit{MAT1-1} | L, S | CPA0062, CPA0063 |
| 582                     | a         | 1                | 2           | \textit{MAT1-1} | L, S | CPA0064, CPA0065 |
| 604                     | a         | 1                | 1           | \textit{MAT1-2} | L | CPA0066 |

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* Soil samples are indicated by sampling site numbers. Independent extractions of soil samples are identified as “a” and “b.” Soils 573 to 582 are from a 10-m transect at a single location.

b Number of strains isolated from each infected mouse. Asterisks indicate that multiple single-colony isolates were obtained from a single lung.

c Mating type idiomorph detected by PCR.

d Type(s) of organ tissue from which strains were isolated: A, abdominal cell wall, L, lung, and S, spleen.
infected mice. Six *Coccidioides* colonies isolated from the lung tissue of a single mouse inoculated with a soil sample from site 500 were screened for *MAT* loci. All isolates were of the same mating type, *MAT1-2*. For all sites, the strains isolated from different organs from a single mouse had the same mating type. For 8 of the 11 sites, only a single mating type was found, with a range of 1 to 11 isolates analyzed per site. Three of these sites, 573, 574, and 582, were part of the 10-m transect and yielded only *MAT1-1* strains. For three sites, strains of both mating types were recovered, indicating coincidental habitation of potentially sexually compatible strains. Site 409B corresponded to *MAT1-1* strains recovered from samples of all three tissue types, lung, spleen, and abdomen, from one mouse and *MAT1-2* strains from the three tissue types from a second mouse. These two mice were inoculated with different aliquots of a single soil extract. For site 578, which was part of the 10-m transect, of five strains recovered, four had *MAT1-1* and one had *MAT1-2*. The *MAT1-2* strain was from a single mouse inoculated with one aliquot of the soil extract, while the four *MAT1-1* strains were from two mice inoculated with a separate aliquot. For site 580, another of the transect sites, of 10 strains recovered, 8 had *MAT1-1* and two had *MAT1-2*. Two separate aliquots of soil from site 580 were made, with one producing a single infected mouse and three isolates, all having *MAT1-1*, while the other resulted in seven isolates from three mice, with two from one mouse having *MAT1-2* and the other five having *MAT1-1*.

The temporal distribution of mating types can be analyzed also (Fig. 5B). Because of the small sample size, it was necessary to combine data from different decades. For this analysis, we looked at only clinical isolates, as multiple isolations from the same soil sites would skew the data. For 1970 to 1979, the *MAT1-1/MAT1-2* ratio was 6:7, which approximates 1:1, despite the small sample size. For 1980 to 1989, the ratio was 43:49, and the ratio was 50:56 for 1990 to 1999. However, for 2002 to 2005, the timing of the recent outbreak of coccidioidomycosis in Arizona, the ratio was 64:44. This finding demonstrates a shift from the normal 1:1 ratio observed in all other analyses to a 1.4:1 ratio. When only strains from 2005, for which we have extremely reliable clinical data and genotypic information, were analyzed, the same ratio (42:30) was found. However, when a χ² test was performed, the difference in the *MAT1-1/MAT1-2* ratio from 1:1 was not significant (χ² = 2.94; P = 0.08).

**DISCUSSION**

Analyses of molecular markers in *Coccidioides* have suggested that rather than reproducing clonally, the species have a recombining population structure. In this report, we provide direct support for sexual reproduction in *C. immitis* and *C. posadasii* by demonstration of the existence of mating type loci characteristic of heterothallic ascomycetes. Sexual reproduction in ascomycetes is controlled by mating type (*MAT*) loci that in heterothallic species are composed of nonhomologous sequences at the same chromosomal positions in compatible strains. These regions, termed idiomorphs (26), have been described previously as varying in size from 1.2 to 5.7 kb and range in gene number from one to three in other filamentous ascomycetes (8). *MAT* loci in all fungi have been shown to contain an HMG domain gene in one idiomorph and an α box gene in the other, while additional genes in the loci vary. In many apparently asexual fungi, mating type loci have been identified via genome sequences or amplification of the two genes that define the idiomorphs to suggest the presence of cryptic sexual reproduction.

In *Coccidioides*, we have identified the idiomorphs and provided evidence for their function, both by transcriptional analyses and by a study of their distribution in the population. Structurally, the *Coccidioides* idiomorphs are unusually large, with the *MAT1-1* and *MAT1-2* loci being 8.1 and 9 kb, respectively. The only other characterized *MAT* loci in members of the *Eurotiomycetes* are those of the homothallic species *E. nidulans* (10) and those of *A. fumigatus* (29). In *E. nidulans*, the two genes that define *MAT* loci are on separate linkage groups in the same strain, while in *A. fumigatus*, idiomorphs that are 2.0 and 2.4 kb in length have been defined, and each were reported to contain only the single genes that define *MAT1-1* and *MAT1-2* loci, although we believe that the *MAT1-2* idiomorph contains an additional gene (see below).

*Coccidioides* and the related fungus *U. reesii* appear to have expanded their *MAT* idiomorphs by the acquisition of two genes, the *APN2* DNA lyase gene and the *COX13* cytochrome c oxidase subunit VIa gene, that are frequently found adjacent to mating type idiomorphs in the flanking regions common to both loci (8). After the acquisition of these genes, the genes in the different idiomorphs diverged dramatically from each other, with the *Coccidioides* genes retaining very limited DNA sequence similarity, the COX13 proteins showing only 76.4% amino acid identity, and the APN2 proteins showing only 53.7% amino acid identity. Phylogenetic analysis of the *APN2* and *COX13* genes shows that the *MAT1-1* idiomorphs of the two *Coccidioides* species are more closely related to the *MAT1-1* idiomorph of *U. reesii* than to the *Coccidioides* *MAT1-2* idiomorphs. This result suggests that the acquisition of the *APN2* and *COX13* genes by the mating type idiomorph predates the divergence of *Uncinocarpus* and *Coccidioides* species and that *Uncinocarpus* is likely to have the same idiomorph structure as *Coccidioides*. Conversely, the *H. capsulatum* *APN2* and *COX13* genes from *MAT1-1* strain WU24 (Broad Institute) fall outside the *Coccidioides/Uncinocarpus* clade containing both *MAT1-1* and *MAT1-2* mating types, suggesting that the acquisition and subsequent divergence of the *APN2* and *COX13* genes in the mating type idiomorphs occurred within the *Onygenales* after the *Histoplasma-Coccidioides/Uncinocarpus* split (Fig. 4).

This conclusion is consistent with the recently reported characterization of the mating type idiomorphs of *H. capsulatum* (4). In contrast, our inferred history of the *APN2* genes in *Coccidioides* differs from that reported by Fraser et al. (13), in which a gene genealogy shows the *MAT1-2* *APN2* genes as basal in a clade including all other *APN2* genes from the *Pezizomycotina*. However, we believe that topology is probably an artifact due to long-branch attraction to the very distantly related outgroup sequence (*APN2* from *Candida albicans*) and is not the result of horizontal gene transfer as speculated. Rather, the origin and diversification of the two *APN2* genes in *Coccidioides* appear to be the result of their incorporation into the *MAT1-1* and *MAT1-2* idiomorphs in a common ancestor of *Coccidioides* and *Uncinocarpus* and subsequent sequence di-
vergence. Part of this divergence may be due to the positive selection that Fraser et al. report to be acting on these genes as detected by a PAML analysis (13).

Interestingly, the order and orientation of the APN2 and COX13 genes relative to the α box gene in the sequenced MAT1-1 strain of *H. capsulatum* are inverted compared to those in *U. reesii* and *Coccidioides* (data not shown). Whether this inversion event is connected to the acquisition of the APN2 and COX13 genes in the mating type idiomorphs is not known. The analysis of additional species within the *Onygenales* more closely related to *Coccidioides* than *Histoplasma*, such as *Auxarthron zuffianium*, *Aphanasacus fulvescens*, *Trichophyton rubrum*, and *Malbranchea dendritica* (24), should allow further definition of how and when these two genes were acquired in the mating type loci.

In *Coccidioides*, both the COX13 and APN2 genes are unique, indicating that their expression in the *MAT* idiomorphs is necessary for cellular function. SAGE of Silveira, a *MAT1-2* strain, indicates that these two genes are the most highly expressed of the *MAT* loci genes (Table 2) and are expressed at all stages examined. COX13 is not essential for cytochrome c oxidase activity in *S. cerevisiae* but appears to play a role in ATP regulation of the complex that modulates this activity (2). APN2 is important in other fungi for DNA repair via the base excision repair pathway in response to DNA damage that produces apurinic/apyrimidinic sites (17).

The roles of the additional genes in the *Coccidioides* *MAT* loci during sexual reproduction are not known. The *MAT1-2* strains of *Coccidioides* and is not associated with *MAT* loci in the other sequenced *Onygenales* or in the genomes of four *Aspergillus* species, *A. fumigatus*, *A. terreus*, *A. nidulans*, and *A. oryzae*. Sequences similar to that of the unknown *MAT1-2* gene are found adjacent to the HMG domain gene, and in the same relative orientation as the gene in *Coccidioides*, within the idiomorphs of four other *Eurotium* species, *A. fumigatus*, *N. fischeri*, *H. capsulatum*, and *P. marneffei* (4, 29, 31, 35), although only those in *A. fumigatus* and *N. fischeri* have been annotated. In *N. fischeri*, the *MAT1-2* homolog is described as a putative mating locus gene, but there is no indication in any system of whether it has a function in mating. It is not found associated with the *MAT* locus in the sequenced strain of *U. reesii* (although this result may be because the *U. reesii* strain is a *MAT1-1* strain) and is completely absent from the *Aspergillus* genomes other than that of *A. fumigatus*. It is possible that this gene represents an earlier acquisition of a gene in the *MAT1-2* locus of heterothallic *Eurotium* species than the APN2 and COX13 genes. Further analysis of other members of the *Eurotium* species is needed to address this possibility. The *MAT1-1* unknown gene is found adjacent to the α box gene in *U. reesii*, *H. capsulatum*, and *P. marneffei* but is not found in the *Aspergillus* genomes, even though *MAT1-1* locus sequences are available for *A. fumigatus*, *A. oryzae*, and *A. nidulans* (14).

The *MAT1-1* gene is found in the same orientation within the *Onygenales*, *Coccidioides*, *H. capsulatum*, and *U. reesii*, but in the orientation opposite that of *MAT1-1* in *P. marneffei*.

An expression of a sexually reproducing organism is that compatible partners will be present in the population in approximately equal numbers, while for a primarily clonally reproducing organism, there is no such requirement. Clearly, in all ways that the *Coccidioides* populations are analyzed, the 1:1 distribution of *MAT1-1* and *MAT1-2* loci is indicative of a sexually reproducing species, supporting previous evidence of recombination. Although the analysis of clinical strains supports the distribution at a geographic level only, our finding of both mating types in three of 11 soil samples that contained *Coccidioides* demonstrates the local potential for sexual reproduction. Further population analyses of these sites should allow testing of recombination at a local level. The equal distribution of the *MAT* idiomorphs among *Coccidioides* clinical isolates suggests that there is no obvious association between mating type and virulence for these strains, in contrast to findings reported for *H. capsulatum* (23). It has been suggested previously that mating type plays a role in virulence in *Cryptococcus neoformans*, but the development of congenic strains suggests that the genetic background of the strain is important in determining whether the mating type locus has an effect (27). Our observation that clinical strains collected from 2002 to 2005 show a slight bias for the *MAT1-1* idiomorph needs further strain analysis to determine whether it is significant.

The role of sexual reproduction in the pathogenicity of *Coccidioides* is not known. Formerly, studies of the epidemiology and ecology of *Coccidioides* have assumed a life cycle involving the production of saprobic asexual spores for dispersal in the environment, with the growth of spherules and endospores during dissemination in a mammalian host. The likely presence of a sexual cycle raises the possibility that ascospores may play a significant role as a disease reservoir or as a longer-term survival propagule.

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ADDENDUM

During the preparation of this paper, it came to our attention that a related analysis of the *Coccidioides* idiomorph structure by Fraser et al. was published online in *Eukaryotic Cell* (13). This paper extends that analysis with identification and expression analyses of all the genes of the idiomorphs, along with a detailed population analysis of the distribution of *MAT* loci.

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