

# Identification of the Mating-Type (*MAT*) Locus That Controls Sexual Reproduction of *Blastomyces dermatitidis*

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*Blastomyces dermatitidis* is a dimorphic fungal pathogen that primarily causes blastomycosis in the midwestern and northern United States and Canada. While the genes controlling sexual development have been known for a long time, the genes controlling sexual reproduction of *B. dermatitidis* (teleomorph, *Ajellomyces dermatitidis*) are unknown. We identified the mating-type (*MAT*) locus in the *B. dermatitidis* genome by comparative genomic approaches. The *B. dermatitidis* *MAT* locus resembles those of other dimorphic fungi, containing either an alpha-box (*MAT1-1*) or an HMG domain (*MAT1-2*) gene linked to the *APN2*, *SLA2*, and *COX13* genes. However, in some strains of *B. dermatitidis*, the *MAT* locus harbors transposable elements (TEs) that make it unusually large compared to the *MAT* locus of other dimorphic fungi. Based on the *MAT* locus sequences of *B. dermatitidis*, we designed specific primers for PCR determination of the mating type. Two *B. dermatitidis* isolates of opposite mating types were cocultured on mating medium. Immature sexual structures were observed starting at 3 weeks of coculture, with coiled-hyphae-containing cleistothecia developing over the next 3 to 6 weeks. Genetic recombination was detected in potential progeny by mating-type determination, PCR-restriction fragment length polymorphism (PCR-RFLP), and random amplification of polymorphic DNA (RAPD) analyses, suggesting that a meiotic sexual cycle might have been completed. The F1 progeny were sexually fertile when tested with strains of the opposite mating type. Our studies provide a model for the evolution of the *MAT* locus in the dimorphic and closely related fungi and open the door to classic genetic analysis and studies on the possible roles of mating and mating type in infection and virulence.

*Blastomyces dermatitidis* is a thermally induced dimorphic fungus associated with severe respiratory and disseminated diseases in immunocompetent humans and other animals (1, 2). It can undergo a temperature-dependent morphological change from mycelium at environmental temperature (22°C) to pathogenic yeast at body temperature (37°C). In the lung, or in culture at 37°C, the conidia germinate into multinucleate, haploid, budding yeast cells, resulting in lung infections and, at times, systemic spread to other organs (1). *B. dermatitidis* is endemic to the Ohio and Mississippi River valleys, and in southeastern states, and causes pneumonia following the inhalation of conidia from the soil and subsequent conversion to budding yeast cells in animal hosts (3).

*B. dermatitidis* can reproduce sexually, and *Ajellomyces dermatitidis* is the teleomorphic form (4). While *B. dermatitidis* sexual structures have not been identified in nature, the teleomorph *Ajellomyces dermatitidis*, McDonough & Lewis, can be generated by pairing opposite mating-type cultures *in vitro* (4). Work by McDonough (4, 5) and by Kwon-Chung (6) in the late 1960s and early 1970s showed the formation of cleistothecia containing asci with eight ascospores each and characterized the mating system as heterothallic and bipolar. Sexual reproduction is pervasive in eukaryotes and is a major force driving the evolution of eukaryotic microbes by significantly increasing genetic diversity and, in some cases, may generate novel genotypes with altered virulence (7, 8). *B. dermatitidis* is generally regarded as a heterothallic species that requires isolates of both mating types to complete the sexual cycle. However, a *B. dermatitidis* isolate of both mating types has been reported from a patient with blastomycosis (9, 10). This suggests that patients might be infected either with two isolates of different

mating types or with diploids heterozygous for mating type. Other possibilities are that *B. dermatitidis* also has a homothallic mating system and that some isolates are self-fertile.

Sexual reproduction may be associated with the virulence and outbreak of infectious diseases. For example, sexual reproduction of *Cryptococcus gattii* has been advanced as a potential contributing factor to the outbreak on Vancouver Island (11, 12). *C. gattii* is a sibling species of *Cryptococcus neoformans* and, while both species can infect both immunocompromised and immunocompetent hosts, *C. neoformans* causes most opportunistic infections in HIV/AIDS patients. The *C. gattii* VGII molecular type is causing an outbreak that began on Vancouver Island in 1999 and has since expanded to the Pacific Northwest region in both Canada and the United States (11, 13, 14). The major outbreak genotype VGIIa is highly clonal, but multilocus sequence typing (MLST) and the identification of an  $\alpha/\alpha$  diploid isolate of the same mating types provides evidence that the outbreak genotype may have been generated via same-sex mating to cause the outbreak and its expansion (12). Recently, studies revealed that the *C. gattii* VGIII molecular type is another common agent causing cryptococcosis in HIV/AIDS patients in southern California (15). The VGIII popu-

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TABLE 1 Primers used in this study

Primer	Sequence (5'→3')	Aim
BD_alpha_box_F	TTCCAAATTTACTTGACCAGCAT	Mating-type determination ( <i>MAT1-1</i> )
BD_alpha_box_R	CGTTGAATTCATTCATAGCCTTC	
BD_HMG_F	ATCGCCTGAGGTGTGTTTATCTA	Mating-type determination ( <i>MAT1-2</i> )
BD_HMG_R	GGAGCGTATTGATAGTCAGGATG	
PI primer 10	AATAGAACCCATCCC	RAPD
PI primer 24	CGTGCAAGGGAGCACC	RAPD
RFLP-1-F	CAGGTTATCTCTTCCGTGTCC	PCR-RFLP
RFLP-1-R	CCCAGACAAACTCATAGTTCC	
RFLP-2-F	GGGGAAATGTGAAATGTTGTT	PCR-RFLP
RFLP-2-R	ACAGCTTGGCTCTCATCAACC	

lation includes two distinct sublineages, VGIIIa and VGIIIb, which differ in virulence in animal models, although both can cause infections in HIV/AIDS patients. Many VGIII isolates are fertile, and recombination has been detected in the VGIII global population (15). These findings suggest sexual reproduction may be an important force maintaining the genetic diversity and virulence of the *C. gattii* VGIII population that is causing disease in HIV/AIDS patients.

In this study, we used comparative genomic approaches to delineate the structure of the *MAT* locus of *B. dermatitidis* and conducted mating assays to demonstrate that the *B. dermatitidis* *MAT* locus controls sexual reproduction.

## MATERIALS AND METHODS

**Strains and cultivation.** *B. dermatitidis* strains ATCC 18187 and ATCC 18188 were obtained from the American Type Culture Collection. The yeast-phase cells were maintained at 37°C on slants of Middlebrook 7H10 agar with oleic acid-albumin-dextrose-catalase (OADC) supplement or on 3 M agar plates. Conversion to the mold form was accomplished on potato dextrose agar (PDA; 2% dry potato flakes, 1% glucose, and 1.5% agar) at 22°C, and mold-phase cells were maintained at 22°C on Sabouraud dextrose agar (SAB; BD Biosciences, Sparks, MD).

**Identification of the *B. dermatitidis* *MAT* locus.** Mating in ascomycetous fungi is governed by a single *MAT* locus with two idiomorphs of highly divergent sequences (16). In the euascomycetes, each *MAT* idiomorph encodes an alpha domain or HMG domain transcription factor (16, 17). The alpha domain and HMG domain genes determine plus and minus strains, respectively, and the corresponding *MAT* locus is designated *MAT1-1* and *MAT1-2*, respectively. The alpha domain and HMG domain genes that determine the mating types of dimorphic fungi are evolutionarily conserved and can be identified by homology searches using genes from the closely related species. To identify the *MAT* locus of *B. dermatitidis*, the alpha domain and HMG domain genes from the *MAT* locus of closely related species *Histoplasma capsulatum* (18, 19), *Coccidioides immitis* (19), and *Paracoccidioides brasiliensis* (20) were used as query sequences to do BLASTn searches of the *B. dermatitidis* genomic sequences using the Broad Institute database ([http://www.broadinstitute.org/annotation/genome/blastomyces\\_dermatitidis/MultiHome.html](http://www.broadinstitute.org/annotation/genome/blastomyces_dermatitidis/MultiHome.html)). The available genome sequences are for isolates ATCC 26199, SLH14081, ER3 (ATCC MYA-2586), ATCC 18188, and ATCC 18187 (GenBank accession numbers AEII01000000, ACBU01000000, ACBT01000000, ADMK01000000, and AJJV01000000, respectively). The top hits and flanking regions were then extracted from the *B. dermatitidis* genomic sequences to identify the *MAT* locus. The alpha domain and HMG domain genes and flanking regions were aligned using the CLUSTALW program to determine the borders of the *MAT* locus.

A syntenic comparison of the *MAT* locus and flanking regions among the four *B. dermatitidis* isolates was performed by using the Artemis and ACT tools (21). Briefly, the *MAT* locus and flanking regions of the four

isolates were compared using local BLASTn and the BLAST results were then entered into the ACT tool to visualize synteny.

**Isolation of genomic DNA (gDNA) and mating-type determination for *B. dermatitidis* by PCR.** Liquid cultures of yeast cells in histoplasma macrophage medium (HMM) were grown to mid-log phase, and  $\sim 5 \times 10^7$  to  $1 \times 10^8$  cells were washed with water two times and resuspended in 4 ml of osmotic medium (0.3 g/ml MgSO<sub>4</sub>, 10 mM NaPO<sub>4</sub> buffer [pH 5.8]) containing 4 mg/ml Yatalase (Fisher Scientific) and 2 mg/ml lysing enzymes (Sigma, St. Louis, MO). The suspensions were incubated at 37°C, with gentle mixing. After overnight incubation, the preparations were checked for protoplasts and washed by adding 2.5 volumes (10 ml) of trapping buffer (0.6 M sorbitol, 0.1 M Tris [pH 7.0]), centrifuged at  $1,000 \times g$  for 10 min, and washed two more times with 15 ml of trapping buffer. gDNA was isolated from the protoplasts using a Qiagen genomic DNA kit, with Qiagen 20/G tips and modifications used for yeast DNA.

The available *MAT1-1* and *MAT1-2* sequences of four *B. dermatitidis* isolates were aligned using CLUSTALW to choose primers for the mating-type determination for *B. dermatitidis* by PCR. In addition, the selected primers were further used as query sequences in BLAST searches of the *B. dermatitidis* genome to avoid potential nonspecific amplification. The primers used for the mating-type determination by PCR are listed in Table 1. PCR assays were conducted in a PTC-200 automated thermal cycler (MJ Research). A total of 300 ng of each DNA preparation was amplified in a 25- $\mu$ l reaction mixture containing 10 pM each primer, 2 mM each nucleotide (dATP, dCTP, dGTP, and dTTP), 2.5  $\mu$ l of  $10 \times$  *Ex Taq* buffer, 0.125  $\mu$ l of *Ex Taq* polymerase (TaKaRa), and an appropriate volume of distilled water. The following conditions were used for the amplification: an initial 3 min of denaturation at 95°C, followed by 35 cycles of denaturation for 30 s at 94°C, an annealing time of 20 s at 54°C, and an extension cycle for 1 min at 72°C. The amplification was completed by an extension period of 5 min at 72°C. Sterile water served as a negative control in each assay. PCR products were analyzed on 1.5% agarose gels.

**Mating assays.** The medium used for the cocultivation of paired mating strains was soil extract agar (SEA). Soil extracts were produced from 350 g of Miracle-Gro African Violet Enriched Potting Soil (obtained from a local nursery), which was placed in 2.1 liters of tap water, autoclaved for 30 min at 15 lb/in<sup>2</sup>, allowed to cool, and filtered through two layers of Miracloth. To make SEA, 1 g of yeast extract and 1 g of D-glucose were added to 1 liter of soil extract and the pH was adjusted to 6.8, prior to adding 15 g of agar. The mix was autoclaved for 10 min at 20 lb/in<sup>2</sup>, with a 15-min exhaust, and allowed to cool, and penicillin and streptomycin were added (to 100 units/ml and 100 mg/ml, respectively) before pouring either 60- or 100-mm plates. Sections of agar ( $\sim 0.5$  by 1 cm) containing mold, from the leading edge of growth on PDA or SAB plates, for pairs of strains of interest, were placed  $\sim 2$  cm apart on SEA plates and incubated at 22°C for 4 to 12 weeks. Plates were examined with a low-power stereomicroscope at 45 $\times$  to visualize cleistothecium formation at the junction of the spreading inoculation. The fertility of cleistothecia was analyzed in one of the following two ways: (i) cleistothecia were scraped into 10  $\mu$ l of lactophenol blue on a microscope slide, squashed under a coverslip, and

viewed at 400 $\times$  to visualize ascospores and (ii) cleistothecia were scraped into 100  $\mu$ l of water in a Microfuge tube, disrupted by vortexing, and then viewed at 400 $\times$  for ascospore release. In some cases, the disrupted cleistothecia were preserved in 2% formaldehyde before microscopy.

To validate the identified *MAT1-1* and *MAT1-2* loci as functional mating-type determinants for *B. dermatitidis*, we conducted mating assays using two isolates that were analyzed in the original work by McDonough to demonstrate fruitful mating in culture: isolates ATCC 18187 and ATCC 18188. In addition, F1 progeny of crosses between the parental strains were backcrossed to the parental strains, and siblings mated with other F1 strains (see below).

**F1 progeny testing.** Two batches (M1 and M2) of F1 progeny from different sites on the mating plate were analyzed in our study. Individual F1 progeny were obtained by scraping several cleistothecia off the agar surface of SEA mating plates after 10 weeks or more and by placing in 100  $\mu$ l of sterile water. After vortexing to release the ascospores, an aliquot was viewed with a hemocytometer at 400 $\times$  to determine the presence of ascospores, their abundances, and the presence of nonascospore cells. Serial dilutions were plated on 3 M medium, and the plates were incubated at 37 $^{\circ}$ C to allow the growth of independent yeast cell colonies. Individual colonies were patched onto 3 M agar and, when grown, were streaked for single colonies to ensure the clonality of individual F1 strains. An individual colony was then selected for each F1 strain and expanded on 3 M agar plates at 37 $^{\circ}$ C. These recloned F1 strain cultures were used to isolate genomic DNA for molecular analyses and were converted back to mold for mating studies.

To test the fertility of F1 progeny, eight *B. dermatitidis* strains, including the two parental isolates ATCC 18187 (–) and ATCC 18188 (+) and six F1 progeny, M1-7 (+), M2-1 (+), M2-2 (+), M2-16 (+), M2-11 (–), and M2-18 (–), were used as tester strains for mating assays. The mating assays were conducted as described in the previous section. The production of cleistothecia with ascospores was defined as a successful mating.

**Identification of SNPs between isolates ATCC 18187 and ATCC 18188.** A total of 824,355,256 reads, with a length of 101 bp, were generated at ATCC 18187 using Illumina HiSeq technology. To identify single nucleotide polymorphisms (SNPs), the ATCC 18187 reads were aligned to the ATCC 18188 assembly using BWA version 0.5.9 with default parameters. Alignments with mapping quality of at least 30 were selected using SAMtools view; these high-quality alignments have an average depth of 11 across all reference positions. Candidate variants were called from the alignments using GATK's Unified Genotyper (GenomeAnalysisTK version 1.0.3471) (22). The resulting SNPs were then filtered to require a depth of 4 or more, a maximum of 1 SNP per 10-bp window, a homopolymer run of the variant in either direction of  $\leq 5$ , and strand bias values of  $\leq 0.0$  (high strand bias values denote more bias in the forward or reverse strand and are more likely to indicate false-positive calls).

The filtered set of 44,353 SNPs were then subjected to a variant recalibration process, using GATK's Variant Recalibrator. The purpose of the variant recalibration process is to assign a well-calibrated probability to each variant call in a call set.

The module evaluates variants in a two-step process. The variant recalibration procedure involves using the Variant Recalibrator walker to create a Gaussian mixture model (GMM) by looking at the different parameters for a high-quality subset of the call set and then evaluating all the variants. For the ATCC 18187 analysis, 29,727 concordant SNPs between the GATK and SAMtools pipelines (23) were used as the high-quality subset. A GMM using five statistics, QD (variant quality divided by depth), MQ (mapping quality), MQRankSum (mapping quality rank sum test), ReadPosRankSum (read position bias rank sum test), and SB (strand bias), was generated. Using the GMM tranche file generated by the previous step, the ApplyRecalibration walker examined each variant's VQS LOD value and decided which tranche it falls in (true positive or false positive). Variants in tranches that fall below the specified truth sensitivity filter level were filtered out. The variant calibration process resulted in a total of 41,892 high-quality recalibrated SNPs. These SNPs were submit-

ted to dbSNP (handle, BROAD-GENOME BIO; dbSNP release build identifier, B136).

**Evaluation of genetic recombination in F1 progeny of *B. dermatitidis*.** The F1 progeny of the cross between isolates ATCC 18187 and ATCC 18188 are expected to demonstrate recombination between unlinked genetic markers. One known polymorphic locus between these lines is, of course, the *MAT* locus. Therefore, PCR primers were generated to yield distinct products representing one or the other mating type (see above). To find other genetic markers, random amplification of polymorphic DNA (RAPD) primers were tested and found to generate DNA products that are polymorphic between the parental strains.

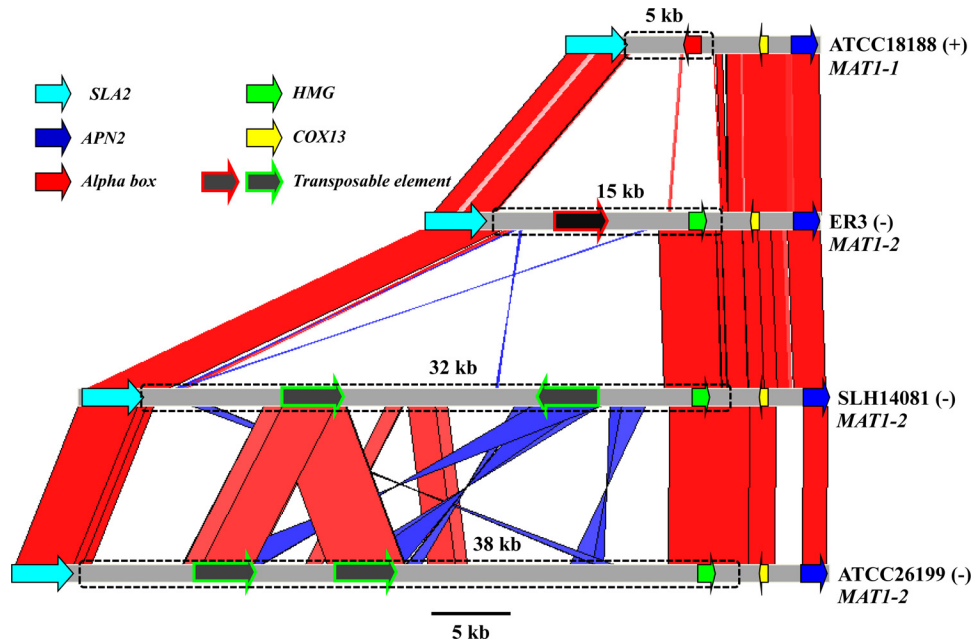
We also used SNPs, as identified above, to design PCR-restriction fragment length polymorphism (PCR-RFLP) assays for genotyping strains. Genomic DNA was isolated from 22 of the F1 progeny (obtained from two different crosses of ATCC 18187 and ATCC 18188) and assayed for these PCR polymorphisms.

## RESULTS

***B. dermatitidis* is heterothallic with two mating types, *MAT1-1* and *MAT1-2*.** By performing BLASTn searches, an alpha domain gene that exhibits 73% DNA sequence similarity with the alpha domain gene of the *MAT1-1* allele of *H. capsulatum* was identified in the *B. dermatitidis* ATCC 18188 genomic sequence. By a similar approach, an HMG domain gene that exhibits 68% DNA sequence similarity with the HMG domain gene of the *MAT1-2* allele of *H. capsulatum* was identified in the genomic sequences of the *B. dermatitidis* isolates ATCC 26199, ER3, and SLH14081. The identified alpha domain and HMG domain genes were then used as query sequences to perform BLASTx searches in GenBank, and the top hits are the *MAT* locus genes of dimorphic fungi and the closely related dermatophyte fungi. This suggests that the alpha domain and HMG domain genes that were identified in the genomic sequences of four *B. dermatitidis* isolates are bona fide *MAT* locus genes. Analyses of the flanking regions further support the identification of the *MAT* locus idiomorphs of *B. dermatitidis* (see below). Only an alpha domain or HMG domain gene (and not both) was identified in the genomic sequence of any individual isolate, providing further support that *B. dermatitidis* is a heterothallic species.

***B. dermatitidis* has a similar *MAT* locus gene architecture as other dimorphic fungi and the dermatophytes.** Like other dimorphic fungi, *H. capsulatum*, *C. immitis*, and *P. brasiliensis* (18, 19, 24), the *B. dermatitidis* *MAT* locus is tightly associated with the *SLA2*, *APN2*, and *COX13* genes (Fig. 1). The *SLA2* gene lies on one side, and the *COX13* and *APN2* genes flank the *MAT* locus on the other side (Fig. 1). A conserved gene order is a common feature of the *MAT* locus of ascomycetous fungi and is often used as a strategy to identify the *MAT* locus genes. However, in contrast to the dimorphic fungal pathogens, in the dermatophytes, the *SLA2*, *COX13*, and *APN2* genes are instead linked on one side of the *MAT* locus (24).

***B. dermatitidis* *MAT* locus size varies significantly due to the presence of transposable elements.** Typically, the fungal *MAT* locus is highly diverged and exhibits significant DNA sequence dissimilarity compared to other genomic regions. The *MAT* locus boundaries and size are determined by DNA sequence alignment and comparison between isolates of different mating types (24–26). The *MAT* locus sizes of *B. dermatitidis* determined by DNA sequence alignment between isolates ATCC 18188 (+) and ER3 (–) are 5 and 15 kb, respectively. However, the *MAT* locus (–) idiomorph sizes as determined by DNA sequence alignment be-



**FIG 1** Syntenic comparison of the *MAT* locus of *B. dermatitidis*. Red bars/lines represent highly syntenic and similar DNA regions, and blue bars/lines represent highly syntenic and similar DNA regions with reverse orientation. The *B. dermatitidis* *MAT* locus is flanked by the *SLA2*, *COX13*, and *APN2* genes, which share high sequence identity among these isolates. Isolate ATCC 18188 is designated plus (+) mating type due to the presence of the *MAT1-1* locus, which is determined by an alpha-box gene. Isolates ER3, SLH-14081, and ATCC 26199 are designated minus (-) mating type, based on the identification of an HMG gene in the *MAT1-2* locus. The genomic regions depicted with boxes in dashed outlines are the *MAT* locus. These TEs result in expansion of the *MAT* locus of *B. dermatitidis*. TEs are present in the *MAT* locus of three minus isolates ER3, SLH-14081, and ATCC 26199.

tween the plus isolate ATCC 18188 and the minus isolates SLH14081 and ATCC 26199 are 32 and 38 kb, respectively. The size variation of the *B. dermatitidis* *MAT* locus is mainly the consequence of the presence of transposable elements within the *MAT* locus. One retrotransposon is identified in the minus isolate ER3, and two different retrotransposons are found in the minus isolates SLH14081 and ATCC 26199 (Fig. 1). These retrotransposons are flanked by terminal repeats and encode a DEE superfamily endonuclease domain and a PRK00247 60-kDa IMP superfamily domain and are also found in other genomic regions. However, no transposable element was identified in the plus isolate ATCC 18188. It is not known if other minus strains lack transposable elements or if other plus strains harbor them.

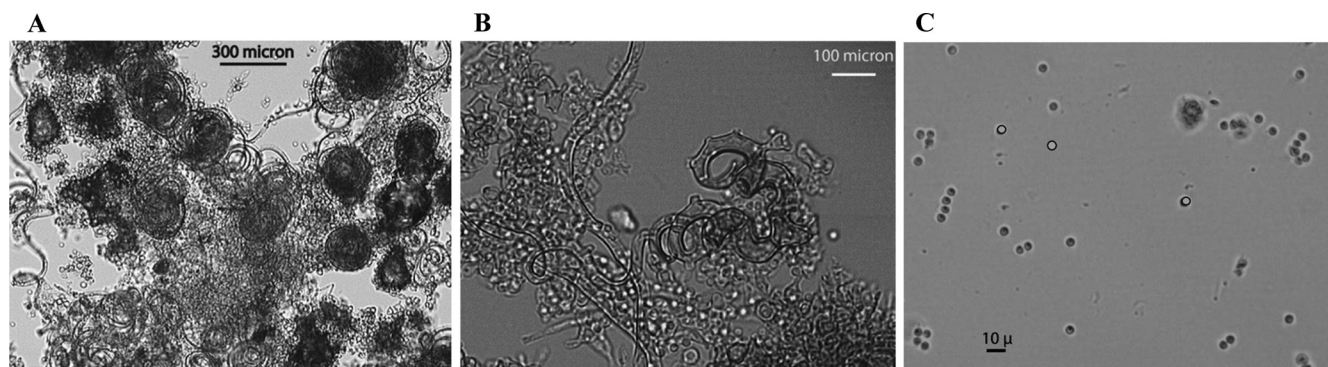
**Sexual reproduction of *B. dermatitidis* isolates of opposite mating types.** The sexual development of *B. dermatitidis* has been reported previously (4, 5). However, the biochemical and molecular basis of the control of *B. dermatitidis* sexual reproduction by the *MAT* locus was not clear at that time. In our study, two heterothallic *B. dermatitidis* isolates of the opposite mating type, ATCC 18188 and ATCC 18187, were cocultured on appropriate mating medium to study the sexual development of *B. dermatitidis* and to confirm that the *MAT* locus controls the sexual cycle. Several types of agar medium were tested to find a medium that would support mating, and SEA (see Materials and Methods section) was found to reproducibly support mating in fertile strains of the opposite mating type. After 3 or 4 weeks at room temperature (22°C), spherical structures representing immature cleistothecia could be seen by low-power light microscopy. With longer incubations, the cleistothecia grew larger and acquired a tan pigmentation. Putative cleistothecia were scraped from the agar surface, squashed on a microscope slide, and viewed under high

power, where we observed sexual structures, including coiled hyphae with greatly thickened hyphal structures, asci, and spores, as reported in previous studies (Fig. 2) (4, 5). Multiple attempts to find cleistothecial structures from self-pairing were unsuccessful.

**F1 progeny are fertile.** The F1 progeny of this mating were isolated and analyzed for two reasons: (i) to confirm that a sexual cycle had been completed and (ii) to demonstrate that the sequence structures, identified as *MAT1-1* and *MAT1-2*, correspond to the functional mating-type determinants. An F1 progeny population allows us to analyze genetic marker recombination to confirm meiosis, and it allows analysis of the linkage between the functional *MAT1* mating phenotype and the PCR-based identity of the *MAT1* idiomorph for each strain.

The fertility of F1 progeny was tested by crossing each back to the two parental isolates (ATCC 18187 and ATCC 18188) and to six sibling F1 progeny. Fertility was monitored microscopically by the production of cleistothecia that release ascospores (Fig. 2C). All of the F1 progeny are fertile (Table 2). They can mate with one or more strains of opposite mating type, but all fail to mate with any strain of the same mating type (Table 2). However, F1 progeny were not sexually fertile in combination with all of the potentially compatible tester strains. We found ~56% (111/198) successful matings of the F1 progeny with strains of the opposite mating type (Table 2). This finding suggests that other genetic modifiers of fertility unlinked to the *MAT* locus remain to be identified.

**Evidence of genetic recombination of the F1 progeny of *B. dermatitidis*.** Meiosis, the hallmark of sexual reproduction, is characterized by chromosome segregation and genetic recombination. To confirm that meiosis had indeed occurred, progeny from two mating experiments (M1 and M2) of ATCC 18188 (+) and ATCC 18187 (-) were isolated and their DNA was tested for



**FIG 2** Sexual development of *B. dermatitidis*. After coculture of *B. dermatitidis* isolates ATCC 18188 (+) and ATCC 18187 (–) on SEA medium for 10 weeks, cleistothecia were scraped off the agar, disrupted in water, and fixed in formaldehyde prior to microscopic examination. (A) 20× light microscopy shows multiple disrupted cleistothecia, with highly spiraled hyphae. (B) 40× image showing thickened, coiled hyphae and specialized vegetative cells attached to the spiral hyphae. (C) 60× image shows multiple ascospores released from asci. The ascospores are characterized by a light halo around the cell.

recombination using five genetic markers: two RAPD markers, two PCR-RFLP markers, and also the mating-type locus itself (as determined by PCR with primers specific for *MAT1-1* and *MAT1-2*). None of the F1 progeny was positive for both of the *MAT1* markers, suggesting that each strain does represent a single-cell

clone (Fig. 3A and B). Based on a combination of the five genetic markers, we detected genetic recombination in 16 progeny out of 22 tested (Fig. 3 and 4; Table 3). All of the progeny in the M2 mating experiment show recombinant genotypes, indicating that they are the products of successful sexual reproduction. In contrast, genetic recombination was only detected in three of the M1 mating assay progeny. The lack of genetic recombination in some M1 progeny may be explained by the limited genetic markers used or by some of the progeny strains representing mitotic cells of the parental culture, because these progeny were not microdissected from asci to ensure that only ascospores were cultured.

Based on the shared conservation of the alpha domain and HMG domain genes of the *MAT1* idiomorphs in related ascomycetous fungi, it is expected that the control of mating in *B. dermatitidis* will be governed by the *MAT1* locus. The results for 16 different F1 strains, for which both mating tests and marker analysis were preformed, indicate complete congruence between the functional mating test phenotype and molecular identification of the *MAT1* idiomorph (Table 3).

## DISCUSSION

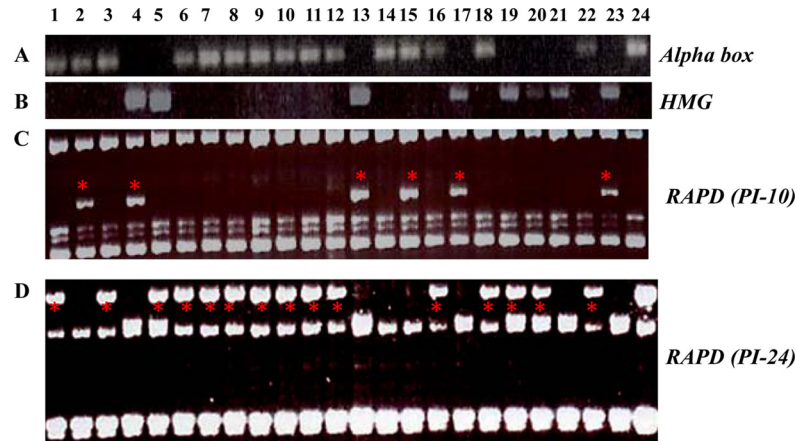
The teleomorphic form of *B. dermatitidis* is *Ajellomyces dermatitidis*. The sexual structures—such as thick-walled structures (cleistothecia) and spiraling hyphae surrounding numerous asci, each containing eight ascospores—were previously observed in *B. dermatitidis* (4, 5, 27); however the molecular characteristics of the *MAT* locus and its control of sexual reproduction were unknown. In this study, we defined the DNA-sequence structure of the *MAT* locus of *B. dermatitidis* and conducted mating assays by coculturing two *B. dermatitidis* isolates of opposite mating types on appropriate mating medium. Our genetic studies reveal a complete sexual cycle of *B. dermatitidis* based on the detection of genetic recombination.

Both homothallic and heterothallic mating systems have been identified throughout the *Ascomycota*. Some studies have hypothesized that the ancestral mating system in the *Ascomycota* was heterothallic (19, 28, 29), but other studies have derived the opposite conclusion, namely, that the ancestral mating system was homothallic (30, 31). This controversy may in part result from a limited number of fungal species with identified *MAT* loci and defined sexual cycles, as well as the challenge in assigning ancestral states to characters subject to recent and ongoing transitions. Our

**TABLE 2** Summary results for mating F1 strains with testers of the same or opposite mating type<sup>a</sup>

Strain	Crossed with tester strains of:	
	Same <i>MAT1</i>	Opposite <i>MAT1</i>
<i>MAT1-2</i> strains		
18187	0/13	23/34
M2-11	0/8	6/17
M2-18	0/13	16/30
M1-4	0/5	1/7
M1-5	0/5	6/7
M2-3	0/5	1/7
M2-17	0/5	2/7
<i>MAT1-1</i> strains		
18188	0/29	6/13
M1-7	0/16	5/9
M2-1	0/30	10/16
M2-2	0/16	7/9
M2-16	0/16	7/9
M1-2	0/7	3/5
M2-4	0/7	1/5
M2-6	0/7	1/5
M2-20	0/7	4/5
Nonrecombinant <i>MAT1-1</i> strains		
M1-1	0/7	3/5
M1-3	0/7	2/5
M1-6	0/7	1/5
M1-8	0/7	2/5
M1-9	0/7	3/5
M1-10	0/7	1/5
Total for both <i>MAT1</i> mating types	0/184	111/198

<sup>a</sup> Results are presented as the number of ascospore-producing crosses/total number of crosses attempted with 8 mating tester strains.

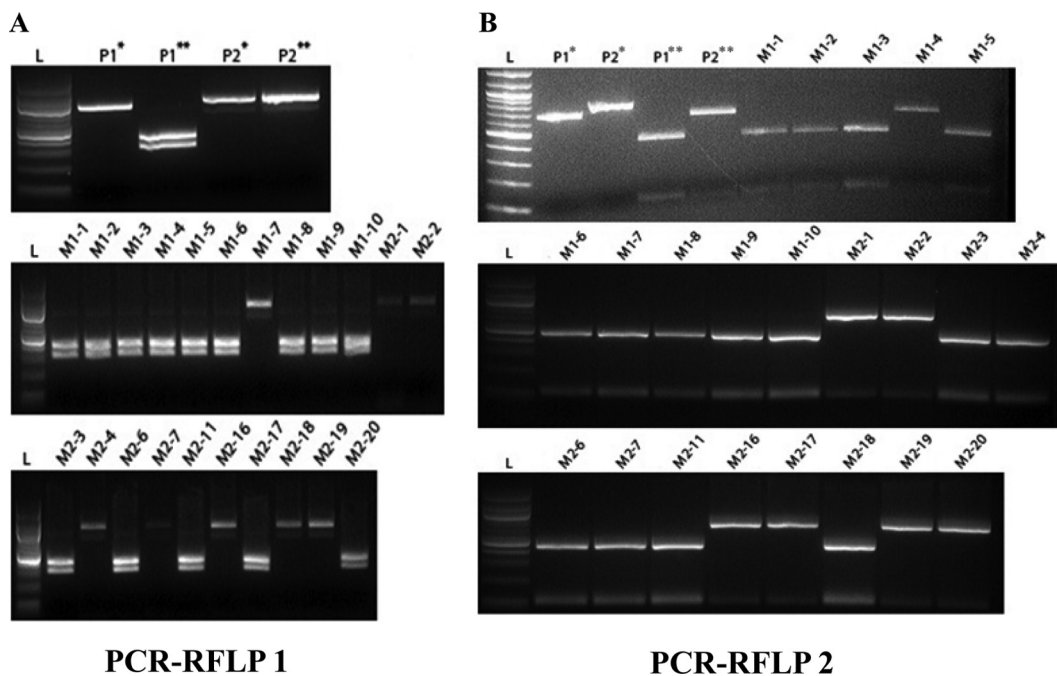


**FIG 3** (A and B) Mating-type determination of *B. dermatitidis* parental isolates (ATCC 18187 and ATCC 18188) and 22 potential progeny by PCR. Primers were designed to specifically amplify the alpha-box (plus mating type) and HMG (minus mating type) genes, which are characteristic of the *B. dermatitidis* MAT locus (see Table 1). (C and D) RAPD analysis of the progeny of isolates ATCC 18188 and ATCC 18187. Two RAPD primers, PI-10 (C) and PI-24 (D), reveal genetic variations (indicated by an asterisk) between these two parental isolates and the potential progeny. Lane 1, M1-1; lane 2, M1-2; lane 3, M1-3; lane 4, M1-4; lane 5, M1-5; lane 6, M1-6; lane 7, M1-7; lane 8, M1-8; lane 9, M1-9; lane 10, M1-10; lane 11, M2-1; lane 12, M2-2; lane 13, M2-3; lane 14, M2-4; lane 15, M2-6; lane 16, M2-7; lane 17, M2-11; lane 18, M2-16; lane 19, M2-17; lane 20, M2-18; lane 21, M2-19; lane 22, M2-20; lane 23, ATCC 18187; and lane 24, ATCC 18188.

mating assays suggest that *B. dermatitidis* is a heterothallic species on the basis of the observation of successful mating of *B. dermatitidis* strains of opposite mating types rather than the same mating types or during solo culture (Tables 2 and 3). In addition, either a single *MAT1-1* or a single *MAT1-2* idiomorph, and not both, was identified for each of the four sequenced *B. dermatitidis*

genomes (Fig. 1). Only a heterothallic mating system has been identified in dimorphic fungi and the dermatophytes (18–20, 24). It is therefore likely that the ancestral mating system of the dimorphic fungi and dermatophytes was heterothallic.

In three minus strains of *B. dermatitidis*, we identified two different retrotransposons that are not found in the *MAT* locus of the



### PCR-RFLP 1

### PCR-RFLP 2

**FIG 4** PCR-RFLP analysis of the progeny of isolates ATCC 18188 (+) and ATCC 18187 (-). SNP and insertion/deletion between isolates ATCC 18188 and ATCC 18187 were used to design a PCR-RFLP analysis to detect genetic variation between these two isolates and the potential progeny. An SNP was identified between isolates ATCC 18188 and ATCC 18187 that abolished a single BamHI restriction site in ATCC 18188 (A). Note that for ATCC 18188, digestion results in two distinct bands, while digestion of ATCC 18187 fails to produce any novel fragments. Progeny were scored as either genotype 1 or 2, denoting their relationship to ATCC 18188 or ATCC 18187, respectively (see Table 3). A second SNP identified within a different region abolished an NdeI restriction site in ATCC 18187. However, sequences of the amplified region predicted an additional NdeI restriction site shared by both parents, which accounts for the faint low-molecular-weight band appearing after digestion of ATCC 18187 DNA (B). For panels A and B, parental DNAs representing the amplified region around the SNP prior to and after digestion are indicated by one asterisk and two asterisks, respectively. L, DNA ladder.

TABLE 3 Evidence of genetic recombination of the progeny of *B. dermatitidis* ATCC18187 and ATCC18188<sup>a</sup>

Isolate	PCR-RFLP 1	PCR-RFLP 2	Mating type	RAPD 1	RAPD 2	Recombination
18188 (P1)	1	1	1	1	1	Not applicable
18187 (P2)	2	2	2	2	2	Not applicable
M1-1	1	1	1	1	1	Not detected
M1-2	1	1	1	2	2	Yes
M1-3	1	1	1	1	1	Not detected
M1-4	1	2	2	2	2	Yes
M1-5	1	1	2	1	1	Yes
M1-6	1	1	1	1	1	Not detected
M1-7	2	1	1	1	1	Yes
M1-8	1	1	1	1	1	Not detected
M1-9	1	1	1	1	1	Not detected
M1-10	1	1	1	1	1	Not detected
M2-1	2	2	1	1	1	Yes
M2-2	2	2	1	1	1	Yes
M2-3	1	1	2	2	2	Yes
M2-4	2	1	1	1	2	Yes
M2-6	1	1	1	2	2	Yes
M2-7	2	1	1	1	1	Yes
M2-11	1	1	2	2	2	Yes
M2-16	2	2	1	1	1	Yes
M2-17	1	2	2	1	1	Yes
M2-18	2	1	2	1	1	Yes
M2-19	2	2	2	1	2	Yes
M2-20	1	2	1	1	1	Yes

<sup>a</sup> The mating types were determined by PCR assay (see Fig. 3). Amplification of an alpha-box gene is determined as mating type 1 (*MAT1-1*), and amplification of an HMG gene is designated as mating type 2 (*MAT1-2*).

one plus isolate analyzed in our study (Fig. 1). Further genomic analyses will need to be done to determine the frequency of retrotransposons among *B. dermatitidis* strains. The transposable elements and additional nonsyntenic DNA in the *MAT* locus of some strains significantly and variably increase the *MAT* locus size in the four *B. dermatitidis* strains studied, 5 kb, 15 kb, 32 kb, and 38 kb. In other fungi, transposons have been found within or flanking *MAT* loci, such as in the homothallic fungus *Neosartorya fischeri* (29) and in the *MAT* locus of *C. neoformans* (26) and *P. brasiliensis* (24), but the influence of transposon transposition on *MAT* locus genes or mating ability is unknown. There is evidence that insertion of a transposon in an avirulence gene caused a gain of virulence in the rice blast fungus *Magnaporthe grisea* (32). Transposition of transposons into or around the *MAT* locus may significantly increase the sequence diversity between the two idiomorphs and decrease recombination of this genomic region if sexual reproduction is still possible. Insertion and local transposition likely also provide homologous sequences driving recombination events at the *MAT* locus.

Compared to closely related fungal species (including the other dimorphic fungi *H. capsulatum*, *C. immitis*, *Coccidioides posadasii*, and *P. brasiliensis* and the dermatophytes *Microsporium gypseum*, *M. canis*, *Trichophyton equinum*, *T. rubrum*, and *T. tonsurans*), *B. dermatitidis* has a similar structure of the *MAT* locus that harbors either an alpha-box gene or an HMG domain gene and is flanked by the *SLA2*, *COX13*, and *APN2* genes. Previous studies suggested that the *MAT* locus of dimorphic fungi is expanding based on the comparison of the *MAT* locus between dimorphic fungi and the dermatophytes (24). In the *Cryptococcus* species complex, the *MAT* locus has also expanded by capturing adjacent genes into the *MAT* locus (25, 26, 33). This fungal *MAT* locus expansion is mainly caused by the capture of adjacent genes into the *MAT* locus

and, as a result, increases the DNA sequence similarity between isolates of the opposite mating type (7). Herein, we present another mechanism of fungal *MAT* locus expansion involving the incorporation of transposable elements.

Both mating types were found in the natural population of *B. dermatitidis*, and they have a similar frequency in nature (10, 34). In two other dimorphic fungi, *H. capsulatum* and *Coccidioides*, both have a similar ratio of the two mating types in nature (35, 36), and a recent study performed by Torres et al. reveals an ~1:1 distribution of the *MAT1-1* and *MAT1-2* strains of *P. brasiliensis* in South America (20). The approximately equal proportion of the two mating types in nature argues that dimorphic fungi may have a sexual cycle in nature. Studies of population genetics further support that these dimorphic fungi reproduce sexually in nature (21, 37–40). Strain typing results based on 27 microsatellite markers suggest genetic recombination and sexual reproduction of *B. dermatitidis* in nature (10). The identification of the *B. dermatitidis* *MAT* locus that exhibits a highly similar structure with other fungi and the population genetic studies indicate that *B. dermatitidis* may reproduce sexually in the environment. Sexual reproduction is linked with virulence in some pathogenic fungi. For example, the  $\alpha$  mating type dominates the *Cryptococcus neoformans* population and is more virulent than  $\beta$  mating-type isolates in some genetic backgrounds (41, 42). Moreover, spores produced by sexual reproduction of *C. neoformans* are infectious propagules providing another link between sex and virulence in this species (43–45). Many pathogenic fungi have the genes encoding sexual machinery in their genomes, but no known sexual cycle has been observed in nature or the laboratory (7). It is not clear whether *B. dermatitidis* reproduces sexually in nature and, if so, whether the sexual spores are infectious propagules. Animal experiments using sexual spores dissected from the sexual repro-

duction of *B. dermatitidis* in the laboratory and virulence comparisons of strains of the opposite mating type could be useful to understand the possible associations of sex and virulence. In addition, comparison of virulence and mating ability between strains with and without transposon insertions in the *MAT* locus can help to understand the function or consequences of transposons in sexual reproduction and pathogenesis.

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