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A Single Mating-Type Locus Composed of Homeodomain Genes Promotes Nuclear Migration and Heterokaryosis in the White-Rot Fungus *Phanerochaete chrysosporium*▽†

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The white-rot basidiomycete fungus *Phanerochaete chrysosporium* (Agaricomycetes) is a model species that produces potent wood-degrading enzymes. The mating system of the species has been difficult to characterize due to its cryptic fruiting habit and lack of clamp connections in the heterokaryotic phase. By exploiting the draft genome sequence, we reevaluated the mating system of *P. chrysosporium* by studying the inheritance and segregation of putative mating-type gene homologues, the homeodomain transcription factor genes (*MAT-A*) and the pheromone receptors (*MAT-B*). A pattern of mating incompatibility and fructification consistent with a bipolar system with a single *MAT* locus was observed, but the rejection response was much weaker than that seen in other agaricomycete species, leading to stable heterokaryons with identical *MAT* alleles. The homeodomain genes appear to comprise the single *MAT* locus because they are heterozygous in wild strains and hyperpolymorphic at the DNA sequence level and promote aspects of sexual reproduction, such as nuclear migration, heterokaryon stability, and basidiospore formation. The pheromone receptor loci that might constitute a *MAT-B* locus, as in many other Agaricomycetes, are not linked to the *MAT-A* locus and display low levels of polymorphism. This observation is inconsistent with a bipolar mating system that includes pheromones and pheromone receptors as mating-type determinants. The partial uncoupling of nuclear migration and mating incompatibility in this species may be predicted to lead to parasexual recombination and may have contributed to the homothallic behavior observed in previous studies.

The mushroom-forming fungi, Agaricomycetes (Basidiomycota), have a mating system in which compatible haploid mycelia (homokaryons) exchange nuclei to form mated mycelia (heterokaryons) that are comprised of cells with two compatible nuclear types (mating types). Once mated, heterokaryons function as genetic diploids, but the two nuclear types divide together in an unfused state until karyogamy occurs in the basidial cells of the fruiting body immediately prior to meiosis and spore formation. In the model mushroom species (e.g., *Coprinopsis cinerea* and *Schizophyllum commune*), heterokaryons are termed dikaryons because each cell contains exactly two nuclei, but in a large number of Agaricomycetes species (e.g., 20 to 30% of the nongilled forms [10]), the hyphal cells of heterokaryons are multinucleate. In these fungi (variously termed multinucleate, plurinucleate, or holocenocytic), each cell presumably contains both mating types but a variable number of nuclei per cell, for example, up to 12 nuclei per cell were observed in the termite symbiont *Termitomyces* (15) and as many as 69 per cell in *Wolfiporia extensa* (34).

In dikaryotic fungi, differentiating homokaryotic from heterokaryotic phases is readily accomplished by observing the characteristic clamp connections or hook cells that function in the maintenance of the binucleate state (43). In contrast, species with multinucleate heterokaryotic cells present a challenge when studies of their life cycles and mating systems are attempted. Although several of the multinucleate species have clamp connections in the heterokaryotic phase, e.g., *Heterobasidion annosum*, many multinucleate species lack clamp connections altogether or have clamp connections in both homokaryotic and heterokaryotic phases (10), making it difficult to distinguish homokaryons from heterokaryons. The mating systems of multinucleate fungi have been investigated using visual scoring of mycelial interactions or by pairing putative heterokaryons with the original homokaryotic isolates and scoring incompatibility reactions versus intermingling (50, 71, 79). Because the genetic basis for incompatibility is unknown in these studies, approaches directly examining genotypes rather than phenotypes would be advantageous because isolates may be somatically compatible but not mating compatible (9, 70).

The processes of nuclear migration and the maintenance of a heterokaryotic state are controlled by mating-type loci. In tetrapolar species, these are separated into two loci, the *A* and the *B* mating-type loci (*MAT-A* and *MAT-B* [62]). The functions and contents of the mating-type loci are known from studies of the model tetrapolar species, *C. cinerea* and *S. commune.* The *MAT-A* genes encode homeodomain transcription factors that regulate synchronized nuclear division and the formation of the clamp connection (11, 65). Each *MAT-A* haplotype typically encodes two different classes of homeodomain transcription factor proteins (HD1 and HD2 types), and the active dimer of the two types can only form in nonself combinations but not self combinations (44). The *MAT-B* locus encodes small peptide pheromones and pheromone receptors

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that together control nuclear migration and the fusion of the clamp connections (11, 65). As in the *MAT-A* proteins, only nonself interactions between pheromones and receptors stimulate nuclear migration through a G protein-coupled signaling pathway (21, 27, 58, 68). Much less functional information is available for mating-type loci in bipolar species. For at least two species, it appears that the single mating-type locus in these species encodes only the *MAT-A* homologues, yet *MAT-B* homologues are still present in the genome (37, 84). Heterologous expression of a pheromone receptor from the bipolar species *Coprinellus disseminatus* in *C. cinerea* demonstrated that the protein was still able to function in clamp connection fusion and the completion of the sexual cycle (37). The organization of mating genes in bipolar Agaricomycetes differs from what is observed in the bipolar and dimorphic early diverging Basidiomycetes, such as *Ustilago hordei* and *Cryptococcus neoformans*, in which the single *MAT* locus contains genes for pheromones and their receptors, as well as homeodomain genes (8, 22).

One such species that exemplifies the difficulties of understanding mating systems in multinucleate Agaricomycetes is the fungus *Phanerochaete chrysosporium* Burdsall. The mycelium of *P. chrysosporium* is comprised of multinucleate cells that lack clamp connections at any stage. In nature, the fungus has been collected as fruiting bodies on the undersides of decaying logs and also isolated from wood chip piles (13). The species produces simple, crust-like fruiting bodies bearing basidia directly on the surface of a smooth or wrinkled hymenium. *P. chrysosporium* also produces ample conidia in culture, and its asexual form had been previously described as a separate anamorphic genus and species, *Sporotrichum pulverulentum* (13, 59). Its mating system has yet to be resolved unambiguously. The production of basidiocarps and basidiospores by strains derived from single basidiospores has led some authors to conclude that the mating system of *P. chrysosporium* is homothallic, or self-fertile (4, 5, 16, 42). However, the mating behavior of a set of progeny from one strain (ME446) suggested that mating was controlled by a single locus, i.e., bipolar heterothallism (79). Additional support for a heterothallic mating system in *P. chrysosporium* derives from the observation that the most common laboratory strains are heterokaryotic (24) and that basidiospores of heterozygous strains produce recombinant, homokaryotic offspring (4, 61). The segregation of markers has been developed for use in strain selection and breeding studies, including the construction of a genetic map that has localized the mating-type locus onto the largest linkage group (61), a placement consistent with the general observation that the *MAT-A* locus is often found on the largest chromosome of Agaricomycetes (30, 56). Despite the fact that the two most widely used strains (ME446 and BKM-F-1767) appear to have a bipolar, heterothallic mating system, the mating behaviors of few strains have been carefully investigated.

Although functional studies of mushroom mating-type genes using gene transformation have provided the crucial basic knowledge on the complex protein machinery involved in controlling mating incompatibility (29), the recent emergence of genomics provides the opportunity to look beyond model species and to study the evolution of mating systems in Agaricomycetes. Genome sequencing has shown that the *MAT* gene homologues are found in every species examined (51–53), that most genomes have pheromone receptor-like genes that are not a part of *MAT-B* (56, 68, 76), and that there is conserved synteny near the *MAT-A* locus (35). The draft genome sequence of the *P. chrysosporium* homokaryotic strain RP-78 was no exception to these general findings, with a single genomic region encoding *MAT-A* homeodomain motif homologues and three additional genomic regions encoding putative pheromone receptors (53). The present study investigated the inheritance of these putative mating-type regions using laboratory pairings and population genetic analyses. Our goal was to clarify the mating system of *P. chrysosporium* and to develop a framework for understanding mating behavior and nuclear migration in multinucleate agaricomycete species that lack clamp connections.

TABLE 1. Strains used in the present study

Strain	Ploidy[a]	Origin
BKM-F-1767	$n + n$	Almaty, Kazachstan
FP-104297-Sp	$n + n$	Etchison, MD
FPLV-170G	$n + n$	Atholville, NB, Canada
ME-OC-11	$n + n$	Brunswick, GA
SF-4	$n + n$	Hixton, WI
Gold-9-419-4	$n + n$	Beaverton, OR
FP-102074	n?	Hawesville, KY
SB11-SB59	n	Basidiospore progeny of BKM-F-1767
ME-sb1-12	n	Basidiospore progeny of ME-OC-11
ME-OC-11_c2	n	Conidiospore progeny of ME-OC-11
ME-OC-11_c7	n	Conidiospore progeny of ME-OC-11

[a] $n + n$ isolates are heterokaryotic, and n isolates are homokaryotic.

MATERIALS AND METHODS

Strains and culture conditions. The strains used in these experiments included 7 wild isolates; all except FP-102074 were shown to be heterokaryotic (Table 1). Segregation and mating analyses utilized single basidiospore and single conidiospore isolates derived primarily from heterokaryon BKM-F-1767 and secondarily from ME-OC-11. One strain used in the experiments (RP-78) is the protoplast-regenerated homokaryon derived from heterokaryon BKM-F-1767, which was used for genome sequencing (77). Cultures were routinely propagated on malt extract agar (MEA) containing 1.5% malt extract and 1.5% agar. Fruiting assays were conducted using the protocol of Dhawale and Kessler (16). After 2 weeks at 25°C, the plates were photographed and the presence of fruiting bodies was recorded.

Genetic crosses and confrontations. A half-diallel cross (cross 1) of 19 homokaryons and one heterokaryon, including 15 single-basidiospore isolates (sbi) derived from BKM-F-1767, was conducted in order to test the segregation of nuclear markers with incompatibility. A second cross (cross 2) among 12 single-basidiospore progeny of ME-OC-11 was also conducted. In the crossing design, each homokaryotic isolate was paired with all the others, including self-pairings, on the medium YMPG plus V8 (containing [per liter] 2 g of yeast extract, 10 g of malt extract, 2 g of peptone, 10 g of glucose, 2 g of KH_2PO_4, 1 g of $MgSO_4 \cdot 7H_2O$, 1 mg of thiamine, 1 g of $CaCO_3$, 177 ml of V8 juice, and 15 g of agar). Crosses were performed on 10-cm petri dishes, and the inocula of the two strains were placed approximately 2 cm apart in the center of the dish. The petri dishes were placed in plastic sleeves and grown in darkness at 37°C for 8 days. The plates were then removed from the sleeves and placed at room temperature under ambient laboratory light for 2 weeks. The pairings were photographed, and the mycelial interactions were scored according to the method of Thompson and Broda (79): uniform intermingling and formation of secondary mycelium (crust-like growth and compact aerial mycelium), unilateral overgrowth following secondary mycelium formation, a band of antagonism marked by heavy conidiation, deadlock or ceased growth, uniform intermingling without secondary mycelium formation, and formation of a lytic zone between the isolates.

Analysis of putative heterokaryons. For the cross 1 pairings, a subset of isolates ($n = 10$) were subjected to further analysis of heterokaryon formation

using molecular markers. For crosses among these 10 isolates, three subcultures were taken from the mating plates: one from the zone of interaction where the two mycelia met, and one from each homokaryotic side, approximately 1 cm distal to the original inoculum. The subcultures were transferred to fresh MEA plates after 24 days of coculture. Hyphal-tip isolation was accomplished by inoculating these subcultures onto cellophane overlying MEA. After 2 to 3 days of growth, subcultures of single hyphal tips were obtained by transferring only hyphal cells that descended from a single cell after all other hyphae were scraped away with a fine blade. The subcultures were transferred to MEA and then again transferred to MEA overlaid with cellophane for DNA extraction and molecular analysis. For the cross 2 pairings, 6 crosses were examined in detail by immediate subculturing 21 days after crossing onto cellophane overlying MEA for genotyping. The same three subculture locations were used as for cross 1.

Analysis of nuclear migration. Nuclear migration was assessed in various combinations of homokaryotic isolates by placing a block of inoculum from a potential nuclear donor (paternal strain) onto a culture acting as a nuclear acceptor (maternal strain). The maternal mycelium was prepared by inoculating a 5-mm-diameter plug into the center of a 14-cm petri dish of MEA. The maternal strain was allowed to grow for 5 days, after which it completely colonized the surface of the medium. A 5-mm-diameter plug of paternal inoculum was removed from an actively growing culture, transferred to fresh MEA, and allowed to precondition for 24 h at 25°C. The maternal inoculum plug was removed from the maternal strain and replaced with the preconditioned, inverted paternal inoculum. The plates were then placed at 25°C (12-h light cycle) and subcultured every 24 h for 4 days to detect the movement of nuclei. The subculturing involved removing four small plugs of inoculum along a single random radius from the central point of inoculation. The plugs were taken using sterilized Drummond Wiretrol precision bores of 2-mm diameter at even intervals: 1.5, 3.0, 4.5, and 6.0 cm from the inoculum. The plugs from the nuclear-migration plates were placed onto cellophane overlying MEA, and DNA analysis was performed to determine the rate of nuclear migration. Five strains were utilized as both maternal and paternal strains in all possible combinations: SB11, SB34, SB37, SB43, and ME-OC-11_c2.

Growth rate estimation. Growth rates were estimated using the same inoculation and growth procedures used for the nuclear-migration study. Three replicates of each strain were used, and the growth rate was measured at 24, 48, and 72 h as the distance from the inoculum along 2 random radii.

DNA techniques. DNA was isolated from cultures growing on cellophane overlying MEA by grinding fresh mycelia in a 2× cetyltrimethylammonium bromide (CTAB) buffer (38). Cells were disrupted by grinding them with a microcentrifuge pestle or with glass beads (a mixture of 3 and 0.3 mm) in a Retsch MM301 ball mill. Homogenates were extracted twice with (24:1) chloroform-isoamyl alcohol, and DNA was precipitated with a two-thirds volume of isopropanol. Several DNA polymerase mixtures, restriction enzymes, primers, and annealing temperatures were used to study the inheritance and polymorphism of the *MAT* regions and ribosomal internal transcribed spacer (*ITS*) and elongation factor 1α (*EF1*α) regions (see Table S1 in the supplemental material for PCR primers and conditions). PCR was conducted on a Mastercycler pro S thermocycler (Eppendorf) using the annealing temperatures shown in Table S1 in the supplemental material. Markers to study segregation and inheritance were developed by sequencing multiple homokaryons or by direct sequencing of heterokaryons to identify polymorphisms as chromatograms with double peaks. PCR products were purified for sequencing using ExoSAP-IT (USB) and run on a 3730 XL sequencer (Applied Biosystems) at the University of Michigan Sequencing Core. Extracting haplotype information from heterokaryons was facilitated by PCR subcloning using pCR4-TOPO (Invitrogen) and sequencing with M13 primers following colony PCR. Heterozygosity and segregation of the *MAT-B* homologues and a *MAT-A*-linked marker encoding a hypothetical protein (*Pc-HP2*) was determined using PCR-restriction fragment length polymorphism (RFLP) assays (see Table S1 in the supplemental material for restriction enzymes). Genotyping of the *MAT-A* locus was also done using allele-specific multiplex PCR. These assays utilized pairs of primers that were highly specific to one of the *MAT-A* alleles in the sample and that produced PCR amplicons of different sizes. The *MAT-mplex-1* multiplex assay was used for cross 1 with three primer pairs at a concentration of 0.167 μM each primer and target PCR amplicon sizes of 344, 548, and 635 bp. The *MAT-mplex-2* assay was used for cross 2 with two primer pairs at a concentration of 0.25 μM each primer and target PCR amplicon sizes of 452 and 635 bp.

Sequencing of *MAT-A* haplotypes. Additional sequences of the entire *MAT-A* region were obtained by amplifying across the highly variable region using long PCR. Primers were designed to target the flanking loci (*MIP* [the gene encoding mitochondrial intermediate peptidase] and *Pc-HP2*) after sequences for homokaryons and heterokaryons were obtained. The primers for long PCR varied depending on the strain (see Table S1 in the supplemental material for primers and amplification conditions). The amplicons were ligated into pCR2.1-TOPO or pCRXL-TOPO (Invitrogen), and the sequences of the resulting plasmids were determined from subclones of the plasmids containing random insertions of a transposon encoding chloramphenicol resistance using either the GeneJumper kit (Invitrogen) or the HyperMu <CHL-1> Insertion kit (Epicentre Biotechnologies). All plasmid minipreps were done using the QIAprep Spin Miniprep Kit or the QIAprep 96 Turbo Miniprep Kit (Qiagen).

Nuclear staining. The numbers of nuclei in conidia were assessed by staining them with DAPI (4′,6-diamidino-2-phenylindole) with SlowFade Gold (Invitrogen) and viewing them on a Zeiss Axioimager A2. For each strain, 50 conidia were investigated.

DNA sequence analyses. Sequence chromatograms were edited and assembled using Sequencher 4.10.1 (Gene Codes). DNA sequences were aligned and translated using MacClade (49), and *MAT-A* homeodomain proteins were aligned automatically using MUSCLE (18). Basic statistics of nucleotide and amino acid diversity and substitution patterns were analyzed using MEGA 4 (78) and DNAsp v. 5.10.01 (47). The ITS phylogeny was constructed using PHYML v2.4.4 (25). Searches for coiled-coil dimerization motifs were conducted using COILS (48), and searches for nuclear localization sequences were done using WoLF PSORT (31). Amino acid identity was calculated using the PROTDIST module of PHYLIP (19).

Nucleotide sequence accession numbers. The DNA sequences have been deposited in GenBank under accession numbers HQ188379 to HQ188443.

RESULTS

Nuclear numbers in conidia and fruiting potential of wild strains. The characteristics of fruiting and numbers of nuclei in conidia have been investigated as a means of differentiating homokaryons from heterokaryons in *P. chrysosporium* with variable results (5, 16, 79). We tested our 7 wild strains for the ability to form basidiospores using the method of fruiting developed by Dhawale and Kessler (16). Obvious fruiting was observed as the formation of hymenial patches on the surface of the agar plate and the deposition of forcibly discharged basidiospores on the lid of the petri dish (Fig. 1). Five of the tested isolates displayed vigorous fruiting (FP-104297-Sp, FPLV-170G, ME-OC-11, SF-4, and Gold-9-419-4), while two strains produced only a few basidiospores (BKM-F-1767 and FP-102074). None of the putative single-basidiospore isolates tested ($n = 6$) displayed evidence of fruiting. The wild strains and two homokaryotic isolates (ME-OC-11_c2 and SB43) were examined for numbers of nuclei in blastoconidia by staining them with DAPI. Based on counts of 50 conidia, the values show a mean of 2.02 and a range of 1 to 4 nuclei per conidium. No consistent differences between nuclear numbers per conidium between wild strains and single-basidiospore strains were observed.

***P. chrysosporium* is comprised of at least two species.** The strains used in this study have been deposited in the Forest Products Laboratory Center for Mycology Research culture collection (FPL) under their teleomorph name, *P. chrysosporium*. The anamorphs associated with a *P. chrysosporium* Burdsall teleomorph are *Sporotrichum pulverulentum* Novobr. and *Sporotrichum pruinosum* J. C. Gilman & E. V. Abbott, and the synonymy of the two species is debated (12). The ITS rDNA spacer region was sequenced from each of the wild strains in order to determine their relatedness. The ITS sequences were of two distinct types, with all strains from the FPL collection, except FP-102074, at least 99.8% similar. FP-102074 was 98.0 to 98.3% similar to this major type. BLAST searches against GenBank revealed that the FP-102074 type was 100% identical to the ITS sequence deposited for the type strain of *S. pruino-*

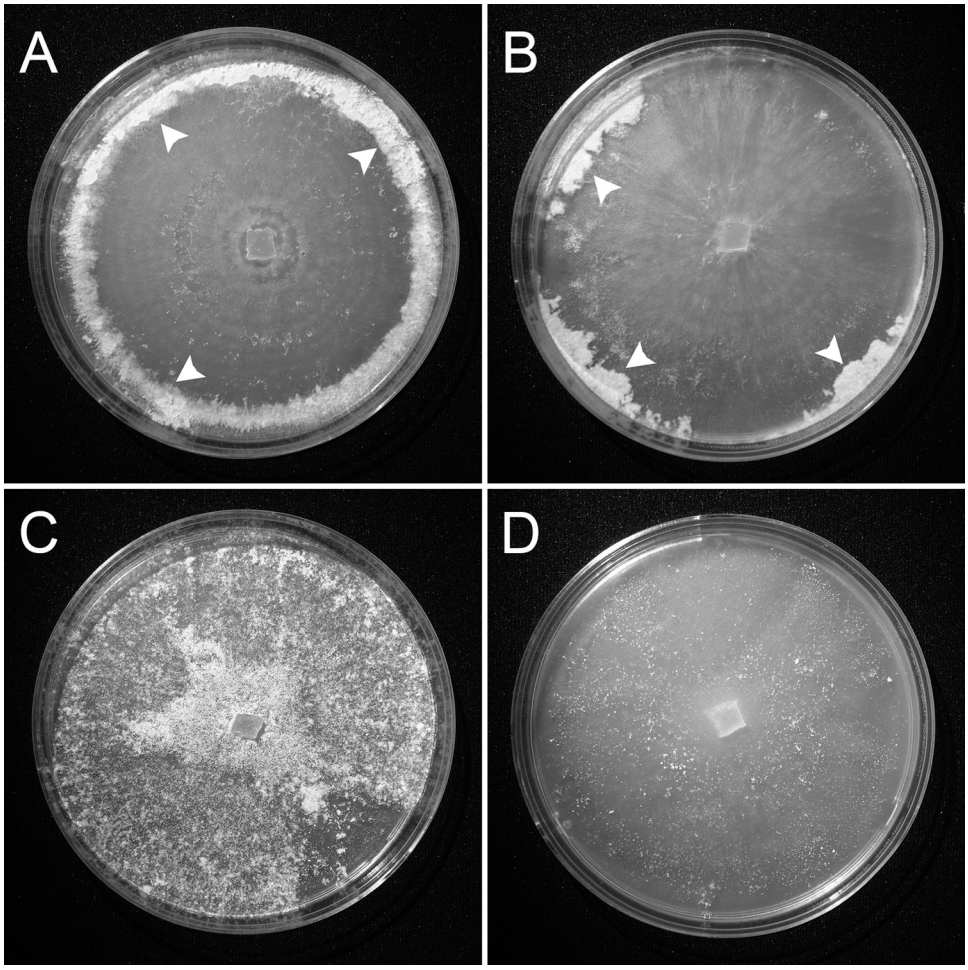

FIG. 1. Morphologies of heterokaryotic (A and B) and homokaryotic (C and D) isolates on fruiting medium (16). The arrowheads indicate the crust-like basidiocarps that typically form near the sides of the petri dish. (A) SF-4. (B) Gold-9-419-4. (C) SB43. (D) SB11.

sum (ATCC 1727; GenBank accession number AB361644). A phylogenetic analysis, including the FPL sequences and additional sequences from GenBank, suggested at least two distinct groups of *P. chrysosporium* sequences (Fig. 2), which we consider likely correspond to the *S. pruinosum* and *S. pulverulentum* species concepts due to the known association of the anamorphs with these cultures. The distinctiveness of the FP-102074 strain was confirmed by sequencing the locus *EF1*α (data not shown).

Mating-type gene homologues of *P. chrysosporium* RP-78. The v2.0 release by the Joint Genome Institute (JGI) of the *P. chrysosporium* genome consists of 35.1 million bp in 232 scaffolds, the majority (90%) of which are assembled into 21 scaffolds (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html). Genes homologous to the *MAT-A* homeodomain genes were found on scaffold 13, positions 1.071 to 1.074 Mb. The *MAT-A* locus is comprised of one HD1 gene and one HD2 gene in a divergently transcribed orientation, as has been observed in other agaricomycete *MAT-A* loci (37, 56, 84). The *MAT-A* region of *P. chrysosporium* displays extensive synteny across Agaricomycetes, a subset of which are shown in Fig. 3. Unlike most Agaricomycetes, in which the HD1 protein flanks *MIP*, the HD2 protein in *P. chrysosporium* is adjacent to *MIP*. By comparison to the most closely related genome species, *Postia placenta*, this inverted arrangement of HD1-HD2 genes relative to *MIP* appears to have been present in the ancestor of these bipolar Polyporales species, as previously suggested (52). The *P. chrysosporium MAT-A* locus is flanked by two genes encoding hypothetical proteins (HP) lacking similarity to any other proteins in GenBank: Pc-HP1 (JGI protein ID 7554) and Pc-HP2 (JGI protein ID 7553).

BLAST searches revealed five pheromone receptors homologous to the STE3 class of receptors, located on three separate scaffolds. Scaffold 9 encodes three adjacent receptors (*PcSTE3.1* to *-3*), an arrangement similar to that observed at other agaricomycete *MAT-B* loci (37, 56). The 20-kb region surrounding the three *STE3* receptors also encodes the PAK kinase *CLA4* (35) and a putative peptide pheromone gene (*Pc-Phb1*), making it the prime candidate for *MAT-B* function. Four additional predicted mating pheromone genes that are not associated with receptor genes have been detected at the end of scaffold 3 and on scaffold 5 (U. Kües, personal communication), and the program PrePS suggests the putative pheromones are all farnesylated like other mating-type pheromones (54). Two additional STE3 pheromone receptor genes (*PcSTE3.4* and *PcSTE3.5*) are found unlinked on scaffolds 5

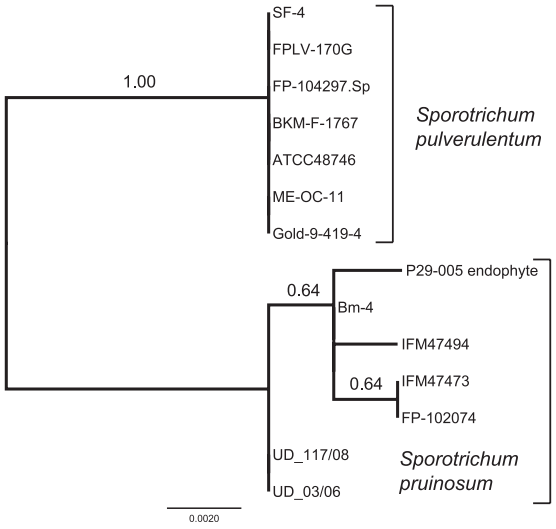

FIG. 2. Phylogeny of *P. chrysosporium* strains based on the ITS rDNA region. Shown is a phylogram estimated using maximum likelihood (GTR + I + Γ) constructed using the program PHYML v2.4.4 (25). The numbers above the nodes show bootstrap support values greater than 50%. The data suggest two distinct lineages that appear to be related to the two described anamorphs, *S. pulverulentum* and *S. pruinosum*. The GenBank numbers for the additional sequences are as follows: ATCC 48746, GU256751.1; P29-005, EU818897.1; Bm-4, GQ280374.1; IFM47494, AB361645.1; IFM47473, AB361644.1; UD 03/06, GQ249876.1; UD 117/08, GQ249875.1.

and 9 (the latter over 700 kb distant from *PcSTE3.1* to *-3* loci), respectively. Each of the putative pheromone receptor proteins possesses 7 transmembrane helices, as observed in other G protein-coupled receptors. The sequences of the putative pheromone *Pc-Phb1* and two pheromone receptors have been manually reannotated and provided as third-party annotations at GenBank (*PcSTE3.1*, BK007880; *Pc-Phb1*, BK007881; and *PcSTE3.5*, BK007882). The remaining three pheromone receptors can be found on the JGI website (*PcSTE3.2*, fgenesh1_pg.C_scaffold_9000315; *PcSTE3.3*, e_gwh2.9.420.1; and *PcSTE3.4*, fgenesh1_pg.C_scaffold_5000290).

Polymorphism of markers reveals heterozygosity and heterokaryosis. The sequences of the putative *MAT* regions were obtained to develop markers for segregation and to assess their levels of polymorphism. PCR and sequencing of the *STE3.1* to *-5* regions was successful for most of the wild isolates, with the exception of *S. pruinosum* strain FP-102074. This strain was excluded from further analyses. Several measures of polymorphism were calculated for the *STE3* genes and compared to the *EF1*α locus (a non-mating-type-specific gene on scaffold 11 [Table 2]). The polymorphism (π, or the average pairwise percent differences) of the *STE3* loci varied over the range 0.007 to 0.026. Looking specifically at silent polymorphisms (π_S) and the ratio of nonsynonymous to synonymous substitutions (ω) reveals evidence for background selection against amino acid replacements. The values of *STE3* π_S are similar to

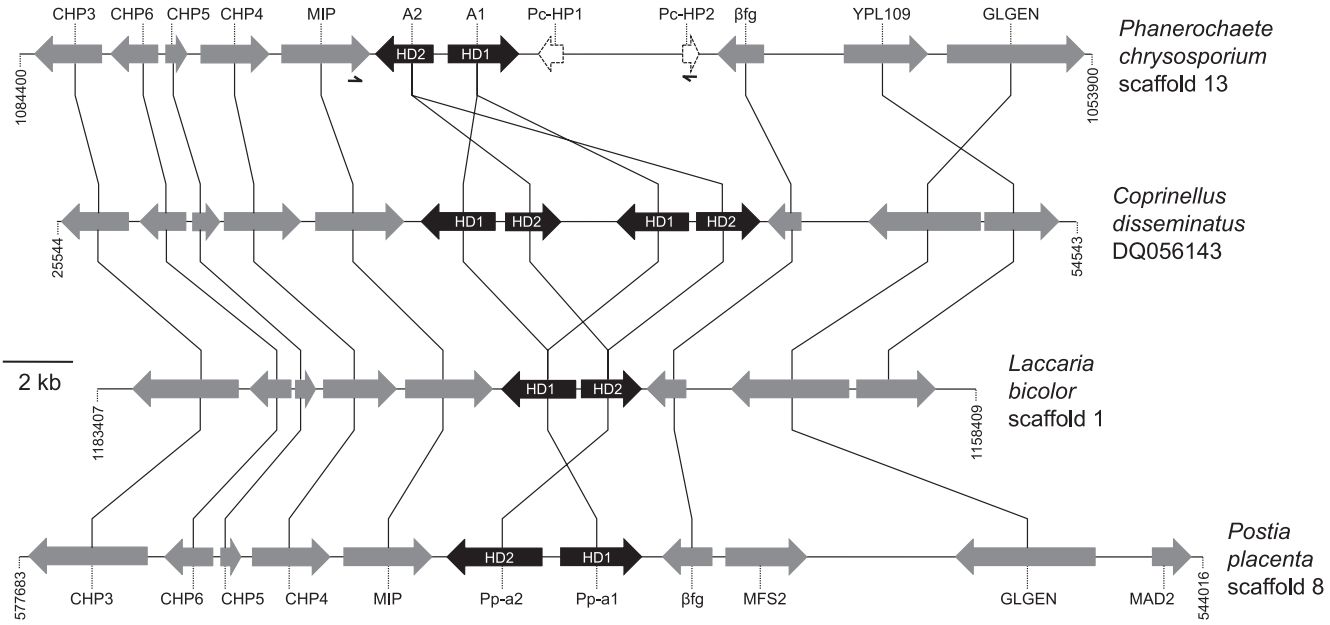

FIG. 3. Comparison of the *MAT-A* region from *P. chrysosporium* demonstrates synteny with other Agaricomycetes. The arrows indicate the direction of transcription. The gray arrows indicate genes that linked to the *MAT-A* region in previous studies (35, 84). The black arrows indicate the actual mating-type-specific homeodomain genes of the two classes of DNA binding motifs (HD1 and HD2 [44]). The lines connect homologous genes. The dashed arrows in the *P. chrysosporium MAT-A* region indicate two hypothetical genes predicted from the automated annotation of the genome but lacking any similarity to other proteins in other organisms. The half arrows underneath the *P. chrysosporium* locus show the locations of the primers used for long PCR of additional mating alleles. CHP3 to -6 are conserved hypothetical proteins of unknown function (35), MFS2 is a member of the major facilitator superfamily of transport proteins, GLGEN encodes a glycosyltransferase family 8 protein involved in the synthesis of glycogen, and YPL109 encodes a conserved kinase of uncertain function. The numbers shown at the beginning and end of each of the schematics indicate the first and last base pair of each region in the current Joint Genome Institute genome browsers for the indicated scaffold or in the GenBank accession (*C. disseminatus*). Data from *P. placenta* are from the latest release on the JGI website (http://genome.jgi-psf.org/Pospl1/Pospl1.home.html), those from *P. chrysosporium* are from http://genome.jgi-psf.org/Phchr1/Phchr1.home.html, and data from *L. bicolor* can be found at http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html.

TABLE 2. Summary of DNA polymorphism in *P. chrysosporium*

Locus[a]	n^b	N^c	S^d	π^e	$\pi_S{}^f$	ω^g
EF1α	9	854	18	0.007 (0.002)	0.051	0.00
STE3.1	9	412	24	0.026 (0.006)	0.041	0.33
STE3.2	10	666	21	0.007 (0.002)	0.014	0.16
STE3.3	10	783	28	0.012 (0.002)	0.029	0.10
STE3.4	11	485	24	0.014 (0.003)	0.078	0.02
STE3.5	10	581	37	0.019 (0.003)	0.035	0.05
MIP	7	509	221	0.278 (0.017)	1.383	0.03
Pc-HP2	9	349	55	0.057 (0.008)	0.117	0.14
a1	7	1429	1254	1.085 (0.026)	2.666	0.32
a2	7	1221	1058	1.047 (0.030)	2.687	0.31

[a] Genes: *a1* and *a2*, homeodomain genes; *STE3.1* to *-5*, pheromone receptor homologues; *EF1α*, elongation factor 1α; *MIP*, mitochondrial intermediate peptidase; *Pc-HP2*, a hypothetical protein linked to *MAT-A*.
[b] *n*, number of sequences.
[c] *N*, number of aligned base pairs.
[d] *S*, number of segregating sites.
[e] π, nucleotide diversity, or the average number of pairwise differences per site (using Jukes–Cantor correction). The nucleotide diversities are followed by standard errors (in parentheses) estimated using 1,000 bootstrap replicates (78).
[f] π_S, nucleotide diversity of silent sites (synonymous and intronic).
[g] ω, ratio of nonsynonymous to synonymous differences between pairs of sequences.

that at *EF1α*, suggesting that polymorphism is not specifically elevated at the *STE3* loci (Table 2).

A higher level of variation was observed in the genes in and near the *MAT-A* region. In order to design conserved primers to amplify the HD1 and HD2 genes from additional wild strains, PCR and sequencing of the flanking genes (*MIP* and *Pc-HP2*) were attempted. No single set of conserved primers could be found to amplify *MIP* from the wild strains, so several primers were utilized. The overall values of π and π_S at *MIP* and *Pc-HP2* exceeded those for the *STE3* genes (Table 2), with extraordinary levels of polymorphism in silent positions observed at *MIP*.

All 6 of the wild isolates were heterozygous at at least one locus, providing strong indication that they are heterokaryons. Using the data from the wild isolates and the RP-78 genome, PCR-RFLP markers were designed to assess the heterozygosity and segregation in single-basidiospore and conidial isolates. Among 28 sbi from two parental strains, only one (SB26) displayed heterozygosity at 2 out of 4 assayed loci, while the remaining sbi were homozygous at all assayed markers. In contrast, out of 76 single-conidial isolates (sci), from 5 parental strains, only 4 sci were homozygous, and all were sci of isolate ME-OC-11. Two of the isolates among the sci with different molecular patterns were designated the mating types of ME-OC-11 (sci 2 and 7). These data suggest that the majority of wild isolates and their conidia are heterokaryons, while the basidiospores produced by them are largely homokaryotic.

Deep sequence divergence of *MAT-A* alleles. Long PCR was used to amplify the *MAT-A* homologues from several of the wild strains, and the resulting amplicons were cloned and sequenced. The *MAT-A* region was considered the region of the genome between the gene encoding mitochondrial intermediate peptidase (*MIP*) and the β-flanking gene (β*fg*), a conserved gene of unknown function (35). *MAT-A* regions varied in size from 8.9 to 18.6 kbp, but each sequenced haplotype encoded only a single pair of HD1 and HD2 homeodomain proteins. All A1 proteins showed high divergence, with an average of 27% identity across the protein and a range of 25 to 30%. A2 proteins similarly showed high divergence, with 30% mean identity (range, 26 to 34%). The highest degree of conservation was observed in the homeodomain regions and a small proline-rich region in the A2 protein sequences (see Fig. S1 in the supplemental material). The C-terminal regions of the proteins were very difficult to align and displayed DNA and amino acid polymorphism (similarity, <25%), suggesting mutation saturation (Table 2; see Fig. S1 in the supplemental material). Scans for coiled-coil motifs indicative of dimerization helices were significantly detected in the N-terminal (putative allele specificity) region of the A1 protein only. Nuclear localization sequences (NLS) were readily detected in the C-terminal half of the A1 protein, and an additional NLS was detected in the A2 protein of Gold-9-419-4. These observations are largely consistent with the model of homeodomain protein structure-function based on the model species *C. cinerea*, in which the N-terminal region of the HD proteins is the specificity-determining region that dimerizes between HD1 and HD2 proteins, and nuclear localization is restricted to the HD1 protein and active HD1-plus-HD2 dimer (75, 80). A major difference between *P. chrysosporium* and *C. cinerea* is that the most divergent regions of the proteins are the C-terminal region in the former and the N-terminal region in the latter (7).

The region between *A1* and the hypothetical gene *Pc-HP2* was scanned for additional genes and similarity using pairwise identity plots in PipMaker (74) (Fig. 4). Whereas the locus for the novel protein Pc-HP2 is conserved in all sequences, the putative *Pc-HP1* gene is undetected in all other haplotypes. The region downstream of *A1* appears to be a region of low conservation and the target of insertion of putative transposable elements (Fig. 4). A large gypsy-like retrotransposon is inserted into this region in ME-OC-11_c7, and an additional repeat region is shared between ME-OC-11_c2 and c7 haplotypes. A novel gene encoding a hypothetical protein (*Pc-HP3*) has been translocated into the *MAT-A* region in Gold-9-419-4. The *Pc-HP3* element displays weak similarity to a transposase and is found in the genome of RP-78 on scaffolds 1 and 14 (JGI protein IDs 710 and 7690). Unfortunately, the Gold-9-419-4 *MAT-A* sequence is incomplete at one end of the amplified fragment due to a truncated subclone that is missing the reverse primer and associated *Pc-HP2* sequence.

Segregation of *MAT* genes and their effects on mating. PCR-RFLP markers were designed to genotype the basidiospore progeny of BKM-F-1767 at the *MAT-A* locus and for the three unlinked *STE3* loci (*STE3.2*, *STE3.4*, and *STE3.5*). Each of the regions showed no evidence for linkage to each other or segregation bias ($P > 0.05$) (see Table S2 in the supplemental material). For cross 1, the basidiospore progeny were paired with each other, with the protoplast derivative RP-78, with two additional single-conidial isolates from another heterokaryon (ME-OC-11_c2 and c7), and with a heterokaryotic isolate (Gold-9-419-4). The phenotypes were scored according to the nomenclature of Thompson and Broda (79). These results show a wide degree of variation in responses between isolates and can be divided into two major groups according to the segregation of markers at *MAT-A* (Fig. 5). In general, the incompatibility responses were rather weak, and a large number of crosses resulted in modest rejection reactions or an absence of clear heterokaryon formation. Crosses between the

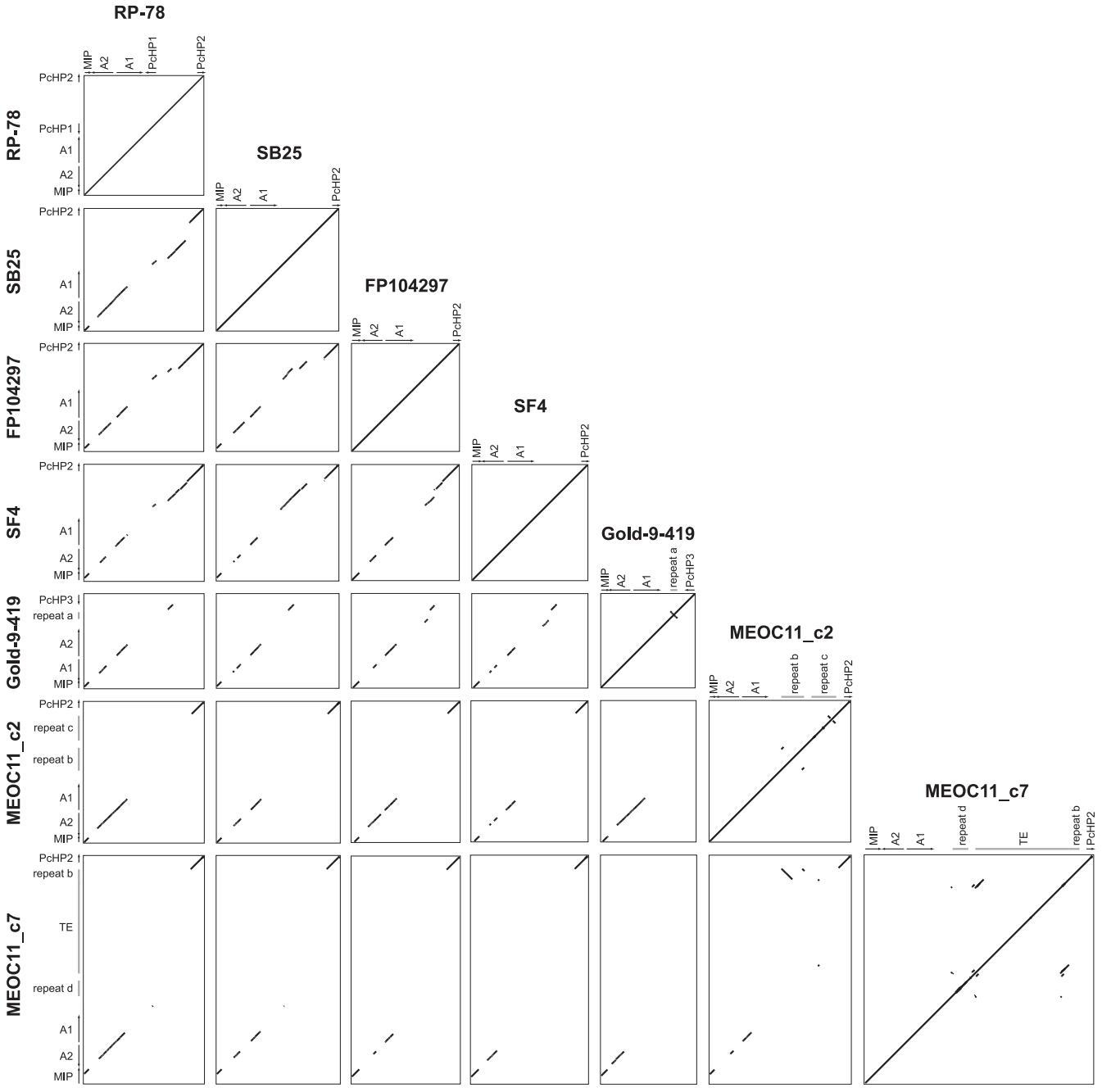

FIG. 4. Pairwise comparisons among *MAT-A* haplotypes reveal hyperdiversity of DNA sequences and insertion of transposable elements. Shown for each comparison is a dot plot of regions of >50% identity calculated by PipMaker (74). Gene annotations for each haplotype are provided along the axes. Also, annotated in gray are four repeat regions (a to d) found in other places in the RP-78 genome, as well as a gypsy-like retrotransposon (TE) similar to those observed in other mushrooms, such as *Tricholoma matsutake* (GenBank accession number BAA78625), flanked by long terminal repeats.

SB isolates and the ME-OC-11 sci showed the clearest indication of heterokaryon formation. Most instances of heterokaryotization resulted in a unidirectional migration of nuclei, and reactions in which a clear secondary mycelium formed throughout the plate were rare.

Because the phenotypes of the crosses are subjective, we chose to further analyze the strains using molecular markers. For a subset of isolates ($n = 10$), subcultures were taken from opposite sides of the interaction, as well as the zone of interaction, and used to generate single hyphal-tip cultures for genotyping using the PCR-RFLP markers. The molecular analysis of cross 1 confirmed that nuclear migration was often unidirectional or limited to the junction zone, or demonstrated that certain strains (SB43 and ME-OC-11_c2) were strong nuclear acceptors (Fig. 6). The heterokaryotic isolate Gold-9-419-4 only donated nuclei and did not accept, as observed in

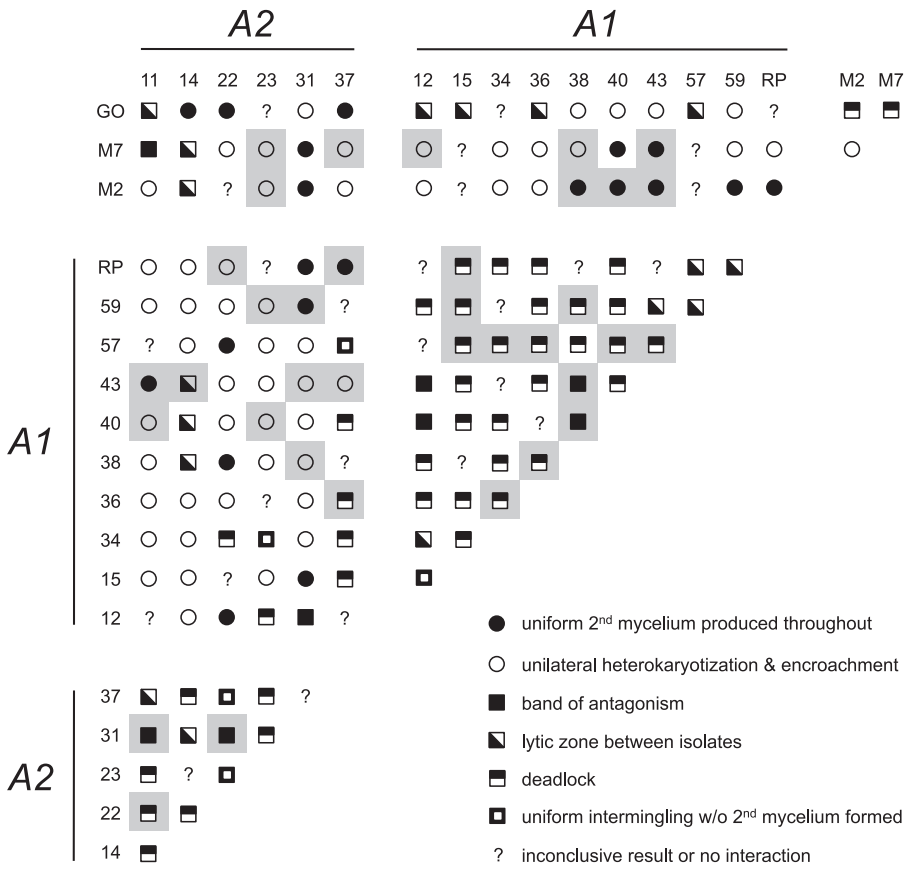

FIG. 5. Morphological characterization of reactions between paired *P. chrysosporium* strains (cross 1). The strains have been grouped according to genotype at *MAT-A*. The numbered strains are single-basidiospore isolates SB11 to -59. The additional strains are GO (Gold-9-419-4, a heterokaryon), M7 (ME-OC-11_c7, a homokaryotic single-conidial isolate), M2 (ME-OC-11_c2, a homokaryotic single-conidial isolate), and RP (RP-78, a homokaryotic protoplast derivative). A number of interactions were scored as "?," which typically appeared as a reaction with few obvious morphological changes. The interactions shaded in gray are those that can be scored with a high degree of confidence.

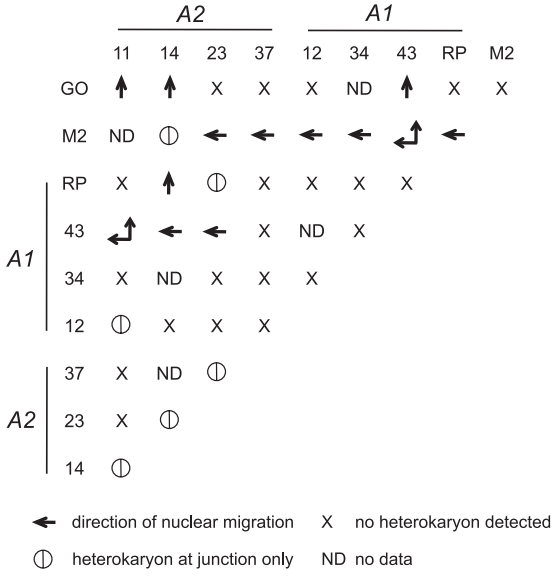

FIG. 6. Molecular characterization of reactions between paired *P. chrysosporium* strains. The strains are a subset of cross 1 (Fig. 5). Subcultures were taken from the interaction zone (junction) and from both homokaryotic sides and bottlenecked to single hyphal tips before being genotyped with markers to detect heterokaryons. Some hyphal-tip cultures did not grow, resulting in some missing data (ND).

many other agaricomycete species (the "Buller phenomenon" [6]). An unexpected result was the finding that strains that shared the *MAT-A2* allele could form stable heterokaryons, but these heterokaryons were limited to the junctions where the two strains interacted. The isolates were also tested for the ability to fruit. Of 135 subcultures tested, 30 subcultures displayed evidence of fruiting, all of which were crosses between sbi of BKM-F-1767 and different *MAT-A* alleles or crosses between sbi of BKM-F-1767 and ME-OC-11 sci or Gold-9-419-4. These data clearly show that heterokaryons with identical *MAT-A* mating types can form, but only heterokaryons with different alleles of *MAT-A* have the potential to fruit.

Cross 2 utilized 12 single-basidiospore progeny of ME-OC-11. Crosses were performed in the same manner as cross 1. The degrees of rejection and secondary mycelium formation were much lower than those observed in cross 1, and this may be related to a much higher variation in morphology among the cross 1 isolates. Most isolates merged completely, with only a faint interaction line observed. Six crosses were investigated using molecular markers by subculturing from the same three regions of the mating plate as in cross 1. Unlike cross 1, subcultures were not bottlenecked to a single hyphal tip but immediately transferred to cellophane overlays of MEA that

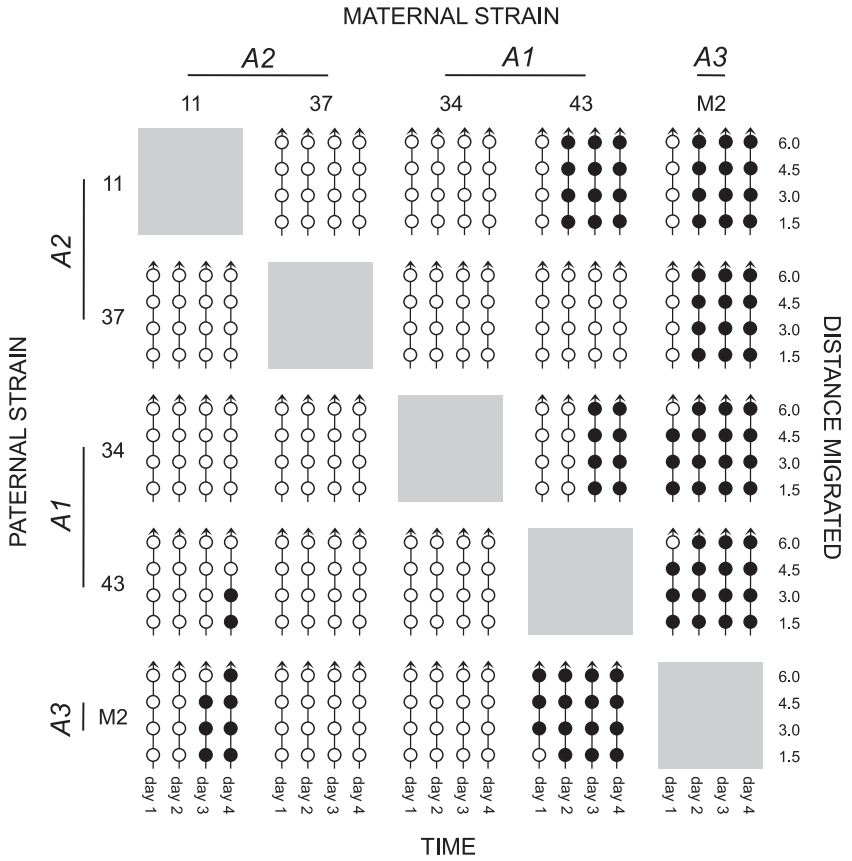

FIG. 7. Assay for nuclear migration among five homokaryotic strains. Paternal strains were placed as a small inoculum on the center of the maternal strain. Subcultures were taken over the next 4 days along a single random radius sampled at four equal intervals. The filled circles indicate the detection of a paternal nucleus using molecular markers. The empty circles indicate no nucleus was detected. The distance migrated is expressed in cm.

were then genotyped. Of the three crosses between strains of different *MAT-A* alleles, all subcultured regions were heterokaryotic. Of the three crosses between isolates of identical *MAT-A* alleles, one resulted in all regions being heterokaryotic, one was heterokaryotic at the junction and a single side of the interaction zone, and one was heterokaryotic only at the junction zone.

Effect of mating type on nuclear migration. The results of the above-mentioned pairings suggested nuclear migration might be influenced by the *MAT-A* locus. We tested this hypothesis by measuring the rate of nuclear migration following implantation of a paternal strain (nuclear donor) onto a maternal strain (acceptor). In order to distinguish nuclear migration from mycelial overgrowth, the growth rate of the homokaryotic strains was investigated. For two of the five strains (SB11 and SB43), a quadratic function displaying an accelerating growth rate over time showed a better fit than a standard linear model ($P < 0.001$). The linear portion of the growth equation ranged from 0.6 to 0.9 mm/h among the five homokaryotic strains. The five strains were then used in all pairwise maternal and paternal combinations for the migration experiment.

These results confirmed the high frequency of nuclear acceptance observed in cross 1 with strains SB43 and ME-OC-11_c2 (Fig. 7). Strains SB34 and SB37 did not allow any nuclear migration when used as maternal strains. Combinations with strain ME-OC-11_c2 as the maternal strain showed a high rate of nuclear migration. However, the maximal migration rate was observed in the combination ME-OC-11_c2 (paternal) and SB43 (maternal), where the migrating nucleus had already traveled the maximum sampled distance by day 1. This allowed a minimum rate of nuclear migration to be calculated as 2.5 mm/h, which exceeds the rate of hyphal extension by over 2.5 times. These data showed that nuclear migration is not entirely controlled by the mating type because, for example, strains SB34 and SB37 differ from ME-OC-11_c2 at all putative *MAT* loci but were incapable of migrating into the ME-OC-11_c2 maternal strain. Furthermore, one combination of strains with identical *MAT-A* mating types (SB34 and SB43) showed nuclear migration by the SB34 nucleus at a minimal rate (0.83 mm/h) that exceeded the strain's growth rate (0.68 mm/h), suggesting that actual nuclear migration had occurred as opposed to hyphal ingrowth.

DISCUSSION

Mating system of *P. chrysosporium*. Previous results have reached conflicting conclusions regarding whether the mating system of *P. chrysosporium* is homothallic or bipolar and heterothallic (4, 5, 16, 42, 79). By analysis of putative mating-type

genes, the present results provide convincing evidence for a bipolar heterothallic mating system for the species but also provide some hints as to why a homothallic mating system may have been inferred.

Below, we systematically discuss the arguments in favor of a bipolar mating system. Heterothallism is suggested because wild isolates and not single-basidiospore isolates were capable of fruiting under the conditions employed. These wild isolates were predominantly heterozygous at several loci, demonstrating that they are heterokaryons, and these loci segregate following basidiospore production. The half-diallel cross of single-basidiospore isolates of the strain BKM-F-1767 identified only two mating types, consistent with a bipolar mating system, and of the putative mating-type loci found in the genome, only one, the *MAT-A* homeodomain region, appears to cosegregate with mating types in cross 1.

Additional support for a single *MAT* locus is derived from evolutionary considerations. *MAT-A* homologues have the pattern of hyperpolymorphism expected for a basidiomycete *MAT* locus, whereas *MAT-B* homologues do not (37, 55) (Table 2). Molecular systematics also supports a placement of *P. chrysosporium* within the "*Phlebia* clade," a group containing exclusively bipolar species, where known (28). Finally, the mating systems of two other *Phanerochaete* spp. have been studied and shown to also display bipolar heterothallism (70, 79).

Nonetheless, several lines of evidence suggest a very reduced mating incompatibility system that may have been the source of mating system confusion. Pairings between isolates that shared the *MAT-A2* allele formed heterokaryons at the junction zone (Fig. 6), and these heterokaryons were stable and not mycelial mosaics, as shown by hyphal-tip analysis. This observation of heterokaryotization of isolates sharing *MAT-A* alleles is not limited to the junction zone and may include nuclear exchange (Fig. 7). One of the two diallel crosses of basidiospore isolates (cross 2) was characterized by very low levels of hyphal rejection or barrage formation among strains, and DNA analysis suggested that the nuclei or hyphae in these crosses between isolates of the same presumptive mating type (*MAT-A* alleles) moved across the junction zone. Finally, previous studies have shown that nutritionally forced heterokaryons can be readily formed between two auxotrophic mutants that are presumably isogenic except for the mutations, and genetic recombination has been demonstrated in some of these heterokaryons (3, 24).

Taken together, these results suggest there is no genetic basis for primary homothallism in *P. chrysosporium* and that the species has an outcrossing population structure. These results do not discount the possibility of facultative homothallism, at least in the laboratory, or for isolates used by previous investigators to be homothallic. The results of Alic and Gold (4) and Alic et al. (5), clearly demonstrate that homokaryotic fruiting does occur in the laboratory, but it must be noted that our methods of fruiting body production differed from those of Alic and Gold (4) and Alic et al. (5). The possibility for secondary homothallism, in which two mating-compatible nuclei are packaged into the same spore, is unknown. In this study, we observed no fruiting-competent single-basidiospore isolates, but one isolate, SB26, appeared to be a heterokaryon. Whether the heterokaryosis in this strain arose from a contaminated spore isolation or from a heterokaryotic basidiospore is impossible to distinguish. Homokaryotic fruiting in laboratory cultures of other basidiomycetes is well documented (14, 33, 64, 72), and if this were to occur in nature, then it can be considered a form of primary homothallism (33). Primary homothallism in Agaricomycetes has also been well documented (10), but the role of mating-type genes in controlling the process is unknown, though *MAT* genes have been shown to be in the genome of at least one homothallic species, *Moniliophthora perniciosa* (45). By analogy to other homothallic fungi, such as the ascomycete *Sordaria*, homothallic agaricomycete species may still retain the ability to outcross. In some agaricomycete species, such as *Stereum hirsutum* and *Sistotrema brinkmanii*, both heterothallic and homothallic populations within the same species have been detected (70, 81). The possibility of polymorphism of the mating system should be considered, as should the possibility of cryptic species, currently unknown in either *S. hirsutum* or *S. brinkmanii*.

Our leading hypothesis is that in nature, heterozygosity of *MAT-A* is required to maintain a stable heterokaryon and to complete the life cycle. Unlike other *Phanerochaete* species, *P. chrysosporium* has an imperfect state, and it is in this imperfect state that the fungus has been most frequently collected from wood chip piles (13). Homokaryons derived from single basidiospores have generally displayed reduced growth rates and enzyme yields relative to the parental strain (60, 83). This suggests that mating may actually be rare and that asexual reproduction by conidia may be more common. However, among the 7 strains investigated, each possessed novel mating types, pointing to a normal outcrossing population able to carry a high diversity of *MAT* alleles.

Heterokaryons are readily formed in pairings of *P. chrysosporium* strains with identical *MAT-A* mating types in the laboratory. Even if such heterokaryons are unable to fruit, their ability to recombine nuclei during vegetative proliferation via a parasexual cycle (6) and dissemination by conidia may provide the potential for recombination among isolates of the same mating type in the wild. These heterokaryons comprised of nuclei with identical mating-type alleles may be analogous to those observed in tetrapolar species, where pairings between isolates with shared *MAT-A* or *MAT-B* alleles can be isolated, and in the case of heterokaryons with common *MAT-B* alleles but different *MAT-A* alleles, these heterokaryons may be relatively stable (43, 62). One difference between the *P. chrysosporium MAT-A*-incompatible heterokaryons and the *MAT-A*-incompatible heterokaryons of tetrapolar species is that in the former, the morphology of the heterokaryons is indistinguishable from those of homokaryons, whereas in many tetrapolar species, the *MAT-A*-incompatible/*MAT-B*-compatible heterokaryons have a characteristic "flat" morphology (62). In another bipolar species that was investigated for common *MAT* heterokaryons, *Fomitopsis* (= *Polyporus*) *palustris*, Flexer (20) found evidence for the formation of *MAT*-incompatible heterokaryons that were also indistinguishable from homokaryons and in certain instances resulted from exchange of nuclei among incompatible partners. This pattern of nuclear exchange between mating-type-incompatible isolates, similar to that observed in *P. chrysosporium*, may be characteristic of the clade of brown rotting bipolar polypore species that includes *Fomitopsis*, *Piptoporus*, *Postia*, and *Phanerochaete* (28, 40).

The genetic basis for reduced incompatibility among isolates

of identical mating type in *P. chrysosporium* is unclear. One hypothesis could be a loss of somatic incompatibility (SI) response in the fungus, perhaps due to a bottleneck in SI allele numbers. Under the model of mating compatibility action proposed by Rayner (69), mating-type compatibility allows the override of the SI response specifically in homokaryons that are heteroallelic for both SI alleles and *MAT* alleles. Fewer segregating SI alleles in cross 2 than in cross 1 could be responsible for differences in the degree of morphological rejection between isolates in these crosses. Another hypothesis for reduced incompatibility could be the *MAT-B* incompatibility pathway, perhaps due to the presence of a self-activating pheromone receptor that was responsible for the origin of the bipolar system from the tetrapolar one. Such "primary" mutations to self-compatibility of the *MAT-B* genes have long been known in the model species *S. commune* and *C. cinerea* (26, 63) and have more recently been shown to be constitutively activating G protein-coupled receptors (58). Of special interest to the present study, many of the secondary mutations that restored self-incompatibility in the primary mutant background created alleles that conferred a unilateral nuclear migration phenotype (66, 67), as observed in many of the *P. chrysosporium* crosses.

Control of nuclear migration in bipolar Agaricomycetes. The bipolar mating system has evolved repeatedly in the Agaricomycetes from a tetrapolar one (28). This trajectory of mating system evolution in mushroom fungi appears to have occurred by loss of incompatibility determined directly by polymorphism of the *MAT-B* loci (2, 37). This is the third independent lineage investigated, and in each species, the *MAT-B* pheromone receptor genes are retained in the genome, but only the *MAT-A* homeodomain genes are polymorphic enough to specify mating types (2, 37). The amount of diversity between the pheromone receptor alleles at the 5 loci in *P. chrysosporium* (0.7 to 2.6%) (Table 2) is similar to or less than that observed between alleles at other "neutral" loci within heterokaryotic strains of *P. chrysosporium* (39, 46). More importantly, the very low levels of amino acid polymorphism among all of the pheromone receptors demonstrate that the population is essentially monomorphic for pheromone receptor proteins, suggesting that differences among strains are not due to allelism at these loci. Genome sequencing has likewise revealed that non-mating-type-specific pheromone receptors are common in Basidiomycetes (57, 68, 76), but it is also unclear what role they play in the sexual cycle, except in the case of *C. neoformans* receptor CPR2, where this non-mating-type-specific pheromone receptor plays a role in cell fusion and sporulation through the same G protein-coupled signaling cascade as the mating-type-specific pheromone receptors (32).

Observations that the *MAT-A* locus is the only mating incompatibility factor in most bipolar species question the relative role of the *MAT-A* versus the *MAT-B* genes in controlling nuclear migration. These *MAT-B* processes, as understood from the model mushroom species, appear to involve nuclear migration and clamp cell fusion (11), as well as nuclear positioning and heterokaryon stability (73). Expression of the non-mating-type-specific *MAT-B* homologues of the bipolar mushroom *C. disseminatus* in a heterologous species showed that these pheromone receptors were still able to drive clamp cell fusion and to complete the sexual cycle (37). Thus, it is likely that these functions are still controlled by the actions of pheromones and their receptors, but in the bipolar species, the *MAT-A* genes are now able to regulate the expression of these genes. More recently, Yi et al. (85) showed that heteroallelism at *MAT-A* alone could drive both clamp cell formation and fusion in the bipolar species *Pholiota microspora*, suggesting some level of regulation of *MAT-A* genes on the normally *MAT-B*-regulated process of clamp cell fusion. Why loci controlling nuclear migration (*MAT-B*) should be regulated separately from the loci controlling conjugate nuclear division (*MAT-A*) is a longstanding mystery, as common *MAT-A* heterokaryons can form in tetrapolar species, but they produce a flat, unfit mycelium (62). Whether bipolar mushroom species with only non-mating-type-specific pheromone receptors can coordinate the process of nuclear migration as effectually as tetrapolar species remains an open question. Nuclear migration in *P. chrysosporium* is rather fast (exceeding 2.5 mm/h), and the species with the highest known nuclear migration rate, *Coprinellus congregatus* (40 mm/h), is also bipolar, suggesting that evolution of bipolarity does not effect the ability for nuclear migration to occur by definition, a result consistent with the retention of pheromones/receptors in all bipolar species known.

In cross 1, only strains that differed in *MAT-A* alleles appeared to have nuclei that penetrated across the interaction zone (Fig. 6). We then tested the effect of *MAT-A* on nuclear migration using a transplantation assay. These results showed that nuclear migration occurred in a strain-specific manner, because certain strains much more readily accepted nuclei as the maternal parent, while two strains were completely "female sterile" (Fig. 7). Unfortunately, this study design is not able to disentangle the effects of *MAT-A* from those of pheromone receptors or other nuclear loci on migration. Most of the combinations in which nuclear migration occurred happened between strains with different *MAT-A* alleles; however, in at least one case, two strains with identical *MAT-A* alleles (SB34 and SB43) displayed nuclear penetration consistent with true nuclear migration. These data are similar to those obtained for the bipolar polypore *Fomitopsis palustris*, in which rapid nuclear migration occurred only among compatible mating types but was nevertheless observed to some extent in crosses of strains with identical mating types (20). We found numerous examples of unidirectional migration of nuclei in our homokaryon pairings (Fig. 5 to 7), which is a phenomenon that is often observed in pairings of other agaricomycete homokaryons (1). In tetrapolar species, unilateral migration can potentially be explained by the degree of complementary interaction between pheromones and their receptors (41). Given the absence of polymorphism at the pheromone receptors (Table 2), it is clear that this unidirectional migration must be controlled by factors other than pheromone receptors, a result consistent with the numerous secondary mutations outside the *MAT-B* locus in *S. commune* that were shown to result in unilateral nuclear migration (17).

Ancient divergence and *MAT* locus degeneration. The *MAT-A* haplotypes sequenced in this study showed extreme sequence divergence (Table 2). This hyperdiversity is explainable as the product of ancient coalescence times between mating types and a low turnover rate of alleles. This strong sequence divergence extends out of the hypothesized *MAT*

region (the HD1 and HD2 genes) into the flanking regions, where synonymous polymorphism at the 3′ end of *MIP* reached over 1, suggesting each position had been replaced slightly more than once (Table 2). The region downstream of the HD genes appears to be mostly devoid of functional genes and diverging at a high rate (Fig. 4). This "nonhomologous hole" also appears to be accumulating transposable elements, in much the way suspected for nonrecombining regions, like sex chromosomes and larger fungal *MAT* loci (23). The polymorphism data suggest that of the two genes encoding hypothetical proteins found in the genome of RP-78 between *A1* and the β*fg* found commonly at one end of the *MAT-A* locus (35), one, *Pc-HP1*, does not appear to be conserved in the other strains and is likely not an actual gene, while the other, *Pc-HP2*, appears to be found in all strains. It is interesting to speculate whether Pc-HP2 functions in mating, but its level of polymorphism is not consistent with its being a mating-type gene (Table 2).

In most mushroom *MAT* HD proteins, e.g., those of *C. disseminatus* (37), *C. cinerea* (7), and *Pleurotus djamor* (36), the amino acid sequence divergence has been reported as much higher in the amino than the carboxyl terminus, a pattern called the "divergence-homogenization duality" by Badrane and May (7). The opposite pattern was observed in *P. chrysosporium*, where the carboxyl terminus displayed higher variability (see Fig. S1 in the supplemental material). We hypothesize that the difference between *P. chrysosporium* and the other species is that recombination suppression between haplotypes of the HD genes is more extensive in *P. chrysosporium*, with possibly no recombination within the entire *MAT* locus and adjacent regions. The observed lower sequence similarity in the C termini could be a product of lower functional constraint. This is consistent with some studies showing that truncation of the C termini, but not N termini, of HD proteins can still result in a functionally active protein (80, 82). Why the region of recombination suppression in *P. chrysosporium* is larger than in other agaricomycete species is unknown, but its consequence is the introgression of the mutational load into the regions that flank the presumptive allele specificity regions encoded by the 5′ regions of the HD genes.

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