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Evolution of the Bipolar Mating System of the Mushroom *Coprinellus disseminatus* From Its Tetrapolar Ancestors Involves Loss of Mating-Type-Specific Pheromone Receptor Function

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ABSTRACT

Mating incompatibility in mushroom fungi is controlled by the mating-type loci. In tetrapolar species, two unlinked mating-type loci exist (*A* and *B*), whereas in bipolar species there is only one locus. The *A* and *B* mating-type loci encode homeodomain transcription factors and pheromones and pheromone receptors, respectively. Most mushroom species have a tetrapolar mating system, but numerous transitions to bipolar mating systems have occurred. Here we determined the genes controlling mating type in the bipolar mushroom *Coprinellus disseminatus*. Through positional cloning and degenerate PCR, we sequenced both the transcription factor and pheromone receptor mating-type gene homologs from *C. disseminatus*. Only the transcription factor genes segregate with mating type, discounting the hypothesis of genetic linkage between the *A* and *B* mating-type loci as the causal origin of bipolar mating behavior. The mating-type locus of *C. disseminatus* is similar to the *A* mating-type locus of the model species *Coprinopsis cinerea* and encodes two tightly linked pairs of homeodomain transcription factor genes. When transformed into *C. cinerea*, the *C. disseminatus A* and *B* homologs elicited sexual reactions like native mating-type genes. Although mating type in *C. disseminatus* is controlled by only the transcription factor genes, cellular functions appear to be conserved for both groups of genes.

MATING in fungi is controlled by the loci that determine the mating type of an individual, and only individuals with differing mating types can mate. Basidiomycete fungi have evolved a unique mating system, termed tetrapolar or bifactorial incompatibility, in which mating type is determined by two unlinked loci; compatibility at both loci is required for mating to occur. The origin of the tetrapolar mating system in the basidiomycetes is likely to be ancient since it is observed in at least two of the three major lineages, the Ustilaginomycetes, or smut fungi, and the Hymenomycetes, primarily the mushroom fungi (Burnett 1975). Also unique to the basidiomycetes is the presence of multiple alleles at the mating-type loci that allows most individuals within a population to be mating compatible with one another. Only the mushroom-forming homobasidiomycetes possess large allelic series at both loci, typically termed the *A* and *B* mating-type loci (Whitehouse 1949; Raper 1966).

The multiallelic tetrapolar mating system is considered to be a novel innovation that could have only evolved once (Raper 1966; Raper and Flexer 1971). For this reason, the ancestor of the homobasidiomycetes is accepted as having a tetrapolar mating system. Although most (~65%) of the homobasidiomycetes possess a tetrapolar mating system, many species (~25%) instead have a bipolar system controlled by a single locus with multiple alleles (Raper 1966). The distribution of bipolar species is scattered throughout the homobasidiomycete phylogeny, and bipolar species appear to have multiple independent origins from tetrapolar mating systems (Hibbett and Donoghue 2001). The population genetic consequence of the bipolar *vs.* the tetrapolar mating system is a difference in the amount of interbreeding permitted between the haploid progeny from a single parent (siblings). Specifically, the potential for inbreeding is higher in the bipolar system because 50% of full-sib progeny are mating compatible, whereas only 25% are in the tetrapolar case. This trend in homobasidiomycetes toward the evolution of increased selfing is similar to the situation in Ascomycete fungi (*e.g.*, Yun *et al.* 1999) and plants (*e.g.*, Takebayashi and Morrell 2001) in which selfing is typically derived from a system of greater outcrossing.

Using the model tetrapolar species *Schizophyllum commune* and *Coprinopsis cinerea* (*Coprinus cinereus*), the *A* mating-type loci of both species and the *B* mating-type locus of *S. commune* were discovered to be composed of

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two tightly linked subloci, the α- and β-subunits (Day 1960; Raper *et al.* 1960). Each unique combination of alleles at the subloci specifies a unique mating type, making the subloci redundant in function. Less information exists on the mating-type loci of bipolar species. One puzzling finding was that attempts to dissect the bipolar mating-type locus into component subloci failed, suggesting a different genetic architecture of the bipolar locus (Raper 1966).

The frequent evolution of bipolar species suggests that the transition from a tetrapolar mating system to bipolar may have a simple genetic basis. One clue to the genetic mechanism is the absence of documented reversals from a bipolar system back to a tetrapolar one. Raper (1966) put forward three plausible hypotheses concerning the origin of the bipolar mating system. One hypothesis is based on the observation that primary mutations at either of the mating-type loci often display a self-compatible phenotype, resulting in bipolar mating behavior of normally tetrapolar strains possessing such mutated alleles. Such mutants have been recovered many times both by spontaneous origin and by mutagenesis studies (Raper 1966). If such self-compatible mating-type alleles reach fixation frequency in a tetrapolar population, the population will be rendered effectively bipolar. A second hypothesis concerns the potential translocation of a chromosomal segment containing one of the mating-type loci into close genetic linkage with the other, leading ultimately to fusion of the two mating-type loci into one nonrecombining region. The suggestion that the bipolar mating-type locus is a single, indivisible locus gives credence to this hypothesis. A final hypothesis is that the function of one of the mating-type loci could be gradually assumed by the other locus. This hypothesis relates to the broadly applicable finding that the *A* and *B* mating-type loci control distinct but interconnected roles in the process of dikaryotic growth and fruiting in mushrooms (Raper 1978).

Although Raper's hypotheses were formulated before any fungal mating-type genes had been cloned, they are equally plausible today. Detailed molecular investigation of the mating-type genes of basidiomycetes has demonstrated that the mating-type genes of the smut fungi and Hymenomycetes are homologous (Casselton and Olesnicky 1998; Hiscock and Kües 1999). The *A* mating-type locus encodes for one or more pairs of homeodomain transcription factors. Each pair is composed of two classes of homeodomain transcription factor proteins, the HD1 and HD2 proteins, which share similarity with the mating-type proteins of *Saccharomyces cerevisiae* (Hiscock and Kües 1999). Heteroallelic but not homoallelic HD1 and HD2 proteins can heterodimerize, creating a transcription unit capable of initiating the *A* mating-type-specific developmental sequence (Kües and Casselton 1992). The *B* mating-type locus of the basidiomycetes was shown to encode both small peptide pheromones and pheromone receptors that are believed to be coupled to a trimeric G-protein complex (Brown and Casselton 2001). As with the *A* locus, pheromones can activate only heteroallelic *B* locus receptors.

Knowledge regarding the molecular sequence, organization, and function of the mating-type genes allows a reassessment of the manner in which a bipolar mating system might evolve from a tetrapolar one. The nature of the self-compatible mutant mating types of *C. cinerea* and *S. commune* has been investigated by DNA sequencing (Olesnicky *et al.* 1999, 2000; Fowler *et al.* 2001). Self-compatible mutants of the *B* mating type of *C. cinerea* were created by single amino acid substitutions in the pheromone receptors that caused either illegitimate interactions with homoallelic pheromone or constitutive activation of the *B* pathway (Olesnicky *et al.* 1999, 2000). For the *A* mating-type locus of *C. cinerea*, two primary mutations causing self-compatible phenotypes were investigated and found to be the result of a deletion/recombination event that caused the in-frame fusion of HD1 and HD2 genes from the same *A* haplotype but from different subloci (Kües *et al.* 1994; Pardo *et al.* 1996). Thus, self-compatible mating types may arise through mutation and provide a simple explanation for the origin of bipolar mating behavior through loss of discrimination by one of the two mating-type loci of a tetrapolar species. However, there is no evidence that self-compatible mating-types are involved in the origin of bipolar mating systems in the basidiomycetes, nor is there any evidence of such alleles in natural populations.

The origin of a bipolar mating system from a tetrapolar one has been addressed only in the smut fungi. In a landmark study, Bakkeren and Kronstad (1994) demonstrated that the bipolar mating-type locus of *Ustilago hordei* was formed from the fusion of the *a* and *b* mating-type loci observed in tetrapolar smut fungi into one nonrecombining mating-type region with two alleles. Thus, genetic linkage through translocation is the best explanation for the origin of bipolar mating in *U. hordei*, conforming to one of Raper's postulated mechanisms.

Recently, the bipolar mating system of the mushroom *Pholiota nameko* was characterized using linkage mapping and DNA sequencing (Aimi *et al.* 2005). These data demonstrated linkage between the mating-type locus and an *A* mating-type homolog but not a *B* mating-type homolog. Although intriguing, these results fail to differentiate among the possible genetic mechanisms of evolving a bipolar mating system, particularly because multiple *B* mating-type homologs exist in most mushroom genomes (this study), and only one *B* homolog was investigated in *P. nameko*.

The mushrooms in the genus Coprinus (*sensu lato*) provide an excellent group with which to study the evolution of mating systems and mating-type genes, because every known mating system is represented by multiple species. Furthermore, the molecular control of mating type in mushrooms has been intensely studied in

C. cinerea, simplifying cloning and comparative analyses. In this article, we investigated the genetic architecture of the bipolar mating system of the common wood-decaying fungus *Coprinellus* (*Coprinus*) *disseminatus*. Using knowledge of the mating-type loci in other homobasidiomycetes, we cloned and sequenced DNA regions containing homologs of the mating-type genes. We then used a population genetic approach to determine what changes in the mating-type genes of the hypothetical tetrapolar ancestor might have occurred during its transition to a bipolar system.

MATERIALS AND METHODS

Study species: *C. disseminatus* (Pers. ex Fr.) J. E. Lange is a common mushroom species that fruits in large troops on stumps, buried wood, tree tip-up mounds, and logs (Buller 1924). Its distribution is probably cosmopolitan, but appears to be divided into at least three divergent phylogenetic groups on the basis of ribosomal DNA sequencing (Ko *et al.* 2001). The dikaryotic mycelium contains sparse but large clamp connections (Lange 1952; Butler 1981). The mating system was determined by Lange (1952) and judged to be bipolar, confirming earlier results by Vandendries and Quintanilha (cited in Lange 1952). Finally, Lange (1952) found one collection from New Delhi, India that appeared to be intersterile with seven European collections, suggesting the presence of multiple biological species within the morphological species.

Culture isolation and growth: Homokaryotic strains were derived from wild-collected fruiting bodies, collected in 2000–2001 from within an ~15-km radius of the Duke University Campus in Durham, North Carolina (Table 1). One fruiting body was collected per stump or mound. After fruiting bodies were collected in the field or greenhouse, they were placed over aluminum foil for at least 1 hr to collect the dark brown spores. Spores were scraped from the foil into H_2O, and a series of dilutions were plated on half-strength Emerson's YpSS (Y/2) nutrient agar plates with 1.5% agar (Stevens 1974). Following 1–2 days of growth at room temperature (RT), hyphal colonies were inspected under the microscope at 100× to verify that they derived from a single spore. Single-spore isolates were subcultured at least twice on Y/2. Long-term storage was on 1.5% malt extract agar slants at 4°. Also included were three homokaryotic European strains and a Japanese dikaryotic strain (IFO 30972). The Japanese strain was fruited in the greenhouse on a substrate composed of sawdust, rye berries, and soil in a 4:1:1 ratio. Two mating-compatible homokaryons from this dikaryotic strain were isolated for further study.

Mating compatibility tests: Single-spore isolates were obtained (n = 5–14) for each of the 24 wild-collected fruiting bodies. Each of these F_1 progeny arrays was intracrossed in all possible combinations to identify suitable testers representing the two mating-type alleles of the F_0 fruit body. Using the tester strains, all homokaryotic strains (n = 51) were paired in a complete cross design to determine if any mating types were repeated in the population sample. All crosses were conducted on 10-cm Y/2 agar plates by inoculation of the two strains in the center of the plate ~1 cm from each other. Plates were incubated in the dark at RT for 1–2 weeks. The formation of the dikaryon in a genetic cross typically results in vigorous growth from the margins of the paired homokaryons. Large clamp connections become apparent, albeit sparse, at cell junctions at 100× magnification. Finally, after ~2 weeks, brownish rhizomorphic strands clearly distinguish pairs that have mated. We verified dikaryotization on the basis of the production of clamp connections in all crosses.

DNA amplification and sequencing: Mycelium for DNA extraction was prepared by stationary growth of isolates in 2 ml of 1.5% malt extract broth until stationary phase. The mycelium was removed from the broth, rinsed in H_2O, and lyophilized. Approximately 50 mg were used for DNA extraction, using a CTAB buffer following the protocol of Zolan and Pukkila (1986).

To study homologs of the putative *B* mating-type genes, we used degenerate PCR primers to amplify *STE3*-like pheromone receptors from homokaryotic strains following standard PCR protocols (James *et al.* 2004a). Primers br1-F and br1-1R were used to amplify two receptors each from C345.1 and TJ01/19.2; in addition, we used primers br2-F and br2-2R to amplify a small *STE3*-like fragment from strain TJ01/19.2 (see James *et al.* 2004b for primer sequences). All degenerate PCR amplicons were gel purified using a QIAquick gel extraction kit (QIAGEN, Valencia, CA), ligated into pCR2.1 (Invitrogen, San Diego), and transformed into *Escherichia coli* strain TOP10 (Invitrogen). Plasmid templates for DNA sequencing were prepared using the QIAprep spin miniprep kit (QIAGEN) and sequenced on both strands using universal forward and reverse M13 primers. Sequencing reactions utilized the BigDye sequencing kit (Applied Biosystems, Foster City, CA) and were analyzed on an ABI3700 DNA sequencer.

We screened our population sample for DNA polymorphisms at gene regions both linked and unlinked to the mating-type locus, using the nondegenerate PCR primers shown in Table 2. All reactions (except *A*α and *A*β) were conducted in a similar manner to that used for degenerate PCR with annealing temperatures fixed at 50°. Amplicons were purified using the QIAquick PCR purification kit (QIAGEN) and sequenced as above.

Cosegregation analyses: We previously studied the cosegregation of *MIP* and mating type among 13 single-spore progeny of field collection TJ00/38 (James *et al.* 2004a). This same progeny array was used to determine whether the *STE3*-like pheromone receptors (*CDSTE3.1*, *CDSTE3.2*, and *CDSTE3.3*) also cosegregate with mating type. Amplification of the genes was accomplished with the PCR primers shown in Table 2. Amplicons were digested using the enzymes *Sin*I for *CDSTE3.1*, *Bam*HI for *CDSTE3.2*, and *Mwo*I for *CDSTE3.3*, following the manufacturer's instructions (New England Biolabs, Beverly, MA; Promega, Madison, WI). Digested amplicons were electrophoresed on 1–2% agarose gels and scored for the polymorphic restriction fragments.

Long-distance PCR and amplicon sequencing: We used long-distance PCR to amplify *A*α and *A*β subloci from homokaryons, using the enzyme *LA Taq* (Takara, Berkeley, CA) following the manufacturer's instructions. The primers for amplification are given in Table 2. The thermocycling parameters used were: initial denaturation at 94° for 1 min; followed by 35 cycles of 94° for 30 sec, 60° for 30 sec, and 72° for 4 min; and finally a 10-min extension at 72°. Amplicons were digested with the enzyme *Msp*I (Promega) to determine allelism, and two subsets (eight of *A*α and three of *A*β) were chosen for DNA sequencing. Amplicons were purified using the QIAquick gel extraction kit and ligated into the vector pCR2.1-TOPO (Invitrogen). The resulting plasmids were sequenced using a combination of standard subcloning procedures, using plasmid pUC119 (Sambrook *et al.* 1989) and the GeneJumper kit containing kanamycin or chloramphenicol resistance transposons (Invitrogen). Plasmid sequencing from both ends of the transposon used the primers GJSeq-A3 and GJSeq-B2 for kanamycin and GJSeq-A3 and GJSeq-B4 for chloramphenicol (James *et al.* 2004b).

TABLE 1

Geographic origin and mating type of homokaryotic strains of *C. disseminatus* used in the population survey

Strain	Mating type	Origin
C345.1	*A1*	France
TJ00/38.3	*A2*	Duke Forest, Korstian Division, North Carolina
TJ00/38.6	*A3*	Duke Forest, Korstian Division, North Carolina
TJ00/89.1	*A4*	Duke Forest, Durham Division, North Carolina
TJ00/89.2	*A3*	Duke Forest, Durham Division, North Carolina
TJ00/91.1	*A5*	Near Northgate Park, Durham, North Carolina
TJ00/91.2	*A6*	Near Northgate Park, Durham, North Carolina
TJ00/94.4	*A7*	Duke Forest, Durham Division, North Carolina
TJ00/94.5	*A8*	Duke Forest, Durham Division, North Carolina
TJ00/99.1	*A5*	Duke University Campus, North Carolina
TJ00/99.3	*A9*	Duke University Campus, North Carolina
TJ00/100.1	*A10*	Innsbruck, Austria
TJ00/100.3	*A11*	Innsbruck, Austria
TJ01/02.5	*A12*	Duke Forest, Durham Division, North Carolina
TJ01/02.7	*A5*	Duke Forest, Durham Division, North Carolina
TJ01/04.1	*A13*	Duke University Campus, North Carolina
TJ01/04.4	*A14*	Duke University Campus, North Carolina
TJ01/05.1	*A15*	Duke Forest, Blackwood Division, North Carolina
TJ01/05.8	*A16*	Duke Forest, Blackwood Division, North Carolina
TJ01/06.2	*A17*	Duke Forest, Blackwood Division, North Carolina
TJ01/06.5	*A18*	Duke Forest, Blackwood Division, North Carolina
TJ01/07.1	*A19*	Duke Forest, Blackwood Division, North Carolina
TJ01/07.6	*A20*	Duke Forest, Blackwood Division, North Carolina
TJ01/08.1	*A21*	Duke Forest, Blackwood Division, North Carolina
TJ01/08.2	*A22*	Duke Forest, Blackwood Division, North Carolina
TJ01/09.1	*A23*	Duke Forest, Blackwood Division, North Carolina
TJ01/09.3	*A24*	Duke Forest, Blackwood Division, North Carolina
TJ01/10.2	*A4*	Duke Forest, Blackwood Division, North Carolina
TJ01/10.3	*A25*	Duke Forest, Blackwood Division, North Carolina
TJ01/11.2	*A26*	Duke Forest, Blackwood Division, North Carolina
TJ01/11.5	*A27*	Duke Forest, Blackwood Division, North Carolina
TJ01/12.4	*A28*	Duke Forest, Blackwood Division, North Carolina
TJ01/12.6	*A29*	Duke Forest, Blackwood Division, North Carolina
TJ01/13.3	*A30*	Duke Forest, Blackwood Division, North Carolina
TJ01/13.8	*A31*	Duke Forest, Blackwood Division, North Carolina
TJ01/14.1	*A32*	Duke Forest, Korstian Division, North Carolina
TJ01/14.2	*A21*	Duke Forest, Korstian Division, North Carolina
TJ01/15.1	*A33*	Duke Forest, Korstian Division, North Carolina
TJ01/15.4	*A34*	Duke Forest, Korstian Division, North Carolina
TJ01/16.1	*A35*	Duke Forest, Korstian Division, North Carolina
TJ01/16.3	*A36*	Duke Forest, Korstian Division, North Carolina
TJ01/17.1	*A26*	Duke Forest, Korstian Division, North Carolina
TJ01/17.6	*A37*	Duke Forest, Korstian Division, North Carolina
TJ01/18.1	*A38*	Duke Forest, Korstian Division, North Carolina
TJ01/18.5	*A39*	Duke Forest, Korstian Division, North Carolina
TJ01/19.2	*A31*	Duke Forest, Durham Division, North Carolina
TJ01/19.4	*A40*	Duke Forest, Durham Division, North Carolina
TJ01/20.5	*A41*	Duke University Campus, North Carolina
TJ01/20.6	*A13*	Duke University Campus, North Carolina
IFO 30972.16	ND[a]	Japan
IFO 30972.17	ND	Japan

[a] Homokaryons isolated from dikaryotic strain IFO 30972 appear to be intersterile with European and North American isolates.

Cosmid library construction, screening, and sequencing: A cosmid library was prepared in the vector SuperCos-Pab1 (Bottoli *et al.* 1999), using the DNA of strain C345.1. This strain has been deposited into the Belgian Coordinated Collections of Microorganisms as MUCL 43037. DNA of C345.1 was prepared from mycelium grown in 1 liter of Y/2 broth under rotary shaking (~125 rpm) at RT. Preparation and screening of the library followed the protocol of James *et al.* (2004b). The library was screened by PCR of bacterial cells for two genes, *MIP* and *CDSTE3.1* (see Table 2 for primer

TABLE 2

PCR primers used to survey genetic variation in *C. disseminatus*

Gene	Primer	Sequence 5′ → 3′
PAB1	CdPAB1-F	CGTACGACTCATTCACACACAA
	CdPAB1-R	ACGGACTCTGGGTGGTACTG
GLYDH	CdGLYDH-F	TATCGACTGGCACTGCAAAG
	CdGLYDH-R	CAACTACCGAGGGCAATCAT
MIP	CdMIP-F	CTGCGGGCAACTGGRAACAA
	CdMIP-R	GAAGGACGTCTCTGGCACATA
CDHH	CdHH-F	ACTGTGGAGGCAAGTCGAAG
	CdHH-R	CAAACTTCTGCCACTCAGCA
CDRF	CdRF-F	GTTCCCATCCCAACAAACTG
	CdRF-R	AATAGGAACGCGTCTGAGGA
YPL109	CdYPL109-F	AGGCACCTTTGAGCCTCTCT
	CdYPL109-R	ACCCATACCCACACCTTCAA
RPB2	CdRPB2-F	AGCCGACGGAGATACATGAC
	CdRPB2-R	CGTATTCGTTACGCACAGGA
CDSTE3.1	CdSTE3.1-F	CATCGCTCCTGTATGGTGTG
	CdSTE3.1-R	CTGGAGAATAGGGACGCAAA
CDSTE3.2	CdSTE3.2-F	TCGATCGTATGGAACGRTAA
	CdSTE3.2-R	AKCGTCTAGGYGTGAGGTTC
CDSTE3.3	CdSTE3.3-F	CCCATTTGGTGTGACATCTG
	CdSTE3.3-R	GGTCAAGAGCTGGCTGAACT
CDPHB1	CdPHB1-F	TACCGAAGAATCAGGCCTCT
	CdPHB1-R	CGATGTTACAGAACGCACCA
*A*α	Cdα-F	GGCGTATATCAGCTGCCACT
	Cdα-R	CCCYTCCTTTCGATCTTTTC
*A*β	Cdβ-F	AGGCCGAAAAGATCGAAAGG
	Cdβ-R	GGCACGGAGAAGATTTYACTGG

sequences). DNA of all cosmid clones was isolated using the QIAprep spin miniprep kit (QIAGEN). Six overlapping cosmids were sequenced for the *MIP*/mating-type locus region, using a random shotgun subcloning method involving partial digestion with restriction enzymes (Zhou *et al.* 1988). A single *CDSTE3.1* positive cosmid, C25.B2.5, was also sequenced using the GeneJumper primer insertion kit with the kanamycin resistance transposon (Invitrogen). Sequencing reactions were accomplished using the BigDye kit and primers GJSeq-A3 and GJSeq-B2. Assembly of sequence traces into contigs was performed with Sequencher v. 4.1 (Gene Codes, Ann Arbor, MI). Gaps in the contigs were filled by primer walking with synthesized oligonucleotides (Operon, Alameda, CA). Approximately 4.0-fold coverage of the 75.5-kb *MIP* chromosomal region and 3.1-fold coverage of the 41.9-kb *CDSTE3.1* cosmid were achieved.

Expression of *C. disseminatus* genes in *C. cinerea*: *C. cinerea* monokaryon 218 (*A3*, *B1*, *trp1.1,16*, *bad*; Binninger *et al.* 1987; Kües *et al.* 2002) was transformed according to the protocol given by Granado *et al.* (1997). The *trp1*$^{+}$ vector pCc1001 (Binninger *et al.* 1987) was used in cotransformations with plasmid pCR2.1-TOPO containing the entire *A*α sublocus from strains TJ00/99.1 and TJ01/16.3 (plasmids 99.1Aα and 16.3Aα) and the entire *A*β sublocus from strains TJ00/99.1 and TJ00/89.2 (99.1Aβ and 89.2Aβ). Also used in cotransformation with pCc1001 were C25.B2.5 containing the two putative pheromone receptor genes *CDSTE3.1* and *CDSTE3.3* and three pheromone genes *CDPHB1*, *CDPHB2.1*, and *CDPHB2.2* and C25_e1.10, a 10-kb subclone of C25.B2.5 containing *CDSTE3.1* and *CDPHB1* in vector pZERO (Invitrogen). Cotransformations used 1 μg DNA for each plasmid. Transformants were picked onto minimal medium (Granado *et al.* 1997) and checked for *A*-regulated clamp cell production (Kües *et al.* 1992) and *B*-regulated subapical peg formation and clamp cell fusion (Badalyan *et al.* 2004) under a microscope. Functional expression of pheromone receptor and/or pheromone genes was further analyzed in mating reactions (O'Shea *et al.* 1998) on YMG/T medium (Granado *et al.* 1997) with monokaryon PS004-2 (*A42*, *B1*; P. Srivilai, unpublished data). Fruiting abilities of dikaryons were tested under *C. cinerea* standard fruiting conditions (Granado *et al.* 1997). A minimum of 20 transformants were analyzed per cotransformation experiment. Control transformations utilized solely pCc1001.

Data analyses: Identification of genes on the sequenced cosmid clones used homology searching of the GenBank database with the BLASTX algorithm (Altschul *et al.* 1997). CDD searches were also used to determine putative conserved domains that helped in the determination of gene function (Marchler-Bauer and Bryant 2004). Searches for the small peptide pheromone genes were attempted using NCBI's ORFfinder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and the software GeneMark v. 2.5, using both *Schizosaccarhomyces pombe* and *Saccharomyces cerevisiae* models (Borodovsky and McIninch 1993).

Multiple sequence alignments were performed using the alignment editor GeneDoc (Nicholas and Nicholas 1997). A phylogeny of the STE3-like pheromone receptors was conducted by analyzing the sequences from *C. disseminatus* with other pheromone receptor sequences retrieved from GenBank. Following exclusion of ambiguously aligned regions, 158 aligned amino acids were used for phylogeny reconstruction. A maximum-likelihood phylogeny was reconstructed using the software PROTML from the MOLPHY package, using 1000 heuristic searches and the JTT-F substitution matrix (Adachi and Hasegawa 1996). Support for nodes was assessed using approximate bootstrap probabilities by the RELL method (Hasegawa and Kishino 1994). Basic statistics of DNA diversity were calculated using the software MEGA v. 3.0 (Kumar *et al.* 2004) for π and DnaSP v. 3.53 (Rozas and Rozas 1999) for π_S.

RESULTS

***C. disseminatus* has a bipolar mating system with multiple alleles:** We obtained single-spore isolates from 24 fruit body collections and intercrossed them to determine the mating types and mating system of the progeny. All of the 24 collections displayed a bipolar mating system with only two mating types among all progeny of a collection. In a few instances some homokaryons among the progeny of a fruit body showed marked inability to mate with other strains. Deviations from the expected pattern of bipolar mating have already been recorded in certain isolates (Lange 1952). We note that these patterns were restricted to crosses within a progeny array and that they were, without exception, due to strains that showed an inability to mate rather than a promiscuous mating pattern. We specifically avoided these strains when we chose testers for a complete population cross.

When all homokaryotic testers were intercrossed, nearly every mating was successful. We found that the Japanese homokaryons (IFO 30972.16-17) were unable to mate with any of the European or North American

isolates, suggesting that the Japanese strains represent a different species or intersterility group. The complete population crossing design allowed us to assign a series of mating types (Table 1) and to estimate the number of total alleles in the species through the observation of mating-type repeats. We use the nomenclature of Raper (1966) in referring to the sole mating type of the bipolar *C. disseminatus* as the *A* mating-type locus. Six mating types were each shared by two strains; one mating type (*A5*) was recovered from three strains. Using the formula of O'Donnell and Lawrence (1984), ~123 mating types were estimated to be in the global population with 95% confidence intervals of 73–254. These data suggest that *C. disseminatus* has a much larger number of mating types than most of the bipolar species studied to date, the majority of which have allele estimates of ≤50 (Murphy and Miller 1997).

The pheromone receptors of *C. disseminatus* are not part of the mating-type locus: We developed two sets of degenerate PCR primers for amplifying the *STE3*-like pheromone receptors from homobasidiomycetes. Amplification using primers br1-F and br1-1R on genomic DNA of *C. disseminatus* strain C345.1 yielded two fragments homologous to *STE3*-like pheromone receptors in the same amplification reaction. One of them, termed *CDSTE3.1*, possesses the same four introns as do most homobasidiomycete *STE3* receptor genes. The other fragment (*CDSTE3.2*) was unique among the known *STE3* sequences in lacking three of the four introns. Amplification of genomic DNA of strain TJ01/19.2 with primers br1-F and br1-1R also produced two *STE3*-like amplicons. One of these was very similar to *CDSTE3.2*, and the other fragment represented a third receptor paralog, *CDSTE3.3*. Amplification of DNA from strain TJ01/19.2 using PCR primers br2-F and br2-2R also yielded a fourth paralog, *CDSTE3.4*. Using specific primers or cosmid sequencing, we were ultimately able to recover homologs of *CDSTE3.1–CDSTE3.4* from strain TJ01/19.2 and homologs of *CDSTE3.1–CDSTE3.3* from strain C345.1, demonstrating at least three receptor paralogs in the two *C. disseminatus* homokaryons tested.

We have previously used PCR to amplify and genotype a small fragment of *MIP* from *C. disseminatus*, and we demonstrated that the *MIP* gene fragment cosegregates with the mating-type locus of *C. disseminatus*, using a small progeny array (James *et al.* 2004a). PCR primers specific to the four *STE3*-like sequences were designed to test whether these putative pheromone receptors also cosegregated with the mating-type locus. The segregation of three putative pheromone receptors (*CDSTE3.1–CDSTE3.3*) was examined among the progeny array of parental dikaryon TJ00/38, and all three of these receptors displayed no apparent linkage with the mating-type locus (Table 3). Two of the loci (*CDSTE3.1* and *CDSTE3.3*) displayed complete cosegregation with each other, suggesting that they are closely linked in the genome. Although segregation data for *CDSTE3.4* are lacking, one piece of evidence suggests that this gene is not mating-type specific. The PCR primers specific to this gene amplified only DNA of two sibling strains (TJ01/19.2 and TJ01/19.4). These two sibling strains also possess different mating-type alleles but have identical sequences at *CDSTE3.4*.

TABLE 3

Segregation of mating type and genetic markers in a homokaryotic progeny array derived from field collection TJ00/38

Isolate	Mating type	*MIP*	*CDSTE3.1*	*CDSTE3.2*	*CDSTE3.3*
TJ00/38.1	*A3*	−	−	+	−
TJ00/38.6	*A3*	−	−	−	−
TJ00/38.7	*A3*	−	−	−	−
TJ00/38.11	*A3*	−	−	+	−
TJ00/38.14	*A3*	−	−	−	−
TJ00/38.15	*A3*	−	−	−	−
TJ00/38.3	*A2*	+	−	−	−
TJ00/38.4	*A2*	+	+	−	+
TJ00/38.5	*A2*	+	+	+	+
TJ00/38.9	*A2*	+	+	−	+
TJ00/38.10	*A2*	+	+	−	+
TJ00/38.12	*A2*	+	−	−	−
TJ00/38.13	*A2*	+	−	+	−

The two genotypes at the DNA loci are arbitrarily assigned to states + and −, reflecting the segregating PCR-RFLP sites.

Structure of the mating-type locus: Having demonstrated that the single mating-type locus of *C. disseminatus* cosegregates with the *MIP* gene but not with any *STE3*-like receptors, we probed a cosmid library for the *MIP* gene under the assumption that the gene would be very tightly linked to the mating-type locus, as it is in model mushroom species (Kües *et al.* 2001). We obtained four unique, overlapping cosmid clones that contained the *MIP* gene from the library; an additional clone was obtained by a short chromosomal walk (c94.K3.1.22). Through subcloning and DNA sequencing of these cosmids, we assembled a restriction and gene map of the *A* mating-type locus (Figure 1).

Immediately adjacent to *MIP* were four genes homologous to the class of homeodomain transcription factors [Figure 1, supplemental Table 1 (http://www.genetics.org/supplemental/)]. The genes were arranged as divergently transcribed HD1 and HD2 pairs, as seen in other *A* mating-type loci (Casselton and Olesnicky 1998). For convenience and comparison with other mushroom mating-type loci, we refer to the two pairs as *A*α and *A*β subunits of the *A* mating-type locus of *C. disseminatus*. As seen with other homobasidiomycete mating-type loci, ~32 kb upstream from the mating-type genes is the *PAB1* gene, encoding for *para*-amino benzoic acid synthase (Kües *et al.* 2001; James *et al.* 2002). The *A* mating-type locus region from *C. disseminatus* was compared with the genome sequence available for the

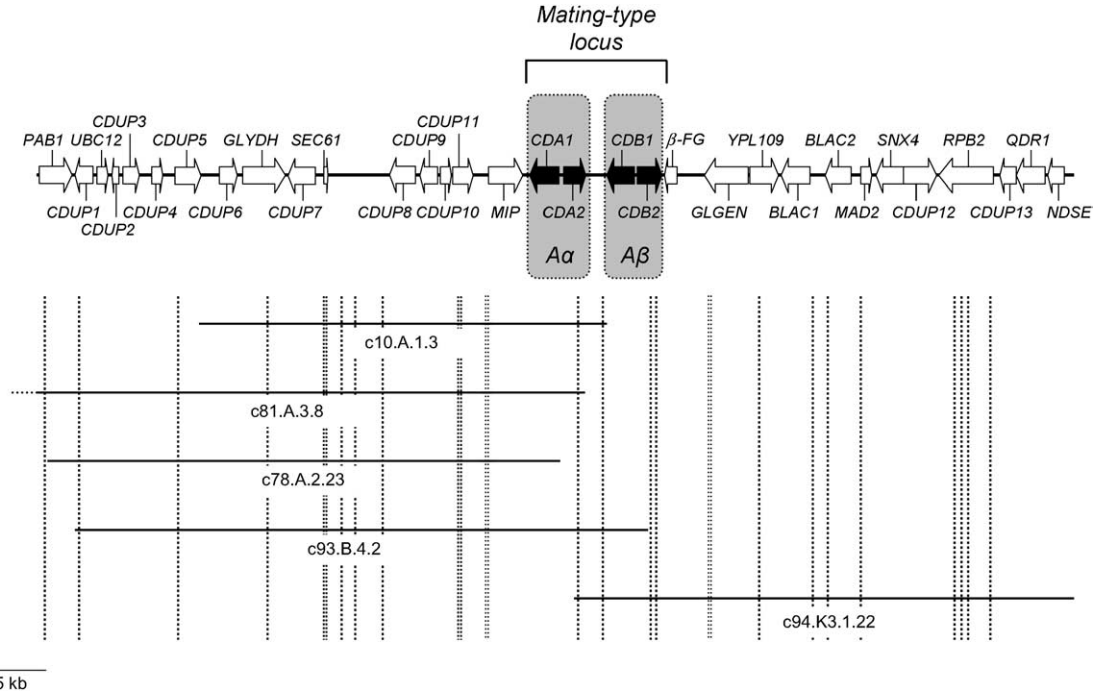

FIGURE 1.—Restriction and gene map of the mating-type locus of *C. disseminatus*. Arrows indicate direction of transcription. Below the gene map is shown the position of the cosmids used to generate the DNA sequences. Genes were identified using BLASTX searches with a cutoff *P*-value of 10^{-4} (see supplemental Table 1 at http://www.genetics.org/supplemental/). Mating-type-specific genes are shown as solid arrows. Predicted genes with no clear cellular function are indicated as *CDUP1–CDUP13*. Vertical dashed lines connecting cosmids represent *Eco*RI cut sites. Clone c81.A.3.8 continues beyond the left border of the map.

related species *C. cinerea* (Figure 2). These data show a very conserved gene order between the two species for nearly the entire 75-kb region with the exception of a duplicated pair of putative drug-binding proteins inserted into the *C. disseminatus* region (*BLAC1* and *BLAC2*). Other genes of known function displaying conserved synteny between *C. disseminatus* and other homobasidiomycetes (JAMES *et al.* 2004b; T. Y. JAMES, unpublished data) are RNA polymerase II (*RPB2*), glycine dehydrogenase (*GLYDH*), and a putative kinase with similarity to yeast protein YPL109 (*YPL109*). In summary, we sequenced a large region of the chromosome surrounding the *MIP* gene, revealing conserved synteny of this region in comparison with a model mushroom species. Importantly, we discovered putative *A* mating-type genes clustered into an ~10-kb region encoding four homeodomain transcription factor genes.

Polymorphism at and near the mating-type locus: Basidiomycete mating-type genes have been shown to have high levels of amino acid polymorphism between alleles with substitutions clustered in the N-terminal regions of the homeodomain proteins, as this region has been determined to function in allele discrimination (YEE and KRONSTAD 1993; BANHAM *et al.* 1995; WU *et al.* 1996; BADRANE and MAY 1999). To determine the extent and location of variability of the putative *C. disseminatus* mating-type genes we used long PCR to amplify the *A*α and *A*β subloci in two separate reactions. Restriction digests of the *A*α PCR products revealed extensive DNA diversity in *Msp*I cut sites (Figure 3). Homokaryons with the same mating type as that determined by genetic crosses shared identical or similar restriction digestion patterns, whereas for different alleles it is difficult even to assess the homology of restriction fragments due to excess polymorphism. A similar result was found for the digests of *A*β amplicons (data not shown).

We subcloned eight *A*α and three *A*β amplicons and sequenced them to determine the pattern of DNA sequence diversity in relation to the functional domains of the proteins they encode. As observed with other mating-type genes, the level of DNA sequence and amino acid diversity between alleles was tremendous. The nine *CDA1* and *CDA2* sequences could be divided into five sequence types (presumably alleles). Similarity among the five heteroalleles at the N-terminal region of the protein before the homeodomain motif ranged from 53 to 69% for CDA1 and from 45 to 65% for CDA2. In contrast, similarity in the C-terminal region after the homeodomain motif ranged from 64 to 79% for CDA1 and from 89 to 97% for CDA2.

If the genomic region surrounding the homeodomain genes in *C. disseminatus* was all part of one nonrecombining region, it would be expected that the same forces of balancing selection that promote sequence divergence of mating-type alleles (MAY *et al.*

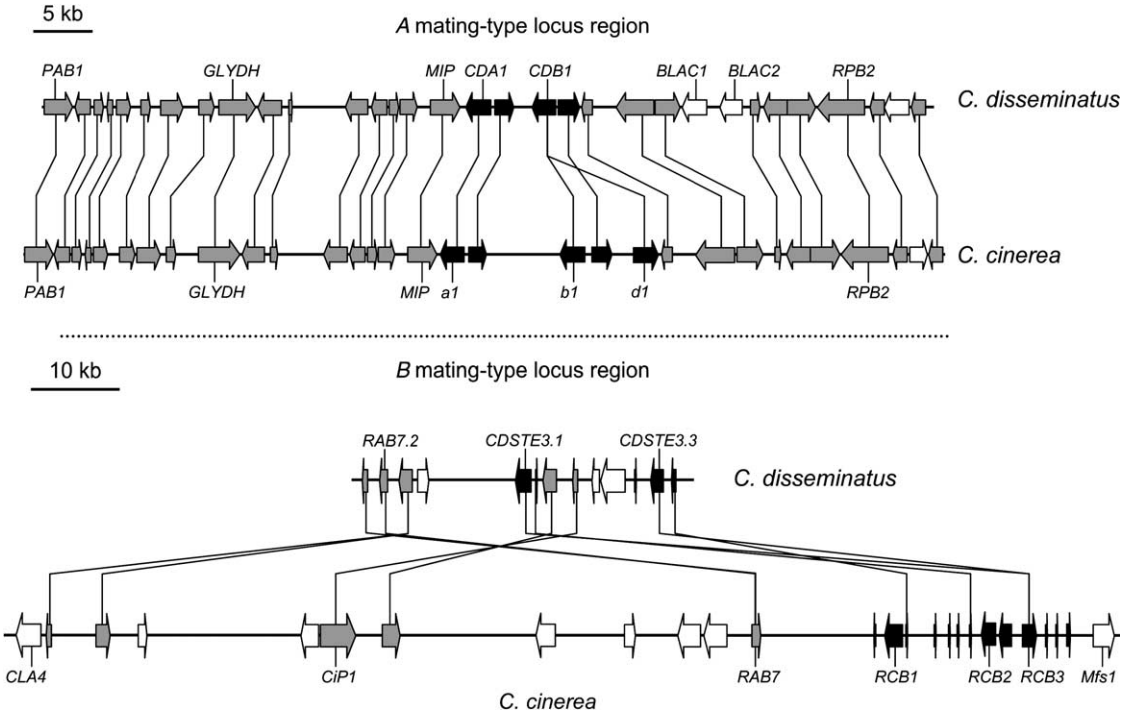

Figure 2.—Schematic comparison of genomic regions from *C. cinerea* to *C. disseminatus A* and *B* mating-type loci. *C. cinerea* mating-type region *A* corresponds to 382–462 kb from GenBank accession no. AACS01000026.1. *C. cinerea* mating-type region *B* corresponds to 50–176 kb from accession no. AACS01000134.1. *C. disseminatus* regions are the same as those shown in Figures 1 and 5. Mating-type gene homologs are shown as solid arrows. Shaded and open arrows indicate genes that are syntenic or not, respectively, between the two species.

1999) would also have a strong effect on increasing polymorphism of the entire region. Thus, we investigated the genetic variation in the genes surrounding the *C. disseminatus* mating-type locus by sequencing seven non-mating-type gene regions (*PAB1*, *GLYDH*, *MIP*, *CDHH*, *CDRF*, *YPL109*, and *RPB2*) spaced over an ~70-kb region centered around the mating-type locus for our sample of 49 homokaryons (non-Japanese isolates). We used these data to estimate DNA polymorphism (π) or the average number of pairwise differences per site between two sequences (Nei 1987). The amount of DNA polymorphism (π) at the mating-type genes was approximately an order of magnitude higher than that of the genes that flank the *A* locus (Table 4). Variation was also significantly higher ($P < 0.05$) at the gene regions directly adjacent to the mating-type locus (*i.e.*, *MIP*, *CDHH*, *CDRF*). However, the substitutions in Table 4 include both synonymous and nonsynonymous changes for protein-coding genes such as *MIP*. We also looked at polymorphism only at silent positions where substitutions have no effect on the encoded proteins (π_S), such that differences in standing genetic variation should reflect only differences in coalescence time due to balancing rather than to positive selection. The DNA diversity at silent positions was similarly low for all genes outside of the mating-type locus (Figure 4); however, the genes upstream of the *A* locus generally had a higher level of silent polymorphism than the genes downstream of the *A* locus. These data suggest that balancing selection on the mating-type loci may have the effect of elevating polymorphism of the neighboring genes, but this effect is greatly reduced over short physical distances, presumably through recombination.

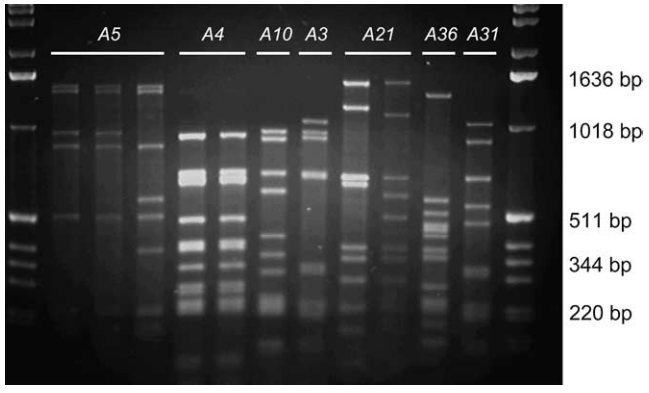

Figure 3.—Restriction digests of *A*α amplicons of *C. disseminatus* homokaryons with the enzyme *Msp*I. In lanes 1 and 13 are size standards with sizes shown to the right. Lane 2, TJ00/91.1; lane 3, TJ00/99.1; lane 4, TJ01/02.7; lane 5, TJ00/89.1; lane 6, TJ01/10.2; lane 7, TJ00/100.1; lane 8, TJ00/89.2; lane 9, TJ01/08.1; lane 10, TJ01/14.2; lane 11, TJ01/16.3; lane 12, TJ01/19.2. Shown above the lanes of the digested amplicons are the mating types of the isolates (Table 1).

Genetic structure of a putative extinct *B* mating type: We probed the *C. disseminatus* cosmid library for clones

TABLE 4

DNA diversity at *C. disseminatus* loci

Gene	*n*	*N*	*S*	π
CDA1 (exons)	9	1863	1024	0.371 (0.010)
CDA2 (exons)	9	1524	213	0.222 (0.009)
CDB1 (exons)	4	1941	948	0.475 (0.015)
CDB2 (exons)	4	1668	920	0.565 (0.016)
PAB1	49	540	25	0.007 (0.002)
GLYDH	49	617	55	0.011 (0.002)
MIP	49	564	84	0.024 (0.003)
CDHH[a]	30	172	19	0.021 (0.006)
CDRF[b]	49	673	121	0.027 (0.003)
YPL109	49	611	18	0.004 (0.001)
RPB2	49	611	12	0.002 (0.001)
CDSTE3.1	9	572	23	0.010 (0.002)
CDSTE3.2	11	475	112	0.085 (0.008)
CDSTE3.3	14	569	13	0.005 (0.001)
CDPHB1	9	201	6	0.011 (0.004)

n, number of monokaryotic samples sequenced; *N*, number of aligned base pairs; *S*, number of segregating sites; π, nucleotide diversity or the average number of pairwise differences per site (using Jukes–Cantor correction). Nucleotide diversities are followed by standard errors in parentheses estimated using 500 bootstrap replicates (Kumar *et al.* 2004).

[a] Noncoding region in between the *A*α and *A*β subloci.

[b] Noncoding region flanking the *A*β sublocus, see Figure 4.

containing the *CDSTE3.1* gene. The complete sequencing of one 41.9-kb *CDSTE3.1* positive cosmid clone C25.B2.5 indicated several genes in this chromosomal region, including two putative pheromone receptors and three putative small pheromone genes [supplemental Table 2 (http://www.genetics.org/supplemental/); Figure 5]. The two *STE3*-like pheromone receptor genes on this cosmid corresponded with the previously identified *CDSTE3.1* and *CDSTE3.3* genes. The predicted proteins encoded by *CDSTE3.1* and *CDSTE3.3* are 536

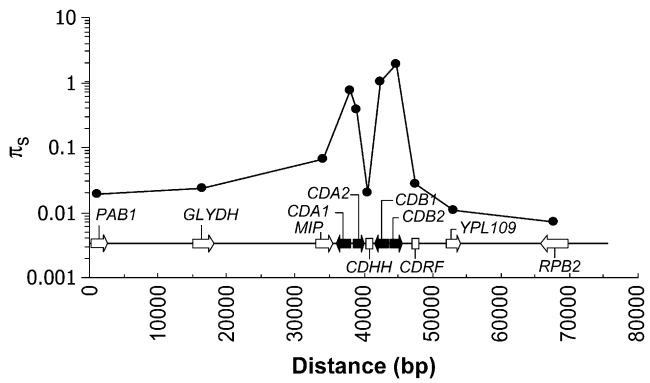

Figure 4.—Plot of silent DNA diversity (π_S) along the *A* mating-type chromosomal region of *C. disseminatus*. The gene map is superimposed below the plot and indicates the position and direction of transcription of the genes in the region. Noncoding loci are shown as open boxes. Data are from the 49 homokaryotic non-Japanese isolates. The values for the non-mating-type genes include intron sequences.

and 426 amino acids in length, both contain the canonical five introns observed in other homobasidiomycete pheromone receptors, and both appear to have seven transmembrane-spanning helices and a long cytoplasmic tail as predicted by the program HMMTOP (Tusnády and Simon 2001). The phylogenetic origin of these receptors is discussed below.

Searches for possible pheromone genes in the *CDSTE3.1* region revealed three genes encoding putative peptide pheromones (*CDPHB1*, *CDPHB2.1*, and *CDPHB2.2*; Figure 5). One pheromone gene is in close proximity to the receptor *CDSTE3.1*, and two are found in the genomic region surrounding *CDSTE3.3*. Only two of the three pheromone genes show a significant match with any published pheromone sequences (supplemental Table 2 at http://www.genetics.org/supplemental/), but all proteins were predicted as probable ORFs or exons using the GeneMark algorithm (Borodovsky and McIninch 1993). Olesnicky *et al.* (1999) suggested that the conserved ER motif located 11 amino acids N-terminal to the modified cysteine of pheromone protein phb2.2 of *C. cinereus* was likely to have a functional role in peptide processing. All three putative *C. disseminatus* pheromones display this pair of amino acids in the positions homologous to the ER motif in *C. cinereus* phb2.2 and other homobasidiomycete pheromones (Fowler *et al.* 2001; Riquelme *et al.* 2005).

The region of the *C. disseminatus* genome containing the two pheromone receptors demonstrates some conserved gene order with the *B* mating-type locus of *C. cinerea* (Figure 2). The gene regions from the two species share four genes (*PERO*, *RAB7*, *CDUP14*, and *CDUP15*) and the pheromone and pheromone receptor genes. However, the amount of gene rearrangement at the *B* mating-type locus is very high compared to the synteny of the *A* mating-type loci of *C. cinerea* and *C. disseminatus* (Figure 2). Genes with known function from the *CDSTE3.1* region include *ERG26*, encoding a putative dehydrogenase involved in ergosterol biosynthesis, *PERO*, encoding a protein with strong similarity to lignin degrading peroxidases, and two *RAB7* genes, encoding putative GTPases involved in vesicle trafficking.

Evolution of the *STE3*-like pheromone receptors: Our analyses of the segregation of the *STE3*-like pheromone receptors demonstrate that these genes are not part of the mating-type locus. A phylogenetic analysis of the basidiomycete pheromone receptor homologs was used to determine if the *STE3*-like genes from *C. disseminatus* actually derive from *B* mating-type receptors of tetrapolar mushrooms. The amino acid sequences of the pheromone receptors of homobasidiomycetes, heterobasidiomycetes, and two Ascomycete outgroups were aligned together with the four *C. disseminatus* putative receptors (see Figure 6 legend for GenBank numbers). Only the STE3 domain (pfam02076) sequence region, containing the seven transmembrane helices, was alignable without ambiguity. The

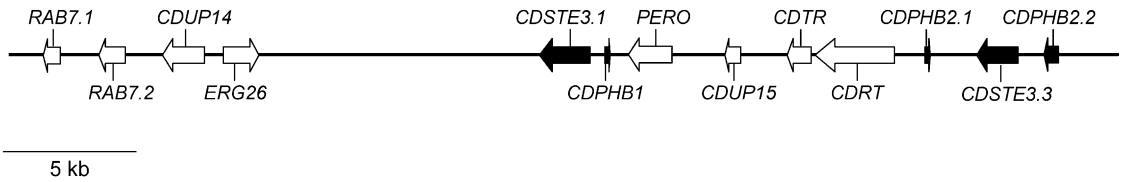

FIGURE 5.—Gene map of the *CDSTE3.1* and *CDSTE3.3* chromosomal region. Arrows indicate the direction of transcription. Genes were identified using BLASTX searches with a cutoff *P*-value of 10^{-4} (supplemental Table 2 at http://www.genetics.org/supplemental/). Genes homologous to other mating-type genes from homobasidiomycetes are shown as solid arrows. Two putative transmembrane pheromone receptors (*CDSTE3.1* and *CDSTE3.3*) and three small peptide pheromones (*CDPHB1*, *CDPHB2.1*, and *CDPHB2.2*) were found. *CDUP14* and *CDUP15* are predicted genes with no clear function.

maximum-likelihood phylogeny estimated using PROTML is shown in Figure 6. The homobasidiomycete receptors comprised two clades, "groups 1 and 2." CDSTE3.1 groups very closely with the three group 1 receptors *S. commun*e BBR2 and *C. cinerea* RCB2.6 and RCB1.3. CDSTE3.2 and CDSTE3.3 are also part of group 1 in a clade with *C. cinerea* RCB3.6 and RCB3.42. Finally, CDSTE3.4 is nested within the receptors of the group 2 clade. These results demonstrate that the pheromone receptors from *C. disseminatus* have specifically diverged from within the family of mating-type-specific pheromone receptors found in other homobasidiomycetes.

The *B* mating-type pheromone receptors of other mushroom species show tremendous DNA and amino acid sequence divergence between alleles (HALSALL *et al.* 2000), presumably due to balancing selection on alleles (MAY *et al.* 1999). Thus, if the pheromone receptors of *C. disseminatus* do not encode for proteins involved in the mating incompatibility response, then the genes should not be very polymorphic in natural populations because they are no longer under strong balancing selection. To test this we obtained partial sequence data for these genes (*CDSTE3.1*–3) and for one putative pheromone gene (*CDPHB1*) from a sample of 9–14 homokaryotic isolates. The results of these analyses are included in Table 4.

The observed DNA diversity (π) at the two tightly linked receptors *CDSTE3.1* and *CDSTE3.3* and at the

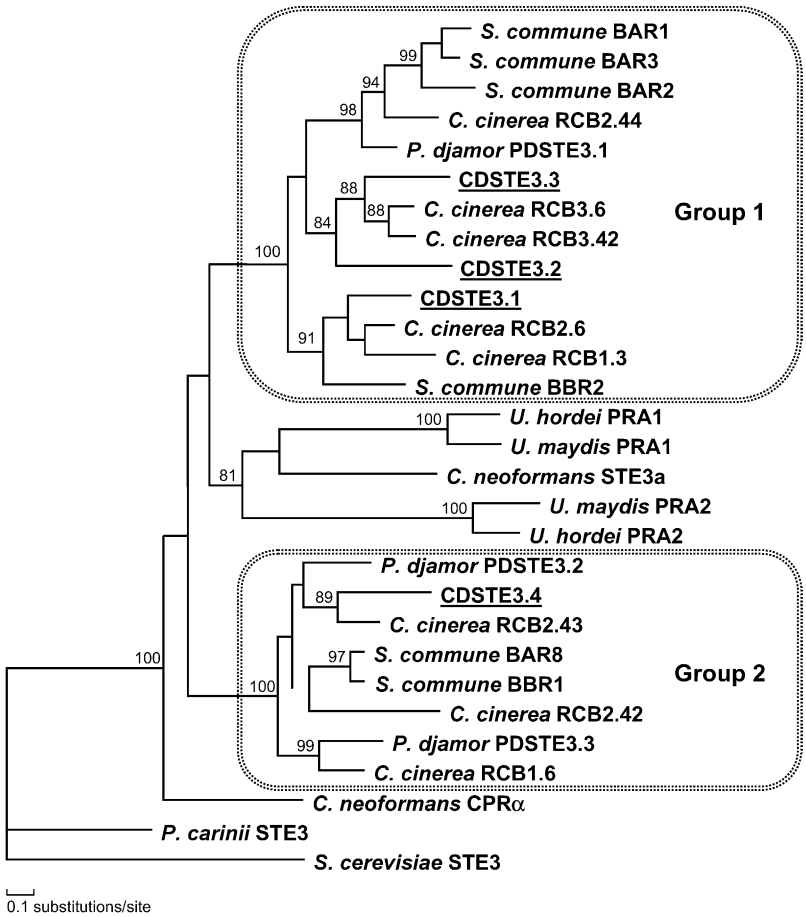

FIGURE 6.—Phylogeny of STE3-like pheromone receptors from basidiomycete fungi. The tree is the maximum-likelihood phylogeny estimated using the program PROTML of the MOLPHY v. 2.3 software package (ADACHI and HASEGAWA 1996). Numbers above nodes indicate bootstrap probabilities estimated using the RELL method (shown only for nodes >80%). Two clades of homobasidiomycete pheromone receptors are observed (groups 1 and 2). The four receptor sequences from *C. disseminatus* are underlined. Other sequences are (with GenBank accession numbers in parentheses): *C. cinerea* RCB1.3 (AAO17255), RCB1.6 (CAA71964), RCB2.6 (CAA71963), RCB2.42 (AAF01419), RCB2.43 (AAQ96345), RCB2.44 (AAQ96344), RCB3.6 (CAA71962), and RCB3.42 (AAF01420); *S. commune* BAR1 (Q92275), BAR2 (CAA62595), BAR3 (P56502), BAR8 (AAR99618), BBR1 (P78741), and BBR2 (AAD35087); *Pleurotus djamor* PDSTE3.1 (AAP57502), PDSTE3.2 (AAP57506), and PDSTE3.3 (AAS46748); *Cryptococcus neoformans* CPRα (AAF71292) and STE3a (AAN75156); *Ustilago maydis* PRA1 (P31302) and PRA2 (P31303); *U. hordei* PRA1 (Q99063) and PRA2 (AAD56044); *Pneumocystis carinii* (AAG38536); and *Saccharomyces cerevisiae* (P06783).

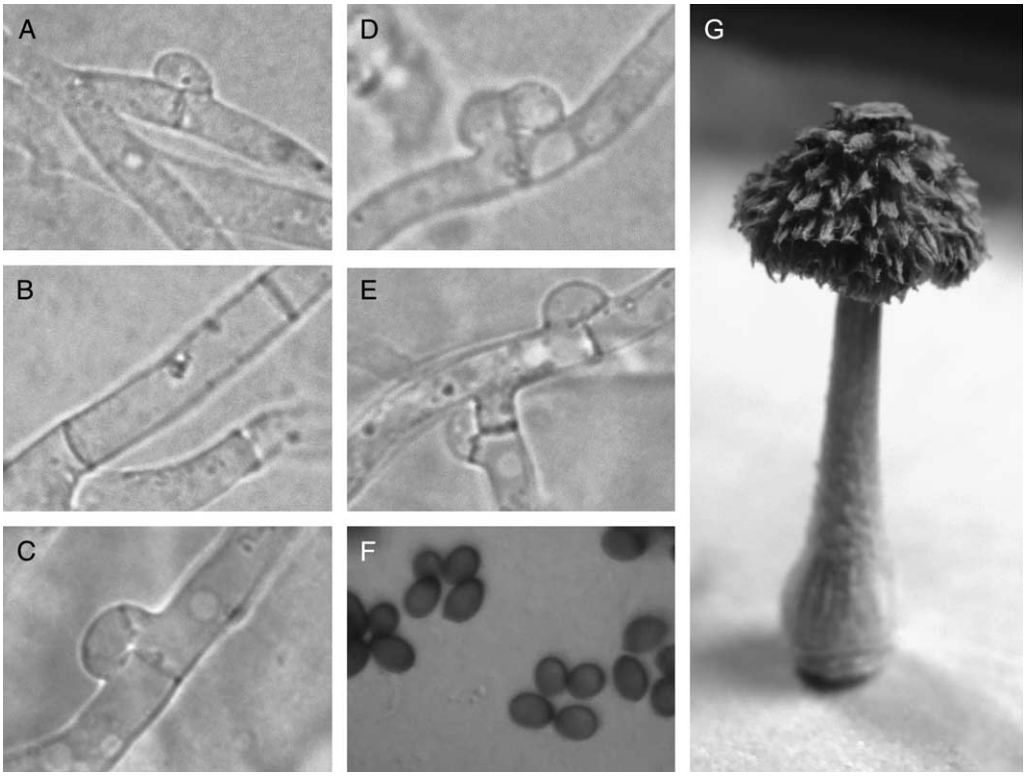

FIGURE 7.—Transformants of *Coprinopsis cinerea A3, B1* monokaryon 218. (A) A transformant of *Coprinellus disseminatus* construct 99.1Aβ with *A* mating-type genes has unfused clamps at hyphal septa. (B) A transformant of C25_e1.10 with *B* homologs from *C. disseminatus* has normal septa. (C and D) Fused clamp cells and apical clamp cells with enlarged subapical pegs have been observed in the mycelium of transformants of construct 99.1Aα (with *A* mating-type genes) and C25_e1.10 (with *B* mating-type genes). Note that all hyphae shown grow from left to right. (E) Upon mating of the clone shown in B to *A42, B1* monokaryon PS004-2, a dikaryon is formed with many fused and unfused clamp cells at hyphal septa. Under fruiting conditions, the dikaryon produces mature basidiospores (F) on the gills of well-developed fruiting bodies (G).

putative pheromone gene *CDPHB1* was similar in comparison with that at the genes near, but not part of, the *A* mating-type locus (Table 4). *CDSTE3.2* was identified as a unique receptor-like gene because it lacks three of the four introns observed in all other homobasidiomycete receptors. Variation at *CDSTE3.2* suggests that it may be a pseudogene because 3 of 11 sequences contained transcripts predicted to be interrupted by stop codons. Furthermore, the amount of DNA diversity (π) at *CDSTE3.2* was higher than average (0.086) for non-mating-type gene regions (Table 4). For two homokaryotic strains, PCR amplification using specific primers for *CDSTE3.2* produced two distinct copies. Separation of the two copies using plasmid subcloning produced two different but closely related sequences. Taken together, the *CDSTE3.2* locus appears to be composed of one or two fast-evolving pheromone receptor genes or pseudogenes.

Function of mating-type gene homologs in *C. cinerea*: When transformed with *A* mating-type genes from its native species, *C. cinerea* monokaryon 218 produces a fluffy mycelium with unfused clamp cells at the hyphal septa. Transformants receiving *B* mating-type genes show retarded colony growth and reduced production of aerial mycelium on complete medium. Transformants receiving both *A* and *B* mating type genes grow fluffy with some septa having fused clamp connections. At other septa, the apical cell displays an unfused clamp while the subapical side produces an enlarged peg (KÜES *et al.* 1998, 2002; BADALYAN *et al.* 2004). Phenotypes of strain 218 transformants receiving *C. disseminatus* constructs 99.1Aα, 16.3Aα, 99.1Aβ, or 98.2Aβ were indistinguishable from phenotypes of transformants with native *A* mating-type genes (Figure 7A). Functional expression of plasmids carrying *C. disseminatus* homeodomain transcription factor genes in host monokaryon 218 was efficient, with transformation rates between 40 and 51%, irrespective of coming from the *A*α or the *A*β sublocus. Transformants of 218 receiving DNA from clones C25 B2.5 and C25_e.10 containing *C. disseminatus* pheromone and pheromone receptor genes had normal septa (Figure 7B) and no special growth phenotype on YMG/T complete medium. When *C. cinerea* monokaryon 218 was cotransformed with the *C. disseminatus A*α and *B* constructs, between 28 and 42% of transformants had fused clamp cells at some septa and apical unfused clamps and subapical pegs at other hyphal septa (Figure 7, C and D).

Transformants of strain 218 containing *C. disseminatus* pheromone receptor genes were crossed with the *C. cinereus B1* mating-type tester strain PS004-2. Nuclear migration was observed in both directions, from and/or into 218 transformants, in 45 and 43% of cotransformants using C25 B2.5 and C25_e.10 DNA, respectively. In most cases, clamp cell production in the formed dikaryons was sparse with the majority of clamps unfused to

the subapical cell (Figure 7E). Native *B* genes have been shown in *C. cinerea* to be active in initiation of fruiting when the *A* mating-type pathway is activated and to be active in fruiting-body maturation at the stage of karyogamy (Kües *et al.* 2002). Dikaryons formed between PS004-2 and 218 transformants with *C. disseminatus B* homologs (two from C25 B2.5 transformants and five from C25_e.10 transformants) with many clamp cells (fused and unfused) initiated fruiting up to completion of fruiting-body development and basidiospore maturation (Figure 7, F and G).

DISCUSSION

We have investigated the genetics behind the bipolar mating system of the common mushroom *C. disseminatus*. Using mating tests, the segregation of molecular markers, and DNA sequencing of large genomic regions, our data suggest that the mating-type locus is composed of two pairs of homeodomain transcription factors (Figure 1). Thus, of the two traditional mating-type loci of homobasidiomycetes, the *A* mating type (homeodomain transcription factor genes) and the *B* mating type (pheromones/pheromone receptors), only one of these (*A*) actually functions in determining mating type. Nonetheless, we have discovered at least four pheromone receptor genes homologous to the *B* mating-type genes of other mushrooms using PCR with degenerate primers (Figure 6). None of the receptors are mating-type specific and none of them show the population genetic signature of balancing selection (*i.e.*, elevated nucleotide polymorphism, Table 4). Furthermore, the heterologous expression of at least one pheromone receptor/pheromone complex in *C. cinerea* suggests that the *B* mating-type homologs of *C. disseminatus* do still function in a manner similar to those of tetrapolar homobasidiomycetes; *i.e.*, they are likely to be involved in controlling clamp cell fusion, subapical peg formation, and nuclear migration.

The switch to a bipolar mating system: Of the three hypotheses put forward by Raper (1966) for the origin of the bipolar mating system in homobasidiomycetes from the tetrapolar system, the hypothesis concerning a chromosomal translocation placing the *A* and *B* mating-type genes in close physical association can be ruled out for *C. disseminatus*. This evidence comes from our analyses that show that homeodomain transcription factor genes cosegregate with mating type and display a level of DNA polymorphism characteristic of other mushroom mating-type genes. In contrast, no such patterns were observed among the pheromone receptor genes found in the *C. disseminatus* genome. These results contrast with the findings in the heterobasidiomycete yeast *U. hordei* (Bakkeren and Kronstad 1994) in which the single mating-type locus is composed of a pheromone gene, a pheromone receptor gene, and a pair of homeodomain genes embedded into a nonrecombining chromosomal segment. Thus, a translocation of the *A* mating-type locus into the *B* mating-type region (or vice versa) was suggested by these data. A similar event must have occurred in the ancestor of the heterobasidiomycete yeast *Cryptococcus neoformans* in which the mating-type locus is a nonrecombining gene-dense region containing many genes important in mating and pathogenesis, including a few pheromone genes, a pheromone receptor gene, and a homeodomain transcription factor gene (Hull *et al.* 2002, 2005; Lengeler *et al.* 2002).

Another hypothesis put forward by Raper (1966) for the origin of bipolar mating systems was that one of the two mating-type loci of a tetrapolar ancestor could mutate to become self-compatible, thus rendering its allelic state meaningless in crosses. The data observed for *C. disseminatus* are generally consistent with this hypothesis. We found the *C. disseminatus* homologs of the *B* mating-type genes of *C. cinerea* through degenerate PCR and cosmid sequencing. Although the identified pheromone receptors appear to be fully functional on the basis of *in silico* predictions, they do not show the characteristic hyperpolymorphism associated with mushroom mating-type genes that are under very strong balancing selection (Table 4).

Raper's final hypothesis that the function of one of the mating types could be gradually assumed by the other mating type is not consistent with our data. Using heterologous expression in *C. cinerea*, we were able to demonstrate that the *A* and *B* homologs of *C. disseminatus* have very similar cellular phenotypes when transformed into *C. cinerea* as do the respective native *C. cinerea* genes (Figure 7), suggesting that genetic control of the *A* and *B* pathways has been maintained separately in *C. disseminatus*. At least one of the pheromone receptors must be involved in the same G-protein-coupled *B* locus pathway as in other tetrapolar homobasidiomycetes because they are able to drive clamp-cell fusion and even fruit body development and sporulation (Figure 7). It remains to be tested whether the receptors or the pheromone genes are constitutively activating or self-compatible mutants or whether they can interact with the native *C. cinerea B* locus proteins. One line of evidence does suggest that CDSTE3.1 may actually be a self-compatible receptor allele. This protein contains a substitution in the third cytoplasmic loop at position 196 of a phenylalanine for a residue that is invariantly aliphatic (valine, isoleucine, or leucine) in all described *C. cinerea*, *S. commune*, and Ustilago spp. STE3 pheromone receptor homologs (data not shown). Moreover, the substitution of glutamine for leucine in precisely the homologous position in *S. cerevisiae* STE3 causes a partially constitutive and hypersensitive **a**-factor receptor (Boone *et al.* 1993).

The mating-type loci of *C. disseminatus* and those of the bipolar mushroom *P. nameko* Aimi *et al.* (2005) are similar in that both species appear to utilize homeodomain

proteins rather than pheromone receptors to determine mating type. The fact that these evolutionary independent lineages may have taken the same course in evolving a bipolar mating system from a tetrapolar one could suggest that loss of *B* mating-type function is easier than loss of *A* mating-type function. Such a process could occur through differences in rates of mutation to self-fertility of homeodomain proteins *vs.* pheromone receptors.

The structure and evolution of the mating-type locus: *C. disseminatus* is the first bipolar mushroom species reported to have a mating-type locus composed of more than a single subunit. We have termed the two separate subloci *A*α and *A*β to facilitate comparison with the traditionally defined mating-type subloci of *C. cinerea* that they closely resemble. Both the *A*α and *A*β subloci encode a pair of divergently transcribed homeodomain genes (Figure 1); the combination of alleles at the two subloci presumably determines the mating-type specificity of an individual. The total number of mating types in the species was estimated to be ~123, using a complete crossing experiment of 49 homokaryotic isolates. This value is rather similar to that observed at the *A* mating type of *C. cinerea*, where 120–164 mating types are estimated (Raper 1966; May and Matzke 1995). We have observed seven *A*α and eight *A*β alleles in *C. disseminatus* on the basis of RFLP patterns with *Msp*I, in small samples of 13 and 16 isolates, respectively (see Figure 3 for a representative gel). These molecular phenotypes suggest a symmetric allele number between subloci. Transformation data using *C. cinerea* as a host show that genes from both subloci initiate clamp cell formation, consistent with the hypothesis that the subloci are functionally redundant and independently contribute to mating-type specificity.

Although we isolated four pheromone receptors from one haploid strain of *C. disseminatus* and have demonstrated that they are not part of the mating-type locus, additional copies of the *STE3*-like pheromone receptors may still exist that were not detected by our PCR-based methods. If such additional receptors exist, then they are not likely to be part of the mating-type locus. Balancing selection can elevate the polymorphism of neighboring genomic regions, but the increase in diversity is a negative function of the recombination distance between the region and the actual target of selection (Hudson and Kaplan 1988). For the genomic region surrounding the *C. disseminatus A* mating-type locus (*i.e.*, *MIP*, *CDHH*, and *CDRF* loci), DNA polymorphism (π) is >0.02 (Table 4), but at distances >10 kb from the mating-type locus, it appears that sites experience little, if any, elevated DNA polymorphism by linkage to the mating-type locus (Figure 4). Such a contrast in nucleotide diversity between the mating-type locus and the regions bordering it likely reflects recombination that separates those sites subject to strong balancing selection from sites that evolve in a more neutral manner.

An indispensable role for pheromone receptors in homobasidiomycetes: Four lines of evidence point to a clear origin of *CDSTE3.1* and *CDSTE3.3* receptors from other homobasidiomycete mating-type-specific pheromone receptors. One line of evidence is that the chromosomal region containing *CDSTE3.1* and *CDSTE3.3* displays some conserved gene order when compared to the *B* mating-type locus chromosomal region of *C. cinerea* (Figure 2). Second, phylogenetic analyses place these proteins among other mating-type proteins of the model mushroom species (Figure 6). Third, the genes exert *B* mating-type-typical function in a heterologous species (Figure 7). Finally, the receptors appear to be each in close physical proximity with one or two putative peptide pheromones (Figure 5).

If the genome of *C. disseminatus* contains both active pheromone and receptor genes that are not polymorphic, what cellular function do they perform? As mentioned previously, it is possible that they encode proteins locked into a self-compatible complex, turning on the *B*-specific developmental pathway through a MAPK cascade, much as their ancestors did for millions of years of fungal evolution. However, it seems quite unnecessary to maintain a functional pheromone/receptor system to turn on a signaling cascade that could be readily turned on by constitutive activation of its downstream partner; *e.g.*, mutations in *GPA1* can constitutively activate the pheromone response system in yeast (Banuett 1998) and mutations in *pcc1* can constitutively activate false-clamp cell production and fruit body development in *C. cinerea* (Murata *et al.* 1998). That *C. disseminatus* has maintained an apparently functional pheromone receptor system suggests that the role of the *B* mating-type receptors is more complex than activating only the MAPK pathway through G-proteins, that the G-proteins interact with more than the pheromone receptors, or that the receptors of *C. disseminatus* have taken on a new functional role.

The homobasidiomycete fungi are unique in that they are the only fungal clade to have evolved a multiallelic pheromone/receptor mechanism to perform incompatibility discrimination between individuals—all other fungi have only a biallelic system. The homobasidiomycete pheromone/receptor systems are also possibly unique because these proteins have not been detected extracellularly (Brown and Casselton 2001). It has been suggested that the pheromone/receptor system of mushroom fungi functions in the recognition between the two compatible nuclei of the dikaryotic cell (Schuurs *et al.* 1998; Debuchy 1999). This function may be indispensable and an intact pheromone/receptor system might be required for proper dikaryon maintenance in all species, bipolar or tetrapolar.

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