

# Three Subfamilies of Pheromone and Receptor Genes Generate Multiple *B* Mating Specificities in the Mushroom *Coprinus cinereus*

John R. Halsall, Michael J. Milner and Lorna A. Casselton

Department of Plant Sciences, University of Oxford, Oxford OX1 3RB, United Kingdom

Manuscript received September 20, 1999

Accepted for publication November 15, 1999

## ABSTRACT

The *B* mating type locus of the basidiomycete *Coprinus cinereus* encodes a large family of lipopeptide pheromones and their seven transmembrane domain receptors. Here we show that the *B42* locus, like the previously described *B6* locus, derives its unique specificity from nine multiallelic genes that are organized into three subgroups each comprising a receptor and two pheromone genes. We show that the three genes within each group are kept together as a functional unit by being embedded in an allele-specific DNA sequence. Using a combination of sequence analysis, Southern blotting, and DNA-mediated transformation with cloned genes, we demonstrate that different *B* loci may share alleles of one or two groups of genes. This is consistent with the prediction that the three subgroups of genes are functionally redundant and that it is the different combinations of their alleles that generate the multiple *B* mating specificities found in nature. The *B42* locus was found to contain an additional gene, *mfs1*, that encodes a putative multidrug transporter belonging to the major facilitator family. In strains with other *B* mating specificities, this gene, whose functional significance was not established, lies in a region of shared homology flanking the *B* locus.

**T**WO sets of multiallelic mating type genes, *A* and *B*, determine mating compatibility in the homobasidiomycete fungus *Coprinus cinereus*. A successful mating occurs only between cells that have different alleles of genes at both loci, converting an asexual mycelium known as the monokaryon into a sexually fertile dikaryon. The dikaryon is a prolonged mycelial stage in which the nuclei from each mating partner do not fuse but remain together in each cell. Both sets of mating type genes play a role in the developmental sequence that leads to the formation of the dikaryon and to the subsequent maintenance of the binucleate cells that characterize it (see Casselton and Olesnicky 1998).

The *A* mating type genes of homobasidiomycetes encode two dissimilar proteins belonging to the homeodomain family (Kües and Casselton 1992; Kües *et al.* 1994; Stankis *et al.* 1992). A compatible mating brings together different versions of these proteins that heterodimerize to generate the active transcription factor complex that initiates *A*-regulated steps in dikaryon development (Banham *et al.* 1995). The *B* genes encode peptide pheromones and their cognate seven-transmembrane domain receptors (Wendland *et al.* 1995; Vaillancourt *et al.* 1997; O'Shea *et al.* 1998). Pheromone signaling apparently plays no role in mate attraction in *C. cinereus* as it does in yeasts (Kurjan 1993) and hemibasidiomycete species (Bölker *et al.* 1992; Spellig *et al.*

1994), but it is evident from studies on *B*-regulated development that it acts after cell fusion to promote the formation and maintenance of the dikaryon.

Following cell fusion in *C. cinereus*, a compatible complement of *B* genes promotes an exchange of nuclei and the migration of donor nuclei through the established cells of each recipient monokaryon. Once the tip cells contain nuclei from both mates, a compatible complement of *A* genes leads to nuclear pairing, the synchronization of nuclear division, and the initiation of a complex tip cell division that involves the formation of a structure known as the clamp connection. Following nuclear division, one daughter nucleus becomes cut off in the developing clamp cell and the other in a newly formed subterminal cell. Compatible *B* genes allow the completion of the clamp connection by promoting fusion between the clamp cell and the subterminal cell.

A remarkable feature of the *A* and the *B* loci is that they both contain several multiallelic genes. The *A* locus of *C. cinereus* contains representatives of three paralogous pairs of genes (Pardo *et al.* 1996) that encode the two classes of *A* proteins. Different allele combinations of the paired *A* genes occur in nature (Lukens *et al.* 1996; Pardo *et al.* 1996) and generate an estimated 160 *A* mating specificities (Raper 1966). Our analysis of a *B* locus suggests that it also derives its many specificities, an estimated 79, from three sets of paralogous genes. In the *B6* locus we identified nine genes arranged into three groups, each encoding a receptor and two pheromones (O'Shea *et al.* 1998). The *B6* and *B3* loci were found to have different alleles of all nine genes, but *B6*

Corresponding author: L. A. Casselton, Department of Plant Sciences, University of Oxford, Oxford OX1 3RB, United Kingdom.  
E-mail: lorna.casselton@plants.ox.ac.uk

and *B42* were predicted to share the alleles of one group of genes on the basis of DNA homology and failure of this group of *B6* genes to activate *B*-regulated development in *B42* hosts. In this report we describe the sequence of the *B42* locus, confirm our prediction of shared genes in *B6* and *B42*, and provide more extensive evidence to support our model for *B* locus organization in *C. cinereus*. We demonstrate that the cloned genes from each group are found together as a functional unit in different *B* loci and that compatible *B* mating specificities require that only one group of genes have different alleles.

## MATERIALS AND METHODS

**Fungal strains and growth conditions:** *C. cinereus* strains used in this study were: *A42B42*: JV6 wild type and LN118 *trp-1.1, 1.6 ade-2*; *A6B42*: PG78 *pab-1 trp-1.1, 1.6*; *A6B6*: H9 wild type and LT2 *trp-1.1, 1.6*; *A5B6*: FA2222 *trp-1.1, 1.6*; *A2B3*: LCO12 *trp-3*; *A6B3*: PR94226 *ade-5 cho-1*; *A43B43*: AT8 *ade-8 trp-3*; *A42B43*: JV56 wild type; *A5B5*: HT8 *trp-1.2*; *A6B5*: TC10 wild type; *A3B1*: 218 *trp-1.1, 1.6*; *A2B1*: 68 wild type; *A40B40*: LCO7 *trp-1.1, 1.6*; *A42B40*: LCO7R wild type; *A41B41*: LCO5 *trp-1.1, 1.6*; *A42B41*: LCO5R wild type; *A44B44*: NL1 *trp-1.1, 1.6*; *A42B44*: NL1R wild type. Media and methods for culturing *C. cinereus* were described by Lewis (1961), with modifications summarized by Mutasa *et al.* (1990). Hosts for transformation were LT2, LCO12, and LN118. *B6* genes were introduced by cotransformation with plasmids pCc1001 or pDB1 containing the *C. cinereus trp-1* (Binniger *et al.* 1987) or *trp-3* gene (Burrows 1991), respectively. Transformation was performed as described by Casselton and de la Fuente Herce (1989). Routinely, 50 transformants were tested for the expression of an introduced *B* gene. *B* gene expression was assayed by the method of O'Shea *et al.* (1998), which involved mating transformants to a tester strain having a different *A* mating specificity from the host but the same *B* mating specificity. If the introduced gene activated the pheromone response pathway in the host, this led to a compatible mating interaction and formation of a dikaryon. Frequency of cotransformation was variable, but a positive mating reaction was always detected in 12–50% of transformants.

**DNA and RNA procedures:** For Southern analyses, DNA was isolated by the small-scale method of Zolan and Pukkila (1986) and the analysis was carried out as described by Melton *et al.* (1987). DNA sequencing was carried out on an ABI 373 (Perkin-Elmer, Norwalk, CT) automated sequencer. Sequence analysis was performed as described by O'Shea *et al.* (1998). RNA was isolated by the method of Hoge *et al.* (1982). Poly (A)<sup>+</sup> RNA was purified from total RNA by oligo(dT) cellulose affinity chromatography (Edmonds *et al.* 1971; Aviv and Leder 1972) or by the PolyATtract mRNA Isolation System (Promega, Madison, WI) following the manufacturer's instructions.

**Plasmids and PCR strategies:** The *B42* locus was isolated from a cosmid genomic library constructed from JV6 wild-type DNA cloned into the cosmid vector LORIST 2 (Mutasa *et al.* 1990). Routine cloning was in pBluescript II (Stratagene, La Jolla, CA) and plasmid amplification was in *Escherichia coli* strain XL1Blue or DH5 $\alpha$ . PCR products were purified by agarose gel electrophoresis and were cloned into pGEM-T and pGEM-T Easy (Promega). Plasmids containing *B6* genes were described by O'Shea *et al.* (1998). Plasmids containing all but one of the *B42* genes were obtained by cloning the following restriction fragments: pJH3, on a 1.7-kb *HindIII* (*phb1.1*<sup>42</sup>);

pJH38, 0.9-kb *EcoRI* (*phb1.2*<sup>42</sup>); pJH31, 2.2-kb *PstI-SalI* (*phb2.1*<sup>42</sup>); pJH25, 1.8-kb *HindIII-EcoRI* (*phb2.2*<sup>42</sup>); pJH30, 4.0-kb *PstI-SalI* (*rcb2*<sup>42</sup>); pJH26, 1.0-kb *HindIII-PstI* (*phb3.1*<sup>42</sup>); pJH10, 1.4-kb *HindIII-EcoRI* (*phb3.2*<sup>42</sup>), and pJH33, 2.1-kb *EcoRI* (*rcb3*<sup>42</sup>). *rcb1*<sup>42</sup> was amplified by the polymerase chain reaction (PCR) using the primers 5'-CCCCGACGGCCTTG TACTGTAGC-3' and 5'-GGTCCGGGAGCAGAGCGAG-3'. The group 3 and group 1 genes from *B3* were amplified from genomic DNA of strain LCO12 using the GeneAmp XL-PCR Kit (Perkin-Elmer) and the primers 5'-GGCAGGTCTAAAGGT AGCCACG-3' and 5'-CCACAAGGGCAGTGACTTGCCTTC-3' for group 3, and 5'-ATCGTGGGAAACGCAGATGGCG-3' and 5'-CGTTCTGTTCGCCAGTTGTGAC-3' for group 1. The initial PCR fragment containing the *B3* group 3 genes also contained *phb2.2*<sup>42</sup>. A fragment containing the group 3 genes only was amplified using the following primers: 5'-GGTTG TCACAGACAGAGAAGG-3' and 5'-GCCTCAAAGCTTGAC GAGTCCG-3'. cDNAs for the *rcb2* and *rcb3* receptor genes and the *mfs1.1* gene were obtained by RT-PCR using the Access RT-PCR Kit (Promega) and the following primers: 5'-TGC GCTTTCCTCGCTGCCATCCTA-3' and 5'-GACGTTACTCG TGAATGCACTCG-3' for *rcb2*, 5'-TCCTCGTCACGAAAGCTG TCCTTC-3' and 5'-GACTTTTGTAGTGTGCGTTAGAGTCCG-3' for *rcb3*, and 5'-CGTTCTGACGTTCTTGGTATGTC-3' and 5'-AGATGAAGAACATGGAGTGATCAGG-3' for *mfs1.1*. RNA was extracted from strain LN118 in which receptor gene transcription was induced by transformation with a plasmid containing genes from the *B6* locus (Halsall 1997).

**Sequence data accession numbers:** GenBank accession numbers are as follows: AF186386, *phb1.1*<sup>42</sup>; AF186387, *phb1.2*<sup>42</sup>; AF186390, *phb2.1*<sup>42</sup>; AF136522, *phb2.2*<sup>42</sup>; AF186388, *phb3.1*<sup>42</sup>; AF186390, *phb3.2*<sup>42</sup>; AF186383, *rcb1*<sup>42</sup>; AF186384, *rcb2*<sup>42</sup>; AF186385, *rcb3*<sup>42</sup>; AF186391, *mfs1.1*; and AF186392, *mfs1.2*.

## RESULTS

**Isolation and sequence analysis of the *B42* genes:** A cosmid genomic library constructed from the *B42* wild-type strain JV6 was screened for clones containing sequences from *B42* using a homologous flanking sequence from the *B6* locus as probe. We used Southern blot analysis to show that several of the clones identified contained DNA sequence that was not present in the genomic DNA of the *B6* strain. Since different alleles of the *B* genes do not cross-hybridize (O'Shea *et al.* 1998), *B42*-specific sequences would be expected to contain *B* alleles not present in *B6*. *B42*-specific sequences adjacent to the common flanking sequence were used to isolate other cosmid clones and to initiate a walk that spanned 60 kb of contiguous DNA sequence, sufficient to contain the entire *B42* locus.

Four cosmids, cJH4, cJH5, cJH8, and cJH10, which together covered the entire 60-kb sequence (Figure 1), were tested for *B* gene function by transformation into two host strains, one having a *B6* mating specificity and one a *B3* mating specificity. Monokaryons expressing compatible *B* genes in *C. cinereus* have no easily recognized phenotype and *B* gene function was assayed by a mating test described by O'Shea *et al.* (1998) and detailed in materials and methods. cJH5 and cJH8 contained genes that altered the *B* mating specificity of both host strains tested, whereas cJH4 contained se-

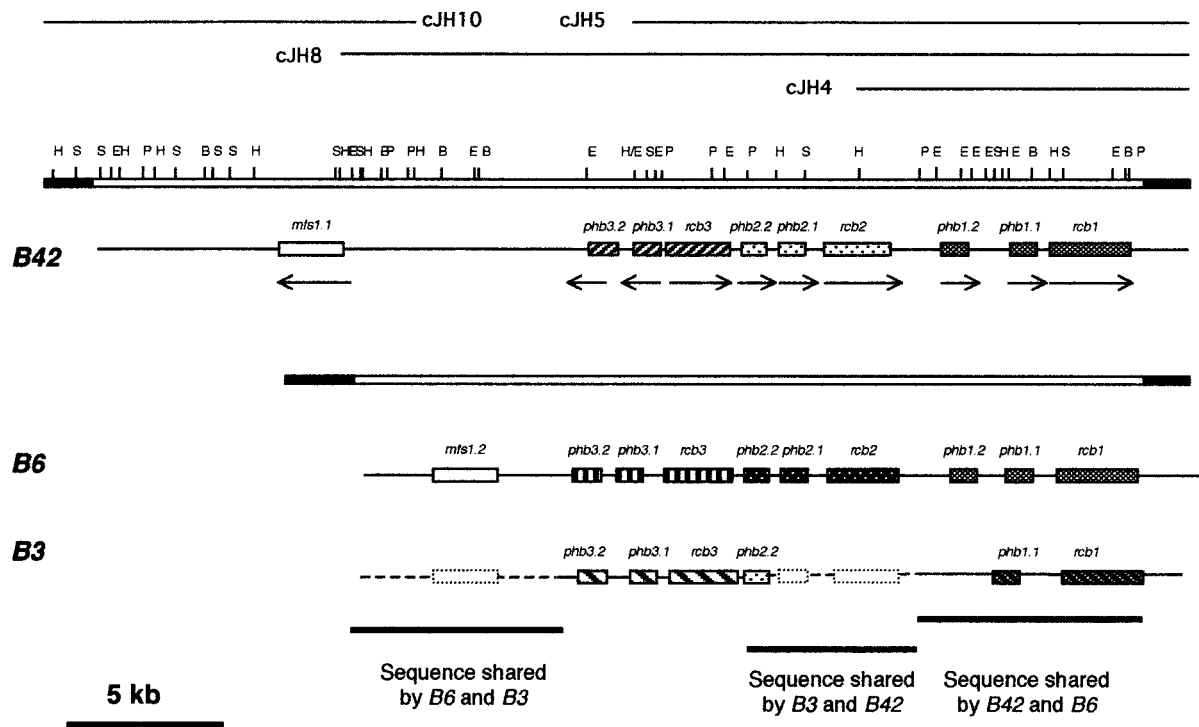


Figure 1.—The genomic DNA sequence of the *B42* locus is covered by the four overlapping cosmids, cJH4, cJH5, cJH8, and cJH10. The physical map was generated using the restriction enzymes *Bam*HI (B), *Eco*RI (E), *Hind*III (H), *Pst*I (P), and *Sal*I (S). The 10 genes identified in the sequence are illustrated as boxes below the map, with arrows indicating the direction of transcription. The three groups of genes determining *B* specificity are differently shaded, and each encodes two pheromone precursors and a pheromone receptor. The relative positions of these 9 genes within the previously characterized *B6* locus and 6 genes in the partially characterized *B3* locus are also illustrated. Dashed boxes and lines indicate genes and sequences detected by hybridization only. Solid boxes represent the homologous sequences flanking the *B* loci. The *mls1* gene encodes a putative multidrug transporter.

quences that only altered *B* mating specificity in the *B3* host. This result would be consistent with cJH4 having genes that we showed previously to be the same in *B42* and *B6* and cJH5 and cJH8 having additional genes whose alleles are unique to *B42*. The sequence present in cJH10, though largely *B42* specific, failed to alter the *B* mating activity of either host.

To identify the sequence corresponding to the *B42* locus we used cloned DNAs to compare the genomic sequences in *B42*, *B6*, and *B3* strains (data not shown). The homologous borders of the *B42* locus were found to flank a 29-kb sequence and a physical map of this is presented in Figure 1. Hybridization analyses identified a 7-kb sequence adjacent to the right border of the *B6* locus that was present in *B6* and *B42* but not *B3*, a 6-kb sequence that was present in *B42* and *B3* but not *B6*, and a 5-kb sequence at the left side of the *B6* locus that was different in all three loci. In addition, the *B42* locus was found to contain 10 kb of sequence that did not cross-hybridize to *B6* or *B3* genomic DNA and did not contain any genes that altered *B* mating specificity in transformation tests.

We predicted previously that the nine genes identified in the *B6* locus belong to three functionally independent or paralogous groups, each comprising a recep-

tor and two pheromone genes (O'Shea *et al.* 1998). The cross-hybridization observed in our Southern analyses is consistent with this interpretation and indicates that *B6* and *B42* share alleles of the three group 1 genes, *B3* and *B42* share alleles of the three group 2 genes, and all three specificities have different alleles of the three group 3 genes. According to this interpretation, we started our sequence analysis of *B42* at the right of the physical map illustrated (Figure 1) where the common *B6* and *B42* genes would be found.

We showed that the *B42* specificity is determined by nine genes, six encoding pheromone precursors, and three encoding pheromone receptors within an 18-kb nucleotide sequence. The genes were organized, like those in the *B6* locus, into three groups each comprising a receptor and two pheromone genes. Significantly, the genes predicted to constitute group 1 were located in homologous sequence shared with *B6*, the genes predicted to constitute group 2 were located in the region of homology with *B3*, and the third group of genes was located in the adjacent 5-kb sequence, which is nonhomologous in all three loci. A comparison of the *B6*, *B42*, and *B3* loci and the relative positions of the genes within each are provided in Figure 1.

The DNA sequences of the three group 1 genes from



TABLE 1  
Comparison, presented as percentage identity/similarity, of predicted *C. cinereus* receptor proteins and those of another hymenomycete, *S. commune*

Receptor	Rcb2 <sup>42</sup>	Rcb3 <sup>42</sup>	Rcb1 <sup>6</sup>	Rcb2 <sup>6</sup>	Rcb3 <sup>6</sup>	Bar1	Bbr1
Rcb1 <sup>42</sup>	53/72	38/64	99/99	37/61	38/64	35/64	49/68
Rcb2 <sup>42</sup>	—	35/64	53/73	37/59	35/61	37/65	46/67
Rcb3 <sup>42</sup>	—	—	38/65	52/73	78/92	49/70	32/58
Rcb1 <sup>6</sup>	—	—	—	36/62	38/65	—	—
Rcb2 <sup>6</sup>	—	—	—	—	52/72	52/72	34/57
Rcb3 <sup>6</sup>	—	—	—	—	—	47/59	34/46

The sequences were aligned with each other using GAP (GCG, version 8.1). Rcb1<sup>42</sup>, Rcb2<sup>42</sup> and Rcb3<sup>42</sup> proteins encoded by *B42* genes; Rcb1<sup>6</sup>, Rcb2<sup>6</sup>, and Rcb3<sup>6</sup> proteins encoded by *B6* genes; Bar1 and Bbr1 proteins encoded in the *Bα1* and *Bβ1* loci, respectively, of *S. commune*.

the *B6* and *B42* loci were found to be >97% identical, indicating that these genes are homoallelic and confer the same mating specificity. All three genes were found to be embedded in a highly conserved sequence present in *B6* and *B42* extending over the 7 kb of homology detected by hybridization analyses. The three genes in group 2 were located in the 6-kb sequence shown to be homologous in *B3* and *B42* but not in *B6*. Comparison of the group 2 genes of *B42* and *B6* showed less conservation in sequence than the group 1 genes, thus indicating allelic differences between the group 2 genes of these specificities. The DNA sequence surrounding the group 2 genes was even less conserved between *B6* and *B42* than that of the genes themselves. This lack of conservation in sequence would act to prevent recombination and would ensure that the three group 2 genes remain together as a functional unit. The group 3 genes and the sequences surrounding them were similarly unconserved between *B6* and *B42*, indicating that these two loci also have different alleles of these genes.

The *C. cinereus* pheromone receptors are predicted to have seven-transmembrane domains and to show homology to the Ste3p  $\alpha$ -factor receptor of *Saccharomyces cerevisiae* and the Pra1 and Pra2 receptors encoded in the *a* mating type locus of the hemibasidiomycete *Ustilago maydis* (Bölker *et al.* 1992; O'Shea *et al.* 1998). A comparison of the proteins encoded by the *B42* and *B6* genes is summarized in Table 1. Overall, the three *B42* receptors have only 35–53% sequence identity and show as little similarity to each other as they do to corresponding receptor proteins encoded by the *B* mating type genes of another homobasidiomycete species, *Schizophyllum commune* (Table 1). Comparisons with the *C. cinereus* *B6* proteins showed that the homoallelic Rcb1<sup>42</sup> and Rcb1<sup>6</sup> receptors were 99% identical. Heteroallelic receptors showed less conservation in sequence, but this is clearly variable with Rcb2<sup>6</sup> and Rcb2<sup>42</sup> displaying only 37% identity but Rcb3<sup>42</sup> and Rcb3<sup>6</sup> showing as much as 78% identity.

The pheromones of *C. cinereus* belong to the family characterized by  $\alpha$ -factor of *S. cerevisiae* (reviewed by

Bölker and Kahmann 1993; Vail l ancourt and Raper 1996). The predicted sequences of the *B42* pheromone precursors are presented in Table 2. Mature peptide pheromones from other basidiomycete species and from yeasts range in size from 9 to 15 amino acids. All the pheromone precursors encoded by the *B42* genes have the C-terminal CaaX motif (CIVA, CVVS, CIIA) that signals C-terminal truncation and carboxymethylation and farnesylation of the cysteine residue. In five of the sequences, there is a conserved amino acid pair, ER, 12–14 amino acids N-terminal to the cysteine residue of the CaaX motif which we predicted to be an internal processing site (Casselton and Olesnicky 1998). Recently we have provided direct evidence to support this prediction by demonstrating that a synthetic pheromone based on the Phb2.2<sup>42</sup> sequence can activate a compatible *C. cinereus* receptor expressed heterologously in *S. cerevisiae* (Olesnicky *et al.* 1999). A possible alternative motif is a DQ found in the Phb3.1 precursor. Based on this prediction, the group 1 and group 2 genes from *B42* encode mature peptides of 12 amino acids, and the members of each pair differ in only 3 amino acids. In contrast, the two group 3 pheromones show less sequence similarity and one is predicted to have 12 amino acids and the other 13.

**Different *B* loci derive their specificities from multiple alleles of three groups of genes:** Using a combination of Southern blot analyses and transformation with cloned genes, we set out to identify whether other *B* loci in our collection shared alleles of genes that we had identified in *B42* and *B6*. To increase the scope of this analysis, we exploited the fact that *B42* and *B3* contain the same alleles of the group 2 genes to isolate fragments containing the group 1 and group 3 genes from *B3* by PCR. Two pairs of PCR primers were designed to amplify the *B3* genes. One primer was designed to anneal to the shared sequence to the left of the *B6* and *B3* loci and another to the *phb2.2*<sup>42</sup> allele shared by *B42* and *B3* (Figure 1). These primers amplified an 8.5-kb sequence predicted to contain the *B3* group 3 genes and preliminary sequence data have con-

TABLE 2  
Pheromone precursors encoded by the *B42* genes

Gene	Amino acid sequence of precursor <sup>a</sup>
<i>phb1.1</i>	MDSFDSLDSLNLVSVEETTLQTLLESMDTTDAASESELDAILVNS <b>ERDPGFTSKGFCVIA</b>
<i>phb1.2</i>	MDSFQQLNLFVEETIQQLSPEAIPSSDSTDGTASERDATPVNT <b>ERHLGFTTKGFVCVVS</b>
<i>phb2.1</i>	MDSFSTLSLPATLGNEEAQQTTVIATVAQPQESPSSSGTPVDS <b>ERPAGKVR AFCIIA</b>
<i>phb2.2</i>	MDNFTVDLATLFEFPELQEIQATASEHCSQDQYGSCEGPPIN <b>QERP GSGV NRAF CVIA</b>
<i>phb3.1</i>	MDSGNTVDLAE L C D M D P N I G F T P D S S A P T E D N V A K Q L V D S D <b>Q R L P G G Y Y G G Q C I I A</b>
<i>phb3.2</i>	MSDAFTTLDTVDLFIEENEQEVVEVPSCPPRRRPSFSSADAESIFLTVEEVNDLPVDY <b>ERRTQGGGGLTWFCVIA</b>

<sup>a</sup> The predicted N-terminal processing site is underlined, and the predicted sequence of the mature pheromone is given in boldface type.

firming that these include a receptor gene and two pheromone genes. Another primer was designed to anneal to the homologous sequence flanking the border to the right of the *B* locus in all strains and another to the *rch2*<sup>42</sup> allele shared by *B42* and *B3*. These primers amplified a 9-kb sequence predicted to contain the *B3* group 1 genes. Sequence analysis has identified only two genes, one encoding a receptor and one a pheromone (Figure 1).

Genomic DNAs from strains having *B1*, *B3*, *B5*, *B6*, *B40*, *B41*, *B42*, *B43*, and *B44* mating specificities were digested with *Hind*III and following Southern blotting, were probed with each of the nine individual genes cloned from *B42* and *B6*. Probes to the group 1 and group 3 *B3* genes were excised from the sequences generated by PCR. The results of these hybridization analyses together with transformation tests using the same cloned genes are summarized in Tables 3 and 4. Where we detected hybridization in Southern blots, all three genes within a particular group cross-hybridized and none was able to activate *B*-regulated development in the host to which hybridization occurred. We conclude that hybridization identifies homoalleles. Where we detected no cross-hybridization, generally all three genes from that group activated *B*-regulated development in the host tested. The only exceptions to this rule involved heteroallelic pheromone genes that could activate receptors found in some specificities but not others (Table 4).

Our previous transformation analysis of *B6* showed that a single compatible pheromone or receptor gene is sufficient to activate the *B* pathway in a compatible host cell. In this study we found that, in some cases, only one of the two pheromone genes within a group was able to activate *B*-regulated development in a particular host. Table 3 summarizes the overall specificity of a particular group and data in Table 4 record the specificities of individual pheromone genes. These data allow us to deduce which *B* specificities have homoalleles of genes we have cloned and this is summarized schematically in Figure 2. We conclude that there are only two alleles of each of the group 1 genes in the nine *B* loci tested, four had the alleles present in *B3*, and

five had those present in *B42* and *B6*. The group 3 alleles present in *B6* were found in *B40* and *B41*, and those from *B3* were found in *B1* and *B44*. The only loci that shared group 2 alleles were *B42* and *B3*. We conclude that, for compatibility, *B* loci need to have different alleles of genes in just one of the three groups; *B1* and *B44* share the same alleles of the group 1 and group 3 genes, yet they confer different *B* mating specificities and must, therefore, have different alleles of their uncharacterized group 2 genes. A similar argument applies to *B40* and *B41*. These data are consistent with our prediction that each *B* mating specificity is derived from a unique combination of alleles of all three sets of genes.

The mature pheromones predicted to be encoded by each group of *B42* or *B6* genes in some cases have very similar sequences, differing in just four amino acids (Table 2; O'Shea *et al.* 1998). In other cases the two pheromones have apparently unrelated sequences, and showed a different spectrum of activity in the host strains tested. This is illustrated by the data presented in Table 4. The *B42* group 3 pheromones encoded by the *phb3.1* and *phb3.2* genes are highly diverged in sequence (Table 2). One pheromone (*Phb3.1*<sup>42</sup>) activated *B*-regulated development in *B1*, *B3*, *B5*, *B43*, and *B44* hosts whereas the other (*Phb3.2*<sup>42</sup>) activated development in *B6*, *B40*, and *B41* hosts. The corresponding *B6* group 3 pheromones are also dissimilar in sequence (O'Shea *et al.* 1998), and although it is not possible to quantify the activity of a gene using our mating test, it was apparent that the mating responses they induced in different hosts varied in strength. When tested in transformation, *phb3.1*<sup>6</sup> induced a much stronger mating response in *B1*, *B3*, *B5*, and *B44* hosts than *phb3.2*<sup>6</sup>, and most notably *phb3.2*<sup>6</sup> failed to activate development in the *B43* host. No differences in host specificity were observed with either the *B42* group 2 or *B42* group 1 pheromones that have similar sequences (Table 2) but a little variation was displayed by the corresponding *B6* pheromones that have unrelated sequences (O'Shea *et al.* 1998; Table 4). Perhaps more surprising is the fact that, overall, the group 2 pheromones encoded by both the *B6* and the

TABLE 3

Southern blot and DNA-mediated transformation analyses identify shared alleles in nine different *B* specificities of *C. cinereus*

Genes	Host strains <sup>a</sup>								
	<u><i>B42</i></u> H/C	<u><i>B6</i></u> H/C	<u><i>B40</i></u> H/C	<u><i>B41</i></u> H/C	<u><i>B3</i></u> H/C	<u><i>B1</i></u> H/C	<u><i>B44</i></u> H/C	<u><i>B5</i></u> H/C	<u><i>B43</i></u> H/C
Group 1 <sup>42</sup>	+/-	+/-	-/+	-/+	-/+	+/-	+/-	+/-	-/+
Group 1 <sup>6</sup>	+/-	+/-	-/+	-/+	-/+	+/-	+/-	+/-	-/+
Group 1 <sup>3</sup>	-/+	-/+	+/-	+/-	+/-	-/+	-/+	-/+	+/-
Group 2 <sup>42</sup>	+/-	-/+	-/+	-/+	+/-	-/+	-/+	-/+	-/+
Group 2 <sup>6</sup>	-/+	+/-	-/+	-/+	-/+	-/+	-/+	-/+	-/+
Group 3 <sup>42</sup>	+/-	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+
Group 3 <sup>6</sup>	-/+	+/-	+/-	+/-	-/+	-/+	-/+	-/+	-/+
Group 3 <sup>3</sup>	-/+	-/+	-/+	-/+	+/-	+/-	+/-	-/+	-/+

<sup>a</sup> *B42* (LN118), *B6* (LT2), *B40* (LCO7), *B41* (LCO5), *B1* (218), *B44* (NL1), *B5* (HT8), *B43* (AT8). H, Southern analysis; C, *B*-regulated development in transformed host. +, DNA hybridization or activation of *B* pathway; -, absence of either hybridization or *B*-regulated development. Underlined entries indicate homoallelism for genes of a given group between different specificities. These data are summarized in Figure 2. Hybridization data were identical for each of the three genes present in each *B42* and *B6* group. Scores for transformation with *B42* and *B6* genes represent the activity of the receptor gene and at least one of the two pheromone genes within each group.

*B42* genes have the same activity spectrum yet they differ markedly in sequence.

The *B42* locus contains a gene encoding a membrane transporter belonging to the major facilitator family: A 10-kb *B42*-specific DNA sequence adjacent to the group 3 genes did not activate *B*-regulated development in either a *B6* or a *B3* host. Northern analysis (not shown) identified a single gene in this region with a transcript

of 2.5 kb. The sequence from genomic DNA and an RT-PCR-derived cDNA identified the gene product as a putative membrane transporter belonging to the major facilitator family. The presence of this gene (*mfs1.1*) within the *B42*-specific sequence prompted us to look for a corresponding gene in *B6* genomic DNA (Figure 1). This gene, *mfs1.2*, was detected in a region adjacent to the group 3 genes of *B6* in a sequence that lacks

TABLE 4

Activity spectra of pheromone gene pairs in different hosts

Pheromone genes	Host strains <sup>a</sup>								
	<i>B42</i>	<i>B6</i>	<i>B40</i>	<i>B41</i>	<i>B3</i>	<i>B1</i>	<i>B44</i>	<i>B5</i>	<i>B43</i>
<sup>b</sup> <i>phb1.1</i> <sup>42</sup> / <i>phb1.2</i> <sup>42</sup>	A	A	+	Group 1	+	A	+	A	A
				+					
<sup>c</sup> <i>phb2.1</i> <sup>42</sup> / <i>phb2.2</i> <sup>42</sup>	A	+	+	Group 2	A	+	+	+	+
				+					
<sup>d</sup> <i>phb2.1</i> <sup>6</sup> / <i>phb2.2</i> <sup>6</sup>	+	A	-/+	+	+	+	+	+	+
<sup>e</sup> <i>phb3.1</i> <sup>42</sup> / <i>phb3.2</i> <sup>42</sup>	A	-/+	-/+	Group 3	+/-	+/-	+/-	+/-	+/-
				-/+					
<sup>f</sup> <i>phb3.1</i> <sup>6</sup> / <i>phb3.2</i> <sup>6</sup>	+ <sup>w</sup> /+	A	A	A	+ <sup>w</sup> /+	+ <sup>w</sup> /+	+ <sup>w</sup> /+	+ <sup>w</sup> /+	+ <sup>w</sup> /+

<sup>a</sup> Host strains as described in Table 3. A, presence of homoallelic genes in host; +, a compatible interaction; +<sup>w</sup>, a weak compatible interaction; -, an incompatible interaction. Where both genes of the pair gave a compatible interaction, this is scored as +; when only one of the pair gave a compatible interaction, the interactions for both genes are given individually as -/+, +/-, +/+<sup>w</sup>, or +<sup>w</sup>/+ as appropriate.

Sequences of mature peptides based on this study and O'Shea *et al.* (1998):

<sup>b</sup> ERDPGFTSKGFC/ERHLGFTTKGFC;

<sup>c</sup> ERPGAGKVRAF/ERPGSGVNRAF;

<sup>d</sup> ERAGPGDTPGGFC/ERYNANKAYAWC;

<sup>e</sup> DQRLPGGYGGQC/ERRTQGGGGLTFWC;

<sup>f</sup> DRRLPDSYGGAC/ERRTHGGNGLTFWC.

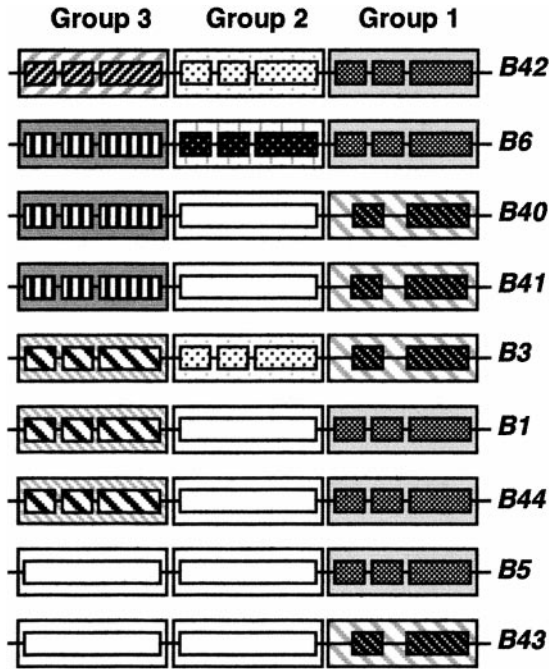


Figure 2.—Identification of shared alleles in nine different *B* loci of *C. cinereus* derived from hybridization and transformation analysis with cloned genes from *B42*, *B6*, and *B3*. Homoalleles are represented by similarly patterned boxes. Uncharacterized alleles are shown as open boxes.

homology to *B42* DNA but shows homology to a corresponding sequence in *B3* and seven other genomic DNAs derived from strains with the different *B* mating specificities listed in Table 3. The DNA sequences of *mfs1.1* and *mfs1.2* are only 60% identical, which accounts for the lack of cross-hybridization in Southern blots, but the predicted proteins show 72.5% identity (83% similarity).

## DISCUSSION

**Paralogous sets of genes confer multiple *B* mating specificities:** We have shown that the genes within the *B42* locus of *C. cinereus*, like those of the previously described *B6* locus, are arranged into three discrete sets each comprising two pheromone genes and one receptor gene. The three genes comprising each group are kept together as an indivisible unit by being embedded in allele-specific DNA sequences. Using a combination of Southern blot analysis and transformation, we have identified shared alleles of genes in nine *B* loci and demonstrated that variation in only one set of alleles is the minimum requirement to generate different *B* mating specificities. We have shown previously that the *A* locus of *C. cinereus* derives its many specificities from three pairs of paralogous genes (Pardo *et al.* 1996), in this case genes encoding the two components of a heterodimeric transcription factor. By making use of natural deletions, we were able to introduce different

combinations of genes into a partially *A*-null background and to demonstrate unambiguously that only different alleles from the same pair of genes were able to generate compatible protein combinations. No comparable *B*-null background exists for us to test individual combinations of receptor and pheromone genes but the genetic data we have presented are consistent with the *B* genes belonging to three functionally redundant and paralogous groups, which are closely linked.

The 79 versions of this locus predicted to occur in nature (Raper 1966) can be generated with just a few alleles of each of the three paralogous groups of genes. We have identified 2 alleles of the group 1 genes and at least 4 alleles of the group 3 genes in the nine *B* loci we examined. With just these few alleles of the group 1 and the group 3 genes it would require only 8–10 alleles of the group 2 genes to generate 79 ( $2 \times 4 \times 10$ ) unique combinations of the three paralogous sets of genes. It would thus require a total family of some 16 different receptors and 30 different pheromones to generate all the predicted *B* mating specificities found in nature.

In the only other mushroom that has been studied, *S. commune*, multiple *B* mating specificities are derived from just two sets of paralogous genes. The genes are separated into two discrete loci known as *B $\alpha$*  and *B $\beta$*  by as much as 4.5 map units. Genetic recombination studies established the functional redundancy of these two loci and the fact that there were nine specificities of each (Raper 1966). Molecular analyses have revealed that the *B $\alpha$ 1* and *B $\beta$ 1* loci each encode a single receptor and at least three pheromones (Wendl and *et al.* 1995; Vail lancourt *et al.* 1997). There is no evidence of shared alleles of receptor or pheromone genes in any of the different specificities.

The evolution of multiple alleles of pheromone and receptor genes in hymenomycetes has necessitated a remarkable degree of specificity in pheromone-receptor recognition. A single receptor may be activated by several pheromones, and each pheromone may activate several receptors. Perhaps it is not surprising to find that multiple pheromones are required to activate the many different receptors. Where receptor sequences are similar, only slight differences in pheromone sequence may be sufficient to distinguish a compatible from an incompatible target. For example, the *C. cinereus* receptors encoded by the *rcb3* genes present in *B42* and *B6* (*Rcb3<sup>6</sup>* and *Rcb3<sup>42</sup>*) are 78% identical in sequence. They are distinguished by the pheromones encoded by the *B42* and *B6* alleles of *phb3.2* (*Phb3.2<sup>42</sup>* and *Phb3.2<sup>6</sup>*), which in their predicted mature version differ in just four amino acids (ERRTHGGNGLTFWC and ERRTQGGGGLTWFC, respectively, this study and O'Shea *et al.* 1998). The two group 3 pheromones encoded in *B42* (*Phb3.1<sup>42</sup>* and *Phb3.2<sup>42</sup>*) are completely different in sequence, and do not activate the same receptors (Table 4). *Phb3.1<sup>42</sup>* activates *Rcb3<sup>3</sup>* and preliminary sequence



data for the *B3* genes indicate that this receptor has only 38% identity to Rcb3<sup>6</sup>. However, pheromones with quite different sequences may activate the same receptor, as occurred when the *B42* and *B6* group 2 pheromones were tested in several different backgrounds. They do not always activate development to the same extent but this is impossible to quantify in the *C. cinereus* mating assay. In *S. commune*, where transformants with an activated *B*-pathway have a recognizable phenotype, attempts were made to assess the response to different pheromones and this was seen to be variable (Wendland *et al.* 1995; Vaillancourt *et al.* 1997). In this highly complex system, multiple genes for pheromones would seem to be necessary to extend the range of receptors that can be activated by an allelic set of genes and, where only weak receptor activation is possible, to allow two or more pheromones to act together to maximize the level of activation.

**A role for a membrane transporter in pheromone activity:** Because gene disruptions are difficult to effect in *C. cinereus*, we cannot at present say whether Mfs1, the predicted multidrug transporter, has a role in mating in *C. cinereus*. The mating pheromones of the basidiomycetes belong to the CaaX-modified class typified by a-factor of *S. cerevisiae*. Processing of the 36- and 38-amino acid yeast a-factor precursor molecule has been well documented and leads to a mature pheromone of 12 residues with the C-terminal cysteine carboxymethylated and farnesylated (Caldwell *et al.* 1994; Tam *et al.* 1998). Then, this peptide is actively transported across the cell membrane by the Ste6p protein, a member of the ATP-binding cassette (ABC) family of transporters. Msf1 belongs to a different family of transporters, the major facilitator superfamily (MFS). The association of *mfs1* with the *B* locus may, therefore, be accidental rather than being required for pheromone secretion.

Genes of unrelated function can become trapped accidentally in the long stretches of nonhomologous DNA that one finds at a locus such as *B*. There are, for example, two genes of unknown function in the corresponding *a2* locus of *U. maydis* that apparently have no relevance to mating (Urban *et al.* 1996). Like other alleles of the *C. cinereus* gene, *mfs1-1* may have resided originally in a homologous sequence flanking the *B42* locus and may have become translocated into it by a DNA rearrangement. Flanked by *B42*-specific DNA, its chances of being recombined into other genomic backgrounds would be reduced, there would be no selection against nucleotide substitutions, and the gene would become fixed in its current position.

We thank Professor Ian Connerton for providing DNA sequencing facilities and Suzanne O'Shea, Pushpalata Chaure, and Heike Koch for help with the DNA sequence analysis. J.R.H. thanks the Biotechnology and Biological Sciences Research Council (BBSRC) for a research studentship, M.J.M. was supported by the Gatsby Technical Education Project by the award of a Sainsbury Studentship, and L.A.C. is a BBSRC Senior Research Fellow.

## LITERATURE CITED

- Aviv, H., and P. Leder, 1972 Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA* **69**: 1408-1412.
- Banham, A. H., R. N. Asante-Owusu, B. Göttgens, S. A. J. Thompson, C. S. Kingsnorth *et al.*, 1995 An N-terminal dimerization domain permits homeodomain proteins to choose compatible partners and initiate sexual development in the mushroom *Coprinus cinereus*. *Plant Cell* **7**: 773-783.
- Binninger, D. M., C. Skrzynia, P. J. Pukkila and L. A. Casselton, 1987 DNA-mediated transformation of the basidiomycete *Coprinus cinereus*. *EMBO J.* **6**: 835-840.
- Bölker, M., and R. Kahmann, 1993 Sexual pheromones and mating responses in fungi. *Plant Cell* **5**: 1461-1469.
- Bölker, M., K. Urban and R. Kahmann, 1992 The *a* mating-type locus of *Ustilago maydis* specifies cell signalling components. *Cell* **68**: 441-450.
- Burrows, D. M., 1991 Transformation studies in the basidiomycete fungi *Coprinus cinereus* and *Coprinus bilanatus*. Ph.D. Thesis, University of London.
- Caldwell, G. A., S-H. Wang, C-B. Xue, Y. Jiang, H-F. Lu *et al.*, 1994 Molecular determinants of bioactivity of the *Saccharomyces cerevisiae* lipopeptide mating pheromone. *J. Biol. Chem.* **269**: 19817-19826.
- Casselton, L. A., and A. de la Fuente Herce, 1989 Heterologous gene expression in the basidiomycete fungus *Coprinus cinereus*. *Curr. Genet.* **16**: 35-40.
- Casselton, L. A., and N. S. Olesnick, 1998 Molecular genetics of mating recognition in basidiomycete fungi. *Microbiol. Mol. Biol. Rev.* **62**: 55-70.
- Edmonds, M., M. H. J. Vaughan and H. Nakazato, 1971 Polyadenylic acid sequences in the heterogeneous nuclear RNA and rapidly-labeled polyribosomal RNA of HeLa cells: possible evidence for a precursor relationship. *Proc. Natl. Acad. Sci. USA* **68**: 1336-1340.
- Halsall, J. R., 1997 Isolation and characterisation of the *B42* mating type locus of *Coprinus cinereus*. Ph.D. Thesis, University of Oxford.
- Hoge, J. H. C., J. Springer, B. Zantinge and J. G. H. Wessels, 1982 Absence of differences in polysomal RNAs from vegetative monokaryotic cells of the fungus *Schizophyllum commune*. *Exp. Mycol.* **6**: 225-232.
- Kües, U., and L. A. Casselton, 1992 Homeodomains and regulation of sexual development in basidiomycetes. *Trends Genet.* **8**: 154-155.
- Kües, U., A. M. Tymon, W. V. J. Richardson, G. May, P. T. Gieser *et al.*, 1994 A mating-type factors of *Coprinus cinereus* have variable numbers of specificity genes encoding two classes of homeodomain proteins. *Mol. Gen. Genet.* **245**: 45-52.
- Kurjan, J., 1993 The pheromone response pathway in *Saccharomyces cerevisiae*. *Annu. Rev. Genet.* **27**: 147-179.
- Lewis, D., 1961 Genetical analysis of methionine suppressors in *Coprinus*. *Genet. Res.* **2**: 141-155.
- Lukens, L., H. Yicun and G. May, 1996 Correlation of genetic and physical maps of the *A* mating-type locus of *Coprinus cinereus*. *Genetics* **144**: 1471-1477.
- Mellon, F. M., P. F. R. Little and L. A. Casselton, 1987 Gene cloning and transformation in the basidiomycete fungus *Coprinus cinereus*: isolation and expression of the isocitrate lyase gene (*acu-7*). *Mol. Gen. Genet.* **210**: 352-357.
- Mutasa, E. S., A. M. Tymon, B. Göttgens, F. M. Mellon, P. F. R. Little *et al.*, 1990 Molecular organization of an *A* mating type factor of the basidiomycete fungus *Coprinus cinereus*. *Curr. Genet.* **18**: 223-229.
- Olesnick, N. S., A. J. Brown, S. J. Dowell and L. A. Casselton, 1999 A constitutively active G-protein-coupled receptor causes mating self-compatibility in the mushroom *Coprinus*. *EMBO J.* **18**: 2756-2763.
- O'Shea, S. F., P. T. Chaure, J. R. Halsall, N. S. Olesnick, A. Leibbrandt *et al.*, 1998 A large pheromone and receptor gene complex determines multiple *B* mating type specificities in *Coprinus cinereus*. *Genetics* **148**: 1081-1090.
- Pardo, E. H., S. F. O'Shea and L. A. Casselton, 1996 Multiple versions of the *A* mating type locus of *Coprinus cinereus* are generated by three paralogous pairs of multiallelic homeobox genes. *Genetics* **144**: 87-94.



- Raper, J. R., 1966 *Genetics of Sexuality in Higher Fungi*. Ronald Press, New York.
- Spellig, T., M. Bölker, F. Lottspeich, R. W. Frank and R. Kahmann, 1994 Pheromones trigger filamentous growth in *Ustilago maydis*. *EMBO J.* **13**: 1620–1627.
- Stankis, M. M., C. A. Specht, H. Yang, L. Giasson, R. C. Ullrich *et al.*, 1992 The *A* alpha mating locus of *Schizophyllum commune* encodes two dissimilar multiallelic homeodomain proteins. *Proc. Natl. Acad. Sci. USA* **89**: 7169–7173.
- Tam, A., F. J. Nouvet, K. Fujimura-Kamada, H. Slunt, S. S. Sisodia *et al.*, 1998 Dual roles for Ste24p in yeast maturation: NH<sub>2</sub>-terminal proteolysis and COOH-terminal CAAX processing. *J. Cell Biol.* **142**: 635–649.
- Urban, M., R. Kahmann and M. Bölker, 1996 Identification of the pheromone response element in *Ustilago maydis*. *Mol. Gen. Genet.* **251**: 31–37.
- Vail l ancourt, L. J., and C. A. Raper, 1996 Pheromones and pheromone receptors as mating-type determinants in basidiomycetes, pp. 219–247 in *Genetic Engineering*, Vol. 18, edited by J. K. Set low. Plenum, New York.
- Vail l ancourt, L. J., M. Raudaskoski, C. A. Specht and C. A. Raper, 1997 Multiple genes encoding pheromones and a pheromone receptor define the B $\beta$ 1 mating-type specificity in *Schizophyllum commune*. *Genetics* **146**: 541–551.
- Wendland, J., L. J. Vail l ancourt, J. Hegner, K. B. Lengeler, K. J. Laddison *et al.*, 1995 The mating-type locus *B $\alpha$ 1* of *Schizophyllum commune* contains a pheromone receptor gene and putative pheromone genes. *EMBO J.* **14**: 5271–5278.
- Zolan, M. E., and P. J. Pukkila, 1986 Inheritance of DNA methylation in *Coprinus cinereus*. *Mol. Cell. Biol.* **6**: 195–200.

Communicating editor: R. H. Davis