Mating in mushrooms: increasing the chances but prolonging the affair

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Finding a compatible mating partner is an essential step in the life cycle of most sexually reproducing organisms. Fungi have two or more mating types, and only cells of different mating type combine to produce diploid cells. In mushrooms, this is taken to extremes, with the occurrence of many thousands of mating types. But, having gone to such extraordinary lengths to ensure that almost any two mushroom mycelia in the wild can mate, cell fusion is not followed by nuclear fusion and true diploidy. Instead, the fused cells form a characteristic mycelium, known as the dikaryon, in which haploid nuclei are paired but actively prevented from fusing. The mating-type genes, which encode pheromones, pheromone receptors and homeodomain transcription factors, have crucial roles in regulating the complex developmental programme by which the dikaryon is formed.

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Sexual reproduction ensures the maintenance of genetic diversity, and most organisms impose genetic barriers to self-mating so that diversity is maximized. Mating, the initial step that brings together compatible individuals, has been particularly well studied in fungi and is regulated by a characteristic set of genes. These encode peptide pheromones and their cognate receptors, and the signal transduction machinery typical of eukaryotes: heterotrimeric G-proteins, mitogen-activated protein kinases (MAPKs), adenylate cyclase and cAMP-dependent protein kinases (PKAs)[1]. Together, these allow fungi to respond to the pheromone signals and integrate this response with cellular physiology. Equally important are the transcription factors that act as master regulators, controlling the expression of other genes required for mating, and conferring on cells a sexual identity known as a mating type.

In ascomycete fungi, there are just two mating types, and the alternative forms of the mating-type locus have completely dissimilar DNA sequences, encoding transcription factors unique to each haploid type[2]. Ascomycetes include the budding yeast, *Saccharomyces cerevisiae*, the fission yeast, *Schizosaccharomyces pombe*, and many filamentous species such as *Neurospora crassa*. *S. cerevisiae* provides a paradigm for understanding how mating type is determined and how this leads to different genes being expressed in different cell types. We have summarized the essential features of this in Box 1 because it provides us with a useful framework for looking at mating in mushroom species[3–7]. Mushrooms such as *Schizophyllum commune* and the inkcap, *Coprinus cinereus*, are basidiomycetes, which are distinguished by the possession of several thousand distinct mating types[8,9].

The mating-type genes in these fungi are multiallelic; individuals have the same genes at the mating-type loci but differ in mating type if they have different alleles of these genes. Equally remarkable is the unusual life cycle of these fungi. Cell fusion is not closely followed by nuclear fusion. Instead, these two events are separated by an extended, and often indefinite, mycelial stage known as the dikaryophase, in which the haploid nuclei from each mate remain paired in each hyphal cell[9]. The mating-type genes have essential regulatory roles in forming and maintaining the dikaryophase. Here, we examine these features in the context of the mushroom life cycle and focus on the evolution of multiple mating types. We shall concentrate mainly on *C. cinereus* because the biology of its dikaryon is well understood, the mating-type loci have been characterized fully and key genes regulating downstream events have recently been identified. Where relevant, reference will be made to non-mushroom basidiomycete species such as the corn smut, *Ustilago maydis*, and the human pathogen *Cryptococcus neoformans*.

The mushroom life cycle

The life cycle of *C. cinereus* can be considered typical of many mushroom species (Fig. 1). The haploid sexual spores germinate to give an asexual monokaryotic mycelium that has uninucleate cells. Mating type does not identify cells before fusion, because any mycelia can fuse; instead it determines events that follow fusion, when the nuclei are in a common cytoplasm. If mycelia have compatible mating types, cell fusion triggers a developmental programme that leads to the formation and growth of a sexually competent dikaryon. Following exchange of nuclei, the incoming nucleus rapidly migrates through the cells of its mate until it reaches a new hyphal tip cell, far from the site of the initial fusion, where it starts to divide in synchrony with the resident nucleus. Tip-cell division is complex and involves the formation of a structure known as the clamp connection, which ensures that every cell of the dikaryotic mycelium maintains its 'dikaryotic pair' of nuclei. One nucleus enters and divides in the developing clamp cell, whereas the other divides in the main body of the cell. Septation generates three cells, a new binucleate tip cell and two uninucleate cells, each containing a genetically different nucleus. Division is completed when the clamp-cell tip fuses with the subterminal cell and its nucleus migrates to join its partner. This programme is repeated at each tip-cell division. When, in response to environmental

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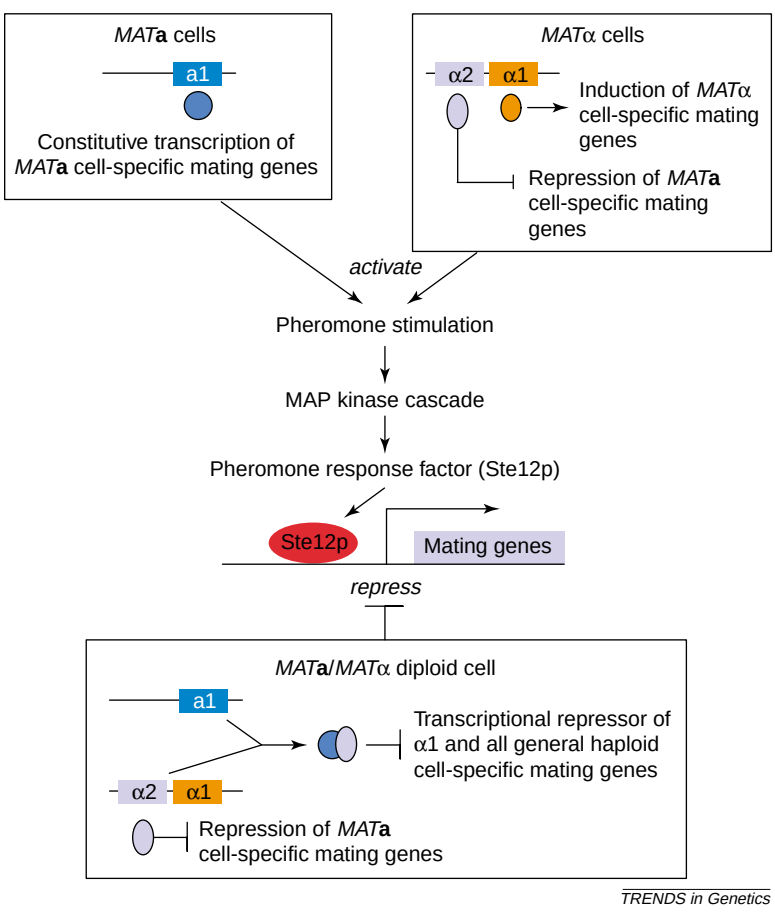
Box 1. A model mating: *Saccharomyces cerevisiae*

Budding yeast provides us with the best-studied system of mating in fungi. *MAT***a** cells express the MATa1 homeodomain protein, which is produced constitutively but does not regulate transcription of *MAT*a-specific genes. *MAT*α cells express the MATα2 homeodomain protein, which represses *MAT*a-specific genes, and the MATα1 protein, which activates *MAT*α-specific genes[a,b]. The crucial haploid-specific genes encode pheromones and pheromone receptors: the Ste2p receptor and a-factor pheromone in *MAT***a** cells, or the Ste3p receptor and α-factor pheromone in *MAT*α cells. Pheromones are secreted as chemi-attractants, causing reorientation of the cytoskeleton, such that compatible cells grow towards each other along the strongest pheromone gradient and then fuse[c,d]. a-factor is derived from a long precursor containing the C-terminal CaaX motif, a signal for post-translational cleavage of the three terminal residues, followed by carboxymethylation and farnesylation of the terminal cysteine[e]. The N-terminus is cleaved to give a mature 12 amino acid lipopeptide that is exported across the plasma membrane by Ste6p, a member of the ABC transporter family. Production of α-factor is through an endoplasmic reticulum/Golgi body secretory pathway and it is not lipid-modified. Ste2p and Ste3p both regulate the same response pathway. Pheromone binding activates a heterotrimeric G protein leading to GDP/GTP nucleotide exchange and dissociation. The G-protein βγ particle then initiates a phosphorelay through MAPK cascade proteins to activate Ste12p, a homeodomain transcription factor that induces mating genes. After mating cells fuse, nuclei fuse and MATa1 and MATα2 homeoproteins dimerize to give a new negative regulator that instigates diploid-specific transcription, and pheromone signalling ceases.



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stimuli, the dikaryon develops into the characteristic fruit body, nuclear fusion finally occurs, but it is immediately followed by meiosis and sporulation. This developmental pathway is not followed when incompatible mating types fuse, and the mycelium rapidly returns to a monokaryotic state.

Mating type is dictated by genes at two unlinked loci known as *A* and *B*, both of which contain multiallelic genes[8,9]. Compatible mates must have different allelic versions of genes at both loci. The *B* genes encode lipopeptide pheromones and pheromone receptors, the activities of which are required to promote nuclear migration following both the initial cell fusion and clamp-cell fusion after the dikaryon is established[10–12]. Clamp-cell fusion is somewhat analogous to cell fusion in *S. cerevisiae*, and it is easy to imagine that pheromones secreted from the clamp-cell activate receptors localized on the surface of the subterminal cell and vice versa. How pheromone signalling promotes nuclear migration is more difficult to envisage. Presumably pheromones encoded by the incoming nucleus trigger receptors in the recipient cell, but whether signalling events also occur elsewhere in the recipient mycelium is currently unknown. *A* genes encode proteins belonging to the homeodomain family of transcription factors[13,14] and are required for synchronized division of the tip-cell nuclei and the formation of the clamp cell.

The complex *A* and *B* loci

Attempts to understand the complicated system of mating-type determination in mushrooms are made easier because the structural organization of the two loci (in *C. cinereus* at least) is similar, even though the genes at each are unrelated[15,16]. The tandemly arrayed genes at each locus are arranged into three

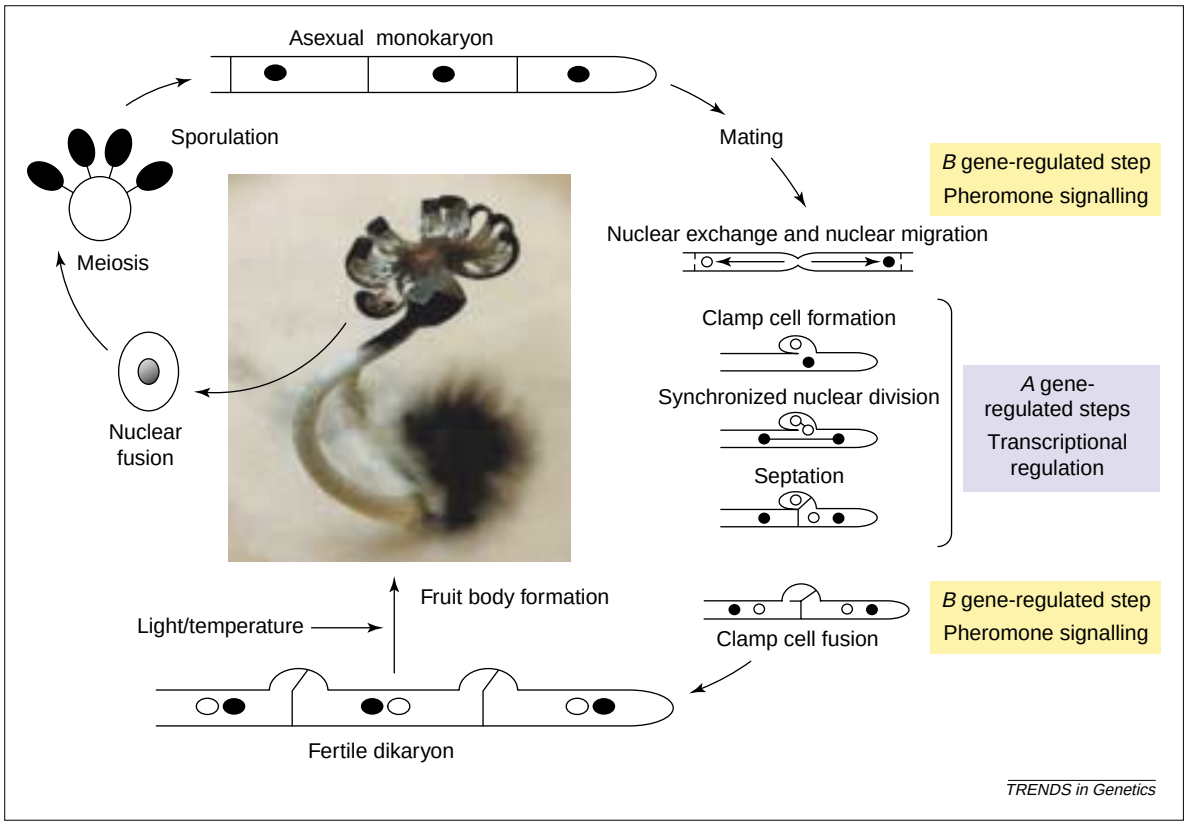

Fig. 1. Life cycle of the inkcap mushroom *Coprinus cinereus*. The *A* and *B* mating-type genes determine compatibility in mating by regulating alternate stages in the formation of the dikaryon, an extended mycelial stage that gives rise to the fruit body.

groups that have clearly arisen by duplication but are now functionally independent (Fig. 2). Each group within the *A* locus encodes two dissimilar homeodomain proteins (HD1 and HD2)[15]. These are the homologues of the *S. cerevisiae* MATα2 and MATa1 mating proteins, respectively (Box 1), that heterodimerize in diploid cells to generate a diploid-cell-specific transcription factor. The A proteins similarly heterodimerize to generate a dikaryon-specific transcription factor[17]. Each group at the *B* locus encodes a pheromone receptor and, usually, two pheromones[12,16]. None of the genes present within a single *A* or *B* locus can activate development because pheromones cannot activate receptors encoded at the same locus, and HD1 and HD2 proteins encoded at the same locus cannot heterodimerize. Dikaryotic development can only occur when mating partners bring together different allelic versions of at least one group of genes. HD1 proteins heterodimerize with HD2 partners encoded by different allelic versions of the same group of *A* genes. Each pheromone can activate a receptor encoded by different allelic versions of the same group of *B* genes, thus activating a downstream signalling pathway.

Locus organization is maintained because the DNA sequence that comprises each group of genes (both coding and flanking sequence) is sufficiently different to prevent homologous recombination between different allelic versions. Thus, each group behaves as a cassette, and the genes remain associated as a single unit, such that compatible gene combinations cannot arise by intralocus recombination. The different cassettes, however, have been randomly mixed during evolution, suggesting that at some stage they have been flanked by a homologous sequence that has directed recombination between loci[18] (a 7-kb sequence still exists between the first two cassettes at the *A* locus). In this way, just a few allelic versions of each group combine to give many genetically different *A* and *B* loci. A population analysis of the *C. cinereus A* locus, for example, identified four allelic versions of group 1, ten of group 2 and three of group 3, enough to generate 120 genetically different *A* loci[18]. Any *A* locus can be combined with any *B* locus to generate more than 12 000 mating types. It is not essential for the genes in different groups to be localized to a single locus as they are in *C. cinereus*. In *S. commune*, both *A* and *B* genes are distributed between distinct loci known as *Aα*, *Aβ*, *Bα* and *Bβ*. In this species, there are an estimated 20 000 mating types[8].

The pheromones and receptors

The mushrooms are probably unique among fungi in having evolved multiple versions of their pheromones and receptors. These signalling molecules are truly remarkable in that a given pheromone can activate several different receptors, and a single receptor can be activated by many different pheromones (although never one that is encoded within the same *B* locus). This provides us with a wonderful resource for learning

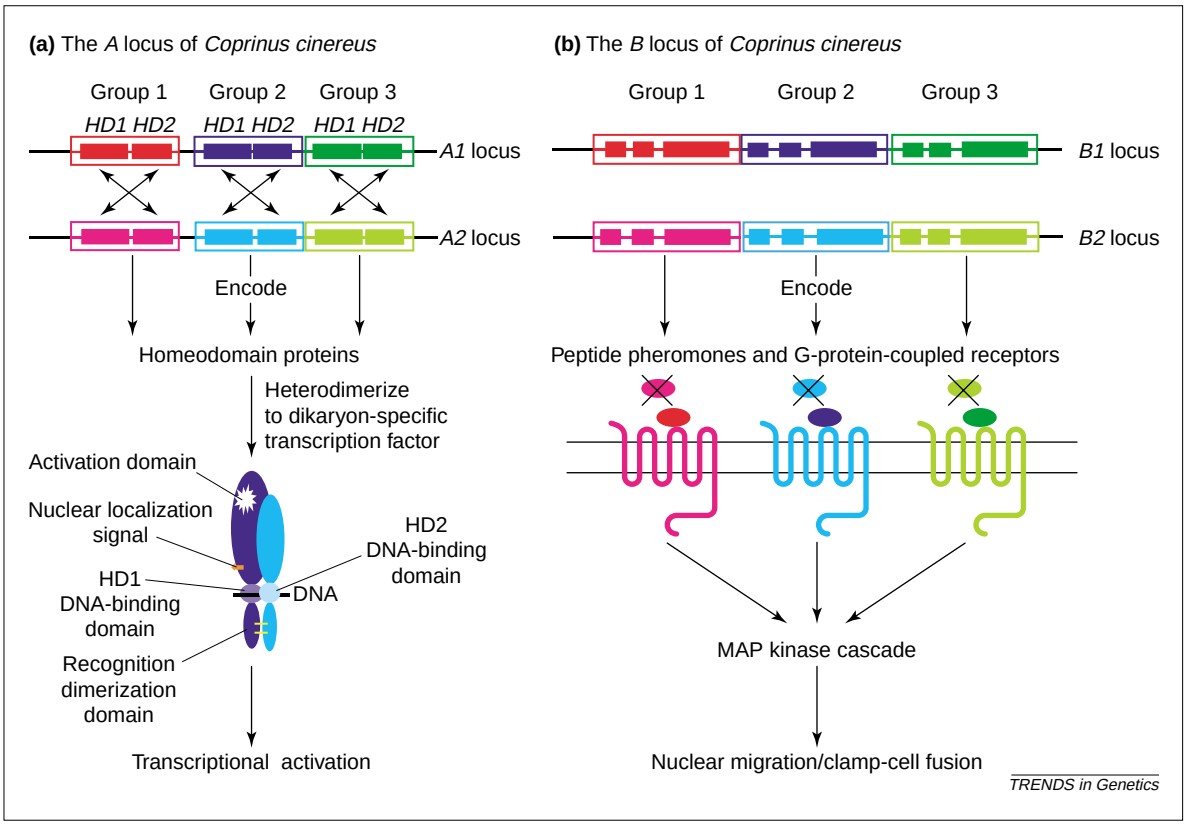

Fig. 2. Structural organization of the *Coprinus cinereus A* and *B* mating-type loci. Each locus contains three groups of genes, represented by different colours. Within the *A* locus, each group encodes two dissimilar homeodomain proteins (a). Each of these is able to heterodimerize with a partner encoded by genes in the same group (dark blue and light blue in this example) but fails to heterodimerize with the partner encoded in the same locus. Crossed arrows indicate this compatibility. Within the *B* locus, each group encodes a pheromone receptor and two pheromones (b). Each receptor is activated by a pheromone encoded by genes in the same group (e.g. those shown in dark green and light green), but not by those encoded in the same locus.

about the structure–function relationships between receptors and their ligands. All pheromone genes identified to date are predicted to encode lipopeptides similar to *S. cerevisiae* **a**-factor (Box 1). These peptides are derived from longer precursor molecules that have a C-terminal CaaX motif. This motif is a signature for C-terminal truncation and modification of the terminal cysteine residue by carboxymethylation and farnesylation[7]. The pheromone precursors in *C. cinereus* and *S. commune* are highly variable in sequence. However, a glutamic acid/arginine (ER) or aspartic acid/arginine (DR) motif is found 12–15 amino acids upstream of the CaaX motif in many pheromone precursors and allows us to predict the probable cleavage site used to generate short mature peptides[9]. When mature pheromone sequences are compared, it is evident that just two amino acids could be sufficient to confer a different receptor specificity (Fig. 3) whereas other pheromones with the same receptor specificity might be totally unrelated in sequence[12,16] (M.J. Milner, PhD thesis, University of Oxford, 2000).

The pheromone receptors belong to the familiar G-protein-coupled receptor (GPCR) family, with seven transmembrane-spanning (7-TM) domains, and are homologous to *S. cerevisiae* **a**-factor receptor, Ste3p (Box 1). Notably, in ascomycete fungi it appears that there are two types of pheromone, as in *S. cerevisiae*[19–22]. There are, correspondingly, two types of receptor, one with similarity to Ste3p and the other with similarity to α-factor receptor, Ste2p. Although there could be a biological advantage in having two types of pheromone in ascomycetes, only the **a**-factor type is known to occur in basidiomycete fungi[23,24].

Pheromone signalling in mushrooms is thought to activate a MAPK signalling pathway similar to that in *S. cerevisiae.* Although this has yet to be confirmed for mushroom species, various components of the MAPK pathway have been identified in *U. maydis* and *C. neoformans*[1].

Heterologous expression of receptors and pheromones in yeast

To study the mushroom pheromones and receptors in more detail, we need to isolate them from the native cellular environment where multiple receptors and pheromones are present in every cell. This has been achieved by expressing the mushroom pheromone and receptor genes heterologously in *S. cerevisiae*[25,26]. Yeast strains have been developed in which the pheromone signalling pathway is engineered to link receptor activation to reporter gene expression[27]. The promoter of *FUS1*, one of the mating genes activated in response to receptor stimulation, has been recruited to drive reporter genes that enable both quantitative (β-galactosidase activity) and qualitative (histidine prototrophy) assays of receptor activity (Fig. 4). When

(a)

S. cerevisiae **a**-factor precursor

MQPSTATAAPKEKTSSEKKDNYIIKGVFWDPACVIA

C. cinereus Phb3.2^{42} pheromone precursor

MSDSFISFDSVVGPAHSEASETIAIVDSQSSQLSAIDPRLSSTS

LDELNDLPVEFERRTQGGGGLTWFCVIA

(b)

Pheromone sequence		B locus	Receptor activated Rcb3^{42}	Rcb3^{6}
(i) Natural pheromones				
Phb3.2^{42}	ERRTQGGGGLTWFC	*B42*	-	+
Phb3.2^{3}	*******S*P*****	*B3*	+	-
Phb3.2^{42}	ERRTQGGGGLTWFC	*B42*	-	+
Phb3.2^{6}	****H**N***FW*	*B6*	+	-
(ii) Pheromone mutants				
Phb3.2^{6}	ERRTHGGNGLTFWC	*B6*	+	-
	****Q*********		+	-
	*******G******		+	-
	**********W**		+	+
	***********F*		+	-
	**********WF*		-	+

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Fig. 3. Sequences of pheromones and precursors. (a) The precursors of *Saccharomyces cerevisiae* a-factor and *Coprinus cinereus* Phb.3.2^{42} pheromones. Phb.3.2^{42} can be processed to active pheromone when expressed in *S. cerevisiae*. The mature a-factor and predicted Phb3.2^{42} sequences are in red. The CaaX motif is underlined, as is the ER motif predicted to be a processing site in *C. cinereus*. (b) The pheromone sequence specificity is determined in part by the FW/WF motif, because a single substitution gives dual specificity, and a double substitution switches specificity. The ability (+) or inability (–) to activate two allelic versions of a receptor (Rcb3) is shown. The natural pheromones show the predicted sequences of mature pheromones encoded by three allelic versions of *phb3.2*. Mutant pheromones are derived by site-directed mutagenesis of one of the natural genes.

mushroom pheromone genes are expressed in yeast cells, a pheromone species is secreted that can activate a compatible mushroom receptor expressed on the surface of the cell, despite the lack of homology with yeast **a**-factor (Fig. 3). Figure 4a illustrates the histidine prototrophy conferred by co-expression of a *C. cinereus* receptor and compatible pheromone. Activation only occurs in *MAT***a** cells, indicating that pheromone maturation is dependent on the **a**-factor processing machinery absent in *MAT*α cells[25].

Although basidiomycete pheromones are processed to active species in yeast, this might not result in the correct structure. Synthetic peptides have been used to identify native structures[25]. A series of peptides of differing length were tested for their ability to activate a *C. cinereus* receptor in the yeast assay (Fig. 4b). The most effective peptide was lipid-modified at the C-terminus with, the predicted ER/DR processing signal at its N-terminus. The availability of the yeast assay, combined with the cloning of many *B* alleles, will soon allow the description of the full family of receptors from both *S. commune* and *C. cinereus*, along with a comprehensive picture of the patterns of pheromone specificity among them. This raises the prospect that comparative sequence analysis can identify crucial invariant residues within receptors corresponding to the ligand-binding site or to residues involved in relaying conformational changes to the G-protein-binding site. This type of analysis, using mutants obtained by saturation mutagenesis, has already led to the development of a sophisticated 3D model of receptor structure, based on another GPCR, the human C5a receptor[28,29].

Rare mutations in the *B* genes lead to constitutive *B*-regulated development (nuclear migration and clamp-cell fusion)[30–32]. A receptor mutation can be constitutively activating such that pheromone stimulation is no longer required to activate the downstream signalling pathway[25]. This type of mutation has been described for many GPCRs, including several causing pathological conditions in man[33], and this propensity reflects a fundamental aspect of the mechanism of G-protein activation. Mutations in either a pheromone or a receptor gene can alter ligand specificity and lead to receptor activation by a normally incompatible pheromone encoded within the same *B* locus[34,35]. This illustrates how subtle the determinants of specificity are and how new compatibility can evolve by minor changes in structure.

The homeodomain proteins

The proteins encoded by the *A* genes also display remarkable specificity, and only those encoded by different allelic versions of genes belonging to the same group can form heterodimers. The HD1 and HD2 proteins have an essential property not required of *S. cerevisiae* MATa1 and MATα2: the ability to discriminate between large numbers of potential dimerization partners. Studies of mushroom A proteins and the equivalent b mating-type proteins of *U. maydis* (which has just a single pair of multiallelic *HD1* and *HD2* genes), have shown that specificity for dimerization resides in the N-terminal domains[17,36,37]. These domains are predicted to contain coiled-coil α-helices that mediate protein dimerization in other transcription factors[38]. As with the receptors and pheromones, specificity is finely tuned and can be compromised by single amino acid substitutions that permit normally incompatible proteins to heterodimerize and activate development[37].

Heterodimerization provides a way of regulating transcription factor function by bringing together different functional domains. Studies on the *S. cerevisiae* MATa1 and MATα2 proteins have highlighted the essential role that dimerization plays in determining the affinity of homeodomains for binding DNA[4]. Induced conformational changes permit these domains to make the appropriate contacts on the DNA[39]. This protein–protein interaction might be more crucial than having both homeodomains, and that of the MATa1 protein has been shown to be sufficient for DNA binding[40]. Similarly, for the mushroom A protein heterodimer (although not the *U. maydis* b protein heterodimer[41]), the HD2 protein has the crucial homeodomain and that of HD1 proteins is dispensable[42,43]. Other studies of the *C. cinereus* proteins have revealed that the HD1 proteins provide

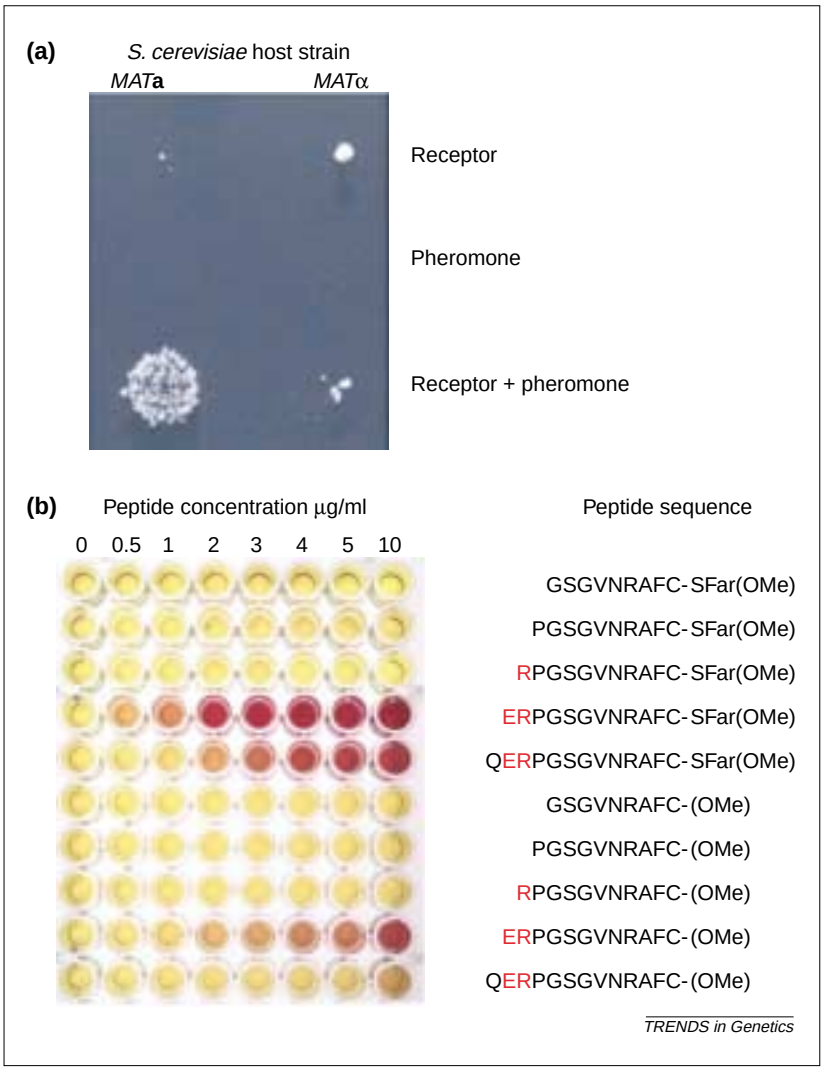

Fig. 4. Activation of *Coprinus cinereus* receptors expressed in *Saccharomyces cerevisiae*. (a) Qualitative assay showing histidine-independent growth of *MAT*a cells expressing a pheromone precursor and a compatible receptor. Yeast cells contain the *FUS1–HIS3* reporter gene and were plated on histidine-free medium so that growth was dependent on activation of the yeast pheromone-response pathway. (b) Quantitative assay showing activation of a *C. cinereus* receptor by synthetic peptides based on the sequence of a compatible pheromone precursor with or without farnesyl modification. Yeast cells contained the *FUS1–lacZ* reporter gene to produce β-galactosidase in response to activation of the pheromone-response pathway. Cells were seeded in liquid medium supplemented with a colourimetric substrate of β-galactosidase, resulting in a yellow to red colour change.

a putative activation domain comprising a negatively charged sequence at the C-terminus[42] and two nuclear localization signals (NLS) that can, in a heterologous assay, direct a reporter protein to the nucleus[44]. HD2 proteins lack an NLS and cannot be localized to the nucleus in this same assay, indicating that they are normally confined to the cytoplasm in unmated fungal cells. The separation of different functional domains onto two proteins represents an elegant strategy to ensure that the mating-dependent developmental pathway is activated only after fusion between compatible mates. A mutation that generated an *HD1–HD2* gene fusion in *C. cinereus* subverts this strategy and generates a single protein that can activate development because it combines all the essential functions of the wild-type heterodimer[45].

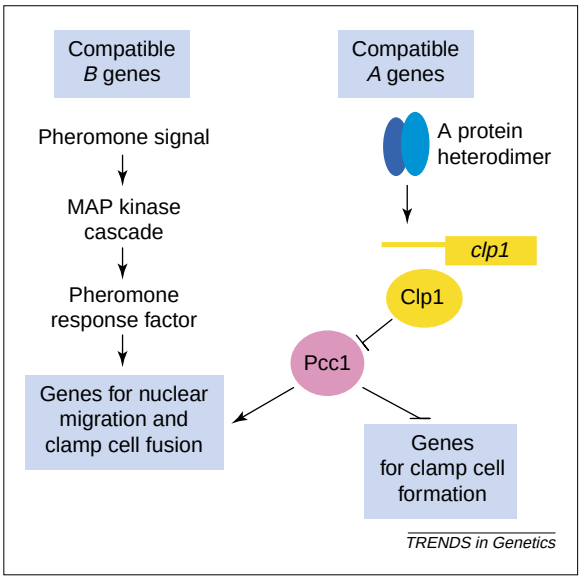

Fig. 5. Proposed interactions between Clp1 and Pcc1 and the *A*- and *B*-regulated pathways of development in *Coprinus cinereus*. Monokaryons lacking Pcc1 constitutively express the pathway leading to clamp cell formation that is normally activated by compatible *A* genes. When mated to compatible monokaryons, *pcc1* mutants are unable to promote nuclear migration or fuse their clamp cells, implying that Pcc1 has an essential function in *B*-gene-regulated development. *clp1* is induced in response to the A protein heterodimer and might relieve Pcc1-mediated inhibition of clamp cell formation.

Coordinating the activities of the *A* and *B* genes

The extended mating ritual we see in the mushroom dikaryon requires the coordination of *A* and *B* gene activity. At present we know little about the way this is achieved, but some clues are beginning to emerge. One of the elements of the pheromone MAPK signalling pathway of *U. maydis* that has been characterized is the target transcription factor (pheromone response factor) Prf1 (Ref. 46). In *U. maydis*, dikaryon development is regulated by a similar set of genes to those found in the *A* and *B* loci of mushrooms. Pheromone signalling leads to enhanced transcription of all these genes and common elements have been identified in their promoters as putative binding sites for Prf1 (Refs 46,47). Similar promoter elements are found in the corresponding genes of *C. cinereus*, and a similar pattern of pheromone-induced transcription is observed (M.J. Milner, PhD thesis, University of Oxford, 2000). Crosstalk between the *A* and *B* pathways thus occurs at the level of gene transcription.

Two recently identified genes in *C. cinereus*, *clp1* (clampless)[48] and *pcc1* (pseudoclamp connections)[49] provide insight into downstream events regulating clamp-cell development and indicate another point at which crosstalk occurs between the *A* and *B* pathways (Fig. 5). *clp1* is a probable target gene for the A protein heterodimer: it is required for clamp-cell formation and is only transcribed in cells having a compatible complement of *A* genes. When expressed from a constitutive promoter, *clp1* is sufficient to activate clamp-cell formation without mating. *pcc1* encodes a

member of the HMG class of transcription factors, and its loss bypasses the need for compatible A proteins and results in constitutive clamp-cell formation. As yet, the Clp1 protein sequence offers no clue to its function. Because of the opposite consequences of mutation in *clp1* and *pcc1*, a plausible hypothesis is that Pcc1 normally inhibits clamp-cell formation in monokaryons[49] and is inhibited by Clp1 following mating[48]. Pcc1 is also implicated in *B*-regulated development, because nuclear migration and clamp- cell fusion are blocked in *pcc1* mutants (T. Kamada and L.A. Casselton, unpublished). Pcc1 thus appears to play a pivotal role in regulating both the *A* and *B* developmental pathways, and it remains to be resolved how an apparent inhibitor of one pathway is required for the activity of the other!

Conclusions

Homeodomain transcription factors and 7-TM receptors are ubiquitous in eukaryotic cells, and their recruitment for mating in fungi presents us with excellent experimental systems for studying their roles in gene regulation. By evolving such huge numbers of mating types, the mushroom fungi have revealed the remarkable specificity that these proteins and peptides can display. As yet, little is known about the role of the cAMP signalling pathway in mating in mushrooms, but it is implicated in mating in *U. maydis* and *C. neoformans*[1]. By linking cAMP signalling to dikaryon morphogenesis in mushrooms, we might gain further insight into how nuclear migration and synchronized nuclear division are regulated – events that are of considerable general interest to developmental biologists.

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Natural selection and the evolution of mtDNA-encoded peptides: evidence for intergenomic co-adaptation

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Mitochondrial DNA (mtDNA) variation is an important tool for the investigation of the population genetics of animal species. Recently, recognition of the role of mtDNA mutations in human disease has spurred increasing interest in the function and evolution of mtDNA and the 13 polypeptides it encodes. These proteins interact with a large number of peptides encoded in the nucleus to form the mitochondrial electron transport system (ETS). As the ETS is the primary energy generation system in aerobic metazoans, natural selection would be expected to favor mutations that enhance ETS function. Such mutations could occur in either the mitochondrial or nuclear genes encoding ETS proteins and would lead to positive intergenomic interactions, or co-adaptation. Direct evidence for intergenomic co-adaptation comes from functional studies of systems where nuclear–mitochondrial DNA combinations vary naturally or can be manipulated experimentally.

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Mitochondrial DNA has been used extensively in studies of population genetic structure and phylogenetic relationships among animals (but not plants[1,2]). Two properties of mtDNA make it particularly useful for such studies: high rates of nucleotide substitution compared with nuclear DNA (nuDNA)[3,4], and maternal inheritance that is not subject to recombination[5] (but also see Ref. 6). Although evolutionary and population genetic studies often assume that mtDNA undergoes NEUTRAL EVOLUTION (see Glossary) or nearly neutral evolution[7,8], the important roles of all 13 mtDNA-encoded peptides in cellular ATP production suggest that mtDNA variation could often have significant metabolic and fitness consequences. Indeed, the functional interactions between mitochondrial proteins encoded by mtDNA and nuDNA might result in strong selection for positive intergenomic interactions, or CO-ADAPTATION. One potential problem for the co-adaptation of the nuclear and mitochondrial genomes is the different substitution rates of these genomes[3,4]. Peptides encoded by the mtDNA show a higher rate of substitution than their co-evolving, nuDNA-encoded counterparts, suggesting that co-adaptation will be driven by the mitochondrial genome.

Two approaches can be employed when attempting to determine the role of natural selection on the evolution of mtDNA-encoded proteins. Either one can attempt to extract information from patterns of DNA sequence variation, or one can seek direct experimental evidence for functional differences among mtDNA variants. With regard to the former, evolutionary models provide testable predictions concerning the patterns of DNA sequence variation within and between species in the absence of selection (i.e. based on the neutral theory of molecular evolution). In fact, predictions derived from the neutral theory have been repeatedly rejected in analyses of animal mtDNA[9–13]. These studies reveal a striking pattern: there is a consistent excess of intraspecies amino acid polymorphism relative to interspecies amino acid divergence.

Although some alternative explanations cannot be eliminated, a leading hypothesis is that the polymorphisms represent the accumulation of slightly deleterious mutations on the nonrecombining mtDNA molecule as GENETIC DRIFT prevents weak PURIFYING SELECTION from eliminating the weakly deleterious NONSYNONYMOUS SUBSTITUTIONS[14]. However, in the absence of recombination, such an underlying mechanism would be expected to affect all coding regions of the mtDNA equally[15]. Ballard[16,17] obtained numerous complete mtDNA sequences from the four species in the *Drosophila melanogaster* group

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