Tetrapolar fungal mating types: Sexes by the thousands

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Abstract

In order to achieve genetic rearrangement in a sexual cycle, eukaryotes go through the processes of meiosis and mating. Different mating types assure that mating is only possible between two genetically diverse individuals. Basidiomycetous fungi display thousands of different mating types that are determined by two genetically unlinked loci. One locus is multiallelic and contains genes for homeodomain transcription factors which are able to form heterodimers. The activation of target genes is dependent on heterodimers formed from the monomeric transcription factor proteins originating from different alleles of this genetic locus. The interactions between the two monomeric transcription factors and the activation of target genes by the heterodimeric proteins make this regulatory system both complex and interesting. The second locus contains a pheromone receptor system: the pheromone receptor is similar to the G protein-linked serpentine receptors in Saccharomyces cerevisiae that activate the pheromone response via a phosphorylation signal transduction cascade in S. cerevisiae. This pheromone perception is a trigger of sexual development and not, as with yeast, itself under control of mating type genes. Rather it directly senses diversity at the mating type loci. Whereas heterobasidiomycetes display a bi-allelic structure for this locus with recognition between one receptor and the opposite pheromone, homobasidiomycetes contain multiple specificities for pheromone receptors and pheromones.

Keywords: Fungal mating type; Tetrapolar incompatibility; Homeodomain transcription factor; Pheromone receptor system

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1. Introduction

The title to this review is chosen in honour of Dr. J.R. Raper who, in the preface of his book [1] published in 1966 explains that he had to compromise on the book title “Genetics of sexuality in higher fungi” because the preferred title “Sexes by the thousands” would not have been considered appropriate at the time. In the light of the current developments in fungal genetics which start to explain the action of thousands of mating types in higher fungi on a molecular level, the title seems appropriate today. The thousands of different mating types found in some higher fungi have intrigued scientists for decades [2-5]. Being used to two genetically determined sexes, even the mating type switching found in yeast [6,7] seemed an unnecessary complication. Yet there are fungi like Schizopyllum commune which display over 20,000 different mating types [1]. The questions of why and how such elaborate systems have evolved have puzzled geneticists and mycologists alike [8-15].

Usually sex implies two alternative genders, as in vertebrates. Such mating systems in which half the population has one mating type and the other half is of the alternative sex are also found in higher fungi. These so-called bipolar mating types are formed by several systems differing at the molecular level (Fig. 1).

Zygomycetes, for example, are truly bipolar [16]. The mating types in these organisms are usually called ‘plus’ and ‘minus’, and it was shown that the two mating types differ in their enzymatic equipment. Both strains are unable to produce trisporic acid which is necessary and sufficient to induce sexual development and zygospore formation. If both strains grow in close proximity, one can use the biosynthetic intermediate excreted by the other to synthesize active trisporic acid. Without this precursor, one essential step in the synthesis of this compound is lacking and trisporic acid is not produced [17,18]. This same system has been found in different genera of the zygomycetes. Other factors must prohibit successful mating between strains of different species. In some cases, a promiscuous mating between species has been reported. One example is the parasitic genus Parastriella growing on an Abisidia host of opposite mating type and forming pseudosexual structures during its parasitic life cycle [19].

Other bipolar organisms are able to switch mating type from one to the opposite sex [20-22]. This phenomenon is investigated in detail in the yeast Saccharomyces cerevisiae [23,24]. Each of the two mating type loci, a and α, encodes transcription factors. In a haploid strain, haploid-specific and either a- or α-specific genes are transcribed, depending on the mating type. Only if both informations are combined in a diploid cell, the haploid-specific genes will be turned off and diploid-specific genes are transcribed. To achieve this regulation, a heterodimer of the transcription factors α1 and α2 is required, which obviously is only present in a diploid cell after mating which combines both loci [25,26]. Mating type switching is possible, since both a and α strains
carry all necessary information for both mating types. Only one information is actively expressed in the MAT (mating type) locus. Silent cassettes for both the a and the α loci reside at silent loci (HML and HMR) in the genome. A gene conversion event is able to achieve mating type switching using recombinogenic double strand breaks and subsequently replacing the active locus with a copy of the opposite sex [7,24,22]. In this event the old copy at the active MAT locus is excised and destroyed and subsequently replaced in a process resembling transposition by a copy of the silent cassette which carries the information of the new mating type. Since this copy is now the only one that is active because of its localization at the active MAT locus, a phenotypic mating type switching is observed [7,24,22].

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**BIPOLAR MATING TYPES**

**zygomycetes**

+ alternative pathways for biosynthesis of trisporic acid leads to physiological complementation

**ascomycetes**

Saccharomyces cerevisiae

mating type a encodes a1 and a2 diploids form a1/a2 heterodimer which is an active transcription factor

mating type a encodes homeodomain transcription factors a1 and a2

mating type switching is seen

Neurospora crassa

mating type A mata-1 induces A pheromone and A-factor receptor

mating type a mata-1 induces α pheromone and α-receptor

the mating type loci are ideomorphic and show no homology

**basidiomycetes**

Ustilago hordei

linkage of independent a and b loci lead to a phenotypically bipolar mating behaviour

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**TETRAPOLAR MATING TYPES**

**basidiomycetes**

Ustilago maydis

bi-allelic a locus each locus encodes a pheromone and a pheromone receptor for the other pheromone

receptor activation leads to cell fusion

multiallelic b locus homeodomain transcription factors b1, b2, b3, b4

active heterodimers if b loci of opposite sexes differ; heterodimers induce filamentous growth

Coprinus cinereus

multiallelic A archetype with 4 pairs of homeodomain transcription factors; active heterodimers induce pseudoclop formation

A1-1 ... A11-1 ... A13-1 ... A19-1 ... A21-1 ... A29-1 ... A31-1 ...

multiallelic B multiple sets of pheromones and receptors; activation essential for fully compatible mating and spore formation

Schizophyllum commune

multiallelic A each locus encodes a pair of homeodomain transcription factors; active heterodimers induce clamp cell formation

multiallelic B pheromone receptors and multiple pheromones; receptor activation leads to nuclear migration and fully compatible mating

A1 ... 9

A11 ... 9

B1 ... 9

B11 ... 9

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Fig. 1. Mating systems in higher fungi. Bipolar and tetrapolar mating type systems of some fungi are listed. The function of the mating type genes is indicated.
In other ascomycetes, a mating type system is found which has been termed ideomorphic [27]. This term was chosen for sequences at the mating type loci which do not show homology between strains of the opposite sexes [28–30]. This system has been analyzed in detail in *Neurospora crassa*, but has also been established for other ascomycetes [31–34].

Another type of bipolar mating is observed when a tetrapolar mating type system is simplified, for example by genetic linkage of the two loci that form the basis of the tetrapolar mating system. This secondary bipolar system will be discussed in the context of tetrapolar mating type systems.

In addition to the systems mentioned above, the fungal kingdom has evolved more elaborate incompatibility systems. With these tetrapolar mating types, a higher outbreeding rate is achieved by the combination of multiallelic loci which govern mating type specificity [1,13].

The term tetrapolar refers to four distinct interactions between haploid mycelia. This observation was attributed to the presence of two genetically unlinked loci working independently to establish mating type specificity [35,36]. Molecular analysis revealed that these tetrapolar fungi have one system based on homeodomain transcription factors, while the second locus codes for a pheromone receptor system. These systems will be discussed in detail. Differences occur between heterobasidiomycetes such as *Ustilago maydis*, which carry a multiallelic transcription factor system but a bi-allelic pheromone receptor system [37], and homobasidiomycetes such as *Schizophyllum commune* and *Coprinus cinereus* which are multiallelic at both loci. This makes these homoba-

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**Fig. 2.** Control of mating in the heterobasidiomycete *Ustilago maydis*. The mating type locus *a* exists in two allelic versions and encodes a pheromone (*mfa*) and a receptor (*pra*) for the pheromone of the opposite mating type. Activation of the pheromone receptor results in mating of the two haploid cells. The *b* locus exists in more than 30 allelic versions and encodes two homeodomain transcription factors, *b*\textsubscript{east} and *b*\textsubscript{west}. Mating between compatible strains combines two allelic versions of *b*\textsubscript{east} and *b*\textsubscript{west} which then constitute active heterodimers activating target genes that induce filamentous growth and pathogenicity. The continuous action of the induced receptor system of the *a* locus is necessary to permit filamentous growth.
sidiomycetes the most prolific outbreeders with over 20,000 different mating types for *S. commune* existing in nature [1].

2. Heterobasidiomycetes

The mating type system of heterobasidiomycetes has been analyzed in detail in the corn smut fungus *U. maydis*. This fungus is biotrophic and can only complete its life cycle during an infection of corn plants [38–42]. The infectious stage is formed when two compatible yeast-like haploid homokaryons fuse and a diploid heterozygote is formed. The compatibility of the two mates is determined by two unlinked loci called *a* and *b*.

2.1. The *a* locus of *U. maydis* is bi-allelic

The *a* locus controls the fusion of the two yeast-like homokaryons and subsequent maintenance of the ensuing filamentous growth [43]. The *a* locus contains genes for a pheromone receptor (*pra* for pheromone receptor *a*) and a mating type pheromone (*mf*a for mating factor *a*), designated *pral* and *mf al* in an *a1*, and *pra2* and *mf a2* in an *a2* strain, respectively [44–46]. Activation of either receptor is achieved by the pheromone of opposite specificity (Fig. 2). The function of this pheromone receptor system is reminiscent of the situation in yeast where pheromones and pheromone receptors are also expressed by the two different mating types that permit fusion of cells with the opposite mating type. In yeast, however, this system is not a master regulator as seen with *U. maydis* or the homobasidiomycetes, but rather itself is under transcriptional control of the mating type genes [47].

2.2. The *b* locus of *U. maydis* is multiallelic

The *b* locus in *U. maydis* [48,49] encodes a multiallelic family of homeodomain transcription factors. Every allele carries two such genes which are transcribed divergently and form heterodimers. The subunits of the heterodimer are termed *b East* and *b West* for their adjacent location but divergent orientation on the chromosome [50–54]. Active heterodimers can only be formed when subunits translated from different alleles are combined. The active heterodimers are transcription factors controlling the development of infectious dikaryotic filamentous mycelium. Physical evidence for the formation of heterodimers has been established in *U. maydis* by immune precipitation and using yeast di-hybrid analyses. It was shown that the formation of heterodimers is possible between *b East* and *b West* proteins of different specificity [55,56].

3. Bipolarity in basidiomycetes

For *Ustilago hordei*, genetic linkage of the *a* and *b* loci has been reported. This leads to a phenotypically bipolar mating type system. However, functional genes for both *a* and *b* are detectable. It has been postulated that tetrapolar mating type systems might be exclusive to the basidiomycetes, while ascomycetes display bipolar mating types. This discussion is kindled by the findings in *U. hordei* and awaits further investigation [57]. In homobasidiomycetes, bipolarity has also been reported [58,59].

4. Homobasidiomycetes

4.1. Two multiallelic sex factors

The mating type system found in tetrapolar homobasidiomycetes such as *S. commune* is closely related to those found in heterobasidiomycetes such as *U. maydis*. However, cell fusion in *S. commune* is seen regardless of the mating type. Only after cell fusion has occurred are distinctive reactions observed dependent on the mating types of the two interacting mycelia. Like in heterobasidiomycetes, two loci, called *A* and *B* in homobasidiomycetes, are found which are functionally completely independent [1,36]. This leads to four possible combinations of the two unlinked mating type loci in spores of one fruitbody. For *S. commune* this system has been analyzed already by Kniep, who established in the 1920’s that two unlinked loci *A* and *B* are responsible for the tetrapolar mating behaviour. The four possible combinations are *A = B =* (incompatible), *A ≠ B =* (semi-compatible *A*, induced for conjugate nuclear division and pseu-
doclamp formation), $A = B \neq \phi$ (semi-compatible $B$, induced for nuclear migration) and $A \neq B \neq \phi$ (fully compatible, formation of fused clamps, fertile) [1,35,36].

The semi-compatible interactions enabled Kniep to define the processes under control of either $A$ or $B$ locus in comparison to development triggered by the simultaneous induction of both $A$ and $B$ in a fully compatible mating. Fusion of cells occurs without regard to the mating type. In matings between strains with different $B$ factors, the hyphae then exchange nuclei which migrate into and throughout the entire mycelium of the opposite mating type [60,61]. With approx. 3 mm per hour this process is very fast in $S.\ commune$ [1,13,61]. For Coprinus congregatus migration has been timed with up to 40 mm per hour in young cultures [62,63]. Following the established dikaryosis in hyphal tips, conjugate nuclear divisions occur which maintain dikaryosis in subsequently developing cells by formation of clamp connections. Whereas the tip cell stays dikaryotic after nuclear division and cytokinesis, the penultimate cell has only one nucleus directly after cell division. The second nucleus is retained in the clamp cell which has to fuse with the penultimate cell in order to re-establish the dikaryotic stage [1].

It has been investigated whether the trapped nucleus is the same in every cell. For $S.\ commune$ predominantly one type of nucleus is found trapped dependent on the $B$ mating type [13,64]. These interesting processes, possibly dealing with some kind of imprinting, lead to a tilted angle in nuclear division resulting in clamp destination of one nucleus.

Whereas nuclear migration is under control of the $B$ genes, the $A$ locus governs nuclear cell division and clamp cell formation. For clamp cell fusion both loci must be active. The phenotypes seen in $S.\ commune$ allow the macroscopic and microscopic distinction between the four possible interactions. This is not true for all basidiomycetes, as the very distinctive phenotype visible in $A = B \neq \phi$ matings of Schizophyllum is not formed, e.g. in $C.\ cinereus$ [1].

4.2. Four types of mating interactions

In $S.\ commune$ homokaryons, or $A = B = \phi$ matings, aerial mycelium as well as substrate hyphae with dolipore septa are formed. The cells are monokaryotic (Fig. 3).

The mating interaction of semi-compatible strains differing at their $A$ loci ($A \neq B = \phi$) does not lead to dikaryosis since nuclear migration is under control of the $B$ locus. Therefore the two mycelia interact only at the zone of mating and only there can a heterokaryon be formed. On complete media, the two mycelia grow in different directions and a zone of repressed growth is formed between the two mates. However, if forced by different auxotrophic markers, the established heterokaryon can be obtained and this heterokaryon will form unfused clamp cells, so called

![Fig. 3. Four mating interactions between homokaryons grown from sibling spores of the homobasidiomycete Schizophyllum commune. The two independent loci $A$ and $B$ govern compatibility in mating interactions. In matings between strains with the same allelic version at the $A$ and $B$ loci ($A = B = \phi$), growth of a normal monokaryotic mycelium is observed. In matings of strains differing in their $A$ loci ($A \neq B = \phi$) growth into different directions is observed. At the hyphal level, pseudoclamps with trapped nuclei are observed at the interaction zone. If the $B$ loci are different ($A = B \neq \phi$) no aerial mycelium is formed and the hyphae show aberrant growth and disturbed nuclear distribution attributed to continuous nuclear migration. In fully compatible matings ($A \neq B \neq \phi$), fused clamps are formed on dikaryotic hyphae and fruitbody formation is induced under the right environmental conditions.](https://academic.oup.com/femsre/article-abstract/18/1/65/588481)
pseudoclamps, at every septum [1]. As will be seen, the same phenotype can be observed if transformants are scored which express a gene conferring Aα mating type specificity in addition to the endogenous locus of the recipient cell [65]. If both Aα loci differ in specificity, the A-regulated development is activated, culminating in pseudoclamp formation. The nuclear distribution is dikaryotic in the tip cell while the pseudoclamps are not able to fuse with the monokaryotic mother cells to release their nuclei. Therefore most cells carry one nucleus with one nucleus trapped in the pseudoclamp. Another type of pseudoclamp forming mycelium is seen in mutants that are rendered self-compatible for the A factor or in partial diploids [66,67]. Molecular analysis of the Coprinus A locus will be discussed later.

In A = B ≠ matings a strong phenotype, called ‘flat’, is observed in Schizophyllum. The mycelia no longer form aerial mycelium, but only grow submerged [35,36]. Examination of a class of genes encoding small, highly hydrophobic proteins located extracellularly which have been termed hydrophobins provide a molecular explanation for this phenotype. The small hydrophobic peptide Sc3 is expressed in aerial mycelium and is thought to be responsible for the hydrophobic surface of aerial hyphae which enables the fungus to emerge into the air. This gene is not expressed in ‘flat’ mycelium [68]. In addition to the macroscopically visible ‘flat’ phenotype, microscopic differences to monokaryotic mycelium can be observed. The hyphae are misshaped and small protrusions appear along the hyphal wall. This is attributed to the continuous expression of cell wall lytic enzymes normally used during the phase of nuclear migration to dissolve septa for the passage of nuclei [1]. Since nuclear migration is persisting in the flat mycelium, expression of the lytic enzymes weakens the cell wall resulting in extrusions created by the cell turgor.

Since nuclear migration is constitutively turned on in this interaction, variable numbers of nuclei per cell are observed, with numbers ranging from none to 12 or more nuclei per cell [69–73]. The process of nuclear migration is under investigation in different species [74–77]. After septal dissolution the newly built septa are simple, not of the dolipore type [78–81]. Because of dependence of the observed nuclear migration on the cytoskeleton, tubulin genes have been investigated. However, no specific isotype or strong induction of tubulin gene expression could be observed [82–88].

The phenotype observed in this semi-compatible mating interaction can be mimicked by partial diploids and constitutive mutations in the B factor, or by DNA-mediated transformation with constructs containing the B mating type locus of another specificity [89–92]. Creating new specificities by mutagenesis has not been successful. Rather, mutants with impaired mating competence were obtained, scored as unilateral maters competent of only donating or accepting nuclei [93,94].

In fully compatible A ≠ B ≠ interactions, nuclear migration occurs into and throughout both mates. After dikaryotic hyphal tips are established, a phenotypic switch occurs from monokaryotic to clamped, dikaryotic mycelial growth, in which both nuclei are faithfully distributed between tip and penultimate cells. The mycelium forms aerial hyphae and can macroscopically be distinguished by the confined growth that is seen at the border of the colony [95,96]. The induction of fruitbody formation is dependent on function of certain genes [97–100], as well as abiotic factors including light and CO₂ [101–109]. Within fruitbodies the hymenium with basidia is formed in which karyogamy and meiosis occur [110]. Certain fruitbody specific genes have been identified and analyzed, among them the genes Sc7 and Sc14 encoding small hydrophilic proteins with extracellular localization and hydrophobins like Sc1 and Sc4 [111–123]. Fruitbody formation and sporulation also can be induced or enhanced by certain factors supplied with the media, e.g. cerebrosides [124–128].

4.3. Both A and B loci are complex

By recombination analysis it was shown that both A and B factors of S. commune are composed of two closely linked, separate loci, each of which is multiallelic [1,13,129–133]. In A, the two loci Aα and Aβ are linked within 8.1 cMorgan (centiMorgan representing recombination rates in percent) with the genetic markers pabl (para-amino benzoic acid synthesis) and ade5 (adenine synthesis) enclosed by the two A loci. Aα and Aβ function independently, but redundantly to activate A-regulated de-
development. Activation of either $A\alpha$, or of $A\beta$, or both is necessary and sufficient to induce $A$-target genes, e.g., capacity for pseudoclamp formation. In a world-wide collection of strains of *S. commune* 9 alleles for $A\alpha$ have been obtained and from 27 alleles found for $A\beta$ a minimal number of 32 alleles existing in nature has been estimated \[1,134\].

In the $B$ factor, two loci, $B\alpha$ and $B\beta$, linked

![Diagram](https://example.com/diagram.png)

**Fig. 4.** The mating type loci and their control of spore production in *Schizophyllum commune*. Both $A$ and $B$ loci are composite and multiallelic ($A\alpha$ with 9, $A\beta$ 32, $B\alpha$ 9 and $B\beta$ 9 alleles). Between $A\alpha$ and $A\beta$ the markers $pab1$ and $ade5$ are located. $A\alpha$ encodes two homeodomain proteins, $Y$ and $Z$, which can form an active heterodimer gene activator protein if two alleles are combined in a mating, as exemplified for $Y3$ and $Z4$. The activation of $A\beta$ through mating leads to the same downstream events. The activated transcription factor then induces pseudoclamp formation. Both $B\alpha$ and $B\beta$ encode pheromone receptors and pheromones. Upon receptor activation nuclear migration is turned on. In a fully compatible mating both pathways converge and lead to formation of fully fused clamps and competence to develop fruitbodies in which basidiospores are formed.
within 2 cMorgan have been established with 9 alleles each. Again, $B_\alpha$ and $B_\beta$ function independently but redundantly to activate a common pathway of $B$-regulated development, e.g. nuclear migration and capacity for clamp cell fusion [13,135,136].

In C. cinereus, composite $A$ and $B$ loci have been established as well by genetic tests. However, linkage is much closer which aided molecular investigation of the $A$ locus [137–139]. As will be discussed in the following sections, molecular data have led to postulate an $A$ locus archetype with four pairs of divergently transcribed genes instead of simply having $A_\alpha$ and $A_\beta$ loci [140].

### 4.4. The $A$ locus codes for homeodomain transcription factors

The first mating type locus from a homobasidiomycete to be identified and analyzed on the molecular level was $A\alpha$ from S. commune. This locus was isolated by cosmid walking from an auxotrophic marker, $pabl$ (para-amino benzoic acid synthesis), which had been localized 0.3 cMorgan from $A_\alpha$. The identification was achieved by transformation assays using a host with an $A\alpha$ mating type different from that of the transforming DNA. Combination of the two different loci in one cell results in the activation of $A$-regulated development normally observed in semi-compatible matings of strains with different $A$ loci. This phenomenon of DNA-mediated transformation to pseudoclamp formation indicative of the cloned $A\alpha 4$ mating type locus was observed within 40 to 50 kb from the start point of cosmid walking, which had been the $pabl$ gene. In $A\alpha 4$ (self) strains no transformation to pseudoclamp formation was observed [65] which had to be postulated for the cloned $A\alpha 4$ locus. Molecular characterization of this clone showed that it harbours two homeodomain protein genes, called $Y$ and $Z$ which may form transcription factors activating target genes involved in pseudoclamp formation. Combination of, e.g. $Y3$ with $Z4$, constitutes an active heterodimer of the homeodomain transcription factors in analogy to $U. maydis b_{\text{East}}$ and $b_{\text{West}}$. Heterodimers of one specificity, like $Y4Z4$, are inactive (Fig. 4). The search for target genes in S. commune is under way. In $A\alpha 1$ only one of the two homeodomain proteins, $Y1$, is present which is interesting in evolutionary terms [141–145] since it may hint at an evolutionary stable loss of the second gene within this specific locus.

In C. cinereus, the closely linked $A$ loci encode several protein products, all with homology to $U. maydis$ transcription factors $b_{\text{East}}$ or $b_{\text{West}}$ and S. commune $Y$ and $Z$ (see Fig. 1). These proteins were classified HD1 and HD2 type proteins [146–152] (HD standing for homeodomain and 1 and 2 indicating the two classes of sequence similarity). From these findings an archetype has been postulated, in which four pairs of genes, all transcribed diver-

![Fig. 5. The archetype of a Coprinus cinereus A mating type locus. In genetic analyses an $A_\alpha$ and an $A_\beta$ locus had been defined, which through molecular analysis could be broken down to four pairs of homeodomain transcription factors in the $a$, $b$, $c$ and $d$ pairs. Each pair consists of a homeodomain type 1 (HD1) and a type 2 (HD2) protein. In strains isolated from nature different compositions of HD1 and HD2 class genes were found.](https://academic.oup.com/femsre/article-abstract/18/1/65/588481)
gently, are able to activate A-regulated development in *C. cinereus* [140]. *Aal-1* corresponds to the HD1-type protein of the first of four gene groups of specificity 1. *Aa2-1* encodes a HD2-class protein of *A1* specificity. Thus, combination of *Aal-1* with *Aa2-3* would constitute an active heterodimer, whereas *Aa1-1* with *Aa2-1* would be inactive because of the same specificity, just as it was described for *U. maydis* and is the case in *S. commune*. The other pairs were consequently named *Ab1-1* through *Ad2-1* (Fig. 5) [153–156]. An interesting feature has been found in a constitutive mutant for the A locus of *Coprinus*: fusion between one HD1 and an HD2 protein by deletion of the fragment separating the two genes in a wild-type strain leads to formation of a constitutively active gene product. This signifies that the interaction of two monomeric transcription factors normally needed to result in an active heterodimer can be overcome by fusion of the two peptides [157]. Interestingly, the constitutive protein has but one homeodomain. Thus, two homeodomains are not essential for function of the DNA binding domain of this class of transcription factors. The same was found to be the case in *Schizophyllum* [141].

In *Coprinus bilantus*, HD1 and HD2 class proteins have also been observed. To identify the homologous genes, probes covering genes at the homologous flanks of the mating type loci of *C. cinereus* were used (Ursula Kües, pers. communication). This was necessary, because no cross hybridization was seen between the homologous genes of different specificity and between clusters. This was also observed with *S. commune*, where no hybridization was seen between *Y* and *Z*, or with homologous genes from different alleles. The degree of sequence conservation is too low on DNA level to allow detection of alleles of different specificity in Southern blot analyses. The underlying cause for this might be that higher degrees in DNA homology could lead to excessive recombination which would destabilize the genetic loci, and thus selection in evolution was directed against such high homology.

A second locus for *A* in *Schizophyllum*, *Aβ*, has been cloned and analyzed and it turned out that this also codes for homeodomain transcription factors, one of which was sequenced [158]. A second active fragment within the locus awaits further analysis. It should be noted that, in contrast to *Aα*, where nine different alleles exist in nature, in *Aβ* 32 alleles are estimated. This higher complexity might be reflected, as is seen with *Coprinus*, by more than one pair of divergently transcribed factors. This question remains to be answered.

### 4.5. The B locus codes for a pheromone receptor system

The *Bα* and *Bβ* loci were cloned from a cosmid library by a strategy which was developed to identify *B* genes [91]. Since transformation with *B* should yield a flat mycelium, screening for flat transformants would have been one way to screen for *B* genes. However, it was not certain whether flat would be easily detectable in transformants. Therefore, transformed cells were plated together with cells containing a different *A* mating type. A transformant would then be induced for *B*-regulated development and if mating with one of the other cells occurred, it would be capable of forming clamp connections and eventually fruitbodies.

This strategy led to the identification of cosmid clones that carry genes responsible for the induction of *B*-regulated development. The first *B* mating type locus to be characterized and sequenced was the *Bal* mating type locus coding for a pheromone receptor system [159] (see below). If fragments were transformed into strains with a different mating type, they were capable of inducing *B*-regulated development. Four different subclones could be identified from an 8.3 kb genomic region, all of which were capable to activate *B* in transformation experiments. One of the active fragments carried a gene for a pheromone receptor which shows homology to other fungal pheromone receptors [45,160,161]. All these receptors belong to the larger class of G protein-linked, seven transmembrane domain, or serpentine, receptors which include e.g. rhodopsin, adrenergic, muscarin and thousands of olfactory receptors [162–166]. These serpentine receptors are spanning the cytoplasmatic membrane with extracellularly located N-termini. The seven transmembrane domains define three extracellular and three cytoplasmatic loops while the C-termini are variable in length and located intracellularly [167–170]. Besides the seven hydrophobic domains spanning the membrane, most
serpentine receptors share other features: three cysteine residues are highly conserved, one at the extracellular face of the third transmembrane domain and a second in the second extracellular loop. These cysteines are supposed to form a disulfide bridge stabilizing the tertiary structure of the protein. A third conserved cysteine is located 12 to 14 amino acids from the last membrane spanning helix in the intracellular C-terminus. It could be shown for the adrenergic receptor that this cysteine is prone to undergo myristylation. These three cysteines are present in the pheromone receptor Bar1 (for B alpha receptor specificity 1) of \textit{S. commune}.

Activation of these serpentine receptors induces specific responses and is dependent on the specific signal. In the mammalian visual system a light-induced conformational change of rhodopsin allows translocation of a proton, whereas in the archaeabacterial membrane halorhodopsin pumps chloride ions across the membrane [171–174].

In other seven transmembrane domain receptors the signal is not directly realized but transduced via a G protein to achieve an intracellular response. For several G protein-coupled serpentine receptors the third cytoplasmatic loop has been implicated in G protein coupling. This follows from mutagenesis, construction of chimeric receptors and competition analyses with short peptides that carry similar sequences [175–181]. However, in yeast the third cytoplasmatic loops of the pheromone receptors Ste2 and Ste3 (ste for sterile, Ste2 being the \(\alpha\)-factor receptor whereas Ste3 recognizes the mating type pheromone \(\alpha\)-factor) are non-homologous although the same signal transduction cascade is induced by both. It therefore was interesting to learn whether two receptors with different specificity of \textit{Schizophyllum} would show sequence identity within this loop. The Bar1 and Bar2 (Bar2 being the receptor of the specificity \(Ba2\)) proteins of \textit{S. commune} reveal high levels of similarity in the parts that are thought to be involved in intracellular factor binding and signal transduction (Fig. 6).

The proximal C-terminus has been implicated in desensitization of the activated receptor. In this process activation-dependent phosphorylation of the receptor by a specific receptor kinase leads to binding of a protein factor (arrestin) subsequently leading to termination of the signal. Also myristylation is involved in desensitization. Internalization by a process of endocytosis is also dependent on binding sites residing in the proximal C-terminus of the receptor [182–189]. This adaptive response is seen if yeast cells are treated with the mating pheromone of the opposite sex. The cells enter G1 phase and are arrested in G1. After some time, however, the G1 block is relieved and cells enter the cell cycle again.

![Fig. 6. The pheromone receptor Bar1 of \textit{Schizophyllum commune}. In analogy to the pheromone receptors known from yeast the seven transmembrane domains (CM: cellular membrane) and cysteins (C) stabilizing the structure are predicted. Comparison to a second allele, Bar2, revealed non-conservative amino acid exchanges at the indicated positions (\(\times\)). High homology is seen in the intracellular parts which are thought to bind to protein factors involved in signal transduction: the extracellular face of the receptors, that must bind the different pheromones, varies.](https://academic.oup.com/femsre/article-abstract/18/1/65/588481)
The intracellular part of Barl shows no homology with the yeast C-terminus involved in adaptation. However, sequence analysis of Bar2 of *S. commune* revealed highest homology between the two proteins in the C-termini, implying cellular factors that recognize and bind to this part of the receptor independently of extracellular specificity domains (Fig. 6).

In other systems involving serpentine receptors, usually only one ligand is recognized. This response may be suppressed by antagonists. In *S. commune* nine different natural alleles are available which presumably all code for a pheromone receptor. Thus the specificities and ligand binding capacities of the serpentine receptors of *S. commune* are of particular interest. The sequence similarities between Barl and Bar2 indicate that the extracellular loops are of considerable divergence. This might be expected for receptors with different specificity since pheromones are prone to bind extracellularly.

Analysis of the mating type locus *Bαl* gave evidence for more than one pheromone. Three small restriction fragments were active in transformation, each carrying a sequence that possibly encodes a pheromone precursor. These preproteins are presumably, like the yeast pheromone a-factor, dependent on N-terminal cleavage and C-terminal modification [190,191]. The three open reading frames each end in a possible isoprenylation signal sequence that could permit addition of a farnesyl tail. A farnesyl group at the C-terminus is found in several fungal pheromones including yeast a-factor, *Schizosaccharomyces pombe* M-factor, the mating factors *mfα1* and *mfα2* of *U. maydis*, or pheromones of *Rhodosporidium toruloides* or *Tremella* [44,192–197]. The three putative *Schizophyllum* pheromone genes were subcloned to fragments of 452, 466 and 933 bp, and these small fragments could be shown to be still active in transformation. A Northern signal was obtained for one pheromone gene, *bapl*(1) (standing for *B* alpha pheromone of specificity 1, 1st gene), that resides on the 452 bp large restriction fragment and activates *B*-regulated development if integrated non-homologously upon transfection. However, other explanations are possible, since different environmental conditions might be needed for induction of the second and third putative pheromone genes. In addition, the putative pheromone genes *bapl*(2) and *bapl*(3) are capable of activating only four and one other specificities, respectively.

Sequence analysis has revealed another pheromone receptor in the other *B* mating type locus cloned, *Bβl*. The gene *bbrl* (for *B* beta receptor of specificity 1) encodes another serpentine receptor with similarity to Barl (Lisa Vaillancourt and Carlene Raper, pers. communication). However, the limited sequence similarity does not allow hybridization in Southern blot analyses. The pheromone response pathway is thought to be activated by a G protein that dissociates upon binding of a compatible pheromone to the receptor. This is postulated in analogy to the known fungal pheromone receptor system found in yeast. It will be interesting to learn whether there are two different G proteins that bind to the respective receptors or whether the independently operating receptor systems of *Ba* and *Bβ* use the same G protein to induce the cascade that permits signal transduction to induce *B*-regulated development in *S. commune*. These questions await the construction of knock-out mutants that allow the investigation of one gene without the background of the native allele.

In *C. cinereus*, the *B* locus also evidently harbours a pheromone receptor system (Lorna Casselton, pers. communication). Whether complexity in that locus is as high as in the *A* locus of *C. cinereus* remains to be seen.

### 5. Fungal mating pheromones

Pheromones are found in different fungal mating systems of which the yeast pheromone response is known best [198–202]. A recent review on fungal pheromones has been published [44]. The two cell-types of different mating type, a-cells and α-cells, produce a-factor and α-factor, respectively. The mating pheromone of one cell is recognized by the receptor expressed in the other mating type and leads
to activation of a signal transduction cascade resulting in cell cycle arrest and competence for mating. This pheromone response pathway in yeast is not a function of the mating type genes, but itself under control of the mating type genes. This is in contrast to the basidiomycetes where it could be shown that the pheromone system is encoded by the mating type loci themselves [45,159] (Lorna Casselton, pers. communication).

In yeast, one of the pheromones, the α-factor, is a peptide of 13 amino acids, whereas the α-factor consists of 12 amino acids with a farnesylated and carboxy-methylated C-terminus [193,194]. For both mating factors high levels of expression are achieved by increased copy numbers: for each of them two genes exist which encode multimeric pheromone precursors. Post-translational cleavage releases four pheromone peptides from the precursor. In the basidiomycetous yeast R. toruloi-des the pheromone genes are similarly arranged. Three genes encode three to five copies of the pheromone peptide that are translated in one precurser and the pheromone of 11 amino acids is released by post-translational cleavage and subsequently C-terminally farnesylated. In addition, in R. toruloi-des the expression can be regulated by transcriptional control. To achieve this regulation, several direct repeats, putative binding sites for a transcriptional activator, precede the genes [192]. In U. maydis the singular pheromone genes contain only one copy of the pheromone precursors, which are farnesylated in both mfa1 and mfa2, before they are excreted [46,55]. The expression is under transcriptional control and a pheromone response element (pre) has been defined, which is seen as 9 bp of direct repeats in front of the genes and a pheromone response factor (Prf1) was identified, which binds to these sequences and may be the regulator of transcription (Michael Bölker, pers. communication). In U. maydis, also a pseudogene for a second pheromone has been found in one of the a mating type loci, a2, reminiscent of a multiallelic rather than bi-allelic system [203]. This led to the idea that in the case of Ustilago, where a multiallelic b locus and a bi-allelic a locus are combined, the evolutionary selection led to loss of the other alleles for a. In the homobasidiomycetes it seems that the multiallelic pheromone mating type loci have promoted the occurrence of several pheromone genes in each locus, which, however, might not all be active as seems possible with Schizophyllum, where bapl(1) alone is able to activate the other alleles and bapl(2) and bapl(3) seem to be redundant in that they confer no further specificity [159]. This is in accordance with the fact that only bapl(1) is preceded by direct repeats which may be pheromone response elements analogous to the situation found in U. maydis.

In S. commune, sequence similarities between the three putative pheromones are not obvious. This would not be expected if the pheromones conferred different specificities. But, as far as we know, this is not the case. The bapl(1) gene was used to transform strains with Bα2 or with Bα3 specificity. This exemplifies that one pheromone alone is able to induce B-regulated development in two different specificities which presumably means binding to two different receptors. It therefore can be assumed that the pheromones and receptors of Schizophyllum are promiscuous, which is in contrast to other serpentine receptor systems. Investigation of cross-reactivity and specificity using these pheromones and their receptors will enhance our knowledge about specific receptor and ligand interactions.

6. A role for the Schizophyllum pheromone receptor system in mating

The mating system in S. commune is not dependent on attraction of mates since cell fusion occurs regardless of mating type [1]. Only after cell fusion is the compatibility of the interaction checked. Therefore, the detection of a pheromone system was quite unexpected. What is a diffusible pheromone good for, if not initial steps of mating leading to cell fusion are controlled? The question might be asked differently. Which genes are under control of the B mating type locus? As discussed, nuclear migration and hook cell fusion are dependent on B. A function for a pheromone system in nuclear migration therefore is postulated. Nuclear migration is, as discussed above, a fast process that makes it likely that the motor proteins and cytoskeleton functions required for nuclear movement and positioning should be produced well in advance. An advance signal to induce these genes could be supplied by a pheromone
that diffuses extracellularly ahead of the migrating nuclei. A nucleus entering a cell already could make use of the machinery induced by the action of the pheromone before septal dissolution has released the nucleus into the cell.

This model allows for a few predictions. The pheromone must be excreted from the cell and the receptor presumably stays bound to the cellular membrane. First evidence for a localization of the pheromone receptor in the plasmalemma is available by immune fluorescence studies (Kothe, unpublished). In addition, the diffusible nature of the pheromone should allow functional distinction of transformants for either receptor or pheromone. Since the pheromone is diffusible it should be able to act on the cell that excreted it — inducing B-regulated development, or nuclear migration — as well as inducing the cells of a mate that carries the same B as the transformation recipient, but a different A mating type. In this case, the mate should be activated for B by virtue of the pheromone and allow nuclear migration into and throughout its mycelium. The different A mating type established by nuclear migration then would allow clamp formation and

![Diagram of pheromone action to induce nuclear migration in Schizophyllum commune.](https://academic.oup.com/femsre/article-abstract/18/1/65/588481 by guest on 21 February 2019)
ultimately fruitbody formation and spore production. This phenotype is seen upon mating transformants transfected with a pheromone gene (Fig. 7).

The receptor, however, is non-diffusible and stays in the cytoplasmic membrane of the transformed cell, where B-regulated development is also induced and nuclear migration turned on. However, a mate would not be stimulated by an advance signal and therefore not be prepared to accept a migrating nucleus. Thus, the mate would not be dikaryotized and only in the zone of direct contact a dikaryon could eventually be established and if subcultured develop into sporulating fruitbodies. Transformants transfected with a receptor gene indeed display this phenotype [159].

7. G protein-mediated signal transduction

The signal transduction pathway is investigated in detail for *S. cerevisiae*. Since homology of *bar1* indicates its function as G protein coupled receptor, a prediction based upon the model established for yeast [198–202] can be proposed. In yeast, activation of the receptor leads to dissociation of the G protein [204,205]. The G protein, a GTP binding and hydrolyzing protein, is bound to the silent receptor and only after a pheromone activates the receptor is the G protein released and dissociates into its α- and βγ-subunits [198–202]. Whether the α-subunit of the βγ-subunit transduce the signal in basidiomycetes remains to be seen. In *Ustilago*, a G protein α-subunit has been identified that seems to be involved in signal transduction, since knock-out mutants for that Ga gene fail to activate pathogenicity, even if placed in a single, b-activated haploid cell. Thus, additional steps not connected to the mating process itself are under control of the pheromone system (Regine Kahmann, pers. communication).
The signal is then transduced via a kinase phosphorylation cascade. The kinases of this cascade are activated by phosphorylation and then are able to phosphorylate their target, another kinase of the pathway. This results in a cascade of phosphorylation and thereby activation of several such kinases (the signal transduction cascade of S. cerevisiae is built by the gene products of STE5, STE11, STE7 and FUS3/KSS1) before phosphorylation of a transcriptional activator (in S. cerevisiae Ste12) is achieved. This is a transcriptional activator for target genes involved in the pheromone response, and also is activated upon phosphorylation (Fig. 8) [206,207].

A homologue to STE7, fuz7 (for fuzzy, because induction of fuzzy growth is achieved by this pheromone response) has been cloned from U. maydis [208]. A functional Ustilago homologue to the yeast Ste12 protein has also been cloned. This pheromone response factor (Prf1) is a transcriptional activator containing a HMG box (for high mobility group because proteins of that family show high mobility in gel electrophoresis) and shows sequence similarity to the S. pombe transcriptional activator ste11+ that is a functional homologue to S. cerevisiae Ste12. Gel retardation and footprint experiments have shown that Prf1 recognizes the pre pheromone response element present in front of the pheromone genes [209,210] (Michael Bölker and Regine Kahmann, pers. communication).

8. Conclusions and evolutionary aspects

It seems that the evolutionary force that drives homobasidiomycetes to establish excessive numbers of alleles in sex factors must be explained. So far, it has been argued that a large number of specificities enhances the outbreeding versus inbreeding rate of the fungus. Whereas in higher animals two sexes allow 50% inbreeding, as well as 50% outbreeding, the two independent loci of Schizophyllum or Coprinus allow 25% inbreeding rate among siblings. However, the large number of alleles existing in nature allow maximal outbreeding rates of, e.g. 98.8% for S. commune.

The independently operating systems of Aα and Aβ, as well as Bα and Bβ, must have evolved by duplication. Duplication, however, and subsequent mutagenesis to yield new specificities, must have led to constitutive alleles before evolutionary pressure might have suppressed the constitutive phenotype. The fact that, in excessive mutational analysis only constitutive alleles have been created, supports this view. The alleles found in nature are not constitutively active, which shows the force that led to suppression. The linkage of the two loci for A and the two B loci might be another hint. Especially if divergently oriented, this chromosomal arrangement leads to recombinational inversion, but avoids the loss of chromosomal sequences.

It had been speculated earlier that both A and B loci must have evolved from one progenitor sex locus, since it was assumed unlikely that multiallelic systems have evolved for two independent sex factors [1,129]. Molecular analysis has answered this question. A and B loci have evolved from completely unrelated sources. Rather evolution has established a second, independent master regulator in tetrapolar fungi using a pheromone system that in yeast had been under control of the homeodomain transcription factor mating type genes. This pheromone receptor system is found in a bi-allelic version in heterobasidiomycetes such as Ustilago, as well as in multiallelic versions in the B mating type loci of homobasidiomycetes like S. commune.

The thousands of sex factors of the tetrapolar mating type system found, e.g. in Schizophyllum give us the great opportunity to investigate interactions of high complexity. Although we now have a much better understanding of the molecular structure that facilitates mating in higher basidiomycetes, much work still remains. As Hans Kniep observed in 1920: “Der Fall Schizophyllum ist auch nicht ganz einfach zu erklären” (The Schizophyllum case remains a mystery).

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