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FUNGAL SEX: THE BASIDIOMYCOTA

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SUMMARY

Fungi of the Basidiomycota, representing major pathogen lineages and mushroom-forming species, exhibit diverse means to achieve sexual reproduction, with particularly varied mechanisms to determine compatibilities of haploid mating partners. For species that require mating between distinct genotypes, discrimination is usually based on both the reciprocal exchange of diffusible mating pheromones, rather than sexes, and the interactions of homeodomain protein signals after cell fusion. Both compatibility factors must be heterozygous in the product of mating, and genetic linkage relationships of the mating pheromone/receptor and homeodomain genes largely determine the complex patterns of mating-type variation. Independent segregation of the two compatibility factors can create four haploid mating genotypes from meiosis, referred to as tetrapolarity. This condition is thought to be ancestral to the basidiomycetes. Alternatively, co-segregation by linkage of the two mating factors, or in some cases the absence of the pheromone-based discrimination, yields only two mating types from meiosis, referred to as bipolarity. Several species are now known to have large and highly rearranged chromosomal regions linked to mating-type genes. At the population level, polymorphism of the mating-type genes is an exceptional aspect of some basidiomycete fungi, where selection under outcrossing for rare, intercompatible allelic variants is thought to be responsible for mating types that may number several thousand. Advances in genome sequencing and assembly are yielding new insights by comparative approaches among and within basidiomycete species, with the promise to resolve the evolutionary origins and dynamics of mating compatibility genetics in this major eukaryotic lineage.

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BREEDING SYSTEMS AND LIFESTYLES IN THE BASIDIOMYCOTA

In the phylum Basidiomycota, a wide variety of lifestyles are represented. These range from well-known and conspicuous wood-decaying mushrooms, plant growth-promoting and mutualistic mycorrhizae, crop destroying smut and rust fungi, to yeast-like human pathogens. Lifestyle differences have consequences for the mating and breeding systems of these fungi (see Box 1 for a glossary of specialist terms used in this chapter), which is reflected in the genetic evolution of mating-type determination. For over a century fungi have been recognized as having diverse breeding systems, from homothallism (i.e. universal compatibility among gametes, including among clonemates) to heterothallism (i.e. mating among haploid gametes carrying different mating-type alleles). The study of breeding systems, for example, led to the discovery of the astounding variability in mating-type alleles among mushrooms, with thousands of different mating-types in some species (1), and to the realization that in many fungal pathogens the process of sexual reproduction is closely linked to infection and pathogenicity (2) (Fig. 1). The importance of basidiomycete fungi and their great research tractability, from ecology to genomics, have brought major insights into the diversification of genetic mechanisms to achieve sexual reproduction.

Diversity and Phylogenetic Relationships – phylum Basidiomycota

From a phylogenetic perspective, the phylum Basidiomycota is the sister group to the phylum Ascomycota, forming together the subkingdom Dikarya. Three independently evolving lineages are strongly within the Basidiomycota (Fig. 2) (3). The subphylum Agaricomycotina contains most of the described species (ca. 21,000), including many mushrooms as saprophytes or mycorrhizal symbionts of plants, the jelly fungi, and a large diversity of yeasts, some of which are important pathogens of humans (*viz. Cryptococcus neoformans*) (4, 5). The subphylum Ustilaginomycotina comprises more than 1,700 species and while many species are pathogens of graminaceous plants (such as the maize smut *Ustilago maydis*), others are commonly associated with human and animal infections and are known from their asexual (anamorphic) states only (*viz. Malassezia* spp.) (4, 6, 7). The subphylum Pucciniomycotina is a sister group of the clade containing Ustilaginomycotina and Agaricomycotina, and consists of more than 8,400 described species (8). Besides the array of saprobic yeast species usually recovered from soils, aquatic habitats, or the phylloplane (e.g., *Rhodotorula* spp. and *Sporobolomyces* spp.) (9), most of the Pucciniomycotina species are plant parasites, such as the obligate pathogenic rust fungi (e.g., *Puccinia* spp.) or anther smut-fungi (*Microbotryum* spp.). Through the diversification of mating and dispersal stages, this huge variety of fungal lifestyles is highly integrated with equally diverse sexual cycles and breeding systems, a theme that has long been the subject of study by influential mycologists (1, 10–13).

Sexual Development and Determination of Cell Type Identity

Most fungi are able to undergo both asexual and sexual reproduction and have evolved tightly controlled mechanisms to regulate the process of mating, with respect to timing, and gamete dispersal, recognition and fusion. In basidiomycetes, the sexual cycle typically involves fusion of genetically distinct homokaryotic hyphae or haploid yeast cells to produce a dikaryon, in which the two haploid parental nuclei are replicated in a coordinated fashion

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without fusion during hyphal elongation, usually involving the formation of clamp connections (i.e. a hook-like structure formed by hyphal cells to ensure proper distribution of the two genetically distinct nuclei during mitotic cell divisions; see below) (14). Nuclear fusion (karyogamy) then takes place in the basidia or in other specialized structures (e.g., teliospores), after which the diploid nucleus undergoes meiosis to generate haploid basidiospores (meiospores) and complete the life cycle (see Fig. 1 for representative life cycles).

Despite the wide variation of sexual cycles in nature, one common underlying feature shared by most fungi is the lack of genetically determined anisogamy: many fungi are isogamous (i.e., where all gametes have the same sizes), and even in anisogamous species, all haploid genotypes produce both types of gamete sizes. This means that there are not individuals of different sexes in fungi (15). Furthermore, many fungi are heterothallic, meaning that syngamy can only occur between gametes of different genetically-determined mating types (15–17).

In members of the sister phylum Ascomycota, mating-type identity is governed at a single genetic mating type (*MAT*) locus (see Section xxx in this volume). In that case, only two mating types segregate in meiosis, defining what is termed a bipolar system. By contrast, basidiomycetes have evolved a breeding system that relies on two genetic *MAT* loci. One locus encodes tightly linked pheromones and pheromone receptors (hereafter referred to as "*P/R* locus") and the other encodes homeodomain-type transcription factors ("*HD* locus" hereafter) determining viability following syngamy. For successful mating and completion of the sexual cycle, haploid cells that conjugate must differ at both *MAT* loci (18, 19). When the two *MAT* loci are unlinked, four mating types can be generated by meiosis among the haploid progeny, defining this as a tetrapolar breeding system. Other basidiomycetes have instead a bipolar system controlled by a single *MAT* locus, either because the *P/R* and *HD* loci are linked or because one has lost its function in mating-type determinism. In mushroom-forming species (Agaricomycetes), there has been a generalized diversification of alleles at both *MAT* loci, in some cases yielding species with hundreds or thousands of possible mating types (1, 20–22). Data compiled from early studies indicate that as much as 65% of the species in the Agaricomycotina are tetrapolar (13, 23), whereas classical mating studies indicate a predominance of bipolar systems in the majority of the Ustilaginomycotina (24) and the Pucciniomycotina (25). In the following sections we summarize current knowledge on the molecular basis of mating-type determination in basidiomycetes and discuss transitions in breeding systems using examples from throughout the Basidiomycota phylogenetic diversity.

MOLECULAR DETERMINANTS OF MATING TYPE

The Pheromone/Receptor Sensing System

In the Basidiomycota, syngamy is governed by, in its simplest form, small 10–15 amino acid-lipopeptide pheromones derived from 35–40 amino acid precursors through post-translation modifications at both the N- and C-termini (26–28). These diffusible pheromones are received by seven-transmembrane domain pheromone receptors, coupled to a G-protein for downstream signal transduction (28, 29). This molecular determination of mating fusion

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is similar in part to the **a**- and α-factor pheromone/receptor (P/R) system in the ascomycetes and is thought to predate the separation of Asco- and Basidiomycota lineages. There is, however, one remarkable difference between the phyla: only homologues of genes encoding the pair of the **a**-factor pheromone and Ste3 pheromone receptor of the ascomycete system appear to exist in basidiomycetes. Therefore, instead of the **a**-factor/Ste3 and α-factor/Ste2 coupled sensing system characteristic of the ascomycetes (described in Chapter xxx), "chemo-sensing" specificity in basidiomycetes is mediated by allelic variants of the same type of genes (18). Mating is often initiated by a reciprocal exchange of pheromones recognized by matching pheromone receptor variants in both mating types, and thus two strains need to carry different alleles of the pheromone and receptor genes at the *P/R* locus.

All active basidiomycete mating pheromones isolated so far are hydrophobic diffusible lipopeptides that undergo farnesylation at the C-terminal cysteine residue of the CAAX motif (where "C" is cysteine, "A" is often an aliphatic amino acid, and "X" is any residue) and amino-terminal processing. This was first demonstrated in the secreted pheromone (rhodotorucine A) of the red-pigmented yeast *Rhodosporidium toruloides* (recently renamed as *Rhodotorula toruloides*) (30, 31) and later extended to many other basidiomycetes (26, 32–36), and also for **a**-factor of the ascomycete yeast *Saccharomyces cerevisiae* that now stands as the model paradigm for the fungal kingdom (37, 38).

Among the basidiomycetes, exceptional pheromone gene structures were revealed in members of the Pucciniomycotina. Where investigated, the pheromone precursor genes each encode tandem copies of the mature peptide moiety (39–43), and an initial step of processing of a spacer region is required to reveal C-terminal CAAX motifs for each repeat (37, 44). In species where pheromones of opposite mating types have been clearly identified (namely in *R. toruloides*, *Sporidiobolus salmonicolor*, *Leucosporidium scottii* and *Microbotryum* spp.), the *MAT A1* pheromone precursor typically presents a greater number of repeats of the peptide moiety (up to six copies in some species), whereas the *MAT A2* pheromone gene appears to encode only one or two copies of the mature pheromone (39, 42, 43, 45). Interestingly, this increased production of pheromone per mole of precursor in *MAT A1* cells, possibly allowing communication with *A2* cells at longer distances, is consistent with the observed asymmetric growth of conjugation tubes in both *R. toruloides* (46) and *Microbotryum* spp. (43, 47), which occurs earlier and to a greater extent from *MAT A2* cells. These findings suggest that the two mating types may have distinct signaling roles during conjugation, despite the absence of morphologically differentiated cells.

In response to the interaction of the pheromone ligand with a suitable pheromone receptor, mating and sexual development is initiated through a heterotrimeric G protein located at the plasma membrane and that triggers a variety of downstream signaling processes. In *U. maydis*, where this has been investigated in greater detail, this signaling is attributed to two interconnected pathways involving a cAMP-dependent protein kinase A (PKA) and a mitogen-activated protein kinase (MAPK) (28, 48–50). The point of convergence of the two pathways is the pheromone response factor (Prf1), which is an HMG-box transcription factor that recognizes and binds to pheromone response elements (PREs) located in the regulatory regions of pheromone-induced genes (51, 52). Interestingly, whereas most of the core components of the MAPK cascade pathway (e.g. Ste11, Ste7, Fus3) are conserved over

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wide evolutionary distances, the key downstream transcription factors that ultimately activate or repress their target genes, are often not conserved among species, and thus are difficult to reveal through candidate gene approaches or sequence comparison.

Homeodomain Transcription Factors: the Second Compatibility Checkpoint

While the *P/R* system controls syngamy, the production of viable mating products requires compatibility at additional genetic components in the basidiomycetes, which is mediated by genes at the *HD* locus. In its most basic configuration, the *HD* locus encodes a pair of proteins of dissimilar homeodomain classes, having protein domains HD1 or HD2, and their coding genes are normally adjacent and divergently transcribed. Development following syngamy depends upon the formation of functional heterodimeric transcription factors where dimerization is restricted to HD1 and HD2 proteins that originate from haploid mates bearing different alleles of the *HD* locus. This requirement ensures that active heterodimers are only formed in the dikaryotic stage and do not arise in haploid cells. Failure to form heterodimers in the haploid is explained by interfering amino acids at HD1 and HD2 proteins from the same allele that disturb their general cohesiveness (53–55). Studies have shown that the relevant interfaces for discriminating HD1–HD2 interactions and determining allele specificity seem to be located N-terminally to the DNA binding motifs and this region is usually highly variable between different alleles of both *HD1* and *HD2* genes (53, 54, 56). Analyses in *U. maydis* of chimeric and mutant alleles and protein-protein interaction studies using the yeast two-hybrid system, revealed that dimerization between HD1 and HD2 proteins involve polar-hydrophobic interactions with variable cohesive contact sites contributing to binding affinity (54, 57, 58). Once formed, the functional heterodimers will bind to gene promoter elements leading to the induction of the specific sets of genes involved in subsequent differentiation, which can include a morphological switch from yeast-like cells to filamentous growth and pathogenicity, as in the smuts, or fruiting body formation in the mushrooms (28, 41, 58–60).

BREEDING SYSTEMS IN THE BASIDIOMYCOTA

The distinction between heterothallic and homothallic compatibility represents a fundamental breeding system classification in fungi and both systems are found across the Basidiomycota. In heterothallic basidiomycete species, sexual reproduction is only possible between gametes carrying different alleles at both *MAT* loci, preventing intrahaploid mating (i.e. selfing at the haploid level) (61). On the other hand, homothallic species produce universally compatible gametes, each being able to undergo intrahaploid mating with their clonemates. We have summarized in Figs. 2 and 3 data regarding the breeding system and the genomic organization of the mating-type genes in representative species of the three major subphyla of the Basidiomycota, some of which will be highlighted in the following sections.

Tetrapolar Systems: *Ustilago maydis* and other smut relatives

The maize smut *U. maydis* (order Ustilaginales, Fig. 2) is one of the most intensively studied basidiomycete model with respect to the organization and function of mating-type genes (62). The fungus has a dimorphic life cycle with a yeast-like haploid saprophytic phase

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switching to filamentous pathogenic growth in the dikaryotic stage following syngamy (Fig. 1A). In this species, the process of mating and completion of the sexual cycle is regulated by a tetrapolar breeding system, i.e. with the *P/R* (or *a*) locus unlinked from the *HD* (or *b*) locus. The *P/R* locus exists in two allelic forms (termed *a1* and *a2*), which are defined by structurally dissimilar DNA regions of 4.5 kb for the *a1* and 8 kb for the *a2*, each comprising one pheromone receptor gene (*PRA1* or *PRA2*) and one pheromone precursor gene (*MFA1* or *MFA2*) (Fig. 3B). The Pra1 receptor only responds to Mfa2 pheromone, while Pra2 can only recognize Mfa1. The observed size difference between the two allelic forms of the *P/R* locus is due to the presence in the *a2* allele of two additional genes (*lga2* and *rga2*) that directs uniparental mtDNA inheritance, as well as a third, non-functional pheromone-encoding gene (26, 33, 52, 63). Interestingly, the presence of this pseudogene led to early speculations about the existence of a complex scenario ancestral to the Ustilaginales in which the *P/R* locus occurred in more than two allelic forms, with this additional cryptic pheromone gene being recognized by a distinct receptor encoded by a third unidentified *P/R* allele (52). This hypothesis was substantiated upon characterization of the breeding system of the closely related head smut fungus *Sporisorium reilianum*. Compared to *U. maydis*, the *P/R* locus of *S. reilianum* exists in three alleles, each containing two distinct pheromone genes and one pheromone receptor (64) (Fig. 3B). Importantly, each of the mature pheromones in one allele interacts specifically with only one of the receptors encoded by the other two alleles. For instance, the pheromones encoded by the *MFA1.2* and *MFA1.3* genes at the *a1* locus interact with the Pra2 and the Pra3 receptors encoded by the *a2* and *a3* alleles, respectively (Fig. 3B). This leads to a scenario where each mating type can detect (and be detected by) the other two, but not itself. Additional support for this ancestral triallelic *P/R* system model was more recently attained in a study where the complete *P/R* loci of additional smut species spanning about 100 million years of evolution in the order Ustilaginales were sequenced and compared (65). Interestingly, the three *P/R* alleles previously described in *S. reilianum* were conserved and generally syntenic among members of the same order. This strengthens a potential scenario where the biallelic *P/R* system as observed in *U. maydis* resulted from the loss of the *a3* allele and loss of one of the two pheromone genes, which is now lacking in extant *a1* and *a2* alleles. Accordingly, a phylogenetic analysis using pheromone receptor sequences from a wider taxonomic range (Fig. 4) shows that all Ustilaginomycotina receptors group together and within one of the three allelic classes previously defined (*PRA1*, *PRA2*, *PRA3*) (65). This result denotes that the triallelic *P/R* system is probably as old as the stem age of the Ustilaginomycotina, about 450 million years (66).

Following fusion of compatible partners, heterozygosity at the *HD* locus is required for dikaryon maintenance and filamentous growth in *U. maydis* (54, 67). The two divergently transcribed *HD* genes encode homeodomain proteins known as bE (for *bEast*; HD1-type) and bW (for *bWest*; HD2-type). However, in contrast to the biallelic and triallelic *P/R* locus of *U. maydis* and *S. reilianum*, respectively, the *HD* locus is highly multiallelic: in *U. maydis* more than 30 intercompatible allelic versions were found in nature [(68); DeVay, cited in (69)], while in *S. reilianum* five alleles have been identified so far (64). The homeodomains and C-terminal regions of the allelic HD1 and HD2 proteins are highly

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conserved, whereas the sequences N-terminal to the homeodomain regions are highly variable and required for the interaction of nonallelic HD1 and HD2 proteins (54).

Variation in Tetrapolar Systems Beyond *Ustilago*/*Sporisorium*

Outside the Ustilaginomycotina, the number of tetrapolar species characterized for molecular polymorphisms at the *MAT* loci has increased in recent years (42, 45, 70–74). *Kwoniella heveanensis* (70) and *Cryptococcus amylolentus* (71) in the Agaricomycotina, and *L. scottii* (42) in the Pucciniomycotina are examples where the *P/R* locus is bi-allelic while the *HD* locus display greater polymorphism (Figs. 2 and 3). In all three, the *P/R* and *HD* loci localize to different chromosomes. Sequencing of the *P/R* locus in each species showed their biallelic nature but, in comparison to *U. maydis* and *S. reilianum*, the *P/R* locus in *K. heveanensis* and *L. scottii* covers a relatively larger genomic region (about 32 kb and 50 kb, respectively; Figs. 3A and 3B), which is highly rearranged between mating types. Included here are the genes encoding *MAT*-specific pheromones (*MFA* or *RHA*) and receptors (*STE3*), as well as genes previously shown to be required for the onset of filamentation in dimorphic species (e.g. *STE20*, *STE12*) (75–77), and also lineage-specific genes not obviously involved in mating (Fig. 3). The *HD* locus in *K. heveanensis* and *C. amylolentus* contains only the divergently transcribed homeodomain genes, *HD1* and *HD2* (named *SXI1* and *SXI2*) (70, 71). In *L. scottii*, contrasting to *K. heveanensis* and *C. amylolentus*, the boundaries of the *HD* locus may have extended further outside from the *HD1/HD2* gene module given the evidence of increased sequence divergence in a region spanning about ~80 kb that encompasses the *HD1/HD2* genes (42). Sequence analysis of this variable region from different *K. heveanensis* isolates provided evidence that the locus is multiallelic with at least six different *HD1*/*HD2* alleles (70). The same holds true for *L. scottii*, but with 28 *HD* alleles being revealed upon inspection of 43 isolates (42).

From an evolutionary standpoint, it is noteworthy that extant tetrapolar dimorphic basidiomycetes have more commonly a biallelic *P/R* locus (26, 70–72, 74, 78). For these, the proportion of compatible mates in the population cannot exceed 50%, irrespective of the number of alleles at the *HD* locus. However, given that compatibility at the *HD* locus can only be assessed after syngamy, increased *HD* allele diversity should be naturally selected when outcrossing is the rule so as to allow more frequent dikaryon viability after syngamy and, in this way, limit the amount of wasted mating opportunities.

Tetrapolar Systems with Multiple Alleles in the Agaricomycetes

A vast repertoire of mating types has been found in most members of the class Agaricomycetes (Fig. 2), such as the wood-rotting mushroom *Schizophyllum commune* (Fig. 1D) with more than 15,000 different intercompatible mating types and in the ink cap mushroom *Coprinopsis cinerea* with an estimated 12,000 distinct mating types (1). Such diversity is likely due to selection for rare alleles under outcrossing: any new specificity will be compatible with all extant mating types and therefore may increase in frequency under this selective advantage (15, 79). Studies have shown that two hallmarks of the *MAT* loci of these and other heterothallic Agaricomycetes account for such astonishing numbers of mating types within a single species. First, a single locus may itself be extremely polymorphic, in some cases with hundreds of specificities (1, 13, 16); secondly, for some

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species, multiallelism in both *P/R* and *HD* loci is generated by rounds of segmental duplication and diversification of *MAT* genes resulting in independent, but functionally redundant subloci, upon which recombination can act to give rise to novel allele specificities, and by definition, new mating types (80).

Recent advances in genome sequencing are now allowing detailed comparisons of *MAT* loci among diverse species and a comparative overview of these genomic regions in several Agaricomycetes is presented in Fig. 3A. A great body of literature exists on the mating-type systems and distribution in Agaricomycetes, preventing a full discussion, and the reader is referred to some exceptional and recent reviews (16, 20). Here, we shall concentrate mainly on the model mushroom *C. cinerea*, which has long served as an invaluable model to understand the molecular underpinnings of multiple mating types and multiallelic *MAT* loci in the Agaricomycetes. Where relevant, reference will be made to other mushroom species, but many of the principles that govern the evolution of multiple mating types in *C. cinerea* seem to also hold in other species.

Initial studies in *C. cinerea* using classical genetics determined that the *HD* locus is multiallelic and composed of two subloci (*Aα* and *Aβ*; Fig. 3A) that can recombine at detectable frequencies despite their close proximity in the genome (81, 82). Molecular genetic studies and genome sequencing later revealed that, whereas the *Aα* sublocus encodes one *HD1/HD2* gene pair, the *Aβ* sublocus encodes up to three pairs and is located at a distance of ~7 kb from the *Aα* sublocus (Fig. 3A) (83–86). Recombination between the two subloci is possible through the ~7 kb-long intergenic region that is highly conserved between alleles. Sequence and functional analysis of five different *HD* alleles led to a theoretical archetypal structure for the *C. cinerea HD* locus with three paralogous *HD1/HD2* gene pairs (known as the *a*, *b* and *d* pairs). This implies that molecular mechanisms akin to gene deletion have been at work during evolution of the *HD* locus because the extant alleles have less than the six *HD1/HD2* genes postulated in model (exemplified by allele *A43* in Fig. 3A) (83–85). At present, four, seven and three distinct alleles have been identified for the *a, b* and *d* pairs, respectively (56, 85), generating 84 different *HD* alleles considering all possible permutations. Although this is about half the number estimated by John Raper of 160 *HD* alleles in nature (1), the identification of one additional allele per group would be sufficient to reach Raper's estimates. However, the actual numbers of alleles in nature is of course unknown.

Similarly, the *P/R* locus in *C. cinerea* is multiallelic and consists of three tandemly arranged but independent groups of paralogous genes (groups or subloci 1 to 3). The sequencing of several alleles revealed that each group contains multiallelic and functionally redundant genes encoding at least one pheromone receptor and up to four pheromone genes (Fig. 3A) (20). In any given group of paralogous genes, the relative order, direction of transcription and number of genes may vary and, importantly, each group has freely interchangeable alleles (80). In *C. cinerea*, from 13 sequenced *P/R* alleles, two, five and seven alleles were identified for groups 1, 2 and 3, respectively. Together, this gives rise to 70 possible combinations, approaching the number of 79 *P/R* alleles estimated to exist in nature (1). Phylogenetic analyses of *C. cinerea* receptors of the three paralogous gene groups and from different alleles added yet another layer of complexity in the evolutionary history. Strikingly,

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the different allelic versions of the pheromone receptors as defined by their position within the *P/R* locus do not always fall into the same clade (20, 80), an observation that led some authors to conclude that gene shuffling occurred within the locus during evolution (80). This pattern has also been observed in *S. commune* (87) and in *Ganoderma lucidum* (88). Such relocations may have restricted intra-locus recombination at some point during evolution, thereby contributing to the fixation of some of the alleles in the populations.

With additional pheromone receptors from other Agaricomycetes added to the phylogenetic analysis, the origin of the paralogous groups of genes within the *P/R* locus can be traced back to an early stage of the evolution in this taxonomic lineage, with a duplication event initially separating these genes into two distinct evolutionary groups (clades 1 and 2 in Fig. 4). More recent duplications (e.g. *CcSTE3.1* and *CcSTE3.3* in *C. cinerea* or *LbSTE3.1* and *LbSTE3.3* in *Laccaria bicolor*; Fig. 4) followed by sequence diversification and recombination (80) are suggested to have led to the high numbers of alleles presently estimated from natural populations of several mushroom species [(16, 20, 41) and references therein].

In *C. cinerea* and other Agaricomycetes, each encounter of two monokaryotic hyphae results in promiscuous cell-cell fusion irrespective of whether or not they are compatible at *MAT* (18, 89). However, for the development and maintenance of the dikaryon, nuclei of interacting partners must harbor different allelic versions of genes in at least one sublocus of both *P/R* and *HD* loci (18, 89–91). Classical genetic analyses of these semi-compatible interactions in *C. cinerea* and *S. commune* have shown that the *P/R* system is involved in events that follow hyphal fusion, namely in the reciprocal exchange and migration of nuclei and clamp cell fusion (Fig. 5). On the other hand, genes encoded at the *HD* locus repress asexual sporulation (92), regulate pairing of nuclei within the dikaryotic tip cell and coordinate nuclear division, clamp cell formation and septation from the subapical cell (1, 18, 93) (Fig. 5). If the two mates share identical alleles at *P/R* and *HD* loci, no nuclear exchange occurs and the fungal individuals resume monokaryotic growth.

Additional pheromone receptor-like genes (i.e. *STE3*-like) have been identified from numerous Agaricomycetes using sequence similarity-based approaches (Fig. 3A) and many fall within one of the three clades of agaricomycete mating-type receptors (subclades A and B of Clade 1 as well as Clade 2; Fig. 4). However, their function remains largely elusive, as reports suggest that these are non-mating-type-specific receptors (94, 95), and experimental evidence in *S. commune* suggest that they are not sufficient *per se* to induce mating-specific development (96). Many of these non-mating-type-specific receptors are located in the close neighborhood of the *P/R* locus while others are unlinked (Fig. 3A). Three distinctive features of these Ste3-like receptors are seen in comparison to the mating-type-specific receptors: (i) they present longer C-terminal cytoplasmic regions, (ii) lack pheromone genes in close association and (iii) show lower levels of intraspecific polymorphism. Non-mating-type-specific receptors have been documented in *C. cinerea* (41), *Phanerochaete chrysosporium* (97), *L. bicolor* (98), *Postia placenta* (99), in several species of the order Polyporales (100) and many more can be expected to be identified from whole-genome sequence data. For example, up to 29 Ste3-like receptors were identified in the genome of *Serpula lacrymans* (Fig. 2). A few theoretical possibilities have been proposed for their function (41). One such

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possibility considers that they may play a role during monokaryotic fruiting, in a way similar to that described for the *C. neoformans* non-mating type receptor gene *CPR2*, whose overexpression elicits homothallic reproduction (101). Another hypothesis suggests that these genes may function in vegetative communication within a species, or in conspecific recognition avoiding hybridization (41). Experimental studies addressing the function of these non-mating type specific receptors are thus critically needed.

Comparative genomics revealed that gene content and organization (synteny) are generally conserved around the *HD* locus in Agaricomycetes. Two genes are usually bordering this locus: one encodes a mitochondrial intermediate peptidase (*MIP*), which has served as a valuable marker for the isolation of *HD* loci from non-model species, and the other is known as beta-flanking gene (*β-fg*) for an unknown protein (Fig. 3A) (94, 100, 102). Although this configuration is found in most species analyzed, exceptions have been reported. In *S. commune*, for example, two large rearrangements at the *HD* locus have taken place causing the separation of the *HD* locus into two clearly distinct subloci that now lie more than 500 kb apart and frequently recombine during meiosis (Fig. 3A) (103, 104). A similar situation is observed in *Flammulina velutipes* (winter mushroom or enoki), but the distance between the two *HD* subloci is about 70 kb. Interestingly, whereas synteny analysis of the surrounding regions indicates that the separation of the two *HD* subloci in *S. commune* emerged via transposition of the two gene clusters, the extant arrangement in *F. velutipes* was driven by two large inversions (95). Other exceptions to the typical *MIP – HD1/HD2 – β-fg* gene arrangement were observed in the cacao pathogen *Moniliophthora roreri*, where both *MIP* and *β-fg* are located 40 and 60 kb upstream of *HD1*/*HD2* (105), respectively, and in the Shiitake mushroom, *Lentinula edodes*, where *MIP* is distant to the *HD* locus (106). Gene order conservation at the *P/R* locus is also observed among Agaricomycetes, although to a lesser extent compared to the *HD* locus. The *P/R* locus in *L. bicolor* was shown to be in a chromosomal region more prone to gene duplication, translocations, and accumulation of transposable elements (98), and in the wood-decay basidiomycete *Botryobasidium botryosum* (order Cantharelalles; Fig. 2), genome inspection revealed the presence of transposons in the immediate vicinity of pheromone and receptor genes (Fig. 3A; this report). Another illustrative example is the position of *STE20* and *STE12* genes, which varies among the species shown in Fig. 3A.

Tetrapolarity with multiple alleles at both *MAT* loci is often suggested to promote outcrossing (107, 108). It is true that tetrapolarity is less favorable to selfing than to outcrossing, as the chances that any two haploid sibling from the same diploid parent are compatible is at most 25% for tetrapolarity, compared to 50% from a bipolar cross (107). However, tetrapolarity also yields only 25% compatibility under outcrossing when both mating-type genes are biallelic, so that tetrapolarity in itself does not favor outcrossing compared to selfing under biallelism. It is multiallelism that may restrict inbreeding, however, and the evolution of high numbers of mating types is suspected to be primarily driven by selection for increased compatibility at syngamy under an already established outcrossing mating system. An evolutionary scenario could be one with ancestral mating-type determinism being biallelic at *HD* locus, the high level of gamete dispersal evolves, leading to widespread outcrossing, which in turns selects for rare alleles, yielding multiallelism at the *HD* locus. Recruiting a biallelic *P/R* locus in mating-type determinism

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would not favour outcrossing, as again compatibility odds will be similar under outcrossing vs. selfing. Different scenarios for explaining the incorporation of the *P/R* locus in the mating-type determinism system are possible, such as its role in syngamy or mate attraction, or again evolution first of multiallelism (e.g. due to population differentiation). Studies addressing the actual pattern of mating in nature, depending on gamete dispersal rates and polymorphism levels at the mating-type genes, will be fundamental to test some of these predictions.

Bipolar Breeding Systems: Genomic Changes and Evolutionary Considerations

Early studies indicated that, although the tetrapolar mating system predominates in the Basidiomycota, a considerable number of species are bipolar. For example, roughly a quarter of the mushroom species are bipolar (1, 13) and, significantly, they occur phylogenetically interspersed with tetrapolar relatives (100). Owing to the occurrence of multiple bipolar species within the basidiomycetes, the origin of tetrapolarity as the ancestral state to this clade is still debatable. However, several lines of evidence argue strongly in favor of this hypothesis. First, the tetrapolar system is architecturally complex making it unlikely to have arisen multiple times (23, 109). Second, the same mating-type-determining genes (at both *P/R* and *HD* loci) are involved in mating-type determinism in species of the three major basidiomycete lineages, implying a common origin (41, 42, 108). Third, all bipolar species investigated so far display genomic signatures suggesting that they derived secondarily from a tetrapolar system (detailed below). Based on these observations, the prevailing hypothesis is that the tetrapolar system evolved in a basidiomycete ancestor following the acquisition of a separate locus (the *P/R* locus) for *MAT* determination that segregated independently of the *HD* locus, and with multiallelism evolving in many lineages (17, 41).

As initially envisaged by John Raper (1), and more recently supported by solid molecular evidence (detailed below), bipolarity in the Basidiomycota appears to be the result of simple genetic changes. There are two general ways this can be achieved: (i) by linkage of the *P/R* and *HD* loci into a single, non-recombining *MAT* region with often only two alleles of each locus being retained; or (ii) by loss of function of the *P/R* locus in mating-type specificity, conceivably through mutations that cause constitutive expression of the *P/R*-regulated pathway or that produce a self-compatible allele at the *P/R* locus.

Transition to bipolarity in smuts and *Malassezia* through linkage of *P/R* and *HD* loci—There are now several direct examples that support the formation of a bipolar system through a chromosomal translocation that places both *P/R* and *HD* loci in tight linkage. In the Ustilaginomycotina, a prime example is provided by *Ustilago hordei*, a bipolar smut pathogen closely related to tetrapolar *U. maydis* (Fig. 2) (110). With the molecular analysis of the *MAT* loci, initially in *Ustilago maydis* (26, 59, 111, 112), the genomic basis to the bipolar mating behavior of *U. hordei* was subsequently revealed: both *P/R* and *HD* complexes were physically linked on the same chromosome, 430 and 500 kb apart on the largest chromosome representing the respective *MAT-2* and *MAT-1* loci (Fig. 3B), and the presence of inversions in between these complexes was proposed to cause the almost complete lack of recombination of the complexes (113–115), likely resulting in a subsequent accumulation of transposable elements. Detailed analyses have since then been

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published on mating types in several Ustilaginomycotina species. Complete genomes of five species of closely-related genera and varying in mating-type organization are available: tetrapolar *U. maydis* (116) and *Sporisorium reilianum* (117), and the bipolar species *U. hordei*, with the *HD* and *P/R* loci 430 – 500 kb apart (118), *Sporisorium scitamineum*, with the *HD* and *P/R* loci 59 kb apart (119, 120) (Fig. 3B) and *Ustilago bromivora*, with the *HD* and *P/R* loci 183 kb apart (121). By comparing chromosomal rearrangements between *U. maydis, U. hordei* and *S. reilianum*, it was suggested that the tetrapolar organization in the *S. reilianum* lineage was ancestral, and that part of *S. reilianum* chromosome 1 harboring the *HD* locus was translocated to its chromosome 20 harboring the *P/R* locus, to generate the bipolar organization in *U. hordei* (118). This potential scenario was strengthened in the recently published bipolar *S. scitamineum* genome: homologous parts of *S. reilianum* chromosomes 1 and 2 seem rearranged to give chromosome 2 in *S. scitamineum* (120). In all these cases, the presence of many repetitive and transposable elements at the breakpoints suggested they may be functionally involved (122). As stated above, the resulting spacing between the *HD* and *P/R* loci in these various species varied between 59 and 500 kb and this was likely a consequence of increasing numbers of such elements due to decreased recombination.

A similar *MAT* loci configuration has also been recently identified in the human skin commensal yeast *Malassezia* species complex that are responsible for dandruff and other skin pathologies. These include *M. sympodialis*, *M. globosa*, and *M. yamatoensis* (123, 124) (Fig. 2). Similar to *U. hordei*, the *P/R* and *HD* loci in these three *Malassezia* species are also located on the same chromosome, and are separated by large chromosomal regions that are >100 kb in size. Interestingly, comparison of the chromosomal regions encompassing the *P/R* and *HD* loci in these species also suggests that the linkage between the two *MAT* loci are the result of two independent events, with one giving rise to the linkage between the two *MAT* loci in *M. sympodialis* and *M. globosa*, while the other rearrangement led to the *P/R* and *HD* linkage in *M. yamatoensis*. Additionally, analyses of natural isolates of *M. sympodialis* suggest that recombination is still occurring between the *P/R* and *HD* loci in natural populations, indicating that linkage between the two *MAT* loci is not tight. In agreement with this, the 170-kb *MAT* region of *M. globosa* does not contain accumulations of repetitive elements as found at the *MAT-1* locus of *U. hordei* (125), as is expected in regions of suppressed recombination. Moreover, a preliminary examination of the *P/R*-containing region in the genomes of two *M. sympodialis* strains of opposite mating types revealed conserved gene order in the immediate vicinities of the *P/R* gene pair, which are found adjacent but inverted in both mating types (Fig. 3B; this report). The breeding system in *Malassezia* species is thus considered “pseudo-bipolar”, a term initially proposed for the breeding system of the red-pigmented yeast *S. salmonicolor* to reflect a situation where multiallelism might occur despite the apparent loose linkage of the two *MAT* regions (45); linkage is expected to be selected for under selfing mating systems, under which rare alleles have no advantage, leading to gradual loss of alleles but two by genetic drift.

Bipolarity in pathogenic *Cryptococcus* species complex and in *Trichosporon* —Outside the Ustilaginomycotina, deviations from the classic tetrapolar system have also been observed in a variety of species. For example, all the species within the human

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pathogenic *Cryptococcus* species complex (Tremellomycetes, Agaricomycotina; Fig 2) (126) have bipolar mating systems. In these species, there are two mating types, *MAT***a** and *MAT*α, defined by two alleles (**a** and α) at a single large *MAT* locus (Fig. 3A). The *MAT* locus in pathogenic *Cryptococcus* species is ~120 kb in size and contains about 20 genes, including those encoding mating pheromones and their cognate receptors and homeodomain transcription factors (*HD1* or *SXI1* and *HD2* or *SXI2*). There is however a distinctive feature regarding the normally two-gene homeodomain function in this group of species: the *HD1* (or *SXI1*) gene is unique to *MAT*α strains, whereas the *HD2* (or *SXI2*) is specific of the *MAT***a** strains (Fig. 3A). This is opposed to other basidiomycetes in which a divergently transcribed *HD1/HD2* gene pair is present in each mating type. Accordingly, each mating type in pathogenic *Cryptococcus* species has been reduced to the minimal number of *HD* genes required to function upon fusion of compatible cells (127). Recessive losses of genes from one mating type could likely occur because each is sheltered in a permanent hemizygous state due to recombination suppression within the *MAT* locus, associated with extensive chromosomal rearrangements. However, gene conversion does occur within these regions and at a frequency that is at least comparable to the crossover frequencies in other chromosomal regions (71). Additionally, it has been shown that recombination hotspots associated with the presence of GC-rich motifs are located at the regions that flank the *MAT* locus in *Cryptococcus* (128, 129), which could potentially function as repressors of crossover within the *MAT* locus. Comparing the *MAT* loci of closely related tetrapolar species such as *Cryptococcus amylolentus* (71) and *Cryptococcus heveanensis* (70), suggests that the single *MAT* locus in the pathogenic *Cryptococcus* species is the result of fusion of ancestral *P/R* and *HD* loci, possibly mediated by repetitive sequences and transposable elements that may act as substrates for non-homologous recombination (130, 131).

A recent genomic study found that in the oil-accumulating yeast *Trichosporon oleaginosus* (recently renamed as *Cutaneotrichosporon oleaginosus*; Figs. 2 and 3A) as well as in some of its sister species, the mating-type regions show significant structural differences from those of other Tremellomycetes that possess tetrapolar breeding systems. Specifically, no homologs of the *HD2* transcription factor could be identified in the genomes of *T. oleaginosus* and *T. asahii*, suggesting that this transcription factor might have been lost during the evolution of the *Trichosporon* species complex (132). Alternatively, this gene is absent from only one mating type like the situation in the *Cryptococcus* species complex; a possibility which cannot be ruled out since the three sequenced strains carried the same alleles of *P/R* and *HD* genes. However, if the latter hypothesis ends up being true, then such gene loss would represent a case of parallel evolution in *Trichosporon* and in the human pathogenic *Cryptococcus* species complex given that the last common ancestor of the two lineages most likely contained the standard *HD1/HD2* gene arrangement. It would be of interest in this scenario to test whether the *HD* gene loss is associated with established genetic linkage between the genomic regions that contain the *P/R* and *HD* genes (Fig. 3A). Unfortunately, the fragmented nature of the available genome assembly of *T. oleaginosus* (132) and the lack of descriptions of a sexual cycle for any *Trichosporon* species, are preventing a more definitive answer at this time.

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Expansion of the mating-type locus in *Microbotryum* following linkage of *P/R* and *HD* loci—The findings in the aforementioned bipolar species support the hypothesis of a tendency toward expansion of the regions of recombination suppression in mating-type regions in fungi (133), although it is not yet clear what underlying factors have been driving this trend, e.g. whether it is simply the linkage of the two mating-type loci. In line with this, a more extreme case of the evolution of bipolarity from tetrapolarity by linkage between the two *MAT* loci is observed in the anther-smut fungus *Microbotryum lychnidis-dioicae*. All species belonging to the genus *Microbotryum* are castrating floral smut pathogens that parasitize plants in the Caryophyllaceae family (Fig. 1B), forming a complex of sibling species highly specialized on different plant species (61, 134, 135). The most studied species is *M. lychnidis-dioicae* parasitizing *Silene latifolia* (136). This fungal species is bipolar, with only one *MAT* locus and two alleles, called a_1 and a_2 (137). This is among the first fungal species where bipolar mating types have been described (138) and also the first fungal species where size-dimorphic mating-type chromosomes were revealed (139). A sex event is required before each new plant infection, and *M. lychnidis-dioicae* displays a highly selfing mating system under heterothallism, performing mostly intratetrad mating (a.k.a. automixis) (140–142). The populations are therefore highly homozygous throughout much of the genome (143, 144).

A recent high-quality assembly of the genome of *M. lychnidis-dioicae* has revealed that bipolarity and mating-type chromosome dimorphism is due to the linkage of the *P/R* and *HD* loci in a very large region of suppressed recombination (145, 146). The centromere is also located in the non-recombining region (145), which leads to the segregation of mating types in the first meiotic division. The mating-type chromosomes of *M. lychnidis-dioicae* thus do not recombine across 90% of their lengths (a_1, 3.5 Mbp; a_2, 4.0 Mbp), with only small pseudo-autosomal regions at both edges that recombine. The non-recombining region is highly rearranged, with at least 210 inversion events. As expected due to the less efficient selection in genomic regions without recombination (147), the cessation of recombination has led to genomic degeneration, with accumulation of transposable elements, of non-synonymous substitutions, biased gene expression and the hemizygous loss of hundreds of genes (145, 148).

Most other anther-smut fungi are bipolar with dimorphic mating-type chromosomes that also show degeneration (148); one clade has recently been found to have a tetrapolar breeding system, in particular *Microbotryum saponariae* and *Microbotryum lagerheimii*, respectively parasitizing *Saponaria officinalis* and *Silene vulgaris* (73). In these closely related species, the two *MAT* loci segregate independently, being located on different chromosomes, but are linked to their respective centromeres (73). This situation may have resulted from a reversion from bipolarity to tetrapolarity, or the independent evolution of linkage of *MAT* loci under automixis in the other lineages. Indeed, both bipolarity with linkage to the centromere, as in *M. lychnidis-dioicae*, and linkage of both *MAT* loci to their respective centromeres on different chromosomes, as in *M. saponariae*, are equally favorable under automixis in anther smut fungi (73, 145).

Transition to bipolarity through loss of function of the *P/R* genes in mating-type determination—It is not surprising that changes in the configuration of the *MAT*

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loci can alter the mating behavior of the individuals, sometimes resulting in transitions between different breeding systems. For example, in tetrapolar heterothallic species, the *HD* locus encodes genes that heterodimerize during mating to form active transcription regulators, ensuring the proper development following syngamy. However, the heterodimer can only form between HD1 and HD2 proteins derived from different alleles. Thus, if recombination occurs between the *HD1* and *HD2* genes resulting in bringing together compatible *HD1* and *HD2* alleles on the same chromosome, a haploid individual bearing this novel *HD* genotype pair would transition from compatibility determined by a two sequential compatibility checkpoints (*P/R* and *HD*) to a single mechanism (*P/R*) inherited in a bipolar manner. This newly formed *HD* allele could also be the first step in a transition from heterothallism to homothallism provided that compatible pheromones and receptors are brought together at the *P/R* locus in a subsequent event. Examples of these types of transition have been reported in *C. cinerea*, where fusion of the *HD1* and *HD2* genes from different subunits results in self-compatibility and constitutive sexual development (149). Alternatively, mutations in a pheromone receptor gene in *C. cinerea* resulted in a receptor that was either constitutively activated (150) or that responded erroneously to a normally incompatible pheromone encoded within the same allele (151), removing its function in mating-type determination.

Studies of several other Agaricomycetes, including *Coprinellus disseminatus* (152), *Pholiota microspora* (153, 154), *Phanerochaete chrysosporium* (94) and *Heterobasidion irregulare* (155) have shown that a transition from a tetrapolar to a bipolar breeding system was indeed due to the uncoupling of the *P/R* from mating-type determining functions. These bipolar species retained nonetheless a multiallelic *HD* locus, with the number of alleles defining the number of mating types (e.g. ~123 mating types are estimated for *C. disseminatus*) (16, 152). Therefore, a new mating type will arise as soon a novel *HD* allele is generated. However, evolving novel *HD* alleles that are still able to heterodimerize with extant alleles, while restricting self-activation, will become increasingly challenging (156). Compared to the tetrapolar multiallelic species such as *C. cinerea* and *S. commune*, and assuming that *HD* alleles are found at equal frequency in the population, the probability of randomly chosen individuals being compatible in an outcrossing event is theoretically greater in bipolar multiallelic species: only a single locus needs to be compatible, rather than two in the tetrapolar situation (16). Furthermore, considering that mating in mushroom-forming fungi is preceded by basidiospore (meiospores) dispersal, outcrossing rather than selfing is more likely to occur (16, 61). Therefore, and because mating-type compatibility is not discriminated prior to syngamy, the costs of landing on an incompatible mate are in this case very high. It can thus be easily envisaged that mutations at the *P/R* locus causing loss of function in mating-type determination may have been selected for, in some circumstances, because the chance of compatibility would be greatly increased, and there is no selective pressure anymore to restrict inbreeding, that is already avoided by gamete dispersal.

Homothallism in the Basidiomycota

In some fungal species, haploid cells are universally compatible for mating, including among clonemates (a.k.a. intrahaploid mating, same-clone mating, or haploid selfing) (156) (15, 79). We call these species homothallic, and several proximal mechanisms have been

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proposed to account for such mating behavior, namely: (i) the presence of compatible sets of *MAT* genes, either fused or unlinked, in one haploid genome (primary homothallism); (ii) mating-type switching (either bidirectional or unidirectional); and (iii) the lack of any gene-based mating-type discrimination (also known as ability for unisexual reproduction or same-sex mating) [reviewed in (157, 158)]. Some other fungal species, referred to as pseudohomothallic, also have the ability to complete the sexual cycle without the apparent need for syngamy (159–161). However, pseudohomothallism is achieved by packaging two nuclei of compatible mating types derived from the same meiosis into a single spore. This process is thus more similar to diploid selfing via automixis or intratetrad mating and has different genetic consequences compared to intrahaploid mating (79, 162).

Homothallism is present in three main fungal lineages (the phyla Mucoromycota, Ascomycota and Basidiomycota) indicating that this form of sexual behavior may provide a selective advantage under certain conditions, for instance, by promoting universal compatibility (79, 162). Compared to other phyla, however, homothallic basidiomycete species are less common. Whether this relates with the complex genetic underpinnings of the mating system in this phylum, as described above, or because they can more easily evolve multiple alleles allowing high frequencies of mating compatibility, is still an unresolved matter. Primary homothallism has been proposed for a handful of species in the Basidiomycota, most notably *Sistotrema brinkmannii* (163), in which homothallic and bipolar heterothallic individuals are found in the same population, and for the cacao pathogen *Moniliophtora perniciosa* (164). A partial genome sequence is only available for the latter species, which upon examination revealed various genes for putative pheromones and receptors and one or possibly two *HD1* genes (165), but their genomic organization as well as their function during the sexual cycle remains undetermined. We present below details in two basidiomycete species where there has been progress in understanding the genetic basis of homothallism.

Homothallic breeding system of *Phaffia rhodozyma*—Recently, inspection of the genomes of three strains of the homothallic yeast *Phaffia rhodozyma*, belonging to the order Cystofilobasidiales (Fig. 2) (166), revealed the presence of pheromone precursor and receptor genes organized in two gene clusters, located ~5kb apart. Each cluster was composed of one pheromone and one pheromone receptor gene (either *MFA1*/*STE3-1* or *MFA2*/*STE3-2*; Fig. 3A) (167). In contrast, a single *HD1/HD2* gene pair was found in the genome that was not linked to the *P/R* locus (Fig. 2) (167). In elucidating the molecular interactions involved in mating compatibility in *P. rhodozyma*, David-Palma et al. (168) pursued targeted gene replacement of the *MAT* genes and genetic crosses using single, double and triple mutants (168). They determined that both *P/R* gene clusters were producing intercompatible gene products, enabling self-signaling, and each pheromone only interacted with the receptor of the other cluster (i.e. Mfa1 activates Ste3-2 and Mfa2 activates Ste3-1; Fig. 3A). Additionally, the single pair of HD1 and HD2 homeodomain proteins were also shown to be required and apparently cooperated for normal completion of the sexual cycle (168). Even though the genetic makeup of *MAT* loci in *P. rhodozyma* is suggestive of primary homothallism, David-Palma et al. (168) pointed out why this configuration departs in several aspects from what would be expected from simply gathering

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in a haploid genome the intercompatible alleles of *MAT* genes. First, although the two pheromone receptor genes exhibited considerable sequence similarity (~50% amino acid identity), phylogenetic analyses indicated that they were more closely related to each other than with receptor genes in other species, and both seemed to derive from the *MAT*α/*A1*-related allele lineage (Fig. 4). This indicates that the two pheromone receptor genes in *Phaffia* have diverged much more recently than in most basidiomycetes (possibly coalescing within the genus; M. David-Palma, pers. comm.), and the extant arrangement may be the result of fusion of two mating-type variants. The second aspect concerns the *HD* genes, in that only one *HD1*/*HD2* pair is present, but both proteins are required for sexual development. Based on evidence of a very weak interaction between the two *HD* proteins in a yeast two-hybrid assay (168), it was proposed that at the generation of a compatible HD1/HD2 pair within the haploid genome there would need to be one or more mutations relaxing the structural hindrance that normally prevents interactions between HD proteins encoded by the same locus in heterothallic species (168). Similar investigations are currently underway in other species of the Cystofilobasidiales in order to reconstruct the evolutionary transitions in breeding system in this lineage (M. David-Palma, pers. comm.).

Homothallism in *Cryptococcus neoformans*—While most homothallic species can initiate sexual reproduction through fusion with any other haploid cell, including clonemates, they do so through the presence of two intercompatible mating alleles within a haploid genome. In the human pathogenic fungus *C. neoformans*, the proximal mechanism for homothallism is different as it concerns haploid cells with a single mating-type allele in their genome (169). Specifically, while *C. neoformans* has a bipolar breeding system where mating typically occurs between *MAT***a** and *MAT*α cells, it has been found that, in response to nutrient limitation, *MAT*α cells can also complete the sexual cycle in the absence of *MAT***a** cells (169). The α-α diploid state can be established either through: (i) cellular and nuclear fusion of two clone mates, (ii) nuclear fusion between mother and daughter nuclei, (iii) endoreplication of the entire genome of a single cell resulting in a diploid nucleus, or (iv) fusion of cells of the same mating type but of different genetic lineages (169). While the first three pathways represent different ways to achieve same-clone mating (haploid selfing) the latter enables outcrossing, and thus the possibility to admix genetic diversity despite identical cell type identity of the mating partners. In all cases, the resulting diploid cell undergoes monokaryotic filamentation and subsequent basidium formation where meiosis takes place to produce the haploid spores as in the heterothallic mating cycle (Fig. 1C). Importantly, recent studies in this model basidiomycete have shown that α-α mating between genetically identical cells can generate phenotypic and genotypic diversity *de novo*, including single-nucleotide polymorphisms (SNPs) (less frequently), chromosomal translocations, and aneuploidy (more frequently) which, although most often likely deleterious, may rarely provide a beneficial novelty (170). Moreover, crossovers have also been detected within the *MAT* locus during meiosis of genotypes resulting from α-α mating (129), presumably because the two *MAT*α alleles are co-linear, unlike **a**-α combinations (130). It should be pointed out that among the *C. neoformans* natural isolates, the vast majority (up to >99%) are of the α mating type (171). Thus, α-α mating could be beneficial when a compatible mating partner for heterothallic cross is hard to find in nature. In turn, it is also possible that α-α mating played a role in generating the highly imbalanced mating-

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type ratio observed among natural isolates. Recently, this type of homothallism determinism has also been identified in the human pathogen *Candida albicans* (172, 173) as well as the tree wound-infecting fungus *Huntiella moniliformis* (174), suggesting it could be more prevalent among fungal species than is currently appreciated.

Evolutionary considerations on the costs of homothallism—Although it has been traditionally accepted among mycologists that homothallism evolved to promote same-clone mating (170, 175), it is important to point out that a potential for same-clone mating of a species as assessed in a laboratory setting does not imply that this is the prevalent mating system of the species in nature. Moreover, the sexual cycle involving clone mates does not result in actual recombination, the supposedly main advantage of sexual reproduction (176), while still incurring some costs (e.g. slower reproduction and maintenance of the meiotic machinery). Same-clone mating is less efficient at purging deleterious mutations: homologous chromosomes carrying different deleterious mutations having occurred in a given large clonal lineage can of course be recombined and produce a chromosome free of deleterious mutations (177), but this constitutes more outcrossing (i.e. mating between two different genotypes) than same-clone mating *per se*. Besides, in a population undergoing only same-clone mating, different deleterious mutations that are fixed by genetic drift in distinct clonal lineages cannot be eliminated from the populations, and Muller's ratchet will inexorably lower population fitness (133). Furthermore, same-clone mating cannot bring together different beneficial mutations having evolved in distinct clonal lineages (Hill-Robertson interference). A transition to asexuality would bypass some of the costs of sex while producing haploid progeny that are genetically the same as the participants in same-clone mating.

Homothallism has been suggested instead to have evolved due to the selective advantage of increasing the number of available mates, as homothallism can be seen as a universal compatibility (15, 79). The frequency of outcrossing in homothallic species under experimental conditions of mate choice has been rarely explored, but outcrossing has still been shown to occur in some homothallic fungi (178, 179). Homothallism would therefore be advantageous through universal compatibility under outcrossing. However, given that same-clone mating incurs costs of sex without some of the major benefits of recombination, heterothallism may be favoured by selection for increasing the chance to produce recombinant offspring when there is limited haploid dispersal (15, 79). Nevertheless, same-clone mating may allow benefit from indirect advantages of the sexual cycle without breaking apart beneficial allelic combinations (180, 181). Such advantages include the production of resistant sexual spores as a life history constraint, changes in ploidy level and physiology, virus elimination, and genome defenses that depend upon the sexual cycle, such as repeat-induced point mutation (182) or meiotic silencing of unpaired DNA(183). Recent studies also showed that same-clone mating can generate *de novo* mutations and new beneficial phenotypes (170).

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New Genome Sequencing Projects: What Can They Tell Us about *MAT* Gene Structure and Evolution

With the exponential growth of data resulting from several large-scale genomics initiatives, such as the 1000 Fungal Genomes Project at the Joint Genome Institute (JGI) (184) or the Fungal Genome Initiative at the Broad Institute (185), it is now possible to identify and compare the genomic organization of *MAT* genes across many fungal species. For example, genome surveys allowed the detection of *P/R* and *HD* genes in important plant pathogens with complex life cycles, such as the rust fungi (Pucciniales, Pucciniomycotina; Fig. 3B) (40, 186). Indeed, because many rust species are macrocyclic (i.e. completing their sexual stage on a different and sometimes obscure or unknown alternate host plant), and sometime unculturable, sexual compatibility has been very difficult to study. There are some reports that have shed light on possible breeding systems: several *Puccinia* and *Uromyces* species may have a simple bipolar system (187) whereas a more complicated tetrapolar system with multiple allelic specificities may be present in *Melampsora lini* (188) and the related oat crown rust pathogen, *Puccina coronata* (189). Whether these and other species have bi- or multi-allelic *MAT* loci remains virtually unknown, but new insight is now arising from different fungal genome sequencing initiatives.

In a recent study, several new wheat rust, *Puccinia* species genomes were analyzed for the presence of mating-type genes (186). In three cereal-infecting rust fungi, *P. triticina* (*Pt*), *P. graminis* f.sp. *tritici* (*Pgt*) and *P. striiformis* f.sp. *tritici* (*Pst*), one divergently-transcribed *HD1*/*HD2* gene pair was found, reminiscent of the organization in other basidiomycetes; two alleles of each were present in the dikaryotic urediniospores which is the cell type produced upon mating. Preliminary analyses of multiple genome sequences allude to the presence of more than two alleles in nature (GB, unpublished). Three homologs for the pheromone receptor (Ste3) were identified in the dikaryotic urediniospores. Phylogenetic analysis, comparing Ste3 proteins from various rusts and other basidiomycetes, revealed that one *STE3* gene (*PtSTE3.2-1*) was present in two allelic forms, whereas the other two (*PtSTE3.2-2* and *PtSTE3.2-3*) likely represented the two mating specificities, one in each haplotype genome (186) (Figs. 3B and 4). This was substantiated by revealing many more SNPs in *STE3.2-1* homologs in *Pt*, *Pgt* and *Pst* among several sequenced genomes and the conclusion, also based on differential expression in various life cycle stages, is that this gene may represent an novel function (186), suggesting that *MAT* pathway components may be repurposed. In further support, a pheromone precursor gene (*PtMFA2*) was identified 650 bp from *PtSTE3.2-2*, divergently transcribed as found e.g. in the *P/R a2* locus of *U. maydis*. However, the current rather fragmented *Puccinia* genomes did not allow to draw further conclusions as to the possible physical linkage of the *HD* and *P/R* gene complexes to constitute a bipolar arrangement, but based on the current assembly and some mapping data in *Pt*, these loci are at least 216 kb apart (186). Current developments of single molecule sequencing by Pacific Biosciences, focusing on generating very long kb-sized reads, are expected to allow improving these genome assemblies in the near future.

The basidiomycete fungi have been found to undertake nearly every permutation of reproductive compatibility mechanisms, and the recent literature reviewed here illustrates that the high diversity at mating compatibility loci has remained for several decades as “the

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most challenging problem in the control of [their] sexuality" (190). The advent to inexpensive molecular tools now provides access to genetic information in a broader range of non-model species, including at the population-genomic level, so that more comprehensive databases are being assembled. Such surveys shed light on the generalities of what *does* happen in nature, beyond our glimpses and speculations at what *can* happen in exceptional cases (vis-à-vis (191). Just as importantly, the large-scale comparative approaches that derive from improved resolution of phylogenomic techniques (192) may provide new understanding of ecological and evolutionary forces responsible for transitions in breeding systems and underlying genetic determinants. Fungi thus provide rich opportunities for the match of tractable study systems with questions pertaining to sexual life histories as a defining characteristic retained among diverse eukaryotic lineages.

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Box 1. Glossary of terms

Breeding system: mechanism determining reproductive compatibility, i.e. with no mating type (homothallism) or mating types determined by one locus (bipolar) or with two independent loci (tetrapolar); it is important to distinguish the breeding system from the mating system, because, even if they likely coevolve, the understanding of their relationships and their evolutionary implications can only be precisely achieved by recognizing they refer to different concepts (15, 17).

Mating system: the pairing of mating participants in nature considering their genetic relatedness, i.e., outcrossing versus inbreeding or selfing (and either haploid or diploid selfing).

Bipolarity: breeding system with a single locus controlling mating type; it is often associated with only two alleles in fungi.

Tetrapolarity: breeding system with two unlinked loci controlling mating type; it is often associated with multiple alleles in fungi.

Homothallism: situation in which an haploid genotype can mate with any other haploid genotype in the population, including its clonemates; homothallism is typically assessed by the ability of a haploid genotype to produce sexual structures alone in a Petri dish; however, it is important to recognize that it does not inform on the mating system actually occurring in natural populations. It is also important to distinguish the terms homothallism and self-fertility, the latter being most often used to refer to the ability of diploid selfing rather than haploid selfing in the scientific literature, which have very different evolutionary consequences. Using self-fertility for homothallism can only lead to confusion in the actual phenomenon that is referred to and therefore on the understanding of concepts and evolution and breeding and mating systems.

Heterothallism: situation in which a given haploid genotype can only mate with another haploid genotype carrying a different allele at the mating-type locus (loci). It is also important to distinguish the terms heterothallism and self-sterility, the latter being most often used to refer to the inability of diploid selfing rather than haploid selfing in the scientific literature, which have very different evolutionary consequences. Even in fungi, many heterothallic species can undergo selfing (e.g. *Microbotryum* anther-smuts; Fig. 1).

Isogamy: situation in a species in which all gametes have similar sizes

Anisogamy: situation in a species with two classes of gamete sizes, one class of large gamete (female function) and one class of small gametes (male function); a single genotype can produce both types of gametes in most anisogamous fungi, so that there are no different sexes.

Sexes: different types of individuals in the population, one type producing large gametes (females) and the other type producing small gametes (males)

Mating-types: different types of individuals or gametes in the population, with gametes of similar sizes, but mating compatibility being usually determined by the exchange of molecular signals and being possible only between different mating types. It is important

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to distinguish sexes from mating-types because the evolutionary causes for their evolution and their evolutionary consequences are very different (15).

Syngamy: haploid cell fusion during mating.

Karyogamy: fusion of haploid nuclei following mating.

Outcrossing: mating among different genotypes, most often unrelated individuals.

Inbreeding: mating occurring among related individuals.

Selfing: mating among meiotic products from the individual; used in the broader literature to mean ‘diploid selfing’

Diploid selfing: mating among meiotic products from a single diploid individual.

Haploid selfing (or same-clone mating, intrahaploid mating): mating among haploid clonemates.

Clonemates: cells with identical genotypes resulting from clonal division of a mother cell.

Automixis: a form of diploid selfing resulting from the fusion among products of a single meiosis (i.e. intratetrad mating).

Homeodomain protein: protein that harbors a characteristic approximately 60 amino acid helical protein folding structure, highly conserved among eukaryotes that binds DNA at so-called conserved homeobox sequences of about 180 base pairs and often regulates transcription of other sets of genes; in basidiomycetes, these transcription factors control a set of genes involved in the mating process, including morphological changes. The term “homeo” refers to the term homeosis designing the replacement of a body part by another body part in animals, as can occur when transcription factors are affected by mutations.

Muller’s ratchet (proposed by Hermann Joseph Muller): a process in which the chromosomes of asexual populations accumulate inexorably deleterious mutations where the lack of recombination prevents regenerating chromosomes free of deleterious mutation from two homologous chromosomes carrying different deleterious mutations.

Hill-Robertson interference (named after the two scientists having first proposed the concept): a phenomenon referring to less efficient selection because of linkage disequilibria (generated either by drift or in clonal population); for example in case of beneficial mutations appearing in different individuals at two different genes in linkage disequilibrium, selection can only fix one of the two.

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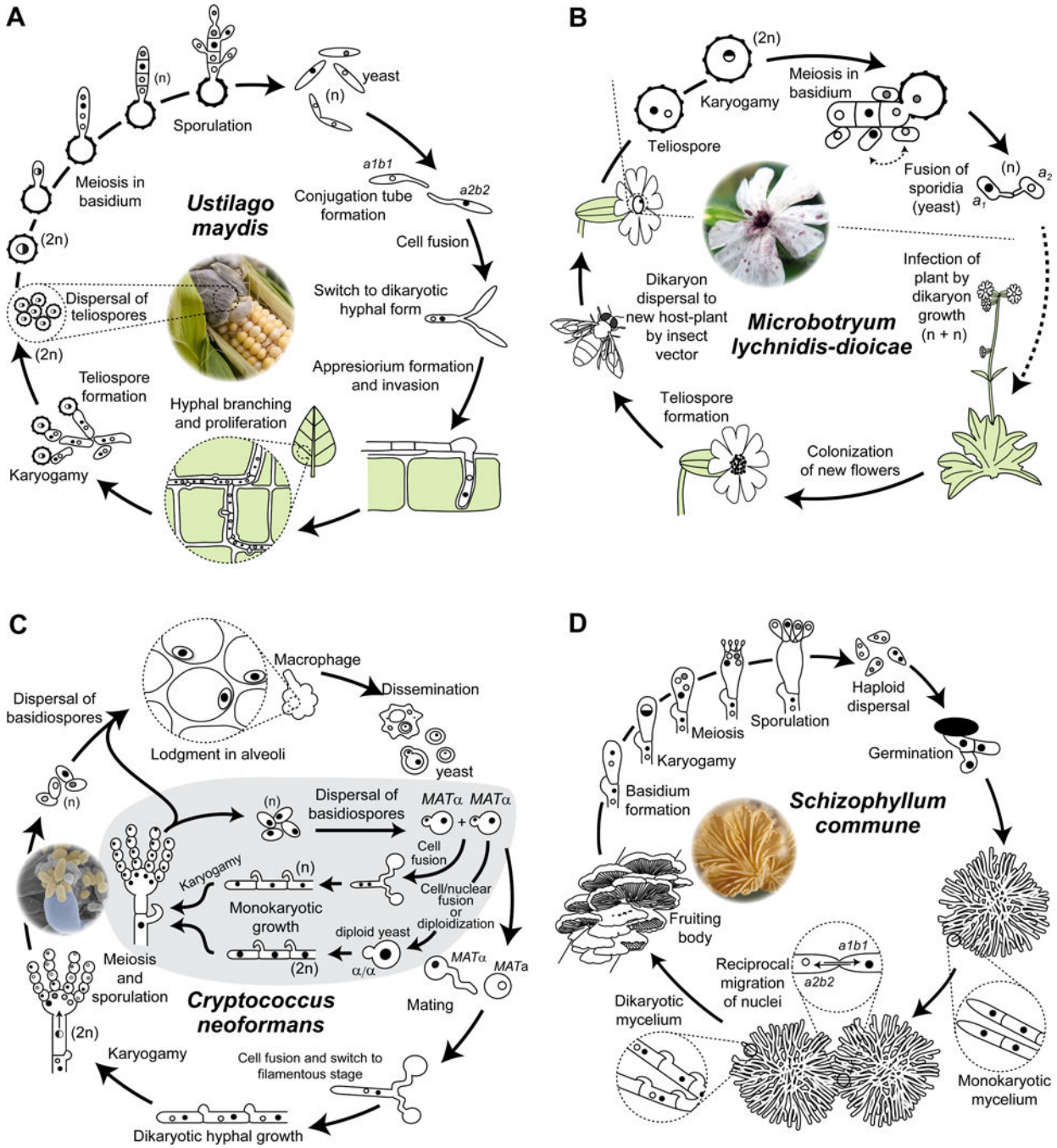

Fig. 1. General life cycles of dimorphic and mushroom-forming basidiomycetes

Three basidiomycetes are pictured where sexual reproduction and a dimorphic switch between a yeast cell and a hyphal form are crucial to infection of plant (A, B) or animal (C) hosts. The haploid yeast form of the maize smut *Ustilago maydis* (A) and the anther smut *Microbotryum* spp. (B) are nonpathogenic and can undergo asexual mitotic vegetative growth. In *Microbotryum*, the yeast stage is, however, short-lived as mating occurs mostly between cells within the same tetrad. Upon mating with a compatible partner, both fungi switch to an enduring infection hyphal form (dikaryon; n + n) that can invade the host plant.

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Proliferation and differentiation of *U. maydis* (A) in the plant culminates with the production of masses of wind-dispersing diploid spores (teliospores; 2n) in large tumor-like tissues, whereas in in *Microbotryum* (B), teliospores are formed in the anthers of infected flowers and transmitted by pollinators onto healthy plants. In the case of *Cryptococcus neoformans* (C), the single-celled yeast form may be free-living or mycoparasitic. A similar dimorphic switch occurs upon mating of yeast cells of opposite mating type (a or α), ultimately resulting in the infectious propagules (basidiospores) that potentially infect an animal host after dispersal. These infectious structures may also be generated by an alternative homothallic mating pathway (depicted in a grey background), where fusion occurs between cells carrying identical *MAT* alleles (α/α diploid is depicted) and form monokaryotic hyphae with unfused clamp connections (see text for details). (D) In mushroom-forming fungi such as *Schizophyllum commune*, germination of haploid spores yields haploid monokaryons capable of independent growth. When two compatible monokaryons meet, a fertile clamped dikaryon is formed which develops into fruiting bodies (mushrooms) triggered upon suitable environmental cues, where basidia arise. In all these and other basidiomycetes, nuclear fusion (karyogamy) is usually delayed until the formation of basidia (or teliospores). Meiosis ensues, generating four haploid nuclei, which will give rise to basidiospores to complete the cycle. Adapted from Morrow & Fraser (2) and Nieuwenhuis et al (17) with permission of the publishers.

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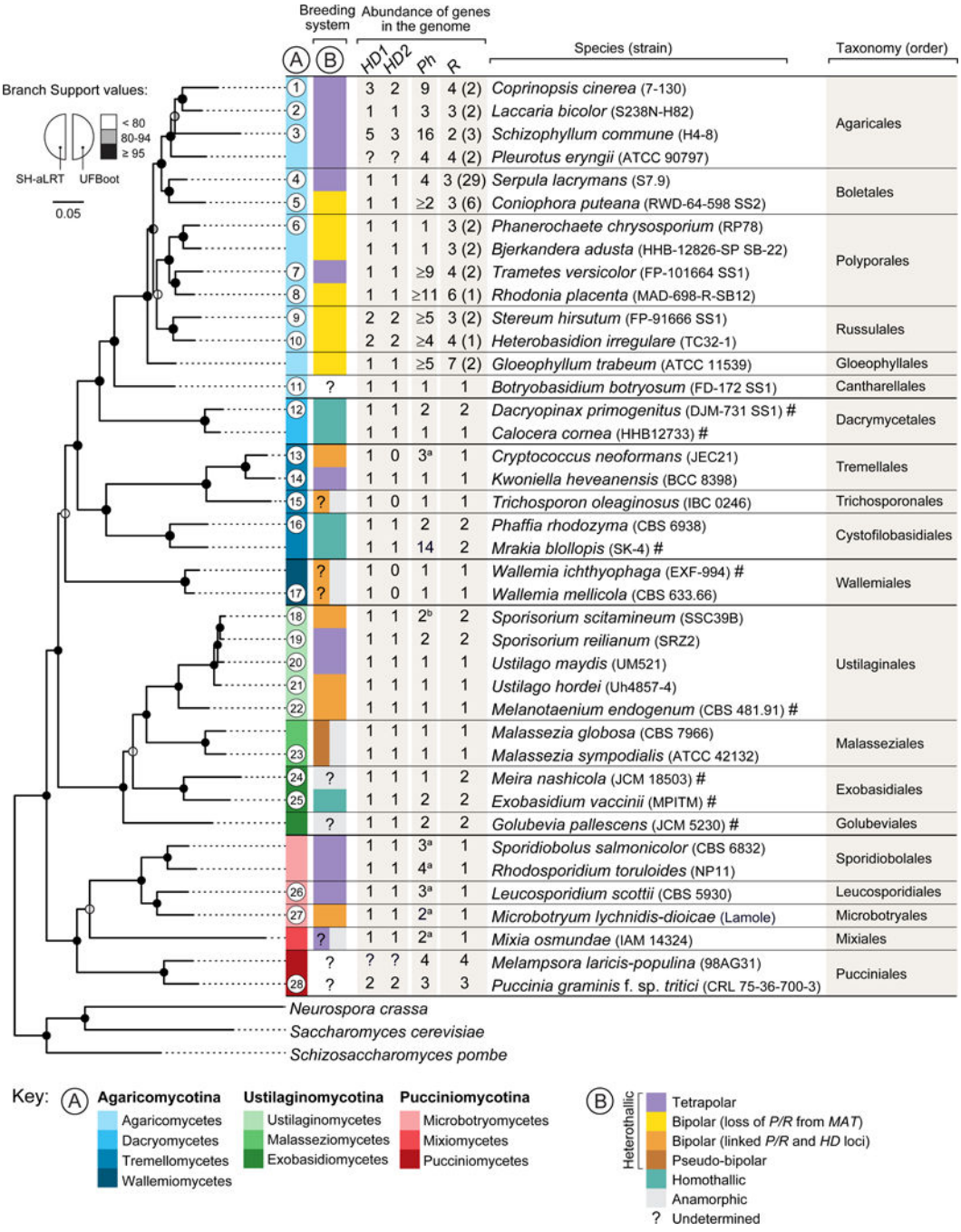

Fig. 2. Phylogeny of the Basidiomycota indicating the breeding system and the number of *MAT* genes across representative species of the three subphyla

The breeding system and the different taxonomic lineages are color-coded as given in the key and are kept consistent in all figures. Gene numbers shown for each species were obtained either from previous reports (20, 41, 100) or from newly surveyed genome data (marked with a hash after the species name). In the Agaricomycetes, values shown in parentheses are putative non-mating type pheromone receptors. A question mark indicates cases where information on the breeding system is not available or is uncertain (e.g. because the sexual stage of a species is unknown). A schematic representation of the *P/R* and *HD*

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loci is given in Fig. 3 for representative species of each lineages marked with numbers enclosed in white circles. Letters in superscript next to the number of pheromone precursor genes indicate that (a) all genes encode the same mature pheromone peptide or that (b) no CAAX motif was detected in one of the putative pheromone precursors. The species phylogenetic tree was constructed in IQ-TREE (193) using a previously described approach (194). Branch support values are shown in the tree nodes as given in the key and were assessed with the ultrafast bootstrap approximation (UFBoot) and the approximate likelihood ratio test (SH-aLRT), each with 1000 replicates. The basidiomycete clade is rooted with sequences from Ascomycete fungi.

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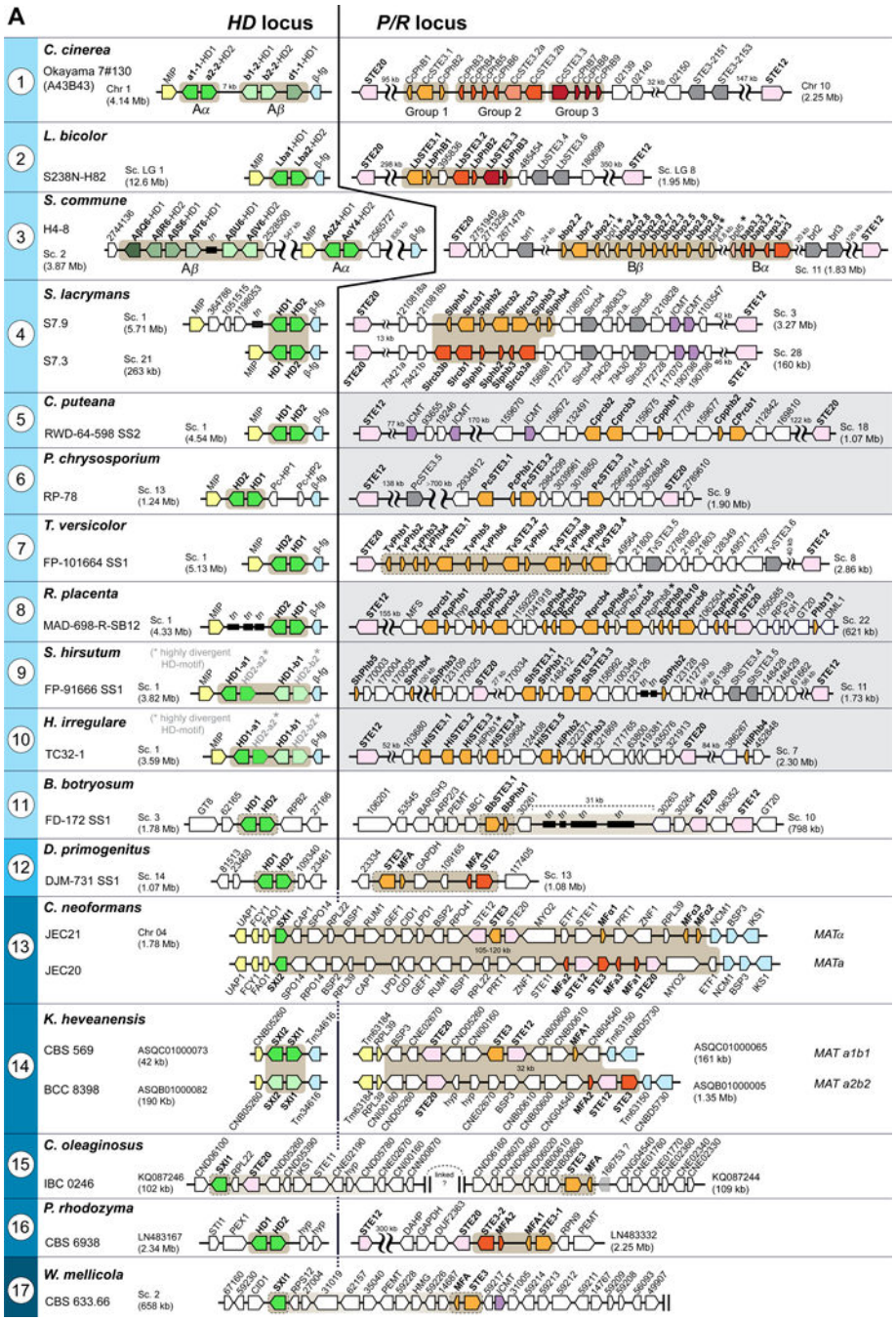

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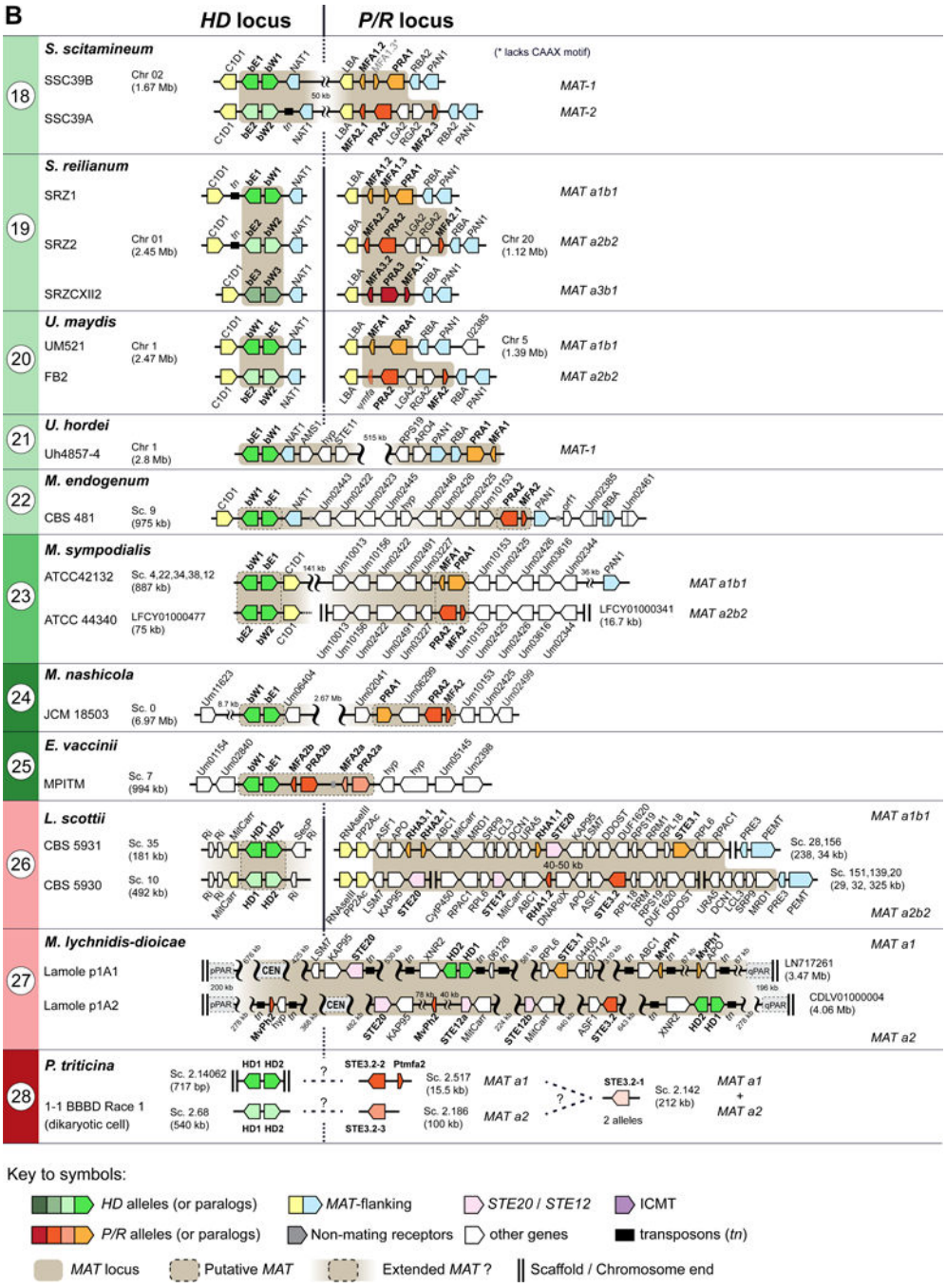

Fig. 3. Schematic showing the genomic structure and diversity of *MAT* loci in representative basidiomycete lineages

The genomic organization of the homeodomain (*HD*) and pheromone/receptor (*P/R*) *MAT* loci is shown for selected species of the Agaricomycotina (A), Ustilaginomycotina and Pucciniomycotina (B). Arrows indicate genes and their direction of transcription. Putative *MAT* loci are shaded in light brown and *MAT* genes are colored as indicated in the key with different color grades representing different alleles (or paralogs). When known, conserved genes flanking *MAT* loci (colored in light yellow or light blue) are shown within each lineage. Genes that encode components of the pheromone response pathway are shown in

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pink and are in many cases within the *MAT* locus. Putative homologues of a protein required for post-translational modification of pheromone precursors (isoprenyl cysteine methyltransferase – ICMT) are colored in purple and appear nearby the *P/R* locus in some species. *P/R* loci no longer determining mating-type specificity in bipolar Agaricomycetes are depicted in a grey background. In *Microbotryum lychnidis-dioicae*, the two mating type chromosomes of are highly rearranged and enriched in transposable elements, so that only a small number of genes is depicted. Of note, whereas in *M. nashicola P/R* and *HD* genes are far apart on the same chromosome, in *E. vaccinii* and *M. endogenum* the two sets of genes are closer together. Other genes or genomic features are colored or represented as given in the key. For citations and additional details, see text. Gene names or their associated protein accession numbers are shown as they appear in their respective genome databases, except in species of Ustilaginomycotina and Tremellomycetes where names were given based on sequence identity to the closest homolog in *U. maydis* and *C. neoformans*, respectively.

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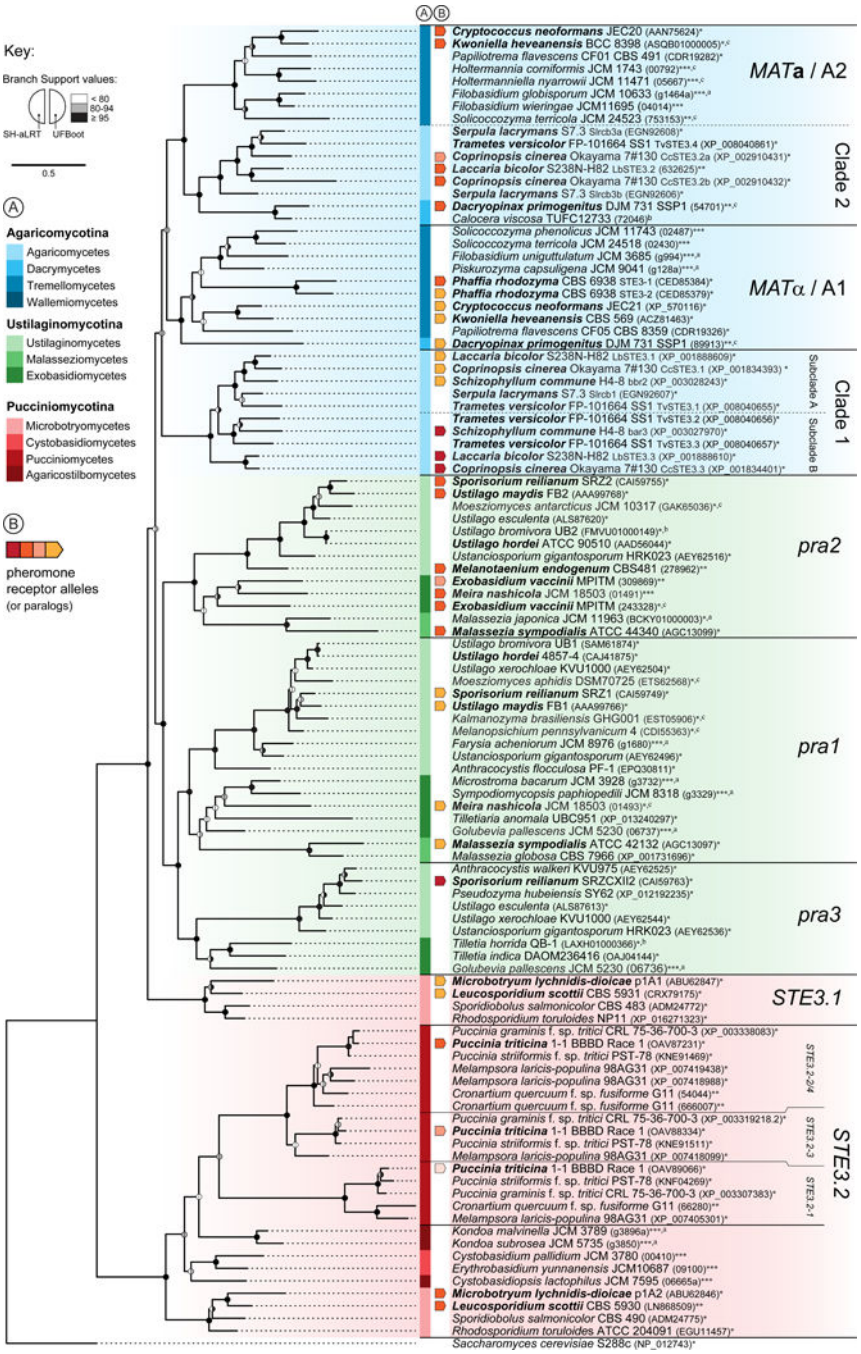

Fig. 4. Phylogeny of Basidiomycota pheromone receptor proteins

Amino acid sequences identified by BLAST from publically available databases or from genome projects were retrieved for representative species of the tree subphyla of the Basidiomycota. A total of 106 sequences were manually inspected, amended where necessary, aligned with MAFFT (195) and poorly aligned regions were trimmed with trimAl (196). The phylogenetic tree and branch support were obtained as in Fig. 2 and the tree was rooted with *Saccharomyces cerevisiae* Ste3p. GenBank accession numbers (∗), JGI protein identifiers (**) and RIKEN/NBRP identifiers (***) and are given after the strain name, with

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letters in superscript indicating: (a) genomes assembled from available raw sequencing data and inspected locally; (b) genomic contigs/scaffolds lacking gene annotation; (c) genes whose annotation was corrected. Species highlighted in boldface are shown in Fig. 3, with arrows before their names indicating the allelic version (or paralog) of the pheromone receptor as colored in Fig. 3. Of note, the *STE3.1* and *STE3.2* alleles in the Microbotryomycetes (Pucciniomycotina) displayed the deepest allelic divergence and trans-specific polymorphism, with the *STE3.1* alleles of the different species branching together rather than each of these alleles clustering with the *STE3.2* allele from the same species (42, 45, 137).

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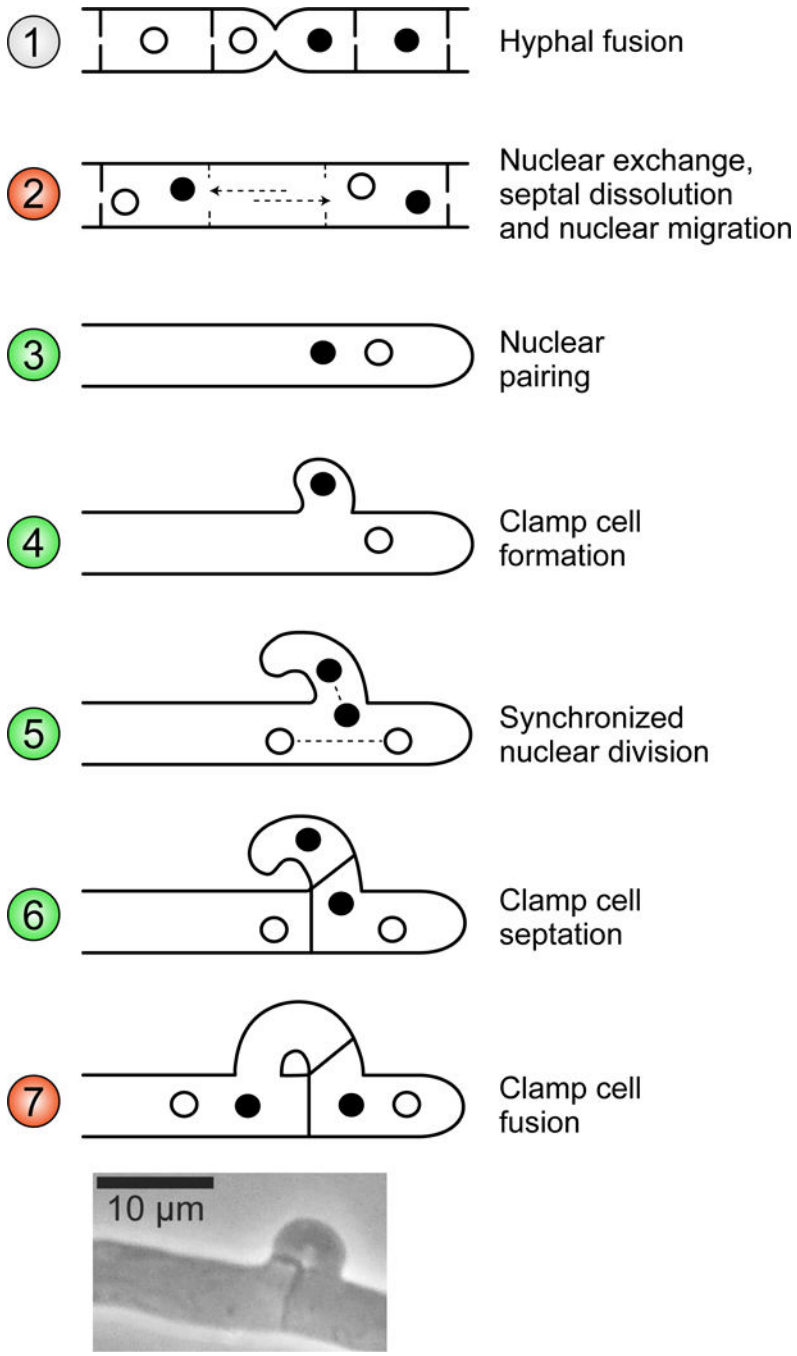

Fig. 5. Roles of *P/R* and *HD* genes on the formation and maintenance of the dikaryon in *C. cinerea*

Pheromone signaling is not required to attract mates and hyphal fusion is mating-type independent (diagram 1). Upon fusion, nuclei enter the mycelium of the other mate and migrate until they reach a hyphal tip cell (diagram 2). During hyphal tip elongation, the two types of haploid nuclei (depicted in white and black, representing different *MAT* genotypes) pair at the tip cell (diagram 3) and, at the place where mitosis will take place, a hook-like structure (called clamp connection) is formed (diagram 4). The two nuclei divide synchronously: one of the nuclei divides in direction of the clamp cell that is growing

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backwards toward the main hyphae, while the other divides along the main hyphal axis (diagram 5). Septa are generated between the dividing nuclei. This way one nucleus stays temporarily entrapped into the clamp cell, one nucleus of the other type is enclosed in the newly formed subapical cell, and a nucleus of each type is maintained in the emerging hyphal tip cell (diagram 6). The clamp cell fuses with the subapical cell and releases the entrapped nucleus from the clamp cell, restoring the dikaryotic state of the subapical cell (diagram 7). In *C. cinerea*, steps controlled by *P/R* (diagrams 2 and 7) and *HD* (diagrams 3 to 6) genes are colored in red and green, respectively. See Casselton et al. (18) and Kües (90) for details. Micrograph at the bottom were obtained from Stajich et al. (86), with permission.