

Mating System of the Anther Smut Fungus *Microbotryum violaceum*: Selfing under Heterothallism[∇]

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Sexual reproduction is of such fundamental importance in eukaryotes that, with unique exceptions, it appears essential for the long-term persistence of species (43). The benefits of sex stem from both the DNA maintenance and repair during meiotic divisions and the impact of various forms of mating upon genetic variation. Meiosis is retained by virtually all sexual organisms, and aside from the meiotic parthenogens, mating is required between distinct and alternate forms, called sexes or mating types (but interesting cases exist in fungi of same-sex mating and sex without evidence of meiosis [17, 76]). Mating compatibility is determined by a great variety of mechanisms. In reptiles, diploid males and females are determined by the incubation temperature during egg development (60). Some fish and plants can switch sexes during their lifetime in response to environmental conditions (4, 70). However, the irreversible differentiation into different sexes or mating types is genetically determined in the majority of species. In many animals and plants, sexual development is established by genes found on a dimorphic pair of sex chromosomes, where females and males differ with regard to being homogametic or heterogametic (i.e., whether or not a diploid carries two copies of the same sex chromosome or one copy of each alternate sex chromosome).

The origin of sex chromosomes is believed to have involved suppression of recombination around the essential sex determining genes on an ancestral autosomal pair (86) and progressive expansion of this region of suppressed recombination through the recruitment of other sex-related factors (92). Recombination suppression and the bias in the extent of heterozygosity between the homogametic or heterogametic sex chromosomes would then drive the accumulation of load loci and the degeneration of sex chromosomes via reduced effective population size and Müller's ratchet (21). The sex chromosomes indeed not only diverge from each other, but they degenerate relative to autosomes, retaining few coding genes and sometimes drastically reducing in size (21).

In fungi, mating compatibility is regulated strictly in the haploid stage by specialized regions of the genome known as

the mating-type loci (MAT). For most species, the successful fusion of gametes can occur only between haploids carrying functionally different mating-type alleles, which is called heterothallism (see Heterothallism and Selfing in Fungi). A key feature of fungal mating types is whether compatibility is determined by alleles at a single locus, a condition called bipolar heterothallism, or by alleles at two unlinked loci, called tetrapolar heterothallism. A small number of fungi are homothallic, meaning that they do not require genetic differences for mating compatibility, the proximal cause being most often that each haploid secondarily possesses two mating types in its genome (75). All ascomycete fungi present a bipolar system regulated by highly dissimilar mating-type genes present at the same locus, referred to as "idiomorphs" rather than alleles due to the uncertainty of the origin by common descent. The most basic MAT configuration is found in filamentous ascomycetes, where the two idiomorphs are called MAT1-1, which encodes a high-mobility group protein, and MAT1-2, which encodes a transcriptional activator protein with an alpha box domain (22). Additional proteins of unknown function may be in complete linkage with the MAT idiomorphs, as in *Podospora anserina* (22). Budding ascomycetes have more complex systems that either do not use high-mobility-group domain proteins or utilize additional homeodomain MAT regulators (17, 20), and in the case of *Saccharomyces cerevisiae*, that involve an unusual mechanism of mating-type switching (75).

By contrast, most basidiomycete fungi present a tetrapolar system, involving two unlinked regions of the genome (13). In *Ustilago maydis* for instance, cell recognition and fusion are regulated by a pheromone-receptor system that resides at the *a* locus, and persistence of the resulting dikaryon, i.e., hyphae with the two haploid nuclei remaining separate in the cells, is determined by heterozygosity for the second mating-type locus, the *b* locus (30). The *b* locus encodes two homeodomain proteins (bW and bE) that function as transcriptional regulators after dimerization. In such a tetrapolar system, the *a* and *b* loci (called B and A, respectively, in some species, like *Schizophyllum commune* and *Coprinopsis cinerea*) are both involved in sexual compatibility (16, 91). There can be four haploid genotypes if both unlinked loci are represented by just two alternate alleles each. However, the alleles at mating-type loci in some tetrapolar hymenomycetes are highly polymorphic, yielding species with thousands of possible mating types (e.g., *C. cinerea* [47, 88]). In contrast, roughly a quarter of basidiomycete species present a bipolar system, which is due to either (i) the tight linkage of the *a* and *b* loci and the existence of a single pair of alternate alleles at each (e.g., in *U. hordei* [12,

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14] and in *Malassezia globosa* [108]) or (ii) the loss of function for the pheromone receptor in mating-type specificity, as in *Coprinellus disseminatus* (59) and *Pholiota nameko* (1). Similar to ascomycetes, these bipolar basidiomycetes typically are not polymorphic beyond the two alternate mating types.

Recent studies on the structure of fungal mating-type loci have revealed features in common with familiar sex chromosomes of animals and plants, principally including regions of suppressed recombination and the accumulation of repetitive DNA elements. These observations have been used to suggest that fungi exhibit processes similar to the early stages of the evolution of sex chromosomes (33–35, 50, 55). While sometimes representing a restricted chromosome region, as in the corn smut *Ustilago maydis*, that spans only ~4 kb (*b* locus) and ~4.5 to 8 kb (*a* locus), mating-type loci can be much larger, as in the barley smut *Ustilago hordei*, in which they cover 500 kb (12, 14), and include genes not directly involved in the mating process. In the basidiomycete *Cryptococcus neoformans*, a ubiquitous human fungal pathogen with a bipolar mating-type system, the mating-type locus contains more than 20 genes spread over 100 kb in length (35). In another basidiomycete and human pathogen, *M. globosa*, the mating-type locus spans 170 kb and encompasses 82 predicted genes. The expansion in size of mating-type loci in these basidiomycetes is due to the evolution of recombination suppression in and around the mating-type locus. Such a suppression of recombination around the MAT locus has also been reported in some *Ascomycetes*, including *Neurospora tetrasperma* and *Cryphonectria parasitica* (37, 82).

Most beneficial to our efforts to understand mating systems are biological models having a strong history of study for both molecular and ecological biology and which can serve as examples for a broad and important group of organisms. In this review, we aim to describe the mating system of the basidiomycete *Microbotryum violaceum*. This fungus is a parasite that causes anther smut disease in more than 100 species in the family *Caryophyllaceae* (103). Although this fungus has similar biology to major crop pathogens (e.g., the corn smut *Ustilago maydis* and the rusts, the *Puccinia* spp.), it has no real impact on human activities. Therefore, no pest control measures have ever been attempted, making it valuable for studies of natural systems. This fungus is a recognized model for the evolution of host-pathogen interactions (8, 62, 102), fungal genetics (38), host-race differentiation, and host shifts (7, 72, 73, 77). *Microbotryum violaceum* is actually a complex of sibling species, highly specialized on different hosts (72, 73). The viability of hybrids has been shown to decrease with the genetic distance between species of the complex, indicating the existence of postmating reproductive isolation (73). The taxonomy of these fungi is currently under revision (see Taxonomy of *Microbotryum violaceum* below). We will review recent investigations of the mating system of *M. violaceum*, in particular of its mating-type genes, its sex chromosomes, its mode of reproduction, the existence of sex-linked deleterious alleles, and the role of the mating system in the reproductive isolation among sibling species of the complex. Also, we will show that *M. violaceum* is a good model for addressing some key points about the relationships between heterothallism, outcrossing, and the number of mating-type alleles (see Heterothallism and Selfing in Fungi below).

HETEROThALLISM AND SELFING IN FUNGI

Genetic mechanisms in such diverse eukaryotes as animals, plants, and fungi have evolved to control reproductive compatibility, and there can be major implications for their mating systems, i.e., the modes of sexual reproduction that range from self-fertilization to outcrossing. For example, animals with chromosomal determination of separate male and female sexes cannot then self-fertilize. However, there are persistent misconceptions about the evolutionary forces acting on the mating systems and genetics of sex determination in organisms such as fungi, some of which we try to clarify below.

Which definition of selfing for fungi? In part, confusion results from using the terms “selfing” and “outcrossing” for fungi in reference to mating compatibility that is determined genetically in the haploid stage. Because no genetic differences are required between mating cells of homothallic fungi and because what may be perceived as the haploid “individual” can complete the sexual cycle alone, cautious analogies were drawn long ago between homothallism and hermaphroditism in animals and plants (6, 29). However, outcrossing is understood in all other eukaryotic organisms as the union of gametes derived from different diploid parents, whereas selfing (or self-fertilization) unites gametes from the same diploid (Fig. 1). This standard definition of diploid selfing has genetic consequences drastically different from those of mating between identical haploids, as can occur under homothallism, resulting in instant genome-wide homozygosity. Specific and different words should therefore be used to designate mating between identical haploids, diploid selfing, and outcrossing in fungi. Fungi are increasingly used as models for population genetics, and the techniques employed by this field are based almost entirely upon the mathematical assumptions of organisms where mating compatibility is determined in the diploid stage, such as in plants and animals. The most common approaches to understanding genetic variation, such as the Hardy-Weinberg equilibrium, the outcrossing index (*t*), or *F* statistics, must be applied to fungi using the same set of assumptions that underlie the models, or otherwise they may lead to potentially invalid conclusions. These approaches are the basis for many further studies beyond only mating systems, including microevolutionary dynamics, metapopulation structure, and the patterns of gene flow, introgression, and speciation. To make their work more approachable and to best integrate the general theory of population genetics, scientists working on fungi should use the same terminology applied to other eukaryotes. Mating between identical haploids, as can occur under homothallism, is very specific to some organisms, including ferns and fungi, and deserves a unique term. “Intragametophytic mating” has been used to designate the phenomenon in ferns (49) and “haplo-selfing” has been used in yeast (68), but “gametophytic” does not apply to fungi and “haplo-selfing” remains ambiguous. We will use hereafter the term intrahaploid mating.

With respect to mating systems, what is recognized by mycologists as the “individual” often differs between ascomycete and basidiomycete fungi, due to the life cycles being predominantly in the haploid and dikaryotic stages, respectively. This makes less intuitive the differences between selfing and intrahaploid mating that can occur under homothallism in ascomycetes. The distinction is more obvious in basidiomycetes, and

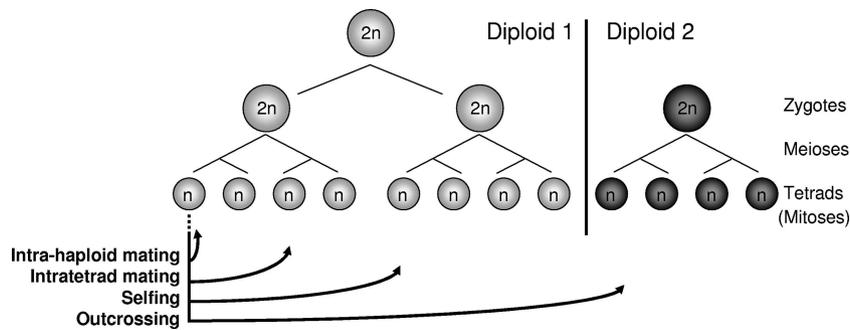


FIG. 1. The diagram shows the products of meiosis from two diploid genotypes, which are often able to proliferate mitotically in fungi and contribute eventually to mating. Various modes of mating are indicated by arrows originating from the far-left meiotic product. Homothallism does not require genetic differences for mating and allows union between any two cells, often including descendants of the same meiotic product as shown here, then called intrahaploid mating and leading to instant genome-wide homozygosity. Intratetrad mating is the union of cells derived from two different meiotic products of the same tetrad. Selfing is most commonly used to indicate mating between cells derived from two different meiotic tetrads of the same diploid genotype (although the previous two modes of reproduction can be considered subcategories of selfing). Outcrossing is the mating between cells derived from meioses in two different diploid genotypes. As explained in Heterothallism and Selfing in Fungi, homothallism and heterothallism have little influence on the mating system.

Microbotryum violaceum is an excellent example. The major portion of the life cycle is dikaryotic, followed briefly by the diploid stage and meiosis, and matings occur preferentially between haploids produced from a single dikaryon, which is clearly selfing. The haploid phase is very brief, and cells can conjugate only with those of the opposite mating type. This fungus is therefore heterothallic but undergoes selfing almost exclusively (see Life Cycle of *Microbotryum violaceum* below).

Heterothallism does not promote outcrossing. A common expression in the fungal literature is that heterothallism is a mechanism to promote outcrossing, while homothallism favors selfing (91). However, because diploids of heterothallic fungi are always heterozygous for the mating-type alleles, meiosis will segregate haploid genotypes that are compatible for mating. If a heterothallic species presents just two alternate mating-type alleles, as is the case for bipolar systems, a diploid will produce compatible haploids in a ratio that is equivalent to the population as a whole (i.e., 1:1), and outcrossing is in no way promoted over selfing in the manner that these terms are commonly understood for most eukaryotes. Still, heterothallism does prevent the mating between identical haploids and the resulting genome-wide homozygosity that is possible in homothallic species, but we have shown that heterothallism does not promote outcrossing in the usual sense of the term: heterothallic species can self as well as outcross.

Homothallism may not promote intrahaploid mating in nature. Furthermore, homothallism has probably not even evolved to allow intrahaploid mating, because mating with an identical haploid has in fact little advantage over asexual reproduction, being unable to create any genetic variation by recombination. Homothallism is more understandable from an evolutionary point of view if it evolved for universal compatibility under outcrossing: in homothallic species, every haploid is compatible with every other haploid in the population. Following this scenario, homothallism would have evolved to allow more efficient outcrossing and not at all for promoting intrahaploid mating. Theoretical models indeed show that a universally compatible gamete often invades populations under an outcrossing system (58, 90). To understand the mating systems of fungi, it is highly useful to think about what occurs

in nature at least as much as the patterns of compatibility in the laboratory. Population genetics studies would be very informative, for instance, if they determined if homothallic species outcross less than or as much as heterothallic ones and whether intrahaploid mating is a frequent phenomenon in nature.

Tetrapolarity does not promote outcrossing. Tetrapolarity is often suggested to promote outcrossing. However, if one considers a tetrapolar system with two alleles at each locus, the chance of mating compatibility is 25%, whether among haploid meiotic products from one diploid genotype or in the population as a whole. For fungi that are polymorphic for large numbers of mating-type alleles, as occurs in some tetrapolar species, the odds of mating compatibility are often greater for outcrossing unions than for selfing. But this would also be true for a bipolar system with multiple alleles. Therefore, it is not bipolarity or tetrapolarity that interacts directly with the mating system through the odds of haploid compatibility; rather, it is the number of alternate alleles at the mating-type loci.

Outcrossing leads to multiple alleles at the mating-type locus, not the reverse. Systems where fungal mating types are polymorphic for hundreds or thousands of alternate alleles may be expected to promote outcrossing because mating compatibility is greater between haploids derived from different diploid genotypes than between haploids from the same diploid (e.g., see reference 59), yet this adaptive explanation is probably not correct either. It is most likely the occurrence of outcrossing in the first place, e.g., from dispersal at the haploid stage, that selects for the high degree of mating-type polymorphism. Indeed, when outcrossing is the rule, any new or rare mating-type allele will have a substantial advantage, being able to confer mating compatibility with all other extant mating types. Such negative frequency-dependent selection should lead to high polymorphism for mating-type alleles, as is observed in the sex-determining system of other eukaryotes (93). In contrast, when selfing predominates, whether the mating-type alleles in the diploid are rare in the population as a whole will make no difference to the compatibility among the haploids. A new allele will therefore provide no advantage in a selfing species, and there will be no selective pressure for increasing polymorphism at mating-type loci.

In tetrapolar basidiomycetes that are polymorphic for multiple alleles at both mating-type loci, it is true that haploids will be compatible with only 25% of those derived from the same diploid genotype, while they will be compatible with the great majority of random haploids in the population as a whole. However, if it is outcrossing (for instance, because of haploid spore dispersal) that has selected for multiple alleles, then such relative odds of mating compatibility between selfing and outcrossing will make little difference, because the organism is already predominantly outcrossing by haploid dispersal. Again, studies addressing the actual patterns of mating in nature, rather than in the laboratory, are essential to the resolution of these evolutionary hypotheses regarding mating compatibility determined genetically in the haploid stage. (For further reading, see references 23a, 83, and 83a.)

TAXONOMY OF *MICROBOTRYUM VIOLACEUM*

Microbotryum violaceum (Persoon) Deml and Oberwinkler (formerly *Ustilago violacea* (Persoon) Fuckel) belongs to subphylum *Basidiomycota*, along with more than 300,000 species representing a great diversity of forms, including the unicellular yeast-like species *Cryptococcus neoformans* and the largest terrestrial organisms, which belong to the genus *Armillaria*. *Basidiomycota* can be terrestrial or aquatic, sexual or asexual, and saprophytic, parasitic, or mutualistic. Some have positive consequences for human endeavors (agricultural mushrooms, antibiotic production, and bioenergy), while others destroy crops and are major causes of human misery and death, particularly among immunosuppressed patients. The subphylum *Basidiomycota* is characterized by the production of a basidium, a cell in which meiosis occurs and from which sexual spores are produced. The union of two compatible haploids, either as single-celled gametes or through the fusion of hyphae, produces a persistent dikaryon of two haploid nuclei residing within the same cell without karyogamy, a genetic condition characteristic of the *Basidiomycota*.

Microbotryum and all smut fungi were for many years classified together in the order *Ustilaginales*, class *Teliomycetae*. The group was characterized by attacks on plant reproductive structures and the formation of dark-colored fungal masses that burst to release the spores. Several species, such as *Ustilago maydis* on maize and *Ustilago hordei* on barley, cause considerable economic losses. The genus *Ustilago* included all species producing uninucleate diploid teliospores, and *M. violaceum* was at that time named *Ustilago violacea*. Later, based on spore mass and color and cellular ultrastructure, as well as phylogenetic studies, the pathogens of dicotyledon host species were transferred to a new genus named *Microbotryum* (26), belonging to the *Microbotryales* in the subphylum of *Pucciniomycotina*. The genus *Ustilago* in the subphylum *Ustilaginomycotina* retained only the species parasitizing monocots (104). The genus *Microbotryum* contains now 77 species (65).

Microbotryum violaceum sensu lato has been further recognized as a complex of sibling species awaiting full taxonomic revision (27, 28, 79). It includes all anther smuts of *Caryophyllaceae* as well as a few plants outside *Caryophyllaceae* (72) and forms a monophyletic group (5, 36, 65, 79). Multiple lines of evidence indicating that *M. violaceum* sensu lato is a complex of distinct species include variation in spore morphology and

color, large genetic distances within *Microbotryum violaceum* sensu lato compared to the distances between and within other *Microbotryum* species (65), congruence among different gene phylogenies (72), and the existence of postmating reproductive isolation (73).

LIFE CYCLE OF *MICROBOTRYUM VIOLACEUM*

Microbotryum violaceum sporulates in the anthers of flowers on diseased plants (Fig. 2). The diploid teliospores are transported to healthy plants by pollinators, giving rise to a frequency-dependent component to disease transmission (19) in addition to passive wind or splash dispersal (2, 11, 94, 95). Once deposited on a new host, the diploid teliospores germinate and produce a short promycelium (septate basidium) into which the nucleus migrates and undergoes meiosis. Because there is the formation of a septation in the promycelium immediately following meiosis I, the result is a linear tetrad that can be isolated by micromanipulation. The fungus is heterothallic with a bipolar system, and the mating types, termed A1 and A2, exhibit complete centromere linkage (53). The postmeiotic nuclei begin to bud yeast-like sporidia by mitosis, which on a culture medium and to a limited extent on plant surfaces, divide in a yeast-like form until conditions are appropriate for mating. In the presence of a compatible mating type and promoted by low nutrients, cool temperatures, and compounds found in the host (i.e., alpha-tocopherol), conjugations occur, after which the fungus initiates a dikaryotic hyphal stage that infects the host tissues, but hyphae cannot be sustained on artificial culture media. The fungus overwinters in the meristematic tissues of the hosts, and the plant becomes systemically infected the next year such that it produces only diseased flowers. Thus, infected hosts are sterilized (referred to as "parasitic castration" [80]), although there are limited effects upon plant mortality. Recovery from disease is rare (3).

MATING-TYPE GENES AND SEX CHROMOSOMES

Conjugation between cells of opposite mating types is required prior to infection of the new host plant, and therefore *M. violaceum* undergoes a sexual generation with every disease transmission event. Studies have suggested a correlation between the rapidity of conjugation and competitive success (105). The developmental processes involved in mate recognition may therefore be of vital importance to the pathogen's life cycle. Putative pheromone receptors have recently been identified in an expressed sequence tag (EST) library of *M. violaceum* collected from the host *Silene latifolia* (109), showing significant protein sequence similarity to fungal *STE3* pheromone receptors (46). A single pheromone receptor was identified from *M. violaceum* cells of each mating type, as expected from the pathogen bipolar heterothallism. Characterization of the full-length *M. violaceum* pheromone receptor genes using a genome walking approach revealed that they were highly dissimilar (109). The A1 pheromone receptor had four exons and three introns (243 amino acids), and the A2 pheromone receptor five exons and four introns (232 amino acids). They could be aligned only when translated into amino acid sequences and even then, had only 28% identity. This suggests that the two pheromone receptors have been maintained for a

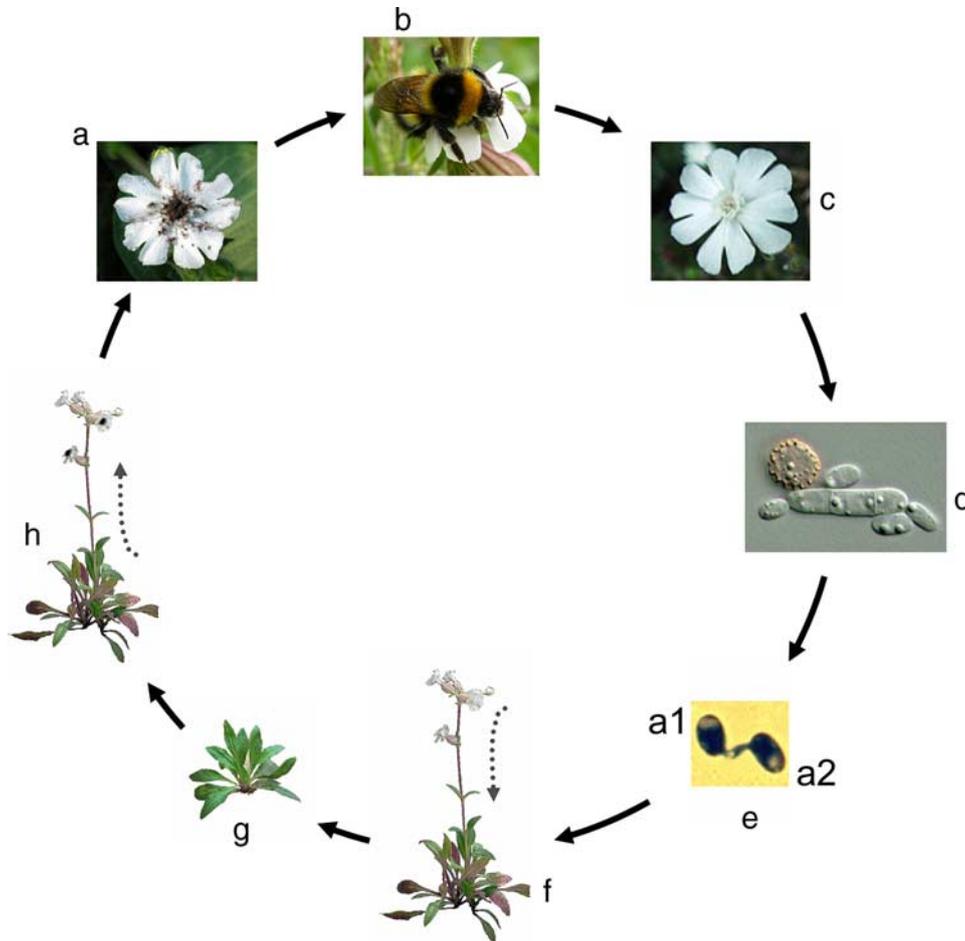


FIG. 2. Life cycle of the anther smut fungus *Microbotryum violaceum* (adapted from reference 78). Diploid teliospores are produced in the anthers (a) and are transmitted by pollinators (b) onto a healthy plant (c). The teliospores germinate, undergo meiosis, and produce yeast-like sporidia (d). Conjugation takes place on the plant between sporidia of opposite mating types (e). Dicaryotic hyphae grow in the plant (f) and overwinter in vegetative tissues (g). The following year, infection is systemic (h), and all flowers produce teliospores (a).

long time by balancing selection, as expected for mating types (81, 93). In fact, the A1 pheromone receptor from *M. violaceum* exhibits a much higher similarity to the *U. maydis* PRA2 and PRA1 alleles (55% identity to both) than to the A2 pheromone receptor from *M. violaceum*, suggesting the existence of transspecific polymorphism. The pheromones of *M. violaceum* have not been characterized yet; however, two homeodomain proteins with similarity to bE and bW proteins from MAT loci in other basidiomycetes have recently been detected in the EST libraries (unpublished data). Investigations are currently underway to determine how these homeodomain proteins differ between the two mating types and if they reside close to the pheromone receptor genes.

Microbotryum violaceum has a haploid genome size of ca. 25 Mb, estimated from electrophoretic karyotypes of strains collected on *Silene latifolia*. The number of chromosomes per a haploid genome estimated from these karyotypes is between 10 and 12 autosomes and one sex chromosome, ranging in size from 1 to 4.2 Mbp (50, 55) (Fig. 3). Karyotypes are highly variable, both among the sibling species and among strains within species (50, 55, 89). The sex chromosomes can be identified by tetrad analysis of electrophoretic karyotypes as the

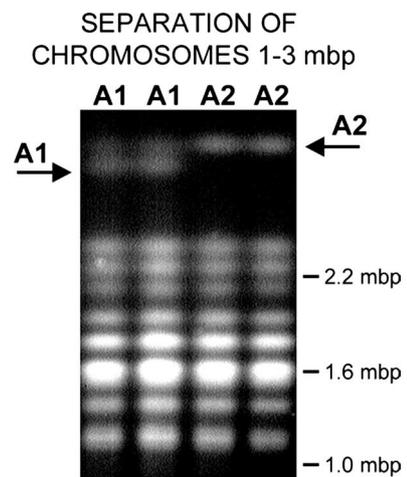


FIG. 3. Electrophoretic karyotype of *Microbotryum violaceum* from host *Silene latifolia*. Four lanes represent the full linear meiotic tetrad, with the structurally homozygous autosomes and size-dimorphic fungal sex chromosomes. Mating-type segregation is at meiosis I and is indicated by the labels A1 and A2. (Reproduced from reference 50 with permission of the publisher.)

only pair of chromosomes whose size dimorphism consistently cosegregates with mating types. The sex chromosomes are among the largest chromosomes in the genome of *M. violaceum* from *Silene latifolia*, ranging from 2.2 to 4.2 Mbp (39). In other species of the pathogen, such as that found on *Silene caroliniana*, the sex chromosomes are intermediate in size relative to the autosomes (55). Across numerous samples of *M. violaceum* from *Silene latifolia*, no evidence was found to suggest any recombination events on the chromosome arms bearing the mating-type locus (50, 55). The *M. violaceum* genome therefore consists of an example of sex chromosomes in fungi with structural dimorphism similar to those in familiar plants and animals. Moreover, the larger A2 sex chromosome contains a lower density of functional genes, suggesting the possibility that the gain of noncoding sequences by the A2 chromosome is the cause for their difference in sizes (55).

Obligate heterozygosity and suppression of recombination are expected to promote degeneration of sex chromosomes mostly through an accumulation of transposable elements and the reduction by half of the effective population size of each sex chromosome relative to the autosome (21). In fact, the sex chromosomes of *M. violaceum* present a higher accumulation of transposable elements than autosomes, while autosomes present twice the density of functional genes (55). The retrotransposon accumulation on sex chromosomes may constitute a reservoir of mobile elements that continue to disperse to the rest of the genome, explaining the high repetitive DNA content in *Microbotryum* (52). Yet, the fungus is not without defense mechanisms: an analysis of retrotransposon sequence variation showed that repeat-induced point mutation, a defense known to attack mobile elements in *Neurospora crassa*, has been very active in *M. violaceum* (57). A large number of transposable element sequences have been identified in a recently published EST library showing that these elements can escape this genomic defense mechanism to some extent, at least regarding their transcriptional activity (109).

HETEROTHALLISM VERSUS OUTCROSSING, SELFING, AND INTRATETRAD MATING

Microbotryum violaceum is one of the first fungi in which bipolar heterothallism was demonstrated, some 90 years ago (67); haploid sporidia are either of mating-type A1 or A2. The obligate heterozygosity at the mating-type locus that results from haploid determination of mating compatibility means that each of the diploid teliospores of *M. violaceum* produces meiotic products of the two mating types in equal proportions. It is therefore as possible for such fungi to mate among the product of the same diploid genotypes (i.e., to self) as to outcross among the gametes from separate diploid genotypes. *Microbotryum violaceum* is therefore an excellent example of bipolar heterothallism in fungi that does not prevent selfing or encourage outcrossing, from the point of view of the diploid stage (see Heterothallism and Selfing in Fungi above). The fungus is able to self while being truly heterothallic, but not secondarily homothallic as it is sometimes classified (75), the distinction being that *M. violaceum* requires the recognition and fusion of separate haploid cells (i.e., gametes), whereas secondary homothallism involves the packaging of compatible postmeiotic nuclei into the same spore product.

In fact, selfing seems to be the dominant type of mating in a natural population of *M. violaceum*. Analyses using microsatellites have shown strong heterozygote deficiencies in *M. violaceum* populations infecting the host *S. latifolia* (25, 39). Further, studies on the meiotic segregation of amplified fragment length polymorphism variation revealed heterozygosity in the genome to be largely restricted to the regions linked to mating type and absent from the autosomes (56). Selfing may result from local spore dispersal or founder effects that lead to a lack of available mating partners. However, several studies have shown the presence of different genotypes of *M. violaceum* in the same population and even in the different stems of individual plants, showing that there are potential partners for outcrossing (25, 39, 78).

Some data indicate a preference for selfing compared with outcrossing (41, 54). To determine experimentally the rates of outcrossing versus selfing of *M. violaceum*, Giraud et al. (41) used mixtures of diploid fungal genotypes homozygous for contrasting microsatellite alleles, and the inocula were deposited together on the plant, where meiosis and mating are prerequisites for infection. This experiment revealed that greater than 70% of infections resulted from selfing events in each of two mixtures of fungal strains under these choice conditions. Such a preference could arise from a selfing propensity due to gamete recognition, and Kaltz and Shykoff (64) found a significant, but weak, preference of sporidia to mate with other products from the same diploid teliospore. The selfing preference may also be mediated in *M. violaceum* through a high frequency of conjugations occurring among contiguous cells of the promycelium (septate basidium) due to developmental programming (54), thus resulting in intratetrad mating, or "automixis." In vitro studies of conjugation patterns between cells in single-teliospore colonies (41, 44, 54) indeed revealed a propensity for infectious hyphae to be produced from mating between promycelial cells. The same result was found in vivo, after the inoculation of teliospores into leaves, flowers, lateral meristems, and cotyledons of *S. latifolia* (54). Such a proximal mechanism of selfing by mating between promycelial cells may be favored if, by abbreviating the haploid stage of the life cycle, genotypes carrying recessive deleterious mutations can continue to shelter such a genetic load without the cost of exposing the mutations to selection in the haploid stage (see Mating-Type Bias and Haplo-Lethal Alleles).

Intratetrad mating by the rapid fusion of the early products of meiosis seems to be the predominant mode of reproduction in *M. violaceum* (Fig. 4) (41, 53, 54) and should perhaps be considered a special form of selfing because of the important consequences for the maintenance of genetic variation and heterozygosity (56). Indeed, the restriction of a haploid mating pool to the four products of a single meiosis leads to a slightly slower loss of heterozygosity than mating among products of separate meioses from the same diploid genotype (66, 111). But more importantly, fungi exhibiting intratetrad mating also tend to show first-division segregation of mating type, for which *M. violaceum* is an illustrative example (66, 110, 111). Centromere linkage of mating type ensures that intratetrad mating will result in the fusion of meiotic products separated at the first meiotic division. Therefore, all other genomic regions that similarly segregate at meiosis I, including regions linked to autosomal centromeres, will also preserve both allelic and

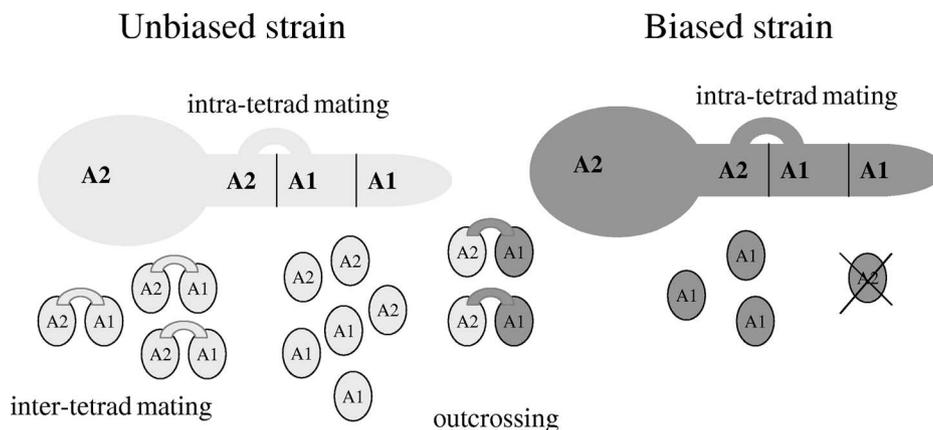


FIG. 4. The different types of conjugation possible in *Microbotryum violaceum*: selfing through intratetrad or intertetrad mating and outcrossing. One of the two mating-type sporidia produced by biased strains presents haplo-lethal alleles. (Reproduced from reference 40 with permission of the publisher).

structural heterozygosities, as observed in *M. violaceum* (56). These patterns of intratetrad mating and centromere linkage of sex-determining genes are not restricted to fungi but can also be found in other organisms, particularly among insects that are meiotic parthenogens. For example, in the bagworm *Solenobia*, females are the heterogametic sex, and there too it is the reunion of first-division products of the same meiosis that ensures the maintenance of heterogamety in females and has similar predicted effects on preserving heterozygosity near autosomal centromeres (74, 97). As genetic load is often associated with the fixed heterozygosity in linkage with the mating type of *M. violaceum* (see below), similar deleterious recessive mutations have repeatedly been found at autosomal centromeres (54, 100). Conventional selfing by the fusion of gametes derived from separate meioses would drive autosomal centromeres to homozygosity at a rate of only 50% per generation due to independent assortment. Thus, as an interesting feedback process, *M. violaceum* may experience the accumulation of load loci at autosomal centromeres that then further restricts this fungus to intratetrad mating to avoid exposing the deleterious recessive mutations to homozygosity (61).

MATING-TYPE BIAS AND HAPLO-LETHAL ALLELES

Associated with the regions of preserved heterozygosity, both on the sex chromosomes and in linkage to autosomal centromeres, *M. violaceum* exhibits high frequencies of deleterious recessive mutations (i.e., load loci) that upon exposure in the haploid stage result in little or no growth in culture. The phenomenon was first observed because of the inability to isolate sporidia of one of the two mating types from certain strains of the pathogen (38, 63, 87, 100). This mating-type bias was investigated for a number of possible causes, including spore killers and meiotic drive (83). However, inheritance in experimental crosses showed that the observed mating-type bias does not result from the presence of segregation distorters, but from the exposure of lethal recessive alleles leading to nonviable haploids (87). Experimental observations of biased and unbiased strains showed that in all cases, the promycelial cells containing the postmeiotic tetrad were initially able to divide mitotically. However, in biased strains, the sporidia pro-

duced from two cells in the tetrad continued growth in vitro and formed large colonies, whereas the other two survived through only a few cell divisions (51, 87) (Fig. 5). Similar “haplo-lethal” deficiencies have long been reported for smuts in the *Ustilaginaceae*, which represent distantly related fungi that infect monocot hosts (24, 31, 45, 85). The similarities in life histories, including haploid sporidia, infectious dikaryons, and the predominance of selfing, contribute to the shared genetic characteristics between *Microbotryum* and *Ustilago* species.

Recessive deleterious alleles are expected to be readily fixed in the region of recombination suppression around mating-type loci, because they will be permanently sheltered in a heterozygous state (42). The existence in *Microbotryum* of haplo-lethal alleles, which are deleterious at the haploid stage but recessive at the dikaryotic stage, can therefore appear not surprising. However, strains with haplo-lethal alleles may still suffer from a selective disadvantage in *M. violaceum* to the extent that there is an expected haploid component to the life cycle. Indeed, because infection probability increases with the number of conjugating sporidia (94), disease transmission is expected to be lower for biased strains that cannot proliferate as haploids (but see reference 44). Given this possible disadvantage of haplo-lethal alleles, the very high frequency of strains with a mating-type bias in natural populations of several

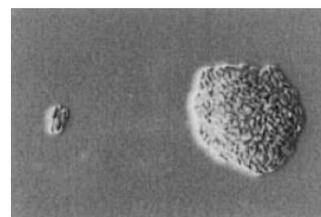


FIG. 5. Colonies produced after teliospore germination of biased strains. The large colony of sporidia (right) was founded by a viable sporidium, and the small colony (left) was founded by a sporidium from the same tetrad, which contains a lethal allele. (Adapted from reference 54.)

Microbotryum species has been the subject of considerable study (63, 87, 100).

To explain the high frequencies of haplo-lethal alleles, it has been proposed that theoretically the mutations may confer a compensatory advantage in heterozygous conditions and are thus maintained by selection at the dikaryotic stage (9). Some biased strains appeared in fact to produce more teliospores than unbiased strains, but in quantities and timing that appeared insufficient to compensate for their infection disadvantage (40). It was also proposed that biased strains could have a competitive advantage against unbiased strains in terms of rapidity of infection due to faster intrapromycelial conjugation. Nevertheless, there is no conclusive evidence to confirm this hypothesis: using different protocols of mixed experimental inoculations, Hood (51) found that biased strains were better at infecting than unbiased strains, whereas Giraud et al. (40) found that under artificial inoculations, unbiased strains were better at infecting the plant *Silene latifolia*, both alone or in competition with biased strains.

Finally, the maintenance of haplo-lethal alleles has been explained theoretically under a metapopulation structure based on founder effects and selection at the population level (98). This model shows that biased strains could stably coexist with unbiased strains in a metapopulation even with a constantly lower transmission rate. The reason suggested was that populations founded only by biased strains experienced a lower extinction rate precisely because of their lower infection ability; strains carrying haplo-lethal alleles spread more slowly and sterilized fewer plants, thus allowing their hosts to reproduce more and therefore to be less prone to extinction at the population level. Haplo-lethal alleles could be maintained in this model only with intratetrad mating rates higher than 0.7, which is consistent with estimates between 0.43 and 0.71 based on experimental studies (41). If a spore production advantage of biased strains was added in the model, biased strains could be maintained even more easily in the metapopulation (99).

REPRODUCTIVE ISOLATION

Sexual reproduction results in genetic novelties and purges deleterious mutations via meiotic recombination but may also produce highly unfit progeny if mating occurs among individuals from different species. Reproductive mechanisms often evolve that decrease the probability of crosses between species in sympatry (41a, 69). Development and signaling that shape the reproductive system can be directly involved in such avoidance of interspecific crosses. For example, in some insects the female pheromones attract only conspecific males (101). Similarly, pheromones and pheromone receptors appear to play a direct role in the avoidance of interspecific crosses in some fungi (15).

The nature of the reproductive isolation among the different sibling species of the complex *M. violaceum* sensu lato has thoroughly been investigated (18, 72, 73, 96, 106, 107). Despite the broad geographic overlap among the pathogen lineages adapted to different hosts, indirect isolation could result if pollinators were specialists and transmitted fungal spores only to members of the same host species. However, although pollinators usually prefer one host species, no insect species has revealed itself to be a true specialist (96, 107). Other repro-

ductive barriers have experimentally been investigated in the *M. violaceum* species complex. For example, in vitro crosses provided no evidence for pre-mating reproductive isolation associated with assortative mating (73): sporidia from alternate mating types of different species conjugated as readily as those from the same species. In contrast, post-mating isolation in the form of hybrid inviability increases with the genetic distance between interspecific mating partners, both when measured as hybrid growth rate in vitro or the ability to cause disease in host plants (73). Hybrid sterility further contributes to reproductive isolation, in particular because meiotic products may have abnormal mating behavior (10; unpublished data).

Pre-mating reproductive isolation was expected to be selected for among sympatric species of *M. violaceum*, because of the hybrid inviability (23), but no evidence of reinforcement has been found when measuring the proportion of successful interspecific conjugations, both at the population and sub-regional levels (unpublished data). One reason could be that pre-mating isolation is promoted by other factors, such as the high propensity of *M. violaceum* for intratetrad selfing. First, as has been invoked in plants (32), a high rate of selfing may be efficient at limiting interspecific matings. Second, the mixture of *M. violaceum* species on a host plant triggers competition between hybrid and nonhybrid genotypes. Indeed, different species of *M. violaceum* have the opportunity to hybridize when they are deposited on the same plant, but among the many teliospores that are deposited together, many of them will undergo intratetrad mating. Hence, there will always be a competition between hybrids and nonhybrids during infection. Because interspecific matings show a reduction in fitness due to post-mating inviability (73), it may be unlikely for hybrids to competitively succeed in expressing disease and realizing the production of spores under natural conditions. Therefore, selfing reduces both the chance of hybridization and the chances for success of the hybrids in completing the pathogenic life cycle. As a result, the lack of hybrid production may result in selection for pre-mating isolation that is insufficient to drive evolution of the reproductive system. Under this hypothesis, the dominance of intratetrad mating in *M. violaceum* would play a significant role as a barrier to interspecific matings.

CONCLUSION

There has been much progress in recent years in the study of many aspects of the *M. violaceum* mating system, such as the genomic structures and genes involved, their evolution, and the actual mode of reproduction of the fungus in nature. However, many important questions remain. First, it will be tremendously informative to have the DNA sequence of the sex chromosomes to assess by which evolutionary events *M. violaceum* acquired bipolarity—via linkage of the two mating-type loci, as in *U. hordei* (12), or by the loss of function of one of the loci, as in *P. nameko* (1). In addition to these proximal mechanisms, it will also be fascinating to explore the evolutionary pressures associated with bipolar heterothallism: in particular, did the mating system (selfing) play a role in preserving, or even selecting for, bipolarity? Indeed, under a highly selfing system, new mating-type alleles are not expected to be selected for, since any diploid individual is able to self, regardless of having rare or frequent mating types. This should lead to bipolarity in

highly selfing fungi, although it remains to be investigated both theoretically and experimentally.

Additional data on the sex chromosome sequences would further allow assessment of how the large regions of these chromosomes have come into linkage with the essential pheromone receptors and whether there exist discrete steps in the evolution of recombination suppression, as has been shown to have occurred in several other organisms, such as humans (71), chicken (48), and *Silene* (84). The balancing selection on the mating-type genes will also be worth investigating further. Comparing the sequences of *M. violaceum* with other fungal taxa will determine the extent to which transspecific polymorphism exists, as would be expected under long-term balancing selection. Functional studies would also be needed to validate the role of pheromone receptors and other genes identified by sequence similarity. Finally, the study of hybrids among *Microbotryum* sibling species and the consequences for the mating behavior may help unravel the functional processes involved in the evolution of these pathogenic fungi.

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