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7 **FROM DIKARYON TO DIPLOID**
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1. Introduction

Most fungi in the Basidiomycota are characterized by a long-lived vegetative dikaryophase, where two genetically distinct nuclei coexist in each cell of the hypha. The dikaryon arises after cell fusion of two haploid homokaryons carrying different mating type alleles. Under appropriate conditions, the dikaryon undergoes a complex developmental program that gives rise to a fruiting body (Kües and Navarro-González, 2015). Differentiation within the fruiting body leads to formation of specialized cells, basidia, where karyogamy and meiosis occur, resulting in production of four basidiospores. Meiosis can be followed by mitosis. The basidiospores can be uninucleate or binucleate. In the latter case, the nuclei may have the same or different genotype at the mating type loci (Hibbett et al., 1994; Raper, 1966). Cell fusion and karyogamy are temporally and spatially separated in the life cycle of the majority of basidiomycete fungi, exemplified by *Coprinopsis cinerea* and *Schizophyllum commune*, which have long been used as model organisms for studies, among others, on mating type determination, fruiting body development, and somatic recombination (Casselton and Olesnicky, 1998; Kües, 2000; Papazian, 1954; Parag, 1962; Raper, 1966; Raudaskoski and Kothe, 2010; Stankis and Specht, 2007).

In dimorphic basidiomycete fungi, the dikaryon arises after cell fusion of two haploid yeast-like cells having different mating type alleles. Karyogamy and meiosis occur in specialized cells without fruiting body differentiation. Cell fusion and karyogamy are also temporally and spatially separated, as exemplified in *Ustilago maydis*, *Cryptococcus neoformans*, and *Microbotryum violaceum*, which have been used extensively as model organisms (among others) for the study of the mating type loci, their role in pathogenicity, ecology of plant disease, and sex chromosome dimorphism (Banuett, 1995, 2002; Feretzaki and

1 Heitman, 2013; Giraud et al., 2008; Hood, 2002; Hood et al., 2013; Hull et al., 2002; Lengeler et
2 al., 2002; Vollmeister et al., 2012).

3 Basidiomycete fungi with a prolonged and stable diploid vegetative phase are of rare
4 occurrence. In these fungi, karyogamy follows immediately after cell fusion. Thus, there is no
5 temporal separation between the dikaryophase and the diplophase. *Armillaria mellea* is the
6 classical example of a basidiomycete fungus with a persistent vegetative diploid phase
7 (Anderson and Ullrich, 1982; Guillaumin et al., 1991).

8 The persistence of a dikaryophase likely provides basidiomycete fungi with a selective
9 advantage (Anderson and Kohn, 2007; Raper, 1966). It has been said that the dikaryophase
10 provides plasticity not possible with diploids. Interestingly, some studies indicate that patterns
11 of gene expression differ when identical mating type information resides in separate nuclei
12 versus the same nucleus in the same cell (Babu et al., 2005). Aside from considerations of
13 evolutionary selective pressures that lead to a persistent dikaryophase, it is of great interest to
14 elucidate the genetic program that controls karyogamy in fungi with a persistent vegetative
15 dikaryophase, and to determine how this program is modified in fungi with a persistent
16 vegetative diplophase.

17 In this review, I discuss dikaryons, heterokaryons, and diploids in selected members of
18 the Basidiomycota, in particular, in *C. cinerea*, *S. commune*, *A. mellea*, and *C. neoformans*
19 (members of the Agaricomycotina), *M. violaceum* (a member of the Pucciniomycotina), and *U.*
20 *maydis* (a member of the Ustilaginomycotina). These fungi have been extensively studied and
21 there are tantalizing observations on the behavior of dikaryons compared with diploids of the
22 same genotype. By no means do I intend to imply that they represent the behavior of all fungi in

the diverse Basidiomycota. For observations on other basidiomycetes, I refer the reader to Raper (1966).

2. Dikaryons and Other Heterokaryons in *C. cinerea* and *S. commune*.

2.1 Mating type (or incompatibility) loci. I provide a brief description of the mating type loci in these fungi as a framework to understand the discussion that follows (for a comprehensive discussion of mating type loci, see [Heitman et al., 2013](#); [Kües et al., 2011](#)).

Both *C. cinerea* and *S. commune* are heterothallic and tetrapolar, that is dikaryon establishment and maintenance are determined by two unlinked multiallelic loci, *A* and *B* (reviewed in [Kamada, 2002](#); [Kües et al., 2011](#)). These loci are complex and consist of two components, α and β ($A\alpha A\beta$ and $B\alpha B\beta$), separable by recombination, and containing two or more genes. Thousands of mating types exist due to the many possible combinations of different mating type alleles (reviewed in [Kamada, 2002](#); [Kües et al., 2011](#)). The *A* locus codes for homeodomain proteins and pairs of non-allelic homeodomain polypeptides interact to form an active heterodimer, which presumably acts as a major regulatory protein to control expression of target genes ([Erdmann et al., 2011](#); [Inada et al., 2001](#); [Kamada, 2002](#)). The *B* locus contains genes that code for pheromone and pheromone receptors, and presumably controls a G-protein coupled receptor (GPCR)-pheromone response pathway resulting in activation of target genes ([Erdmann et al., 2011](#)). Most members of the Agaricomycotina have a tetrapolar mating system, however, examples of fungi with a bipolar multiallelic or biallelic mating system are known ([James et al., 2011](#); [2013](#); [Li et al., 2004](#); [Lengeler et al., 2002](#); [Raper, 1966](#)).

In the Pucciniomycotina a single mating type locus with two alleles appears to be the rule ([Bakkeren et al., 2008](#)) but deviations from the bipolar and tetrapolar pattern (designated

pseudobipolar) have been documented (see, [Coelho et al., 2010](#)). In the Ustilaginomycotina, a bipolar mating type system is common. *U. maydis* and *Sporisorium reilianum* appear to be the exception: both have a tetrapolar mating type system, where one locus is biallelic and the other multiallelic as in *U. maydis*, or both are multiallelic as in *S. reilianum* ([Bakkeren et al., 2008](#); [Banuett, 2007](#); [Schirawski et al., 2005](#); [Vollmeister et al., 2012](#)).

2.2 Compatible, hemicompatible, and noncompatible heterokaryons.

Four types of interaction are possible among haploid homokaryons of fungi with tetrapolar systems: compatible (different *A* and *B* alleles; short notation $A \neq B \neq$), hemicompatible or common *A* ($A = B \neq$), hemicompatible or common *B* ($A \neq B =$), noncompatible or common *AB* ($A = B =$). In bipolar systems, whether multiallelic or biallelic, only two types of interactions are possible, $A \neq$ or $A =$ ([Raper, 1966](#)).

2.2.1. Compatible interactions: the dikaryon ($A \neq B \neq$). The dikaryon is a specialized type of heterokaryon characterized by a 1:1 ratio of the parental nuclei in each cell and the presence of clamp connections between adjacent cells. Clamp connections are involved in partitioning of the two nuclei during synchronous division (see below).

Cell fusion (plasmogamy or anastomosis) between hyphal cells is independent of the mating type loci. Recognition of different mating type alleles occurs intracellularly after cell fusion. Once a bridge is established between compatible hyphae, a reciprocal exchange of nuclei occurs, and the migrant nucleus from one mate moves to the tip cell of the recipient mate, where it pairs with the resident nucleus, establishing the dikaryon ([Raudaskoski, 1998](#); [M. Raudaskoski, this issue](#)). In *C. cinerea* the nuclei maintain a distance of 15-20 μm from each other ([Kamada et](#)

al., 1993), whereas in *S. commune* they appear to be closer (see Schuurs et al., 1998). Movement of the migrant nucleus through the cells of the recipient hypha requires partial dissolution of the dolipore septum (Casselton et al., 1971; Giesy and Day, 1965; Raudaskoski, 1998; M. Raudaskoski, this issue), which is the characteristic septum of fungi in the Agaricomycotina (see for example, Moore and McAlear, 1962; Müller et al., 2000; Orlovich and Ashford, 1994; Raudaskoski, 1998; van Driel et al., 2009). Paired nuclei in the tip cell divide in synchrony. This division involves an intricate process in which a clamp cell (hook) forms near the mid region of the apical cell, at the site of the future septum. The nucleus closest to the tip of the cell (leading nucleus) moves into the clamp cell, where it divides forming a short spindle. The other (lagging) nucleus locates below the hook cell and divides forming a long spindle. Because of the disparate lengths of the spindles, one daughter of the lagging nucleus now becomes the leading nucleus, and one daughter of the leading nucleus now becomes the lagging one. The nuclei switch positions during each conjugate division, so that the leading and lagging nuclei alternate (Iwasa et al., 1998). These events thus far result in a dikaryotic apical cell, a uninucleate subapical cell, and a uninucleate unfused clamp cell. Next, the clamp cell fuses with the subapical cell forming a clamp connection and delivers the nucleus to the subapical cell restoring the dikaryotic state to this cell. The cross walls separating the apical, subapical, and clamp cell contain dolipore septa (M. Raudaskoski, this issue). Early researchers realized that there was a close association between the synchronous division of nuclei in the apical cell and the formation of clamp connections, although, they also recognized that exceptions exist in which nuclear division of a dikaryon occurs in the absence of clamp connections (Raper, 1966).

The *A* and *B* loci control distinct sequential steps during dikaryon establishment and maintenance. Nuclear migration, septum dissolution and fusion of the clamp cell (hook) with the

subapical cell are controlled by the *B* locus. Pairing of nuclei, conjugate division, and clamp cell formation are controlled by the *A* locus. (Casselton and Olesnick, 1998; Kües et al., 2011; Raper, 1966; Raudaskoski and Kothe, 2011; Stankis and Specht, 2007). Transcriptome analysis of strains with an active *A*, active *B*, or active *A* and *B* has led to identification of genes differentially regulated in each of these pathways (Erdman et al., 2011).

2.2.2. Hemicompatible heterokaryons: $A = B \neq$ and $A \neq B =$.

Hemicompatible interactions were analyzed in *S. commune* (Papazian, 1950), *Cyathus stercoreus* (dung-loving bird's nest fungus; Fulton, 1950), and *C. cinerea* (Swiezynski and Day, 1960a,b). These two hemicompatible interactions can be distinguished macroscopically from each other in *S. commune* and *C. stercoreus* but not in *C. cinerea* and other fungi (Fulton, 1950; Swiezynski and Day, 1960a,b; Raper, 1966). A brief description of these different heterokaryons follows.

2.2.2.1. The $A = B \neq$ heterokaryon. In *S. commune*, this heterokaryon produces a sparse mycelium compared to that produced by $A \neq B \neq$ or $A = B =$ heterokaryons due to reduced development of aerial hyphae, giving it a “flat” appearance (Papazian, 1950; Raper, 1966 and references therein). The hyphae can be gnarled, knobby, with short lateral branches (Raper, 1966), and hyphal cells have a variable number of nuclei, from none to many, with a variable ratio of the genetically distinct nuclei. In some cells the nuclei form aggregates. This heterokaryon is capable of nuclear migration, which is consistent with prevalence of anucleate cells in proximity of multinucleate cells. The nuclei migrate at the same rate as compatible nuclei, but do not appear to be as densely distributed as in the dikaryon (Snider and Raper, 1958). In *S. commune*, the $A = B \neq$ heterokaryon gives rise mainly to haploid homokaryotic

colonies when subcultured from single hyphal tips. In this heterokaryon, the nuclei do not remain in close proximity of each other (Schuurs et al., 1998), which may account for its instability. Interestingly, in *S. commune* gene expression of some hydrophobin genes differs when the nuclei are near each other than when they are some distance apart in the same cell (Ásgeirsdóttir et al., 1995; Schuurs et al., 1998).

In *C. cinerea*, common *A* heterokaryons ($A = B \neq$) grow more slowly than the parental haploid homokaryons, and the colonies have an irregular outline due to their uneven growth. The hyphae fail to form clamp connections (Day and Swiezynski, 1960b) as observed in similar heterokaryons in *S. commune*. This heterokaryon exhibits active nuclear migration as evidenced by frequent degeneration of the septal apparatus, resulting in conversion of the dolipore septum to a simple septum (Casselton et al., 1971; Giesy and Day, 1965). Nuclear migration was slower during common *A* heterokaryon formation than during dikaryon formation, but the rate in both cases is faster than hyphal growth (Swiezynski and Day, 1960b). The common *A* heterokaryon is unstable and breaks down into its component homokaryons (Swiezynski and Day, 1960b).

The $A = B \neq$ heterokaryon acts as donor of either nucleus in interactions with any haploid homokaryon, resulting in dikaryotization of the haploid homokaryon, which is converted to an $A = B \neq$ heterokaryon (Swiezynski and Day, 1960b). The common *A* heterokaryon behaves as a dual mater, that is, it mates with two of four haploid testers, those with different *A* alleles (Swiezynski and Day, 1960a). Common *A* heterokaryons form some fruiting bodies in which the progeny show segregation of the *B* alleles (Swiezynski and Day, 1960a).

2.2.2.2. The $A \neq B =$ heterokaryon. Common *B* heterokaryons were obtained from an area of dense mycelium frequently formed at the junction of two mating haploid homokaryons with

different *A* and identical *B* alleles (Swiezynski and Day, 1960a, b). Hyphae from the zone of contact had unfused clamps (false clamps, pseudoclamps) and a few true clamps. In contrast, the dikaryon contains mostly true clamps and a few pseudoclamps. Hyphal tips exhibited dual mating behavior with strains having a different *B* allele. These observations support the notion that these strains are common *B* heterokaryons. Loss of nuclei was less common in common *B* than in common *A* heterokaryons, as determined by growth on different type of media that tests for the presence of auxotrophic markers from both nuclei. The common *B* heterokaryon grew more vigorously than the common *A* heterokaryon (Swiezynski and Day, 1960a, b), and formed fruiting bodies that produce progeny segregating for the *A* alleles (Swiezynski and Day, 1960a).

Common *B* heterokaryons are relatively stable in *C. cinerea* but very unstable in *S. commune* (Parag and Nachman, 1966). Two types of nuclei are present in most tip cells of large radiate hyphae in *S. commune*. Conjugate nuclear division occurs in the tip cell, but in contrast with the dikaryon, the clamp cell does not fuse (pseudoclamp) with the subapical cell and one of the daughter nuclei gets trapped in it, resulting in an uninucleate unfused clamp (hook) cell. Thus the subapical and clamp cell are uninucleate and the apical cell binucleate. The unfused clamp (pseudoclamp) is separated from the apical cell by a septum. There is no nuclear migration in this type of heterokaryon (Raper, 1966).

2.2.3. Noncompatible interactions: the $A=B$ heterokaryon. This type of heterokaryon was first recognized in *C. stercoreus* and *S. commune* (Fulton, 1950; Papazian, 1950). Further studies in *C. cinerea* and *S. commune* rigorously demonstrated that such $A=B$ heterokaryons can be generated (Middleton, 1964; Mills and Ellingboe, 1969; Swiezynski and Day, 1960a, b). Mating of *C. cinerea* haploid homokaryons with identical *A* and *B* alleles and carrying

1 complementing auxotrophic markers was used to select prototrophic common *AB* heterokaryons.
2 This heterokaryon appears to be unstable and recovery of the two parental nuclei was reduced
3 compared to the other heterokaryons. Furthermore, recovery of both nuclei is less frequent when
4 grown on complete than on minimal medium, in tests similar to those used in the analysis of $A=B$
5 $B \neq$ and $A \neq B$ heterokaryons. Nuclear ratios varied widely from 1:1 to ~400:1 ([Raper, 1966](#)).
6 The $A=B$ heterokaryon has no distinctive morphology, and macroscopically and
7 microscopically is indistinguishable from the haploid homokaryon (see [Raper, 1966](#)).
8

9 **3. Diploids in *C. cinerea* and *S. commune***

10 Interest in synthesizing diploids was motivated by the studies of Pontecorvo and
11 colleagues in the ascomycete *A. nidulans* ([Pontecorvo, 1956](#)). Diploid strains in this fungus are
12 unstable and generate somatic recombinants, most of which arise by haploidization, a
13 progressive loss of chromosomes, resulting in recombinant haploid strains (the parasexual cycle)
14 ([Pontecorvo, 1956](#)). Others arise by mitotic recombination, where markers distal to the point of
15 exchange become homozygous. These two processes are independent of each other.
16 Haploidization and mitotic recombination complement meiotic analysis. In the absence of
17 meiotic analysis, they may be the major genetic analysis method. The location of markers to
18 linkage groups can be ascertained from haploidization and the order of markers on a
19 chromosome arm can be determined by mitotic crossing over.

20 Seminal studies by R. Holliday led to the synthesis of diploids in *U. maydis* in the
21 laboratory, the first time this was accomplished in any basidiomycete fungus ([Holliday, 1961a,](#)
22 [b](#)). This important development suggested that it would be possible to obtain diploids in other
23 basidiomycetes such as *C. cinerea* and *S. commune*.

3.1 Diploids in *C. cinerea*.

Casselton (1965) synthesized diploids for the first time in *C. cinerea* using the technique developed by Roper (1952) in *A. nidulans*. *C. cinerea* common $A=B$ diploids derived from an $A=B$ heterokaryon are stable and can be distinguished from haploid homokaryons and common $A \neq B$ heterokaryons by growth rate and colony morphology. The $A=B$ diploid grows at an intermediate rate between that of the haploid homokaryon (faster growing) and the $A \neq B$ heterokaryon (slower growing) on certain media. The haploid homokaryon forms uniformly growing colonies with an even outline, characteristic of *C. cinerea* homokaryons. The $A \neq B$ heterokaryon forms colonies with irregular edges, and the $A=B$ diploid forms uniform colonies with a “feathered” appearance, due to production of long radiating hyphae with numerous short branches. The frequency of $A=B$ diploid formation (diploid oidia produced by the heterokaryon), in *C. cinerea* is $\sim 1 \times 10^{-4}$ to 1×10^{-3} (Casselton, 1965), 100x – 1000x higher than that observed in *A. nidulans* ($\leq 10^{-6}$) (Pontecorvo, 1956), but lower than that reported in *U. maydis* ($\leq 10\%$; see section 5.3) (Holliday, 1961b). Diploids of *C. cinerea* appear to be very stable and rarely undergo haploidization (Casselton, 1965), in contrast to *A. nidulans* diploids (Pontecorvo, 1956). There are no reports of $A \neq B \neq$, $A \neq B =$, or $A = B =$ diploids in *C. cinerea*.

Common A diploids can form dikaryons with haploids and other diploids (Casselton, 1965; Casselton and Lewis, 1966). The $2N + N$ dikaryons form some fruiting bodies, which take longer to mature than those formed by $N + N$ dikaryons, and produce low numbers of basidiospores that have abnormal appearance. Normal basidiospores are elliptical, regular in size, and have a single germ pore, whereas those from $2N + N$ dikaryons are variable in shape, irregular in size, some with two germ pores. Large numbers of aborted spores as well as

1 abnormal tetrads were produced. Basidiospores failed to grow or grew poorly, indicating
2 possibly an unbalanced number of chromosomes. In some crosses, all the markers present in the
3 diploid nucleus were recovered in the progeny, indicative of triploid meiosis, whereas in others
4 only some of the markers of the diploid occurred in the progeny, suggesting that they had been
5 lost by haploidization of the diploid nucleus sometime during dikaryon formation and fruiting
6 body formation. In summary, in $2N + N$ dikaryons markers in the diploid nucleus may persist
7 through meiosis, but more often haploidization of the diploid nucleus takes place (Casselton,
8 1965). Haploidization in *C. cinerea* appears to be due to gradual loss of chromosomes because
9 both aneuploid and haploid nuclei are found. These observations indicate that the diploid
10 nucleus becomes unstable in the presence of a haploid nucleus. This instability is not due to lack
11 of synchrony of nuclear division cycles between the diploid and haploid nucleus in a $2N + N$
12 dikaryon because the diploid nucleus is also unstable in $2N + 2N$ dikaryons (Casselton and
13 Lewis, 1966). Analysis of chlamydospores (asexual spores produced by dikaryons), veil cells
14 (fruiting body), and basidiospores (sexual spores) revealed that haploidization of one or both
15 diploid nuclei in a $2N + 2N$ dikaryon, but more often of one diploid nucleus, occurs sometime
16 after dikaryon formation and fruiting body formation (Casselton and Lewis, 1966). These
17 studies clearly demonstrate that the diploid nucleus is unstable in the presence of a haploid or a
18 diploid nucleus but is stable by itself.

20 **3.2. Diploids in *S. commune*.**

21 An $A \neq B \neq$ diploid arose spontaneously after mating of two compatible haploid strains that
22 had been mutagenized with X-rays to isolate auxotrophic mutants (Koltin and Raper, 1968).
23 Hyphal tips of the presumed $A \neq B \neq$ diploid were prototrophic, whereas the progenitor haploids

1 were auxotrophic at multiple loci. Fruiting bodies obtained from the prototrophic mycelia
2 produced progeny segregating for all 7 allelic loci present in the parental strains. The $A \neq B \neq$
3 diploid has similar morphology to that of a haploid homokaryon rather than that of a dikaryon:
4 the cells are uninucleate and there are no clamp connections. This suggests that processes
5 regulated by *A* are downregulated in this diploid. The diploid mates with haploid strains to
6 establish $2N + N$ dikaryons. The diploid nucleus may become unstable in these dikaryons
7 (Koltin and Raper, 1968). Instability of a diploid nucleus in $2N + N$ dikaryons also occurs in *C.*
8 *cinerea*, as just described (Casselton, 1965; Casselton and Lewis, 1966), and *Armillaria* (see
9 below) (Anderson and Ullrich, 1982).

10 Spontaneous formation of this diploid appears to be due to the presence of a recessive
11 mutation in each mate, designated *dik*⁻ (Koltin and Raper, 1968). It was proposed that the *dik*
12 gene inhibits karyogamy in the vegetative mycelium. Thus, the wild type *dik* gene is necessary
13 for maintenance of the dikaryotic state. Little is known about the *dik* gene, for example, does it
14 code for a protein or a regulatory RNA? How does it control maintenance of the dikaryon? To
15 my knowledge, the map position of *dik* is unknown. The *dik* gene and similar genes may provide
16 clues about the genetic programming that regulates karyogamy in *S. commune* and other
17 basidiomycetes.

18 $A \neq B \neq$ diploids were also isolated from crosses between common *AB* diploids with a
19 compatible haploid (Frankel and Ellingboe, 1976). The diploids exhibited a flat morphology,
20 had mostly uninucleate cells, and fruited spontaneously. In some cases, they gave rise to
21 aneuploid and haploid sectors, indicative of haploidization. These particular diploids appear to
22 be unstable.

1 In *S. commune*, a common *B* diploid strain was obtained from a common *B* heterokaryon
2 (Parag and Nachman, 1966). This diploid is stable. The cells in the mycelium of this diploid are
3 usually uninucleate, except the apical cell, which may be binucleate or uninucleate. Unfused
4 clamps (pseudoclamps) occur at each septum. A cross between the $A \neq B$ diploid and a haploid
5 homokaryon resulted in segregation expected from a $2N \times N$ cross, that is, triploid segregation.

6 Common *AB* diploids were isolated from a common *AB* heterokaryon in *S. commune*
7 (Mills and Ellingboe, 1969). The diploids were obtained from dense, rapidly growing, white fan-
8 shaped sectors that arose from the sparse irregular mycelia of the common *AB* heterokaryon.
9 They are stable upon transfer to different media. The common *AB* diploid morphology
10 resembles that of the haploid homokaryon. Analysis of markers present in basidiospores
11 obtained from crosses between the diploid (*A41/A41 B41/B41*, heterozygous for five auxotrophic
12 markers) with a compatible haploid (*A42 B42*) indicate that haploidization occurs in some but
13 not all crosses between common *AB* diploids and compatible homokaryons.

14 In general, diploids in *C. cinerea* and *S. commune* are relatively stable. Instability occurs
15 in the presence of a haploid or diploid nucleus in $2N + N$ or $2N + 2N$ dikaryons, resulting in
16 haploidization of the diploid nucleus sometime after dikaryon formation and formation of the
17 fruiting body. The molecular mechanism that triggers this instability is unknown.

19 **3.3. Mating interactions between dikaryons/heterokaryons/diploids and homokaryons**

20 One interesting observation is that dikaryons can mate with haploid homokaryons and
21 donate either nucleus to a haploid homokaryon (dikaryon-monokaryon or di-mon matings;
22 Papazian, 1950). This leads to dikaryotization of the haploid homokaryon resulting in formation
23 of a new dikaryon (the Buller phenomenon; Buller, 1930; Quintanilha, 1937). Sometimes both

nuclei of the dikaryon are transferred to the haploid homokaryon and displace the resident nucleus. Dikaryotization of a haploid homokaryon results in new nuclear-cytoplasmic interactions that may confer selective advantage under some conditions or be deleterious under others. The dikaryon is not capable of accepting nuclei. Thus, in these matings there is only unilateral transfer of nuclei in contrast to the reciprocal exchange that occurs during mating between compatible haploid homokaryons. Dikaryons can also interact with other compatible dikaryons but because nuclear migration is blocked in these interactions, new dikaryons arise only from the zone of interaction of the two mycelia. Although both nuclei from a dikaryon can be donated, there appears to be some bias for which nucleus is transferred (Ellingboe, 1964; Nieuwenhuis et al., 2011, 2013; Nogami et al., 2002; Tanesaka et al., 1994). The recipient nucleus appears to determine which nucleus is accepted. In some cases, the outcome is dependent on interaction between the two nuclei (Nieuwenhuis et al., 2011). Nuclear selection also seems to be involved in the formation of trikaryons in basidiomycete fungi with multinucleate cells (James et al., 2009) and in recovery of nuclei upon dikaryotization of a dikaryon (Nieuwenhuis et al., 2013).

Most heterokaryons and diploids can only donate nuclei to a haploid homokaryon; they cannot accept nuclei. The $A \neq B \neq$ diploid as well as the common B ($A \neq B =$) heterokaryon can donate but cannot accept nuclei from a haploid homokaryon. Common A ($A = B \neq$) heterokaryons in *C. cinerea* act as donors and also accept nuclei from compatible or common A homokaryons (Swiezynski and Day, 1960b), whereas the common A diploid acts solely as donor in matings with compatible or semi-compatible haploids (semi-compatible means having a B allele in common with the diploid). Common AB ($A = B =$) heterokaryons and diploids donate and accept nuclei to/from compatible and common A haploid homokaryons. It was proposed that inability

1 of the dikaryon to act as recipient may be due to differences in chemical properties of the septa in
2 dikaryons compared to haploid homokaryons, with the septa in the former being more resistant
3 to chemical degradation (Casselton et al., 1971). It remains to be determined if such chemical
4 difference exists.

5 The haploid homokaryon and dikaryon are characterized by the presence of complex
6 (dolipore) septa between cells (Müller et al., 2000; Orlovich, 1994; Raudaskoski, 1998; van
7 Driel, 2009). In compatible matings and common A matings between haploid homokaryons, the
8 dolipore septum is converted to a simple septum in both *C. cinerea* (Casselton et al., 1971; Giesy
9 and Day, 1965) and *S. commune* (Raper, 1966, and references therein). This presumably
10 facilitates the migration of nuclei, as evidenced by electron microscopic studies showing
11 mitochondria and nuclei traversing simple septa but not dolipore septa (Casselton et al., 1971;
12 Giesy and Day, 1965; Raudaskoski, 1998). Common A heterokaryons have both dolipore and
13 simple septa as do haploid homokaryons during active nuclear migration in dikaryon
14 establishment. In contrast, a common A diploid derived from a common A heterokaryon, has
15 only dolipore septa, indicating that there is no nuclear migration (Casselton et al., 1971). It was
16 proposed that different *B* alleles must reside in adjacent cells, not in the same cell, in order to
17 activate nuclear migration and modify the septal pore (Casselton et al., 1971). Determining if *B*
18 gene expression differs when the nuclei are near each other compared to nuclei that are distant
19 from each other in the same cell, as observed for expression of hydrophin genes under such
20 conditions (Schuurs, 1998; Ásgeirsdóttir et al., 1995), may shed light on these intriguing
21 observations.

22 23 **3.4. Phenotypic differences between dikaryons/heterokaryons and diploids.**

1 Comparisons of dikaryons/heterokaryons and their respective diploids suggest that
2 phenotypic expression differs when the same genetic information is contained in separate nuclei
3 versus one nucleus in the same cell. The $A \neq B \neq$ dikaryon and $A \neq B \neq$ diploid have identical
4 genotypes (with respect to mating type) yet the former has clamp connections and the latter does
5 not. Clamp connection formation is under control of the A locus, and fusion of the clamp with
6 the subapical cell is under control of the B locus (see [section 2](#)). Even though these strains have
7 the same genetic information, different A and B alleles reside in different nuclei in the dikaryon
8 and in the same nucleus in the diploid. A mere speculation is that activation of the A and B
9 regulated pathways requires that the mating type alleles be physically separated from each other,
10 as obtained in the exquisite nuclear movements that occur during dikaryon establishment and
11 maintenance, and that when nuclei come close to each other, the response is downregulated,
12 resulting in an oscillatory cycle of up and down regulation of the A and B loci during each
13 conjugate division. In a compatible diploid, the response would be downregulated all or most of
14 the time.

15 The $A = B \neq$ heterokaryon and the $A = B \neq$ diploid differ despite having the same genotype:
16 the former exhibits nuclear migration and the latter does not. This observation led to the
17 proposal that the difference is due to the distribution of B alleles in individual cells ([Casselton et](#)
18 [al., 1971](#)). In the $A = B \neq$ heterokaryon, the B alleles are irregularly distributed whereas in the $A =$
19 $B \neq$ diploid each cell has both alleles. The observations also indicate that inability of the $A = B \neq$
20 diploid to accept nuclei is correlated with the presence of different B alleles in the same cell.
21 This could mean that disruption of septa, which is necessary for nuclei to migrate through an
22 established mycelium, cannot occur when all the cells have the same two different B alleles,
23 which is also what is found in the $A \neq B \neq$ dikaryon ([Casselton et al., 1971](#)). Recent

transcriptome analysis of strains in which the *A* or *B* pathways are active has identified genes differentially expressed in each of these pathways compared to a strain where both pathways are active (Erdmann et al., 2011). Interestingly, these studies indicate that a pheromone precursor gene is upregulated by *A*. Future comparisons of these expression patterns to those of the corresponding diploids may help explain the phenotypic differences observed between dikaryons/heterokaryons and diploids. In addition, elucidating the molecular regulation of the *A* and *B* loci in cells of dikaryons, hemicompatible heterokaryons, and the respective diploids may provide insights about the genetic circuitry operating when identical genomes are compartmentalized differently in the same cell.

4. *Armillaria* – a basidiomycete with a persistent vegetative diploid phase.

A. mellea (Vahl ex Fr.) Kummer, the honey fungus, and other *Armillaria* species are phytopathogenic fungi of worldwide distribution that cause root rot of forest and fruit trees and other plants (Baumgartner et al., 2011; Guillaumin et al., 1991). The genus also includes bioluminescent species and some species that are “among the largest and oldest living organisms” (Smith et al., 1992). *A. mellea* is a heterothallic fungus with a tetrapolar mating type system. Mating type is determined by two unlinked mating type loci, *A* and *B*, which are multiallelic (Anderson and Ullrich, 1982; Korhonen and Hintikka, 1974; see Anderson and Kohn, 2007), similar to mating type determination in *S. commune* and *C. cinerea* and other basidiomycete fungi. Most species of *Armillaria* are heterothallic with a tetrapolar mating system but species with a bipolar mating system, as well as homothallic isolates have been described (Abomo-Ndongo et al., 1997; Baumgartner, 2012; Guillaumin et al., 1991; Ota et al., 2000).

1 The compatible fertile mycelium of *Armillaria species* consists of uninucleate cells
2 lacking clamp connections (Korhonen and Hintikka, 1974; Tommerup and Broadbent, 1975).
3 This mycelium forms fruiting bodies, which produce basidiospores showing expected Mendelian
4 segregation ratios of mating type alleles and other markers. Mating between compatible haploid
5 homokaryons leads to formation of a transient dikaryon with clamp connections, as observed in
6 many but not all *Armillaria* species (Anderson, 1982; Guillaumin et al., 1991; Korhonen and
7 Hintikka, 1974). This dikaryon is unstable, and the two nuclei fuse readily to form a diploid
8 nucleus. The resulting diploid mycelium is the persistent vegetative stage of *Armillaria* species
9 (Korhonen and Hintikka, 1974; Tommerup and Broadbent, 1975). In most *Armillaria* species,
10 the fruiting body is also diploid, but dikaryotic hyphae in the fruiting body have been
11 documented (Tommerup and Broadbent, 1975; Grillo et al., 2000; see below).

12 The only distinguishing feature between compatible and other interactions is colony
13 morphology. Common *A* ($A = B \neq$), common *B* ($A \neq B =$), and common *AB* ($A = B =$) interactions
14 produce a fluffy colony morphology indistinguishable from that of the haploid homokaryon,
15 whereas compatible interactions ($A \neq B \neq$) produce a flat crustose mycelium consisting of
16 uninucleate diploid cells (Anderson and Ullrich, 1982; Korhonen and Hintikka, 1974; Ullrich
17 and Anderson, 1978). Because common *A* and common *B* interactions are indistinguishable
18 from each other and from the common *AB* strain as well as from the haploid homokaryon, a
19 specific function cannot be assigned to each of the mating type loci.

20 To isolate compatible, common *A*, common *B*, and common *AB* diploids in the
21 laboratory, auxotrophic derivatives of four haploid tester strains (*A1 B1*, *A2 B2*, *A2 B1*, *A1 B2*)
22 were mated in all pairwise combinations with selection for prototrophy (Anderson and Ullrich,
23 1982; Ullrich and Anderson, 1978). Prototrophs were obtained in all instances and shown to be

1 uninucleate, not dikaryotic or heterokaryotic, which was taken as further evidence that the
2 vegetative state of *Armillaria* is diploid. Mating reactions with four different haploid testers can
3 be used as a criterion to differentiate between the hemicompatible diploids. $A \neq B \neq$ diploids
4 cause the fluffy testers to become crustose, indicating that the diploids dikaryotize the haploid
5 homokaryons (similar to the Buller phenomenon; [Buller, 1930](#); see [section 2](#)). The $A = B \neq$ strain
6 mates with testers having a different *A* allele (dual mater), whereas the $A \neq B =$ strain mates with
7 testers carrying a different *B* allele (dual mater). In contrast, the $A = B =$ diploid is compatible
8 with only one of the testers, that containing different *A* and *B* alleles, and thus behaves as the
9 haploid homokaryon ([Anderson and Ullrich, 1982](#)). Thus, in mating reactions, diploids of *A.*
10 *mellea* behave similarly to those of *S. commune* ([Koltin and Raper, 1968](#); [Parag and Nachman,](#)
11 [1966](#)) and *C. cinerea* ([Casselton, 1965](#); [Casselton and Lewis, 1966](#)). The ability to generate all
12 four types of diploids in *Armillaria* (albeit under strong selective pressure) indicates that cell
13 fusion is independent of the mating type alleles present in the mates as observed in *S. commune*
14 and *C. cinerea* (see [section 3](#)). Furthermore, these studies indicate that karyogamy is also
15 independent of the mating type loci, at least under the conditions used ([Anderson and Ullrich,](#)
16 [1982](#)).

17 Because synthetic diploids of *A. mellea* are very stable, and because fruiting body
18 formation occurs rarely in the laboratory, an alternative way of performing genetic analysis is by
19 induction of somatic segregation. Various chemical agents and *UV* irradiation are known to
20 increase somatic segregation in diploids of other fungi. Benomyl treatment of compatible,
21 common *A*, and common *B* diploids of *A. mellea*, heterozygous for auxotrophic markers, induces
22 haploidization as evidenced by the generation of segregants with a different mating behavior
23 than that of the parental diploid, near haploid DNA content, and loss of heterozygosity at several

1 loci. These features are indicative of haploidy or aneuploidy ([Anderson, 1983; Anderson et al.,](#)
2 [1985](#)).

3 Matings between diploids and haploids result in haploidization of the diploid nucleus
4 ([Baumgartner et al., 2010; Korhonen, 1983; Peabody and Peabody, 1985; 1987; Peabody et al.,](#)
5 [2000](#)). Using restriction fragment length polymorphisms to analyze $2N + N$ dikaryons of *A.*
6 *gallica*, it was shown that the diploid nucleus replaces the resident haploid nucleus. In other
7 cases, the diploid nucleus is recombinant and carries markers from both the diploid and haploid
8 nucleus, suggesting that haploidization takes place ([Carvalho et al., 1995](#)). It was proposed that
9 haploidization of the diploid nucleus is followed by fusion with the haploid partner to produce a
10 diploid. In a few cases, the nucleus appears to be triploid, as evidenced by the retention of
11 markers from the diploid and haploid.

12 Interestingly, haploidization is not limited to $2N + N$ dikaryons. It appears to occur
13 during fruiting body (basidiocarp) formation in *Armillaria* species. In some parts of the
14 developing basidiocarp, homokaryotic and dikaryotic hyphae are present, the former lack clamps
15 and the latter have them ([Tommerup and Broadbent, 1975](#)). Hyphae with terminal cells
16 containing 5 – 11 nuclei, which appear to be actively dividing, are present at the site of origin of
17 the gills (see [Kües and Navarro-Gonzalez, 2015](#), for details on basidiocarp developmet in
18 basidiomycetes). These multinucleate cells produce dikaryotic branches, and eventually form
19 clamped basidia containing two nuclei, which fuse and undergo meiosis to produce four
20 uninucleate (haploid) basidiospores ([Tommerup and Broadbent, 1975](#)). It was proposed that
21 haploidization occurs in the multinucleate cells ([Tommerup and Broadbent, 1975](#)). The nuclei in
22 the dikaryotic and multinucleate cells are significantly smaller than those in the diploid cells.
23 DNA content is consistent with haploidy ([Peabody and Peabody, 1985; 1987](#)), supporting the

1 notion that haploidization of the diploid nucleus takes place in fruiting-body primordia
2 ([Tommerup and Broadbent, 1975](#)).

3 Studies in *A. tabescens* also indicate that the fruiting body contains dikaryotic hyphae and
4 clamped basidia. Haploidization of the diploid nucleus was demonstrated using random
5 amplification of satellite markers (RAMS), mating type reactions with sets of testers, and colony
6 morphology ([Grillo et al., 2000](#)). Haploidization results in formation of a dikaryon, which
7 resembles in all respects the dikaryon produced in compatible matings. Homokaryotic
8 components isolated from dikaryotic fruiting-body primordia show fluffy morphology compared
9 to the flat morphology of the compatible diploid. Upon mating with a compatible isolate, these
10 homokaryons form a dikaryon that has clamp connections and is unstable; the haploid nuclei fuse
11 and give rise to a diploid nucleus. The homokaryons obtained from dikaryotic cells of the
12 fruiting body had recombinant mating types and DNA markers, supporting the notion that the
13 diploid nucleus haploidizes during fruiting body formation ([Grillo et al., 2000](#)). The mechanism
14 of haploidization and the signals that trigger it remain unknown. It is intriguing that the diploid
15 nucleus undergoes haploidization in the fruiting bodies to then form a dikaryon and ultimately a
16 diploid nucleus that undergoes meiosis. Somatic haploidization is proposed to contribute to
17 genetic variability of *Armillaria* populations ([Peabody et al., 2003](#)).

18 In summary, members of the genus *Armillaria* are unique among basidiomycetes because
19 of their persistent vegetative diploid phase. I next describe dikaryons and diploids in some
20 dimorphic basidiomycetes.

22 **5. Dikaryons and diploids in representative dimorphic basidiomycetes.**

1 **5.1. *Cryptococcus neoformans*** is an opportunistic pathogen of immuno-compromised patients
2 that causes cryptococcal meningitis. It is characterized by a yeast and a filamentous form.
3 Mating is determined by a bipolar system: a single mating type locus with 2 alleles, *MATa* and
4 *MAT α* . The *MAT* locus is complex clustering genes with diverse functions, including genes for
5 pheromone and pheromone receptor, signaling components, homeodomain transcription factors,
6 and others, and encompasses ≥ 100 kb ([Lengeler et al., 2002](#)). Fusion of an *a* cell with an *α* cell
7 leads to formation of a dikaryotic filament with clamp connections as in other basidiomycetes.
8 The dikaryon does not form fruiting bodies as in *C. cinerea* or *S. commune* but the apical cell of
9 the dikaryotic filament differentiates into a basidium, where karyogamy occurs followed
10 immediately by meiosis and rounds of mitoses to produce four chains of haploid basidiospores
11 on the surface of the basidium.

12 Diploids arise spontaneously from certain crosses, and can also be synthesized in the
13 laboratory, and occur in the environment. I first describe diploids obtained in the laboratory and
14 then those that found in the environment. In certain crosses between *a* and *α* haploid strains
15 carrying complementing auxotrophic markers, diploid basidiospores are obtained, which form
16 filaments with uninucleate cells and unfused clamp cells (pseudoclamps) ([Sia et al., 2000](#)). Thus
17 the diploid differs from the dikaryon in that the former has uninucleate cells and pseudoclamps,
18 and the latter has cells with two nuclei and clamp connections. The nuclei in cells of the diploid
19 are approximately twice as large as those in the haploid parental strains, and have a DNA content
20 twice that of the parental haploids, indicating that the nuclei are diploid or almost diploid. The
21 diploid filaments form basidia and produce haploid basidiospores with the expected Mendelian
22 segregation ratios of auxotrophic markers, and segregation of *MATa* and *MAT α* (although with a
23 skewed segregation ratio), indicating that the strain is a *MATa/MAT α* diploid, capable of

undergoing meiosis. The a/α diploid filament has similar morphology to that of haploid α filaments during monokaryotic fruiting: both have uninucleate cells and unfused clamps (pseudoclamps). The a/α diploid strain exhibits a temperature-dependent dimorphic switch, and grows as yeast at 37°C and as filaments at 24°C (Sia et al., 2000).

MATa/MATa and *MAT α /MAT α* diploid strains were generated by mixing strains of identical mating type carrying complementing auxotrophic markers with a strain of opposite mating type (helper strain) with auxotrophic markers in common with the other two strains of opposite mating type. This selects for rare prototrophic diploids homozygous for *MATa* or *MAT α* , while counterselecting for the formation of *MATa/MAT α* diploids (Hull et al., 2002).

These diploids were not able to filament under conditions where the a/α diploid does (V8 medium) (Hull et al., 2002). The α/α diploid can filament and undergo fruiting under the same conditions as α haploids do. The a/a diploids do not filament, at least in the D serotype background (C. Hull, personal communication). The α/α filaments have unfused clamps (pseudoclamps). They produce basidiospores, the majority of which are diploid (80%) and the remainder (20%) haploid or aneuploid (C. Hull, personal communication). The haploid spores appear to be recombinant. Both α/α and a/a diploids mate with haploids of opposite mating type and with each other and form 2N + N or 2N + 2N dikaryons, respectively. Viable haploid and diploid basidiospores were recovered from both types of dikaryon. The haploid spores produced by 2N + N were not recombinant. The diploid spores produced by 2N + 2N dikaryons were recombinant. It has been proposed that karyogamy does not occur in the 2N + N dikaryon, but does occur in the 2N and 2N dikaryon (C. Hull, personal communication). It remains to be determined if the diploid nucleus becomes unstable in a dikaryon and undergoes haploidization as occurs in other basidiomycetes.

1 It is interesting that a/α and α/α diploids have unfused clamps (pseudoclamps). As we
2 have seen in *S. commune*, diploids heterozygous at both mating type loci lack clamp connections
3 or unfused clamps (false clamps, pseudoclamps) (see [section 3](#)). Common *B* but not common *A*
4 diploids have unfused clamps (pseudoclamps), and common *AB* lack clamp connections or
5 unfused clamps (pseudoclamps).

6 *C. neoformans* diploids occur in nature, and are of genotype a/α or α/α . The a/α
7 diploids in nature are usually of hybrid serotype (AD), and the α/α diploids are mostly of
8 serotype A. The hybrid serotype diploids can also be synthesized in the laboratory. They
9 produce few if any viable basidiospores, probably because of impaired meiosis due to the
10 divergence of serotype A and D ([Lengeler et al., 2002](#)). In contrast, a/α diploids of identical
11 serotype undergo meiosis and produce viable *MATa* or *MAT α* basidiospores. Hybrid $\alpha A/\alpha D$
12 diploids, likewise, do not produce viable basidiopores but if they are of the same serotype (D),
13 they undergo meiosis and produce viable spores ([Lin et al., 2007](#)). The $\alpha A/\alpha D$ diploids mate
14 with *a* strains (serotype D), and produce dikaryotic filaments with clamp connections.
15 Compatible 2N + N dikaryons also form clamp connections in *S. commune* and *C. cinerea* (see
16 [section 3](#)). The two nuclei in the *C. neoformans* 2N + N dikaryon alternate positions at each
17 conjugate division ([Lin et al., 2007](#)), as described for dikaryons in other basidiomycetes (see
18 [section 2](#)). Basidia of these 2N + N dikaryons contain one or multiple nuclei and, some have
19 four chains of basidiospores, but the spores are inviable (fail to germinate), which was
20 interpreted to be due to abnormal meiosis of a triploid nucleus. The fact that the progeny is
21 inviable may be indicative of extensive aneuploidy ([Lin et al., 2007, 2009](#)), perhaps due to
22 haploidization. a/α diploids of hybrid AD serotype are found in some geographical locations,
23 whereas a/a diploids are not ([Lin et al., 2009](#)). The finding that $\alpha A/\alpha D$ and $\alpha A/\alpha A$ diploids are

found in nature in diverse geographical locations, and that the majority are $\alpha A/\alpha A$ lends support to the notion that same sex mating is widespread in nature (see [Heitman and Sun](#) this issue, for in depth discussion of same sex mating in fungi). It was postulated that same sex mating may be an adaptation to the preponderance of α haploid isolates in the environment, or may be the source of the abundance of α cells ([Lin et al., 2007](#)).

5.2. *Microbotryum violaceum* (formerly *Ustilago violacea*) is a plant pathogen that induces anther smut disease in members of the Caryophyllaceae ([Thrall et al., 1993](#)). It is a dimorphic fungus belonging to the subphylum Pucciniomycotina ([Aime et al., 2006](#)), with a yeast-like nonpathogenic form and a filamentous pathogenic form. It has a single mating type locus with two alleles, *a1* and *a2*, thus it has a bipolar mating type system ([Giraud et al., 2008](#); [Hood, 2002](#); [Whitehouse, 1951](#)). Interestingly, the sex chromosomes exhibit length dimorphism (see for example, [Hood et al., 2013](#)). Haploid cells fuse readily under laboratory conditions but the dikaryon is unstable and buds off haploid cells. Day and Jones ([1968](#)) synthesized *a1/a2* diploids which form colonies that are more slimy and translucent than those formed by haploids. Several criteria indicate that they are diploid: presence of a single nucleus per cell, increased length and volume of cells, with a mean volume ≥ 2 that of haploids, ability to induce disease when inoculated singly into plants, and Mendelian segregation ratios of all markers present in the parental haploids, indicating that the markers were present in a heterozygous condition. The frequency of diploid formation is 3×10^{-4} ([Day and Jones, 1968; 1969](#)), similar to that for *C. cinerea* (see [section 3](#)), and much greater than the value observed for *A. nidulans*, but lower than that reported for *U. maydis* (see [section 5.3](#)) ([Casselton, 1965](#); [Holliday, 1961b](#); [Pontecorvo, 1956](#)).

1 The *a1/a2* diploids do not mate (see below) (Day and Jones, 1968), however, *a1/a1* or
2 *a2/a2* diploids, obtained from an *a1/a2* diploid by *UV*-induced mitotic recombination, mate with
3 compatible haploids to establish stable triploids (*a1/a1/a2* or *a1/a2/a2*, respectively), or with
4 each other to form tetraploids, which are unstable in culture. Formation of $2N + N$ or $2N + 2N$
5 dikaryons was not reported. It is interesting that tetraploids, although unstable, were obtained.
6 Tetraploids are very rare in basidiomycetes. An intriguing observation is that the *a2* allele
7 appears to exert dominance over *a1* in *a1/a2* diploids in an age-dependent manner. The *a1/a2*
8 diploid can mate as *a2* with an *a1* strain during the log phase of growth, but is unable to mate
9 during stationary phase. Diploids homozygous at the mating type locus can mate during
10 stationary phase (Cummings and Day, 1973; Day, 1972; Day and Cummings, 1973). One
11 plausible explanation is that the *a2* locus (or chromosome) induces a growth-phase-dependent
12 epigenetic modification (silencing) of the *a1* locus (or chromosome).

13 *UV* irradiation stimulates karyogamy and diploid formation (Day and Day, 1974).
14 Conditions that favor photoreactivation or dark repair reduce the effects of *UV*. Four *uv*
15 sensitive loci showed different effects on karyogamy. From studies of these mutants, it was
16 concluded that there is an inhibitor of karyogamy, whose transcription is reduced after *UV*
17 treatment. Could this inhibitor be analogous to the *dik* gene in *S. commune*? (see section 3). The
18 molecular nature of the *uv* loci is not known.

19 Spontaneous mitotic recombination and haploidization of diploids are rare but both can
20 be stimulated with different treatments (Day and Jones, 1969; Kokonis and Garber, 1983).
21 Irradiation with *UV* leads to recovery of segregants that can mate with the opposite mating type,
22 and are likely homozygous for one of the mating type alleles. It is also possible that the cells are
23 aneuploid for the chromosome where the mating type locus resides. Treatment with DL-p-

1 fluorophenyl alanine (PFP) readily produced haploids, as observed in *Aspergillus*. These
2 haploids arise as papillae from the diploid colonies plated on PFP medium. The cells in these
3 papillae were inferred to be haploid because of their size and ability to mate. The haploids had
4 recombinant and parental markers (Day and Jones, 1969). Because the life cycle requires growth
5 in the plant to produce diploid spores (teliospores) able to undergo meiosis, and because these
6 spores are produced only when the host attains sexual maturity, which may take several months,
7 haploidization and mitotic recombination provided a very useful alternative to meiotic analysis in
8 *M. violaceum*, and contributed to the generation of linkage maps and other findings (Day and
9 Jones, 1969).

10
11 **5.3. *Ustilago maydis*.** Diploids were described in *U. maydis* (Christensen, 1931) long before
12 Pontecorvo and colleagues generated them in *Aspergillus*. Synthetic diploids of *U. maydis* were
13 the first ones to be obtained in any basidiomycete fungus (Holliday, 1961a, b), and have been
14 extensively used in *U. maydis* research (see for example, Banuett and Herskowitz, 1989, 1994;
15 1996; Day et al., 1971; Puhalla, 1968; Ruiz-Herrera et al., 1995; Spellig et al., 1994). Diploids
16 played a key role in pioneering studies on mitotic recombination, allelic recombination, mitotic
17 gene conversion, and DNA repair (Holliday, 1961b, 1966, 1974; Holliday et al., 1976; reviewed
18 in Holliday, 2004). Here I provide a brief historical account of how diploids were first
19 identified, their synthesis in the laboratory, and their use in understanding the role of the mating
20 type loci in cell fusion, filamentous growth, pathogenicity, and meiosis.

21 *U. maydis* (DeCandole) Corda is a plant pathogen belonging to the subphylum
22 Ustilaginomycotina (Begerow et al., 2006) with a host range restricted to maize (*Zea mays* L.)
23 and teozintle (*Z. mays* subsp. *parviglumis* or *Z. mays* subsp. *mexicana*) (Christensen, 1963;

Banuett, 1995). It exhibits a yeast-like unicellular, uninucleate form that is nonpathogenic and a filamentous dikaryotic form that is pathogenic. Cell fusion of haploid unicellular yeast-like cells results in a dikaryon, whose growth in the host leads to formation of tumors. Karyogamy and diploid formation take place in the tumors, resulting in formation of teliospores. (Fig. 1) (Banuett and Herskowitz, 1996; Snetselaar and Mims, 1994). Teliospores germinate and undergo meiosis to produce haploid progeny (basidiospores) (Fig. 2) (reviewed in Banuett, 1995, 2007; Christensen, 1963; Holliday, 1961b).

A tetrapolar mating type system controls the life cycle. The *a* locus is biallelic and the *b* locus is multiallelic (Holliday, 1961a, b; Puhalla, 1968; Rowell and DeVay, 1954). Completion of the life cycle requires that haploid cells that fuse carry different alleles at both *a* and *b*. The *a* locus controls cell fusion (Banuett and Herskowitz, 1994; Rowell and DeVay, 1954; Rowell, 1955; Snetselaar, 1993; Snetselaar et al., 1996). It codes for allele-specific components of a pheromone response pathway: a pheromone precursor (*mfa*) and a pheromone receptor (*pra*) (Bölker et al., 1992; reviewed in Banuett, 2007; Vollmeister et al., 2012). The *b* locus is a major regulatory locus that controls filamentous growth (together with the *a* locus), pathogenicity, and meiosis. It contains 2 genes (*bE* and *bW*) that code for distinct homeodomain polypeptides. Interaction between two non-allelic homeodomain polypeptides (bEx-bWy) triggers filamentous growth and pathogenicity (Gillissen et al., 1992; reviewed in Banuett, 2007; Vollmeister et al., 2012). The *b* heterodimer is a major regulatory protein that directly or indirectly controls other regulatory proteins whose function is more directly involved in *b*-dependent processes (Brachmann et al., 2001; Heimel et al., 2010).

The filamentous dikaryon in culture consists of a long tip cell (>130 μ m), with 2 nuclei near each other in the middle of the cell, and basal septate compartments devoid of cytoplasm

(Banuett and Herskowitz, 1994; Steinberg et al., 1998; Valinluck et al., 2014). In culture, it is unbranched and lacks clamp connections, whereas *in planta*, it is branched and has clamp connections, suggesting that plant signals influence fungal morphogenetic events (Banuett and Herskowitz, 1996; Scherer et al., 2006; Heimel et al., 2010). The filamentous dikaryon is short-lived under laboratory conditions. Instability may result from inability to maintain the two nuclei in close proximity, as observed in *M. violaceum* (see section 5.2).

5.3.1. Spontaneously-occurring diploids. Diploids arise spontaneously in certain crosses (Christensen, 1931; Holliday, 1961a, b; Puhalla, 1968; Rowell and DeVay, 1954; Day et al., 1971). In some crosses the frequency was as high as 10% (Holliday, 1961a), whereas in others it was <1% (Puhalla, 1968). The reason for the high frequency of spontaneous diploid formation in certain crosses is not known but is likely due to failure of meiosis, as first inferred by Christensen (1931). The possibility that diploids arise by fusion of two diploid nuclei followed by tetraploid meiosis is less likely but cannot be excluded. In crosses between compatible strains, Christensen (1931) recovered progeny that induce disease when inoculated singly into plants. He coined the term “solopathogens” to describe these strains, which arose in 2.5% of the teliospore progeny analyzed. Because the solopathogens produced progeny that segregated for mating type, he inferred correctly that they were diploid and had arisen due to failure of meiosis in the teliospore.

Seminal studies by Rowell and DeVay (1954) demonstrated the existence of two mating type (or incompatibility loci) in *U. maydis*. Teliospores from a particular cross segregated the four expected combinations of *a* and *b* alleles (a tetratype tetrad), whereas independent teliospores from the same cross produced two distinct types of progeny. One was

1 solopathogenic, and the other was nonpathogenic but exhibited a dual mating pattern (able to
2 mate with two of four haploid tester strains), clearly distinct from the behavior of the haploid
3 segregants, which mate with only one of the four testers. They concluded that the former were
4 diploids heterozygous at *a* and *b*, and the latter were diploids heterozygous at *a* and homozygous
5 at *b*. Because the latter were nonpathogenic, they concluded that the *b* locus is necessary for
6 pathogenicity (Rowell, 1955, b; Rowell and DeVay, 1954), a conclusion subsequently confirmed
7 by others (Banuett and Herkowitz, 1989; Holliday, 1961a, b; Puhalla, 1968; Day et al., 1971).

8
9 **5.3.2. Synthesis of diploids.** Diploids were first obtained from immature tumor tissue, before
10 teliospores are detectable. The strains recovered were solopathogenic, had a DNA content twice
11 that of haploids, and meiotic progeny segregated as expected for a diploid heterozygous for the
12 mating type loci and other markers. The strains were stable (Holliday, 1961a, b). These diploids
13 segregate auxotrophic markers spontaneously with a frequency of $10^{-3} - 10^{-4}$, which can be
14 increased 10- to 100-fold by *UV* irradiation and other treatments. Spontaneous and *UV*-induced
15 segregation appears to be the result of mitotic recombination, not haploidization. The
16 auxotrophic diploid derivatives recovered were those predicted on the basis of mitotic
17 recombination (markers distal to the point of exchange become homozygous). The presence of
18 mosaic colonies, which represent reciprocal mitotic recombination events, is further evidence
19 that mitotic recombination was involved in generation of auxotrophic diploid segregants. In a
20 mosaic colony, approximately one half of the colony represents one product and the other half
21 the other. For example, one half of the colony is prototrophic and the other auxotrophic, or in
22 other cases, each half is of a different color due to a different auxotrophy present in the
23 respective recombinants, which confers a particular color to the colony (Holliday, 1961a, b;

1966; 1974; Holliday et al., 1976). The diploid auxotrophic segregants were mostly solopathogenic, indicating that they were heterozygous at both mating type loci ($a1/a2\ b1/b2$). Others induced tumors when inoculated with either $a1\ b1$ or $a2\ b1$ (dual maters) but not with $a2\ b2$ or $a1\ b2$, from which it was inferred that they were homozygous for $b2$ ($a1/a2\ b2/b2$), as previously described by Rowell and DeVay (1954) for spontaneously arising diploids (see above).

Studies with diploids led to the finding that spontaneous allelic recombination is about 1000-fold lower than mitotic recombination but is strongly stimulated by low doses of UV irradiation or other treatments. Allelic recombination appears to be largely the result of mitotic gene conversion (Holliday, 1966). Diploids homozygous for a mutant defective in excision of pyrimidine dimers have an enhanced frequency of UV-induced gene conversion. Heteroallelic diploids homozygous for $rec1-1$ and $rec2-1$ have a rec^- phenotype and allelic recombination is abolished. Additional studies also provided evidence for an inducible repair system long before the inducible repair system of *E. coli* was established (reviewed in Holliday, 2004).

Development of a plate-mating assay greatly facilitated synthesis of diploids (Puhalla, 1969), obviating the need for plant inoculations. Compatible ($a \neq b \neq$) and common b ($a \neq b =$) diploids were obtained by matings between compatible haploids, and between haploids with identical b alleles, respectively. Common a ($a = b \neq$) and common ab diploids ($a = b =$) were not obtained after the appropriate matings (Puhalla, 1968), a not surprising result given that the a locus was proposed to govern cell fusion (Rowell, 1955; Rowell and DeVay, 1954). A set of congenic diploids ($a \neq b \neq$, $a = b \neq$, $a \neq b =$, and $a = b =$) was synthesized using a modification of Puhalla's method (1969) (see Fig. 3) (Banuett and Herskowitz, 1989). These diploids are stable. The $a \neq b \neq$ diploid was solopathogenic and the $a \neq b =$ diploid was nonpathogenic, confirming

1 results by others (Holliday, 1961a, b; Puhalla, 1968; Rowell and DeVay, 1954). On charcoal
2 medium, mating of haploid strains ($a1\ b1$, $a2\ b2$, $a2\ b1$, and $a1\ b2$) and common a or common
3 b diploids with four standard haploid testers results in a now classical reaction (Fig. 3). The $a=$
4 $b\neq$ and $a\neq\ b=$ diploids do not form mycelial colonies on this medium but are dual maters and
5 form a fuzzy reaction with haploid strains that have different a or different b alleles, respectively
6 (Fig. 3) (Banuett and Herskowitz, 1989), similarly to the mating pattern of common A and
7 common B diploids/heterokaryons in other basidiomycetes (see sections 3 and 4). These
8 hemicompatible *U. maydis* diploids can also be distinguished at the cellular level (Banuett and
9 Herskowitz, 1994). The $a= \ b=$ diploid behaves as a haploid and forms a fuzzy reaction with
10 only one of the testers (Banuett, unpublished). On charcoal medium it cannot be determined if
11 $a\neq\ b\neq$ diploids mate because they form mycelial colonies, however, in a cytoduction assay $a\neq\ b\neq$
12 diploids mate with haploids at a frequency 100-fold lower than that between compatible
13 haploids, and $a\neq\ b=$ diploids can fuse with a haploid with a different b allele at a frequency
14 similar to that between compatible haploids. These observations led to the conclusion that
15 heterozygosity at b attenuates cell fusion (Laity et al., 1995). The $a\neq\ b\neq$ diploid is more stable
16 in culture than the dikaryon (Banuett and Herskowitz, 1989). Symptoms produced by the $a\neq\ b\neq$
17 diploid solopathogen have been reported to be weaker than those produced by the dikaryon on
18 some maize inbred lines (Babu et al., 2005; Christensen, 1963; Holliday, 1961b) but not on
19 others (Banuett and Herskowitz, 1996).

20 Analysis of the $a= \ b\neq$ diploid in culture led to the conclusion that different a alleles are
21 necessary for filamentous growth. Because this strain is solopathogenic and produces filaments
22 *in planta*, it was concluded that different a alleles are not required for filamentous growth in the
23 plant or for pathogenicity. This diploid produces teliospores and segregates markers in the

1 expected Mendelian ratios, indicating that different *a* alleles are not necessary for meiosis
2 (Banuett and Herskowitz, 1989, 1996). Studies with different diploids contributed to a better
3 understanding of the role of the mating type loci in filament formation, pathogenicity, and
4 meiosis (Banuett and Herskowitz, 1989; 1996).

5 Synthetic diploids homozygous at *b* were used to isolate *b* mutant alleles (*bmut*) (Day et
6 al., 1971), that is, alleles that behave as having a different *b* allele specificity from that of the
7 parental allele. The diploids containing *bmut* (*a1/a2 b/bmut*) were solopathogenic. The tumors
8 produced were smaller, took longer to mature, and contained fewer teliospores than those
9 produced by *a*≠ *b*≠ solopathogenic strains. Additional studies with *bmut* strains led to the
10 conclusion that *b* is necessary for meiosis and basidiospore formation, in addition to its
11 previously defined role in tumor induction (Day et al., 1971).

12 Diploids in *U. maydis* are stable, as are those in *C. cinerea*, *S. commune*, *C. neoformans*,
13 and *M. violaceum*, and do not haploidize and give rise to haploid segregants as occurs in
14 *Aspergillus*. Little is known of the behavior of a diploid nucleus in 2N + N or 2N + 2N
15 dikaryons in *U. maydis*. Development of conditions for long-term culture of the dikaryon would
16 facilitate analysis of such dikaryons.

17 In summary, studies with diploids in *U. maydis* confirmed the critical role of the *b* locus
18 in filamentous growth, tumor induction, meiosis, and basidiospore formation; uncovered a role
19 for the *a* locus in maintenance of filamentous growth via an autocrine response (Banuett and
20 Herskowitz, 1989, 1994; Spellig et al., 1994), and demonstrated that the presence of different *a*
21 alleles are not necessary for pathogenicity or meiosis in strains that bypass cell fusion (Banuett
22 and Herskowitz, 1989, 1996). Diploids played a key role in cloning of the mating type loci by
23 functional complementation (Froeliger and Leong, 1991; Kronstad and Leong, 1990; Schulz et

al., 1990), in purification of the mating pheromones (Spellig et al., 1994), in elucidation of the role of signaling components downstream of the pheromone and pheromone receptors (Banuett and Herskowitz, 1989, 1994, 1996), and in identifying key residues within the b protein which are important for protein-protein interactions (Kämper et al., 1995).

6. CONCLUDING REMARKS.

A recurring theme in this discussion of dikaryons, heterokaryons, and diploids in diverse basidiomycete fungi is that dikaryons (heterokaryons) and diploids of identical genotype (with respect to mating type loci) exhibit phenotypic differences. This is rather surprising and has led to the view that gene expression differs when the genetic information is contained in two nuclei versus one nucleus in the same cell. As described above, examples include the formation of clamp connections in dikaryons of *S. commune* and their absence in the diploid; nuclear migration in the common A heterokaryon and its absence in common A diploids; differences in symptom development between dikaryons and diploids in *U. maydis*; differences in stability of dikaryons and diploids in culture as observed in *U. maydis* and *M. violaceum*. The phenotypic differences may result from differential gene expression as suggested by the studies of Saville and colleagues (Babu et al., 2005) who used whole genome expression profiling to compare gene expression between a dikaryon and a diploid in *U. maydis* grown under the same conditions. These studies indicate that the two genetically identical strains exhibit differences in gene expression, which may account for some of the phenotypic differences between dikaryons and diploids. This result lends credence to the notion that differential gene expression results when two genomes are present in two separate nuclei versus a single nucleus in the same cell. If so,

1 the challenge is to figure out why this compartmentalization of the genomes results in differential
2 gene expression.

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FIGURE LEGENDS

Figure 1. Life cycle transitions in *Ustilago maydis*.

Three basic forms characterize the life cycle of *U. maydis*: a yeast-like cell, a filamentous form, and a spore (teliospore). The transition from one form to the other is accompanied by changes in ploidy (N to N + N to 2N), growth habit (saprophytic to parasitic), and ability to induce tumors (non pathogenic to pathogenic), and entails three processes: meiosis, conjugation, and karyogamy, respectively. These transitions are governed by two unlinked mating type loci, *a* and *b*, by the environment and by nutrients. The fungus undergoes additional morphological changes in the host that are not observed in culture, suggesting that host signals play an important role in fungal differentiation ([Banuett , 1995](#)).

Figure 2. Teliospore germination and meiosis.

The teliospore is a diploid spore, resistant to adverse environmental conditions. It is formed within the tumors following a discrete developmental program during which changes in morphology take place and karyogamy occurs ([Banuett and Herskowitz, 1996](#)). Upon germination, a short filament (the promycelium) emerges into which the diploid nucleus migrates

1 and undergoes meiosis to form a septate promycelium consisting of four haploid cells, which bud
2 off yeast-like cells ([Banuett, 2002](#)).

3
4 **Figure 3. The fuzz reaction of haploid and diploid strains on charcoal agar.**

5 Haploid and diploid strains carrying different *a* and *b* alleles produce a white fuzziness due to the
6 formation of filaments in combination with haploid tester strains ([Banuett and Herskowitz,](#)
7 [1989](#)). Saturated cultures of tester strains (vertical lines) were cross-streaked versus haploid (top
8 four horizontal lines) and diploids (bottom four horizontal lines). The testers are from left to
9 right: *a1 b1* (FB1), *a2 b2* (FB2), *a1 b2* (FB6b), and *a2 b1* (FB6a). The horizontal lines
10 represent, from top to bottom, strains *a2 b2*, *a1 b1*, *a2 b1*, *a1 b2*, *a1/a2 b1/b1* (class I), *a1/a2*
11 *b2/b2* (class II), *a1/a1 b1/b2* (class III), and *a2/a2 b1/b2* (class IV). (From [Banuett and](#)
12 [Herskowitz, 1989](#), with permission).

Figure 1

FIGURES

Figure 2

Figure 3