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Genetics and Breeding of Mushrooms — from Bensaude and Kniep to Molecular Genetics

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1 CONTROL OF THE LIFE CYCLE

1.1 *Schizophyllum and Coprinus*

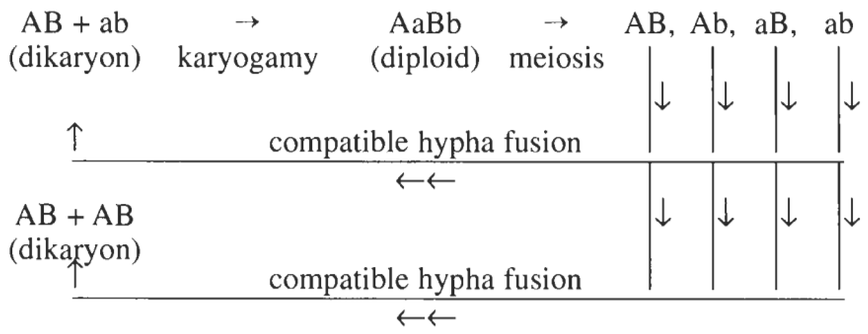
In 1904, Blakeslee had demonstrated sexuality in *Phycomyces* by confronting monosporous mycelia and observing that zygospores were formed only in matings involving different strains that he referred to as plus and minus. However, the mechanism of control of sexuality in Basidiomycetes was not discovered until the period of World War I with the investigations of Kniep (1920) in Germany and Bensaude (1918), a Portuguese woman studying for her Ph.D. at the University of Paris. Kniep and Bensaude both followed the procedure of Blakeslee by obtaining single basidiospore mycelia and confronting these mycelia in all possible combinations. The criterion for compatibility was the formation of clamp connections, for it had been observed that mycelia that formed fruiting bodies of *Schizophyllum* and *Coprinus*, or mycelia that were derived from the fruiting bodies, bore clamp connections. Clamp connections can be determined microscopically and are readily distinguished from unclamped hyphae, which have a simple cross wall, by the bulges (hook cell, buckles, or clamps) which are present in the clamped, dikaryotic hyphae. The dikaryotic hyphae contain two unfused, compatible nuclei in each cell. This condition is maintained by the process of clamp connection formation in which there is an outgrowth from an apical cell in which the dikaryon is located. The outgrowth is the hook cell which grows backward and eventually fuses with the penultimate cell. The compatible nuclei that form the dikaryon undergo simultaneous division, and one of the daughter nuclei passes into the hook cell. Septa form across the main hypha at the place where the hook cell arises and across the base of

the hook cell at the same place. The nuclear situation is such that two compatible nuclei are in the tip cell, one in the hook cell and one in the penultimate cell. With the fusion of the hook cell to the penultimate cell, the nucleus in the hook cell passes into the penultimate cell, establishing a dikaryon there. It is thought that the function of the clamp connection is to provide a passageway to maintain this dikaryotic condition, since the nuclei are large relative to the diameter of the hypha and they could not otherwise pass one another to maintain the dikaryotic condition in the cells. The dikaryotic condition is important in that the dikaryon acts in genetic complementation as does a diploid nucleus.

Bensaude and Kneip worked independently of each other without any knowledge of the other's findings because of the war situation, which curbed scientific communications between Germany and France. Bensaude worked with a species of *Coprinus* which she referred to an tetrapolarity with a very small number of single basidiospore isolates. She also described the nuclear situation in clamped hyphae.

1.2 *The concept of mating type*

In tetrapolarity, two mating type loci are involved and for compatibility, a heteroallelic condition is necessary at both loci. It was Kniep who gave a more complete explanation of tetrapolarity, however, with his studies of a larger number of monosporous mycelia of *Schizophyllum commune*. Essentially, what Kniep demonstrated was this:



The basidiospores are of four different mating types as indicated above. (When we speak of mating type we are referring to sexual differences which are due to genetic factors, there being in most basidiomycetes no morphological sexual distinctions.) Kniep mated the

mycelia arising from single basidiospores in all possible combinations as shown in the following table:

	AB	Ab	aB	ab
AB	–	–	–	+
Ab	–	–	+	–
aB	–	+	–	–
ab	+	–	–	–

In this table the minus indicates an absence of clamp connections and the plus indicates the presence of clamp connections. It can be seen that clamp connections are only formed when there is a heteroallelic condition at both mating type loci.

One of the earlier discoveries following the studies of Kniep and Bensaude on tetrapolarity (now commonly referred to as bifactorial heterothallism) was that whereas the progeny from two individual fruiting bodies of a species might each display tetrapolarity, when single spore progeny from one fruiting body were confronted with those of the other, there might be complete compatibility. Although this was denoted by the term geographical races, it was later discovered that the distance between the fruiting bodies might be quite small — the opposite sides of a horse dung ball for *Coprinus* (Brunswik 1924), or in close proximity on a tree trunk for *Schizophyllum commune* (Miles *et al.* 1966). However, when

Fruiting body I

	AB	Ab	aB	ab
AB	–	–	–	+
Ab	–	–	+	–
aB	–	+	–	–
ab	+	–	–	–

Fruiting body II

	A'B'	A'b'	a'B'	a'b'
A'B'	–	–	–	+
A'b'	–	–	+	–
a'B'	–	+	–	–
a'b'	+	–	–	–

monosporous mycelia from FB I were crossed with monosporous mycelia from FB II, clamped mycelia were formed. For example, AB x A'B' → +, and when single spores were collected from the fruiting body formed from the dikaryotic mycelium of this "hybrid fruiting body," it was predicted that the following mating types should be obtained: AB, AB', A'B and A'B'. This was confirmed by mating the progeny from the "hybrid

fruiting body" against testers from fruiting body I and fruiting body II as seen as follows:

		Hybrid testers			
		AB	Ab	aB	ab
FB I	AB	–	–	–	+
testers	Ab	–	–	+	+
	aB	–	+	–	+
	ab	+	+	+	+

		Hybrid testers			
		A'B'	A'b'	a'B'	a'b'
FB II	A'B'	+	–	–	–
testers	A'b'	+	+	–	–
	a'B'	+	–	+	–
	a'b'	+	+	+	+

Obviously, there are multiple alleles of the mating type factors. Kniep (1923) had discovered some exceptions in the mating reactions of *S. commune*. An occasional isolate turned up which was compatible with not just one, but with two of the four mating types. If we now use the simplified terminology introduced by the late Professor John R. Raper of Harvard University, this can be illustrated as follows. From the cross A1B1 x A2B2, following testers are obtained, A1B1, A1B2, A2B2. When the isolates of the cross are mated with these testers, results such as shown in the following table might be obtained.

		A1B1	A1B2	A2B1	A2B2
Monosporous	A1B1	–	–	–	+
isolates	A2B2	–	–	+	–
	A2B1	–	+	–	–
	A2B2	+	–	–	–
	A*B1	–	+	–	+
	A1B*	–	–	+	+

Knief suggested that the asterisked mating type factors were the result of mutation, but they appeared in higher frequency (*ca.* 2%) than one would expect for spontaneous mutations, and these mutations only occurred in cultures newly obtained from spore germlings. The origin of these asterisked mating types are not determined until the 1960's, but there are a few other findings that we should consider first.

Kniep attempted to obtain tetrads from the basidia of *S. commune*, but he found them too difficult to obtain, so he used *Aleurodiscus polygonia*, whose basidia discharged basidiospores in a cluster from which single basidiospores could be isolated that gave rise to monosporous

mycelia. From a single fruiting body, Kniep obtained 35 tetrads, and the members of each tetrad were mated in all possible combinations. Some of the tetrads were parental ditypes, some were non-parental ditypes, and some were tetratypes. This, along with the equal number of the four mating types indicated the existence of two factor pairs located on different chromosomes.

There were other interesting discoveries concerning sexuality in Basidiomycetes that soon appeared with the increased interest in these matters of sexuality which will now be mentioned but not described in detail.

1.2.1 *Bipolarity* (= bipolar sexuality or unifactorial heterothallism) is the situation in which there is a mating type factor pair at a single locus. Multiple alleles exist at this single locus.

1.2.2 *"Buller Phenomenon"* (= di-mon mating). This is the situation where homokaryotic mycelia are dikaryotized by dikaryons — *i.e.*, unclamped mycelia form clamp connections when confronted by clamped mycelia. The phenomenon has been extensively studied by a number of scientists and still has experimental uses. These di-mon reactions are referred to as being either "legitimate" or "illegitimate." A compatible legitimate reaction is one in which the monokaryon is compatible with both nuclei of the dikaryon. In the case of only one nucleus of the dikaryon being compatible with the monokaryon, it is called a hemicompatible reaction. Illegitimate di-mon reactions occur rarely when the nucleus of the homokaryon is not compatible with either of the nuclear components of the dikaryon.

1.2.3 *Reactions of monokaryotic mycelia other than those forming dikaryons.* One of the first recognizable reactions of this type was the formation of false clamp connections in matings in which the *A* alleles are different, but the *B* alleles are similar, known as common-B reactions. The false clamp connection differs from the true clamp connection in that the hook cell does not fuse with the penultimate cell. This reaction was found in nature by Vandendries and Brodie (1933) in *Lenzites betulinus* in what they called "barrage sexuals," where there was a line of sparse growth in which an apparent aversion occurred between the two mated strains bearing a common-B allele. False clamp connections are characteristically found in the barrage reaction.

It was not until 1950 that other non-clamp forming reactions were demonstrated in basidiomycetes. Papazian (1950), using a weak medium and confrontations made on agar in Petri dishes, showed that there were regular, predictable reactions in *S. commune* in matings involving com-

mon-A alleles and unlike B alleles, and also in matings involving different A alleles and common-B alleles. The former common-A mating showed relatively sparse growth with gnarled hyphae and was called a "flat reaction" because of the absence of aerial growth of the mycelium. The latter was called a "barrage reaction" because of the sparse growth of hyphae at the line of interaction. The common-A reaction was demonstrated to be heterokaryotic and is sometimes referred to as the common-A heterokaryon. In the common-B heterokaryon, heterokaryotic hyphae are found only in the line of interaction. It should be mentioned that Fulton (1950) reported a similar heterokaryotic condition in *Cyathus stercoreus*, but *Cyathus* was less amenable for genetic studies than *S. commune*, so most of the future developments of this nature were findings involving *S. commune* or *Coprinus cinereus*.

The anomaly reported by Kniep involving what he called mutations at the mating type locus could now be investigated because the common-A and common-B reactions indicated that these mating type alleles were identical; whereas, previously, all that could be determined was that the mating type alleles were different (the compatible A≠B≠ reaction giving rise to true clamps) For example, if As x Ay=+ and Ax x Az=–, this only tells us that Ax is different from Ay, but if Ax x Az=F (flat rx.), it is demonstrated that Ax and Az are identical alleles and different from Ay. This information was used by Raper and his students (1958) to study the genetic structure of the mating type loci.

2 THE MATING TYPE FACTORS

2.1 *Genetic control*

Papazian (1951) recognized the Kniep's suggestion of mutated mating type factors where he reported "favored classes of new factors" and frequent "back mutations" might be better explained by intrafactor recombination as the origin of the different mating type alleles. Experimentally, by both tetrad analysis and random spore matings of *S. commune* involving two known A alleles, he recovered factors that were compatible with both parental A factors. This suggested that the A incompatibility factor is controlled by at least two closely linked genes. The question at hand was whether there were a few loci with multiple alleles or many loci with pared alleles. Using a larger sample than Papazian, Vakili (1953) recovered about 3% nonparental A factors and observed that a higher recombination frequency was obtained at 33°C than at 23°C — information which facilitated future studies.

Raper's group, having now moved from the University of Chicago to Harvard University, continued these studies on the structure of the incompatibility loci with increased energy, since it was evident that the large number of mating type alleles were not the result of mutation, but of recombinational events. The study was enlarged to include different pairings from various locations, and progeny were collected from each of these pairings, and test crosses made to identify recombinant mating types. These non-parental A factors were then cross-mated in all intrastock and interstock combinations, as well as being mated with previously distinguished A factors obtained from a worldwide sample which had yielded 96 A factors (Raper *et al.* 1958). Unfortunately, this plan had to be aborted because of the frenquent spontaneous occurrence of a morphological mutation (*thin*) which carried with it unilateral nuclear migration under the conditions employed. Matings of a nuilateral mutant with another unilateral mutant would give no reactionsince a unilateral mating strain does not accept nuclei. The alternative plan developed was to use a strain that was stable in ragard to the *thin* mutation. This strain was then mated against each of the homokaryons from the worldwide sample, fruiting bodies obtained, and the progeny collected from each fruiting body screened for non-parental A factors by appropriate matings. For example, from a mating of A41 x A42, progeny were collected which were then tested by mating with tester strains that had the factors A41 and A42 as follows:

	Testers	
Progeny	A41	A42
A41	–	+
A42	+	–
A*	+	+

A* represents a non-parental A factor, as mentioned previously, these nonparental factors were then cross-mated in all intrastock and interstock combinations as well as with all the 96 factors previously identified from nature. Recombinants occurred in 9 crosses and in each case the recombinants could be assigned to 2 self-sterile, cross-fertile classes, Interestingly, the members of the 2 classes were approximately the same in number. There were 15 different classes of non-parental A factors that were identified, and 5 of these were identical to factors from nature!

2.2 *Refinement of structures*

With this information at hand, Raper (1966) proposed a model for the genetic structure of the A factor. In this model the A factor consists of 2 linked loci with multiple alleles at each locus. These subunits of the A factor were designated Aα and Aβ. Strain #699, which was stable to the *thin* mutation and against which all other strains were mated, was designated as $A41\alpha_1\beta_1$. When A41was mated with A51, two recombinant factors were obtained, and thus A51 was designated $A51\alpha_2\beta_2$. Strain A49 also gave recombinant classes when mated with A41, but one of the classes was the same as one of the recombinant classes from the cross of $A41\alpha_1\beta_1$ x $A51\alpha_2\beta_2$. Therefore, A49 was given the designation $A49\alpha_2\beta_3$ since $A41\alpha_1\beta_1$ x $A51\alpha_2\beta_2$ produced recombinants $\alpha_1\beta_2$ and $\alpha_2\beta_1$, and A49 x $A41\alpha_1X_1$ gave recombinants that could be identified as $\alpha_1\beta_2$ and $\alpha_2\beta\neq_1$. The β allele different from β_3 so that A49 is $\alpha_2\beta_3$. The model was successfully tested by recovering parentals from matings involving recombinants. That is, parental x parental gave recombinants, and, when these recombinants were mated, the parental A types resulted. This confirmed that the specificity of the A factor is determined jointly by the subunits Aα and Aβ, and that 2 A factors are fully compatible when there is heterozygosity at either or both of the subunits Aα and Aβ.

Now the subunits of the mating type alleles have been examined at a more highly resolved level by the use of molecular genetic techniques. Alleles $A\alpha_1$, $A\alpha_2$, and $A\alpha_3$, of the Aα mating type of *S. commune* have been cloned and sequenced by Ullrich's group (Giasson *et al.* 1989). The different Aα alleles reveal considerable sequence heterogeneity and strongly differ in sequence from Aβ, Bα, and Bβ alleles.

Again in *S. commune*, the cloning of the B mating type factor has been accomplished with the interesting finding of a DNA sequence that is capable of inducing development of fruiting bodies when integrated into the genome of unmated, non-fruiting strains. This DNA sequence has been isolated and partially characterized and has been found to override the normal requirement of a mating reaction for fruiting (Horton and Raper 1991, Raper and Horton 1993). It has been suggested that mushroom-inducing genes (FRT genes) may be isolated from the genomes of edible mushrooms. Such a cloned sequence linked to its own promoter might then enhance the normal fruiting process in transformants of this gene and improve crop production and even produce synchronous flushes.

2.3 *Role in development*

One of the questions frequently raised about the mating type factors of *S. commune* had to do with the role of the mating types in development.

This problem was carefully addressed by J.R. and C.A. Raper (1968) by making use of a number of modifier mutations affecting the morphogenetic sequence of dikaryosis. Their findings can be summed up as follows:

	Control by MT factors	
Morphogenetic event	Different A	Different B
Nuclear migration		x
Nuclear pairing	x	
Conjugate division	x	
Hook-cell formation	x	
Hook-cell septation	x	
Hook-cell fusion		x

3 BREEDING

3.1 *Objectives and requirements*

The objective of a mushroom breeding program is to bring together desired traits from two different individuals. In addition to this, breeding programs include the creation and selection of desired traits. It is, however, the assembling in one stock of the best aggregation of genetic material for the production of mushrooms of high quality and yield that is the goal of mushroom breeders. The basic requirements in mushroom breeding include a knowledge of the life cycle of the species, the ability to manipulate this genetically, and the availability of a method for the selection of the hybrid.

For decades, an empirical approach has been used by mushroom breeders that included the selection of individual mushrooms (fruiting bodies) that had a desired characteristic, or characteristics, such as pileus color or resistance to disease. The breeder might make spore prints from the fruiting body of interest, obtain a mycelium from the germinated spores and propagate this mycelium vegetatively for subsequent use as spawn. Some success has also been obtained by mixing different spawns, but the hybrids obtained in this way were rare, and, without selective markers, difficult to obtain. Selection of spontaneous mutations has in some cases resulted in useful cultivars (*e.g.*, the white strains of *Agaricus bisporus*).

3.2 *Problems in breeding*

3.2.1 In *Agaricus bisporus* the life cycle was not undersood until 1972 when it was shown to be a secondarily homothallic species (Raper *et al.*

1972). As indicated by the specific epithet, *bisporus*, the basidia are two-spored with each spore receiving two of the four meiotic products, and, as a consequence of which, a large percentage of the spores germinate to produce self-fertile heterokaryotic mycelia. It has proven difficult to obtain fertile heterokaryons of mating mycelia derived from the infertile homokaryotic spores.

3.2.2 Many of the characters useful for breeding are multigenic, complicating the selection process for the character.

3.2.3 The straw mushroom, *Volvariella volvacea*, is a primary homothallic species, and thus it is impossible to manipulate by standard breeding methods.

3.2.4 In a number of important species, clamp connections are not produced and this makes the identification of compatible reactions a problem. For example, the compatible reaction of *Armillaria mellea* is recognized by the depressed, crustose mycelium of the diploid stage, with the haploid mycelium being aerial and fluffy. In *Morchella crassipes* there is a line of heavier growth, called a "meld" or "overlap," between compatible strains. In *Agaricus bitorquis* there is also heavier aerial growth at the line of interaction between compatible strains.

3.3 *Techniques for selection of hybrid*

The selection of the hybrid is essential in any breeding program. This may be accomplished by the use of *auxotrophic markers* (strains which will not grow on a chemically defined minimal medium without specific supplementation). Complementation of auxotrophic strains permits selection of the sought after hybrid. Similarly, *mutants resistant to chemical inhibitors* of growth can be used on selective media to confirm heterokaryosis or obtain the hybrid strain. The use of mutants resistant to chemical inhibitors of growth is a simpler procedure, since the auxotrophic markers are more difficult to select and identify; whereas the chemically resistant mutants are readily identified just by their growth on a medium containing the chemical for which the strain is resistant.

4 BREEDING SUCCESSES

Although spawn manufacturers are constantly bring forth improved cultivars, these have commonly been the result of the selection of spontaneous mutations and less frequently from the combining of desired traits by hybridization. However, one example of breeding success involves the

production of a white cultivar of *Flammulina velutipes* (Kitamoto *et al.* 1993).

4.1 *Agaricus bisporus*

As previously mentioned, *Agaricus bisporus* has certain inherent difficulties for a breeding program, but with the increased development of molecular techniques and markers available for genetic and genotypic analysis, it is expected that breeding progress in *Agaricus bisporus* will continue to increase. Kerrigan (1993) summed up some of these advances pointing out that with allozyme markers, strains could be individually genotyped, identified and compared, and matings could be confirmed. Restriction fragment length polymorphism (RFLP) markers and random amplified polymorphic DNA (RAPD) markers consistute DNA markers that are now available in number for *A. bisporus*. Of course, the germplasm is the working material for genetic studies and a breeding program.

4.2 *Heterothallic basidiomycetes*

The heterothallic basidiomycetes are more amenable to genetic control and breeding. These include *Lentinula edodes*, species of *Pleurotus*, and *Flammulina velutipes*. Vigorous breeding programs are being carried out with *L. edodes*, and some success has been obtained in creating spore-deficient cultivars of *Pleurotus*.

5 THE FUTURE

With the constantly growing popularity of edible mushrooms and with their tremendously increased use for mushroom products in commercially significant amounts, the incentive is there for the breeding of improved strains, and the molecular methods are available to rapidly advance this quest. In the past four years, there have been several international conferences concerned primarily with the breeding of edible mushrooms. These conferences have been held in China, Hong Kong, the Netherlands, and Japan, and they have dealt with the breeding of *Agaricus* and the so-called specialty mushrooms (*Lentinula, Pleurotus, Flammulina*, etc.) which are gaining in popularity in the Western world. We have the opportunity at the current conference in Pennsylvania to learn even more about recent developments in the genetics and breeding of edible mushrooms and to obtain a glimpse at what the future may bring. Our debt to the earlier workers such as Bensaude, Kniep, Papazian and Raper is huge, but

the future consequences of the application of molecular biology to mushroom genetics and breeding, which had their basis in those early studies, will be even greater.

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