BREEDING AND STRAIN PROTECTION IN THE BUTTON MUSHROOM *AGARICUS BISPORUS*

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ABSTRACT

The button mushroom *Agaricus bisporus* is one of the most widely cultivated edible mushroom species in the world. Being the main species cultivated in the Western hemisphere, its popularity also increases in Eastern Countries such as China and Korea. The world production level for 2009 is estimated at ca. 4 million tons with an economic value of ca. 4.7 $ billion. Despite its economic relevance, it is surprising to see that breeding effort in this species is low. The main reasons for this low effort are the typical life cycle that hampers breeding and the difficulty to protect strains.
The complete life cycle of *A. bisporus* was unravelled in the early 70-ties of the previous century. After an apparently normal meiosis, predominantly bisporic basidia are produced, each containing two non-sister post meiotic nuclei. Upon germination these spores generate heterokaryotic mycelia. Only spores from the rare four spored basidia contain one haploid nucleus and can be used to generate hybrids in breeding programs.

Release of the first commercial hybrid dates back to 1980. Subsequent commercial hybrids were identical or very similar to the first hybrid. It is clear that some "new" varieties were generated by making copies of the first hybrid via tissue cultures of mushrooms. It is, however, unclear how other varieties were generated with apparently identical genotype but nevertheless some clear differences in phenotypes. Recent research indicates that offspring can be generated without recombination between homologous chromosomes. A pairing of non-sister nuclei on bisporic basidia will thus result in a redistribution of homologous chromosomes over the constituent nuclei. This redistribution appears to have phenotypic influence. This phenomenon thus allows for a relatively easy way of generating new varieties.

This paper will present opportunities of this typical meiotic behaviour for breeders but also addresses what consequences it has for strain protection.

Keywords: *Agaricus bisporus*, breeding, meiosis, essentially derived varieties

INTRODUCTION

The button mushroom *Agaricus bisporus* (lange) Imbach is the most widely cultivated mushroom in the USA, Europe and New Zealand/Australia. Its popularity is also increasing in Eastern countries as China and Korea. The world production of button mushrooms in 2009 is almost 4 billion tonnes with an estimated value of 4.7 billion dollars at farm gate (Table 1). Despite the long tradition of cultivation, professionalization of the cultivation system and the economic value it is surprising that efforts in breeding of this species is minimal. The first hybrid varieties for white button mushrooms were released in 1981 [1] and new varieties released afterwards were either identical of very similar to these first hybrids.

Recently, the whole genome of the first hybrid variety (Horst U1) has been sequenced (http://genome.jgi-psf.org/). This allowed the generation of large numbers of markers (SNP's) that have been used recently to study meiosis in the button mushroom and screen the genotypes of

traditional and present-day varieties. Although meiosis of the different subvarieties of the button mushroom has been studied previously [2, 3, 4, 5], the use of SNP markers has generated new data on marker segregation in especially the bisporic subvariety that represents all traditional and present-day commercial varieties and most wild accessions. This subvariety produces mainly two spored basidia and preferentially non-sister nuclei are paired in one spore [6]. Only rarely, four spored basidia are produced in which each spore receives one haploid nucleus. We will illustrate in this article what opportunities the complete absence of recombination offers for breeding. In addition, the SNP analyses will illustrate the likely origin of the present-day white strains and these should be considered as essentially derived varieties.

Life cycle of the button mushroom. Homobasidiomycetes are characterized by the fact that they are haploid during most of their life cycle. Fusion of nuclei only takes place in basidial cells just before spores are produced. Each diploid nucleus produces four haploid nuclei after meiosis and these are distributed to the four spores formed by each basidial cell.

Table 1. World production of button mushrooms in 2009.
The origin of the data are from: 1) Groupement Européen des producteurs de champignons (GEPC); 2) Dedicates estimates; 3) Australian Mushroom Growers Association; 4) New Zealand Growers; 5) American Mushroom Institute; 6) Chinese Chamber of Commerce (CNFA).

Country	Tonnes	Value ($) *1000	Country	Tonnes	Value ($) *1000
Poland[1]	250000	345000	Romania[2]	17000	34000
Netherlands[1]	230000	317400	Serbia[2]	10000	20000
France[1]	102400	141312	Slovakia[2]	3000	6000
Spain[1]	93500	129030	Croatia[2]	5000	10000
Italy[1]	64500	89010	Bosnia[2]	3000	6000
Germany[1]	58000	80040	Macedonia[2]	1500	3000
Ireland[1]	55000	75900	Bulgaria[2]	3000	6000
UK[1]	43000	59340	Russia[2]	9751	19502
Bulgaria[1]	40000	55200	Turkey[2]	30000	60000
Belgium/Luxemb.[1]	34000	46920	Ukraine[2]	30000	60000
Hungary[1]	20000	27600	Australia[3]	61000	310000
Denmark[1]	2300	3174	New Zealand[4]	8500	43197
Austria[1]	700	966	South Africa[2]	20000	40000
Others[1]	24000	33120	USA[5]	356936	924860
			China[6]	2181053	1308632
			South Korea[2]	190000	380000
Total production (tonnes) in 2009: 3,923,000					
Total value (x 1000 $) in 2009 : $ 4,602,100					

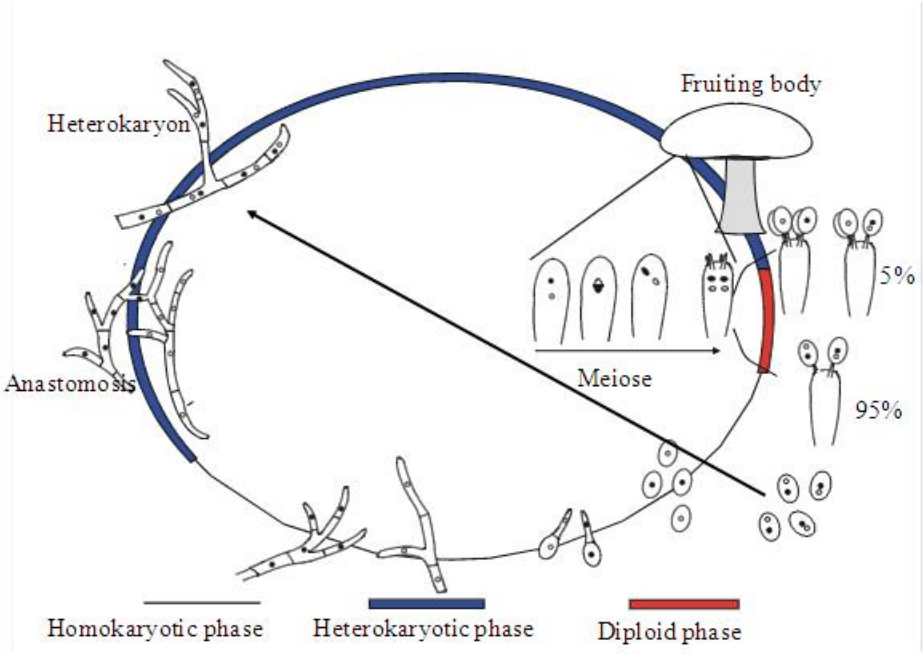

Figure 1. Typical life cycle of *Agaricus bisporus* var. *bisporus*. Most basidia produce 2 spores, each receiving non-sister nuclei. Due to the low recombination frequency between homologous chromosomes, these spores retain (almost) all alleles of the parental nuclei. The homologous chromosomes have an altered distribution over the constituent nuclei.

The spores germinate into haploid mycelium that cannot produce fruiting bodies. These infertile mycelia are designated as homokaryons. Homokaryons with different mating type can anastomose and subsequent exchange of nuclei leads to the formation of heterokaryotic (dikaryotic) mycelium. The presence of both mating types within one mycelial cell triggers a developmental process leading to the formation of fruiting bodies provided environmental conditions are favourable. This non-self compatibility or heterothallism is controlled by one or two unlinked loci. The outbreeding potentials of basidiomycetes are high because they possess numerous distinct mating types [7]. The majority of Homobasidiomycetes show this heterothallic life cycle. The button mushroom *Agaricus bisporus* deviates from this life cycle. Most basidia produce only two spores and the four post-meiotic nuclei are distributed over two spores in such a way that non-sister nuclei are paired in one spore [8, 2] (Fig. 1). This usually leads to mycelia with two different mating types and thus to fertile heterokaryons. This type of life cycle is designated as secondary homothallic. This phenomenon is also referred to as automixis or intra-tetrade mating, a form of selfing where mating occurs among the products of a single meiosis. Only rarely basidia are formed that produce three or four spores. Only on these basidia spores are produced with one haploid nucleus that generate homokaryons and can be used for cross breeding. Two decades ago, a novel variety has been found in de Sonoran desert of California [9]. This variety produces predominantly four spored basidia and each spore germinates into homokaryotic mycelia.

Recombination between homologous chromosomes. Recent breeding programs have shown remarkable differences in recombination frequencies between homologous chromosomes in homokaryotic offspring of the subvarieties *bisporus* and *burnettii*. Whereas in the four-spored subvariety *burnettii* on average eleven recombinations per individual per generation are found [5], a much lower recombination frequency is seen in the two spored *bisporus* variety. Recent analysis of different offspring of the first commercial hybrid Horst U1 showed recombination frequencies

varying from 0.08 to 1.3 per individual per generation, i.e. approximately 100 times lower than offspring of *bisp* x *burnettii*. That indicates that in many individuals most chromosomes are inherited unchanged from either one or the other parent. Present breeding programs indicate that this low recombination frequency might be common in the bisporic variety. Offspring were examined of two hybrids, each having one parent derived from Horst U1. The other parent has been isolated from a wild bisporic variety via protoplasting. These two other parental lines were derived from genetically unrelated wild accessions. Recombination frequencies in these offspring were 0.57 and 0.35 respectively, a similar low frequency as observed in offspring of Horst U1.

Mazheika et al [10] have analysed microscopically meiosis in both the bisporic and tetrasporic variety. They observed incomplete and abnormal axel elements and synaptonemal complexes during meiosis in the bisporic variety whereas in meiosis in the tetrasporic variety all stages can be seen clearly without obvious abnormalities. That could indicate that one or more genes involved in meiosis are mutated. This is obviously a recessive mutation since recombination frequency in a hybrid between the bisporic and tetrasporic variety is considerably higher.

Obstacles and opportunities for breeders. The low recombination frequency between homologous chromosomes in meiosis forms an obstacle for breeding programs since recombination is a prerequisite for mapping QTL's and reducing linkage drag in introgression breeding programs. All traditional and present-day varieties and most of the available wild collected strains are bisporic and it is likely that they all rarely will show recombination between homologous chromosomes in meiosis I. As previously mentioned, alleles responsible for this abnormal behaviour in meiosis are likely recessive. This allows mapping of genes involved and thus offers opportunities to generate advanced breeding stock with normal recombination frequencies. Mapping of these genes, however, is a laborious task since the phenotype. i.e. recombination frequency, has to be analysed for each individual.

Generation of offspring without recombined homologs offers interesting opportunities for breeders. Most steps of meiosis in *A. bisporus* var. *bisporus* seem conventional and a few steps are exceptional. The nuclear numbers per cell are reduced in basidia to two, one for each mating type. After fusion of both nuclei, alignment of homologous chromosomes is rare or incomplete [10] preventing recombination between homologues. In the first reduction division of the meiosis, chromosomes are distributed over two nuclei with an independent distribution of homologues over the two daughter nuclei (although frequently a skewed segregation is seen for some chromosomes). The absence of recombination combined with independent distribution of homologues leads in each basidium to two nuclei with a redistribution of homologues compared to the parental nuclei (Fig. 2). Each nucleus produces subsequently two sister nuclei that are exact copies of each other. The four nuclei can thus be distributed over two, three or four spores. In the latter case, each spore will receive one haploid nucleus in which each chromosome can be of one or the other parental type. This offspring can be used to generate homokaryons in which one chromosome is substituted compared to the parental nuclei. This can be done by crossing these homokaryons with either one or the other parental homokaryon and subsequently selection of chromosome substitution lines in a set of non- or rarely recombining offspring. These chromosome substitution homokaryons can all be mated with one and the same compatible tester homokaryon in order to produce mushrooms. The differences seen in phenotypes will likely be caused by the chromosome that has been substituted since all of these homokaryons have an identical genetic background. In this way, phenotypic effects for each chromosome can be tested. If a trait of interest is located on one chromosome, the introduction of this trait to another variety can be done by only transferring the relevant chromosome to an acceptor line.

In the absence of recombination between homologues, there are two ways in which the four post-meiotic nuclei can be distributed over two spores on bisporic basidia (Fig. 2).

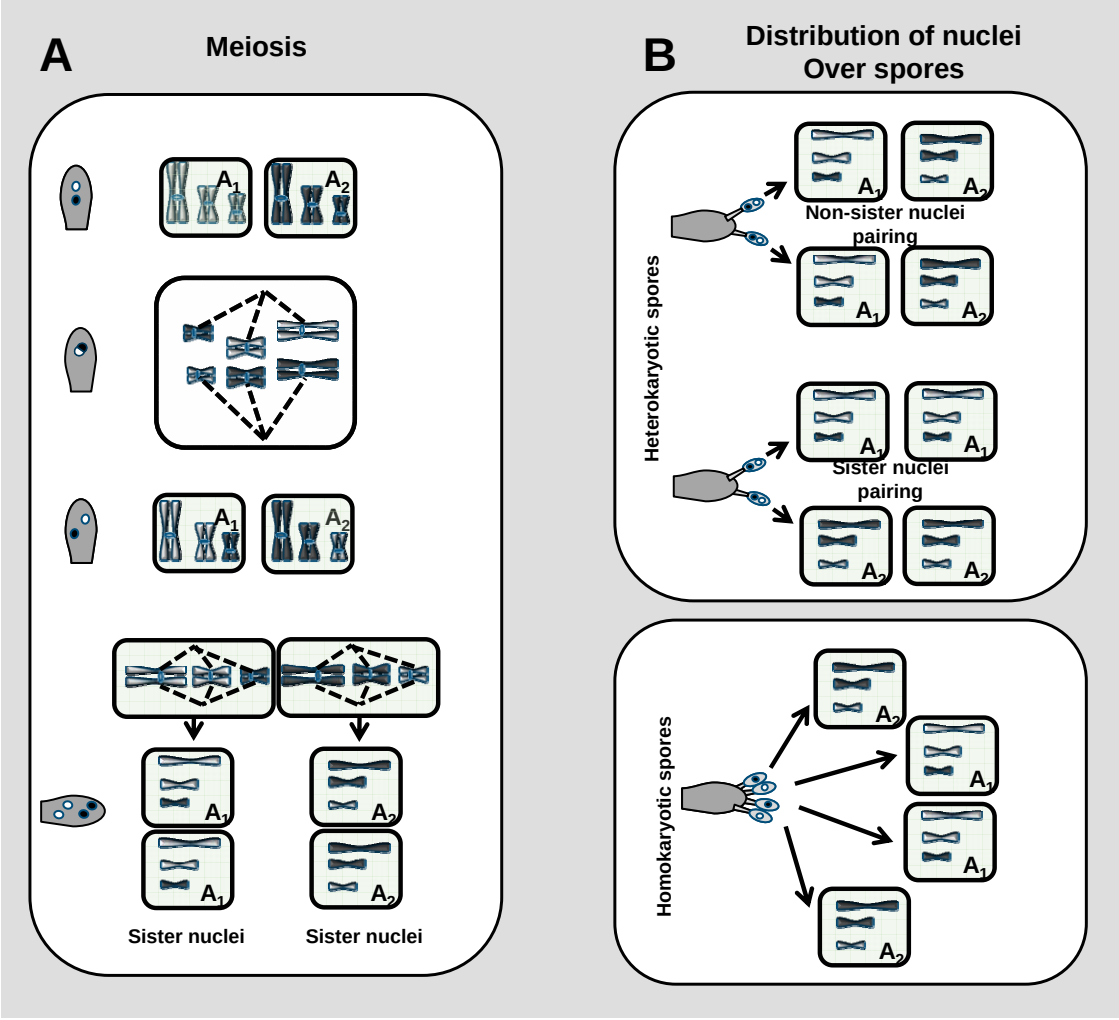

Figure 2. Schematic representation of meiosis (panel A) and distribution of nuclei over spores (panel B) in *A. bisporus* var. *bisporus*. For the simplicity only 3 of the 13 chromosomes are depicted. The different mating types are designated as A_1 and A_2. Without recombination between homologous chromosomes, meiosis leads to four haploid nuclei with 2 sets of sister-nuclei and within each set genetically identical sisters. Previous research indicates that preferentially non-sister nuclei are directed to spores on 2-spored basidia. Without recombination, these two nuclei have the same genetic constitution and show an altered distribution of homologs over the nuclei compared to the parental nuclei. When sister nuclei are distributed over 2 spores, each spore will receive two identical nuclei and will germinate into homokaryons (one nuclear type and one mating type). On 4-spored basidia, each spore receives one haploid nucleus in which chromosomes are substituted.

Either sister or non-sister nuclei are paired in one spore. The pairing of sister nuclei leads to a spore with two identical nuclei and thus one mating type. These will germinate into homokaryons, indistinguishable from homokaryons arisen from four-spored basidia. The pairing of non-sister nuclei leads to spores receiving nuclei that are complementary in chromosome constitution compared to the parental nuclei. All alleles found in the parental heterokaryon are also present in these types of offspring. Compared to the parental nuclei, homologs are redistributed

over the two nuclei. Upon germination, these types of heterokaryons will show the same genotype as the parental heterokaryon. Only by recovering the constituent nuclei via protoplasting, redistribution can be visualized. Analyses of randomly isolated single spore isolates indicate that preferentially non-sister nuclei are paired in one spore in two-spored basidia. How often sister nuclei are paired in one spore is not known but Elliott [8] has shown that this does occur occasionally.

Possible origin of the present-day varieties of white button mushrooms. The genome of the first hybrid Horst U1 has been sequenced recently. One of the parental homokaryons of Horst U1, i.e. H97, has been sequenced by the Joint Genome Institute (http://genome.jgi-psf.org). The other parental homokaryon, i.e. H39, has been sequenced by ServiceXs (http://www.servicexs.com/) using the next sequencing generation techniques of Illumina. The size of the genome and scaffolds generated correlate with the previous observation on genome size (31 Mb) and number of chromosomes (13) in *A. bisporus* variety Horst U1 [11]. Comparison of these two genomes revealed the presence of more than 280,000 single nucleotide differences. This means that on average one out of 110 base pairs differs between these two parental genomes.

Table 2. Strains genotypes with 600 SNP markers developed for alleles in the parental nuclei of the first commercial hybrid Horst U1. In the third column the average percentage of SNP markers is indicated for which both alleles of Horst U1 are present.

Origin	Type	% both alleles identical to Horst U1	# strains tested
Wild accessions	Tetra-sporic	1.3	1
	Bi-sporic	29	16
Traditional varieties	Off-white	50.1	
(used before 1981)	White	45.7	
Post Horst U1 varieties	Present-day white varieties	99	9
(used after 1981)	Brazilian/Chinese commercial	32.4	1

We have used these differences to generate single nucleotide polymorphic markers (SNP's). Six hundred markers were selected evenly distributed over the whole genome. These markers were subsequently used to genotype a number of traditional varieties, present-day commercial varieties and wild accessions [12]. The traditional varieties were used before Horst U1 and Horst U3 were commercially available (1981). Since breeding by hybridisation between homokaryons of different varieties was not common (or not done at all) before 1981, it is expected that these varieties are generated via tissue cultures of superior mushrooms or multi spore cultures derived from one mushroom. The latter is a traditional technique often used to "rejuvenate" strains. The traditional commercial white varieties can be divided in two subvarieties, i.e. white and off-white strains. In these varieties 46 and 50% of both alleles present in Horst U1 were found, respectively (Table 2). Since Horst U1 is a hybrid between a white and an off-white strain, this is an expected outcome. In the bisporic wild accessions on average for 29% of all 600 SNP markers both alleles were found. In the only tetrasporic wild accession tested, only 1.3 % of both alleles of all SNP's were present. This clearly shows that wild accessions are more distantly related to commercial varieties and that germplasm of tetrasporic varieties is not present in the commercial varieties. All tested commercial varieties that were released after Horst U1 was produced show a striking similarity to Horst U1, i.e. for 99% of all 600 SNP markers both alleles are found. Since the SNP marker

scoring has an error of at least 1%, we consider these varieties as identical to Horst U1 and identical to each other. The origin of these varieties is unclear and there are two possibilities, either these varieties are full copies of Horst U1 or they are obtained as fertile single spore isolates of Horst U1. As stated in the previous paragraph, most basidia of the bisporic varieties produce two spores and recombination between homologs is very low. The
pairing of non-sister nuclei in one spore thus results in a preservation of most if not all alleles and an altered distribution of homologs over both nuclei. The latter can be checked by recovering both parental types in these commercial varieties via protoplasting of vegetative mycelium. This procedure was carried out for two commercial varieties. Vegetative mycelium was protoplasted and both nuclear types were recovered as homokaryons. All four homokaryons were genotyped using the SNP markers. The analysis showed clearly that all alleles that grouped in one chromosome in Horst U1 also grouped in one chromosome in nuclei of these commercial varieties, i.e. no recombinations were found between homologs. The distribution of homologs, however, was different from that in Horst U1 and both commercial varieties showed different distributions (Fig. 3). This indicates that it is very likely that these commercial varieties were derived from the first hybrid Horst U1 via selection of heterokaryotic single spore cultures.

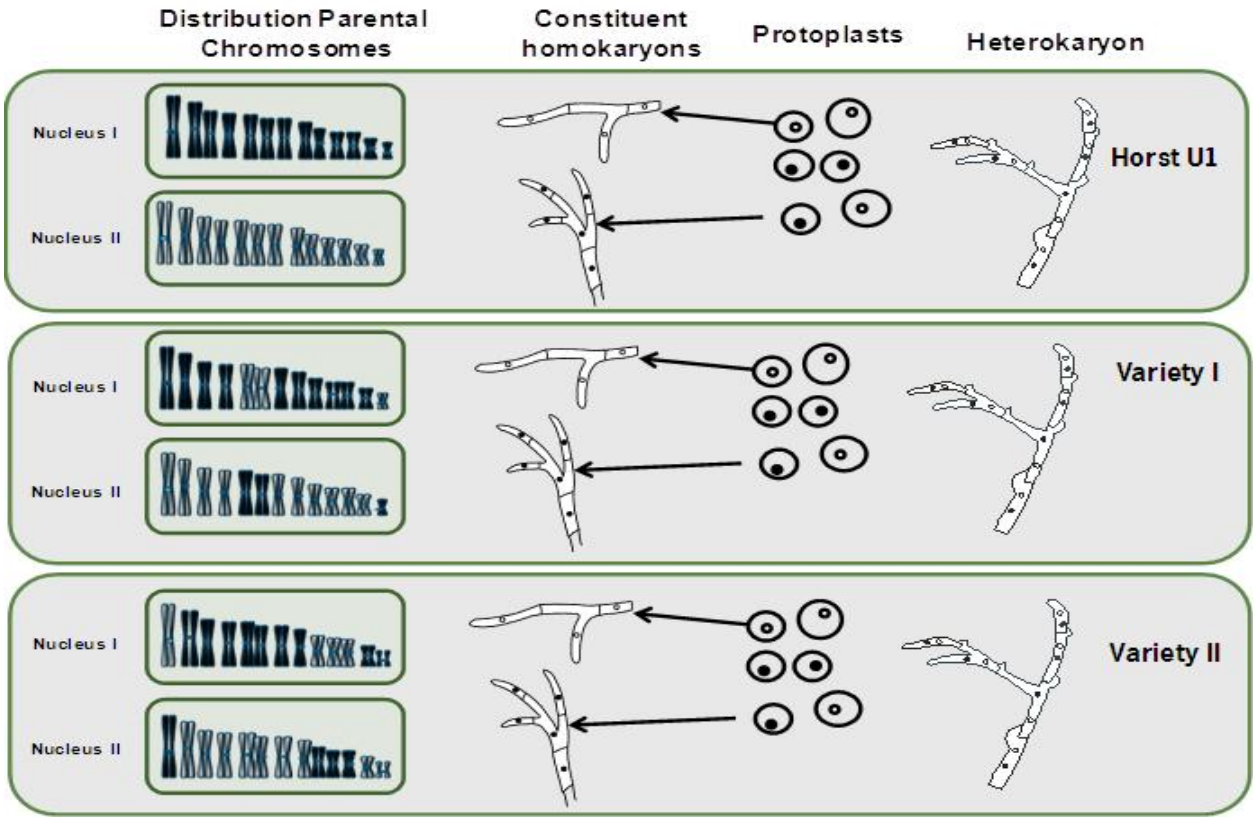

Figure 3. Distribution of homologous chromosomes over the constituent nuclei of the varieties Horst U1 and two other varieties marketed after Horst U1 was released. The constituent nuclei of these varieties were recovered as homokaryons via protoplasting. Six hundred SNP markers, evenly distributed over all chromosomes were generated for the two parental nuclei of Horst U1. These markers were subsequently used to genotype the constituent homokaryons of the two varieties. Alleles that grouped per chromosome in Horst U1, also grouped per chromosome in all homokaryons of the two varieties, indicating that no recombination has occurred. Compared to Horst U1, the homologous chromosomes show an altered distribution over the nuclei. These varieties might have been obtained by isolating fertile single spore cultures from an offspring of Horst U1 that shows no recombination and a normal independent segregation of homologous chromosomes in meiosis I.

It is expected that all other commercial varieties with an identical genotype as Horst U1 have been derived in the same way. It is interesting to see that one strain that is used commercially in Brazil and has an origin in China (Jos Buth, personal communication) has only 32% allele similarity with the other commercial varieties. This variety might have been obtained by true breeding but further genotyping has to be done to exclude the possibility that this strain is identical to one of the traditional commercial varieties.

Despite the striking genetic similarity of the present-day commercial varieties to the first released hybrid Horst U1, phenotypic differences can be seen in scaling, pinning or size of mushrooms. What causes these differences is unknown. It is possible that gene interaction differs between genes within one nucleus compared to genes between different nuclei and that this epistatic effect is the main effector for phenotypic differences between strains with identical alleles. The origin for phenotypic differences could also be epigenetic. One of the epigenetic mechanisms is methylation of nucleotides, usually cytosine. C-methylation does occur in *A. bisporus* and most transposons are methylated (Sonnenberg, unpublished). Whatever the mechanism is, the opportunity to change the distribution over the constituent nuclei and generate minor differences in phenotype is an interesting tool for breeders to "tune" traits of varieties.

Strain protection and definition of essentially derived varieties (EDV's). Although the opportunity for exchange of homologs between nuclei and its effect on phenotype is interesting from a scientific and breeders' point of view, it offers an obstacle to invest in breeding programs. Introducing new traits in varieties by hybridisation of commercial varieties with wild accessions is a laborious task. It involves the assessment of traits which should not be underestimated since many traits are influenced by climate conditions and the quality of substrate. This means that sufficient replications have to be done. Especially efforts have to be put in isolation and analysis of segregating offspring. The latter is a laborious task for several reasons. All present-day hybrids and most wild accessions have mainly bisporic basidia. Large numbers of single spore cultures have to be isolated and analysed to find sufficient homokaryons, since only homokaryons can be used for matings. In addition, due to the low recombination frequency, even more homokaryons have to be isolated and analysed to find sufficient individuals with at least one recombination. Including the repeated backcrossing needed to introduce the trait without too much linkage drag we estimate that the length of a breeding program can vary from four up to ten years. Once released, these bisporic varieties can be used to generate heterokaryotic offspring. These offspring will have all (or almost all) alleles of the original variety and will differ in distribution of homologs between the constituent nuclei. From the phenotypic differences found, an interesting one can be selected and used to market a new strain. Since these varieties have been through meiosis, they are considered to be generated via breeding. This way of breeding, however, will take months or at the most two years. Such new varieties will compete with the original variety as we have seen with "new" varieties marketed after the release of the first hybrid Horst U1. That means that there will be no or insufficient return of investment in large breeding programs. This, together with the typical life cycle of the button mushroom, is the main reason why no new varieties were released for the white button mushroom in last three decades. A solution to this problem would be to define the heterokaryotic offspring of A. *bisporus* var. *bisporus* as essentially derived varieties as defined by the UPOV convention. This does not mean that generating varieties via heterokaryons should be prohibited. They can be useful for growers and processors but a breeder of such derivatives should be tributary to the breeder of the original varieties and negotiate for a licence fee. A good definition and a general acceptance of essentially derived varieties for button mushrooms will certainly help to get investments in breeding program and thus offer the opportunity to generate new varieties that contribute to a much-needed innovation in the mushroom industry worldwide.

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