Breeding for quality of button mushrooms: genetically dissecting bruising sensitivity and quality-related traits of *Agaricus bisporus*

Wei Gao

Thesis committee

Promotor
Prof. Dr R.G.F. Visser
Professor of Plant Breeding
Wageningen University

Co-Promotors
Dr A.S.M. Sonnenberg
Scientist, Wageningen UR Plant Breeding
Wageningen University and Research Center

Dr J.J.P. Baars
Scientist, Wageningen UR Plant Breeding
Wageningen University and Research Center

Other members
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Dr K. Burton, East Malling Research, Kent, UK

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Wei Gao

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Chapter 1

General Introduction

Button mushroom cultivation

The button mushroom, *Agaricus bisporus* (var. *biporus*), is one of the most widely cultivated mushrooms worldwide. The first recorded cultivation of button mushroom was in France in the 18th century (Tournefort, 1707), and mushrooms were grown on a large scale in France at the end of the 19th century (van Griensven, 1988). The development of cultivation was based on the discovery that spent horse manure based substrate could be used as an inoculum for non-inoculated manure. A very important contribution to the further expanding of button mushroom cultivation is the development of spawn. Spawn consists of a sterile nutritious carrier (usually grain) inoculated with a pure culture of an edible fungus. After full colonization, it forms a stable and reliable inoculum that reduced the business risk for mushroom cultivation considerably compared to using grown compost from a previous cultivation as an inoculum. Spawn was produced initially from multi-spore cultures or by tissue culture. The discovery that productive spawn could also be prepared from single spore cultures leads to a more reproducible spawn quality and strain improvement. The main progress in the cultivation of button mushrooms from its early days up to late in the 20th century was, however, due to an improvement of the substrate. With the use of pasteurization, a high-grade and reproducible composting method was developed in 1950 (Sinden & Hauser, 1950). The availability of reliable spawn and a reproducible composting method provided the basis for the development of mushroom cultivation (van Griensven, 1988). Automation was gradually introduced into the whole process of mushroom cultivation since the 1980s, i.e., filling the growing room with colonized compost, casing application and climate control. Nowadays, cultivation technique of button mushroom has been very well developed all over the world, especially in the European countries and the USA. The whole process of commercial button mushroom cultivation can be divided into five steps, i.e., spawn preparation, composting, casing, cropping and harvesting (Figure 1). In the Netherlands, these steps are centralized by commercial producers. Spawn companies provide spawn for the compost company. Mushroom growers directly fill their growing rooms with full spawn-grown compost and casing supplied by the compost company or specialized casing soil producers. Based on trial experiences of many years, these companies are producing spawn, compost, and casing with generally standard operating procedures. Mushroom growers are able to harvest the first flush of mushrooms within 3 weeks after filling the growing rooms with full-grown compost and casing.

Dutch mushroom industry

The Netherlands is one of the largest producers of button mushrooms in the world. According to the world production volume of button mushrooms in 2009, the Netherlands having a production volume of 230 million kg were the fourth largest button mushroom producers worldwide, following China, the USA and Poland (Sonnenberget al., 2011). The competitive strength of the Dutch mushroom industry is more and more challenged by upcoming markets, *e.g.,* Poland has overrun the Netherlands becoming the third largest mushroom producer worldwide since 2009. One reason of this is the high production costs in the Netherlands, in particular the labor costs during harvesting. Nevertheless, the high labor costs can be substantially reduced by replacing handpicking with mechanical harvesting. For instance, in general labor costs accounted for more than 30% of the total production costs (Meulen et al., 2013). These labor costs can be reduced by 65% using mechanical harvesting equipment (Noble et al., 1997b).

With severe competition from the other countries and the development of cultivation techniques, the production of button mushrooms in the Netherlands was centralized by most large commercial growers. The number of mushroom farms in the Netherlands has decreased sharply by 83% (from 750 in 1992 to 133 in 2012) during the last 20 years (Table 1). The cultivation area decreased by about 30%, while the gross yield increased by about 25%. This can probably be attributed to the improvement of cultivation techniques of the Dutch mushroom industry. The price of button mushrooms remained more or less stable over all the years. Comparing 2012 to 2000, the consumer price of button mushrooms in the

Netherlands only slightly increased. However, the more and more expensive labor costs limits the profits of mushroom production. Strategies will have to be figured out to increase the profits of button mushroom production and promote the development of the Dutch mushroom industry. The current situation provides mushroom breeders with both challenges and opportunities. New strains and supporting techniques will have to be developed to meet the various demands of the industry and market. However, mushroom breeding is still relatively new compared to plant and animal breeding. The production of button mushrooms is still small compared to other plant products. Only a limited number of companies worldwide perform breeding and supply cultivars. This is not only because of the small market of button mushrooms but also due to the difficulties of strain (variety) protection by breeders' rights. Mushrooms can be easily isolated as tissue cultures, which are used directly as inoculum of spawn preparation and sold under a different name. In addition, due to the atypical life cycle of button mushrooms it is easy for a 'new' variety to be derived from fertile single spore offspring. The former Mushroom Experimental Station (nowadays part of Wageningen UR Plant Breeding) produced the first hybrid in 1980 (Fritsche, 1981). Most "new" strains, which appeared subsequently on the market, were either identical or very similar to the first hybrid. This is probably the main reason that mushroom breeding lags behind plant breeding; investments in time and money are difficult to recoup given the ease by which 'new' varieties can be isolated from existing successful varieties.

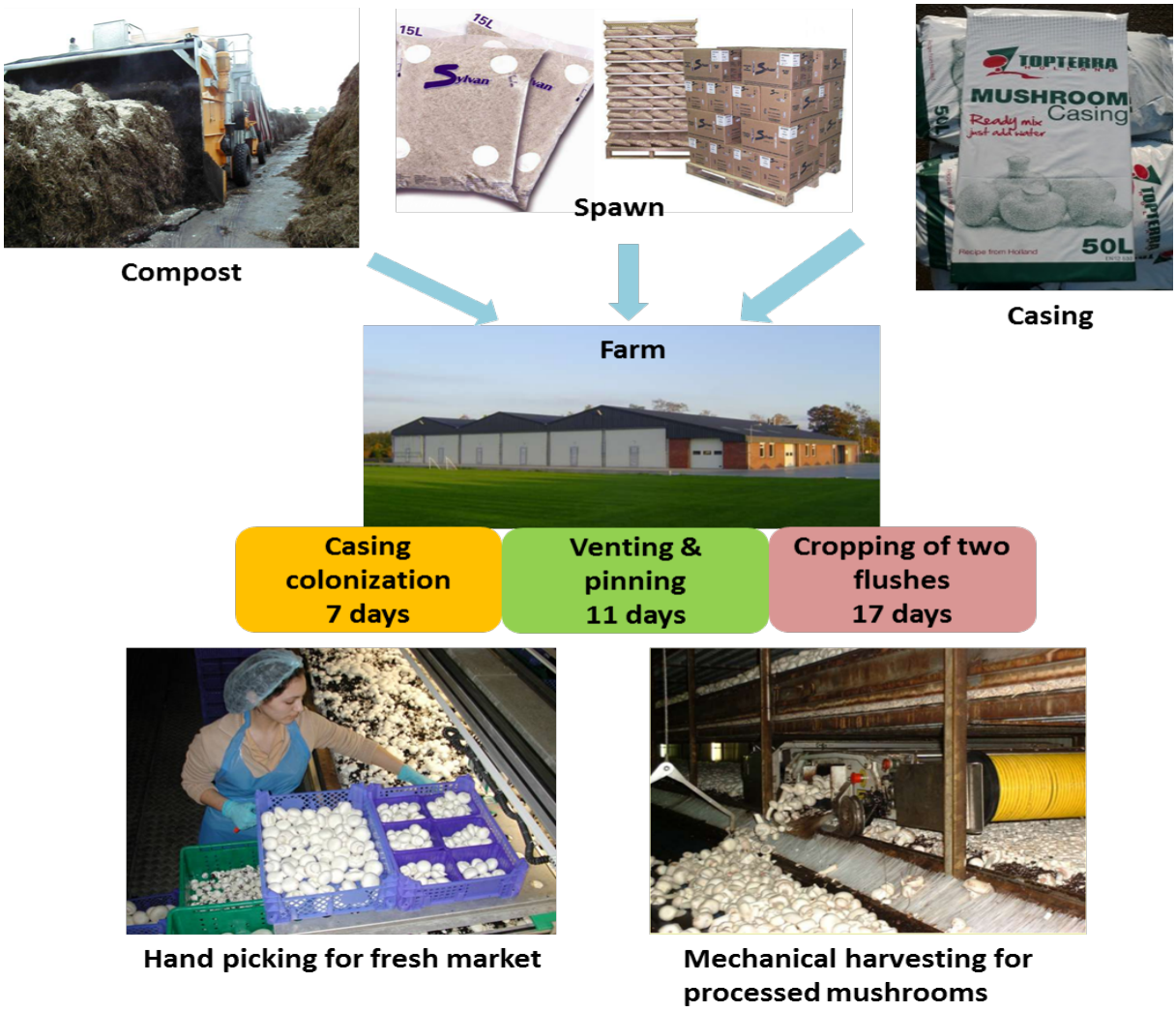

Figure 1. An overview of Dutch mushroom cultivation

Table 1. Number and size changes in Dutch mushroom farms over a 20-year period.

Year	1992	1998	2000	2002	2003	2005	2008	2010	2011	2012
Number of farms	750	583	524	-	400	300	-	-	166	133
Cultivation area (Ha)	-	98	-	93	88	77	77	73	68	66
Gross yield (million kg)	-	246	-	270	263	240	255	266	304	307
Consumer Price (euro)	-	-	0.89	0.98	0.93	0.87	0.93	0.93	0.89	0.93

Source: CBS Statline

Mechanical harvesting and Mushroom discoloration

Since mechanical harvesting is efficient and cost effective compared to handpicking, it is more and more used in button mushroom industry. The capacity of the mechanical harvesting systems (Champignons - (Mushroom machine) - Havatec BV) is up to 12,000 kg/h. However, during mechanical harvesting and packaging most mushrooms are bruised and become discolored and, therefore, are mainly used for canning and processing. Mechanical harvesting is not suitable for the fresh market for which high-quality standards are demanded. Mushrooms on the fresh market are mainly handpicked. At present, 60% of the Dutch mushroom production is harvested mechanically, and 40% are handpicked (Straatsma et al., 2007). Quality is an important factor enabling mushrooms to compete with other plant products and to maintain the status of a high-value crop. Mushroom quality as it is judged by the consumer depends on a number of factors: color, texture, cleanliness, maturity and flavor(Burton, 2004). A consumer survey in the UK has identified good quality characteristics of mushrooms as (ordered by importance) freshness, whiteness, cleanness, uniformity and closed cap. The main factors which contribute to loss in quality after harvest are mushroom discoloration and development, losses in weight and changes in texture (Burton & Noble, 1993). Discoloration is the first visible attribute of a perceived bad quality.

Physiological and biochemical explanations for mushroom browning

Mushroom discoloration develops as a result of biochemical degradative processes caused by either senescence or mechanical bruising. These processes lead to the oxidation of natural phenols, which is catalyzed by the enzymes, tyrosinase and other polyphenol oxidases. The products of this reaction, quinones, chemically react with themselves and with other constituents of the cell to form melanin, and the brown color is due to the dark chemical melanin (Jolivet et al., 1998, Burton, 2004). Burton (1986) suggested that tyrosinase and its phenolic substrates are located in different sub-cellular compartments of mushroom cells as in higher plants, i.e., cytoplasm and vacuoles. Both physiological ageing and bruising would cause disintegration of the intracellular membranes between compartments but at different time points. Exudate released from the mycelium cell in the coalesced mycelia was observed in a damaged cap surface (Rama et al., 2000). It was then suggested that the oxidation of exudate was responsible for the brown discoloration on the cap surface. However, no cell leakage was found in ageing mushrooms suggesting that membrane integrity is not lost during senescence (Braaksma et al., 1994). There was no statistical evidence of differences in the rate of discoloration between bruised and non-bruised mushrooms indicating that the rate of discoloration by postharvest aging is not affected by bruising-induced discoloration(Burton & Noble, 1993). The lack of interaction between bruising-induced discoloration and postharvest discoloration may indicate differences in the mechanism with stimulating tyrosinase activity. The mechanical process that causes bruising is known as “slip-shear” (a downward force combined with a sideways movement). Only the outer layer of cells of the skin are involved in bruising. In all three strains tested by Burton (1993), skin tissue had greater tyrosinase activity, higher protein levels, and higher levels of phenolic compounds than the flesh tissue.

The main natural phenols present in button mushroom are glutaminyl-4-hydrozxybenzene, p-aminophenol, phenylalanine and tyrosine (Jolivet et al., 1998). Tyrosinase is a copper-containing enzyme, which can be in an inactive state. The proportion of the total active tyrosinase was variable in previous studies, ranging from less than 1% (Flurkey, 1989) to 30-40% (Burton, 1988a). Two genes of tyrosinase from mushrooms have been cloned and characterized (Wicherset al., 2003). Tyrosinase can be activated *in vitro* by sodium dodecyl sulfate (SDS) (Yamaguchi *et al.*, 1970) or by proteolytic action (Burton, 1988a). A convergence of activated and native tyrosinase activities occurred during storage, indicating a possible *in vivo* action. Protease activity in skin and flesh tissues of the sporophore increased during storage, while protein levels fell. The increase of protease activity was shown to be a postharvest event (Burtonet al., 1993, Burton, 1988b). Proteinase level was found to be fluctuating during the flushing pattern of mushroom cropping (Burton et al., 1994), and a very large increase in the level of a particular proteinase, serine proteinase, has been observed in the mushroom after harvest (Burtonet al., 1997).Serine proteinases have been shown to activate tyrosinase *in vitro*(Espin et al., 1999). Burton suggested that the increase in tissue browning in mushrooms after harvest could be caused by the increase in serine proteinase activity leading to an activation of tyrosinase (Burton, 2004). Meanwhile, the extent of browning might also be determined by the phenol levels present in the sporophore tissues, as there appeared to be an excess of tyrosinase activity relative to phenol levels in his study and the phenol levels of the less discolored strain were lower than that of the other two strains.

Melanin is the chemical responsible for the brown color. Several melanin pathways were modeled incorporating many possible enzymes and substrate compounds in previous studies. After reviewing many studies about enzymes and phenols present in *Agaricus bisporus*, a melanin pathway was modeled in *Agaricus bisporus* (Jolivet et al., 1998, Weijnet al., 2012). With the availability of the genome sequence of *Agaricus bisporus* 40 relevant genes involved in the melanin pathway were identified by Weijn (2012a) across the genome. All tested genes have expression in all tissues, but particular gene expressions were observed to be more specific for different tissues (Weijn et al., 2012). Nevertheless, since gene expression has not been studied in association with phenotype segregation, the key factors that determine the bruising sensitivity or postharvest discoloration of *A. bisporus* remain unknown.

Environmental factors influencing mushroom quality

Mushroom bruising sensitivity is mainly caused by genetic differences, but also by environmental factors (Burton, 2004). Button mushrooms contain more than 90% water. This water is taken up from the compost, casing layer and irrigation; 60-70% of the substrate and 55-65% of the casing layer is water (Kalberer, 1983). Mushroom discoloration is the first sign of a bad quality. A number of studies were conducted to test the influence of cultivation conditions on levels of bruising sensitivity and postharvest discoloration, which were defined by the CIELAB (L, a, b) color system. Relevant factors in this are the airflow rate and the relative humidity in the growing room, the casing depth, the water potential of the casing, the depth of compost, etc. These factors all have an impact on the appearance of mushrooms on the growing beds.

Climate conditions

The climate conditions in the growing room are defined by a number of parameters, *e.g.* the temperature, the relative humidity, the carbon dioxide concentration, etc. A balanced airflow can regulate the level of the above-mentioned parameters. The variation in airflow in different areas of the growing room is a common occurrence in commercial mushroom farms (Bowman, 1987). Mushrooms in areas of reduced airflow appeared to be smoother and whiter than mushrooms on trays exposed to high airflow (Bartley, 1991). It was suggested that high airflows probably induces surface discoloration of mushrooms on growing beds due to tissue damage and release of polyphenoloxidase enzymes. In addition, mushrooms

(flush 1 & 2) grown in high humidity (92%) were less sensitive to bruising than those grown in low humidity (85%) (Burton, 2004).

Compost composition and depth

Compost is the substrate of mushroom production, and it provides the source of all nutrition for mushroom growth. Different composts varying in different levels of straw degradation were tested for their influence on mushroom bruising sensitivity. Mushrooms grown on the less degraded, straw-like compost were slightly but significantly less discolored (high L value) than those grown on more degraded compost (Burton, 2004). Mushroom number and dry matter content were higher in deeper compost, but compost depth had no influence on the susceptibility of the mushroom surface to bruising (Noble et al., 1997a). Meanwhile, the degree of discoloration of blocks of mushroom tissue was observed to be greater in the shallow compost treatment. Nevertheless, no significant relationship was observed between the susceptibility of whole mushrooms to bruising and the degree of discoloration of blocks of mushroom tissue (Noble et al., 1997a).

Casing composition and depth

After the mushroom mycelium has fully colonized the compost, a layer of casing soil has to be placed over the compost to initiate the fruiting of mushrooms. The functions of casing layer are usually considered as follows: a source of water for growth and development of mycelium and fruit bodies, allowing a balanced water evaporation, protecting the compost layer against drying-out and against too rapid disappearance of metabolic products which can fulfill a useful function later on, forming an environment in which both mushroom mycelium and certain bacteria indispensable for fructification can develop, and providing an environment with low osmotic value. Two casing compositions varying in different content of sugar beet lime and peat were tested together with three levels of water potential. With casing composition of 30% sugar beet lime (70% Peat), mushrooms grown in dry casing were significantly less sensitive to bruising than those grown in medium and wet casing. The water potential of casing with a composition of 9% sugar beet lime (91% peat) did not significantly influence the bruising sensitivity (Burton, 2004). Flush 1 mushrooms grown in wet casing discolored significantly less (higher L value) than mushrooms grown in medium and dry casing; flush 2 mushrooms did not show response to different level of casing water potentials; flush 3 mushrooms grown on dry casing discolored less than those from medium casing, which in turn was less discolored than those from wet casing. Burton (2004), therefore, suggested that to reduce bruising sensitivity, mushrooms should be grown wet (casing water and humidity) and allowed to dry out somewhat towards the end of the crop period. Casing depth also has an influence on bruising sensitivity. Mushrooms grown on 25 mm casing (shallow) showed a greater degree of discoloration as a result of mechanical damage than mushrooms grown on 40 or 55 mm casing which had similar b value ($P<0.001$). However, this significant difference was not observed using $\log_e(100\text{-}L)$ value. Besides, it was observed that deep casing decreased the firmness of mushrooms (Noble et al., 1997a).

Flush

Compost and casing conditions in different cropping flushes represent different environmental factors. Mushrooms from the second and third flushes of the cropping cycle were progressively darker prior to harvest and browned at a faster rate during postharvest storage at 12 °C than those from the first flush (Bartley, 1991). However, it was found that second flush mushrooms were significantly less discolored after bruising than those from flushes 1 and 3 (Burton & Noble, 1993). Mushroom maturation and the rate of weight loss were observed to be highly dependent on temperature, being slow at 5 °C and rapid at 18 °C. There were no differences in maturation between flushes at 5 °C, while at 18 °C clear flush differences were established. Mushrooms from flush 1 matured more rapidly than those from the second

flush and third flush. Besides, bruising has no effect on maturation rate. During 5°C storage, mushrooms from the third flush lost weight faster than the second flush, which in turn lost weight at a greater rate than the first flush. At 18°C mushrooms from the first and the second flush had similar rates of weight loss, which was smaller than those from the third flush (Burton & Noble, 1993). Additionally, flush is also a factor affecting disease resistance, i.e., first flush mushrooms were less resistant to *Pseudomonas tolaasii* than those of the second and the third flush (Olivieret al., 1997).

Irrigation and postharvest quality

Chemicals are used to improve the mushroom color and slow the rate of postharvest discoloration. The addition of Oxine (stabilized chlorine dioxide) and calcium chloride to irrigation water resulted in a significant improvement of mushroom color prior to harvest and a significant decrease in browning rate during postharvest storage (Bartley, 1991, Miklus et al., 1996). Although it is possible to reduce cap discoloration by the use of calcium chloride, the current market trends favor fresh products containing no chemicals (Rama et al., 2000). The best method to minimize postharvest browning was suggested to be cooling since cooling reduces nutrient use and movement, and especially vacuum cooling had received a lot of interests(Burton et al., 1987). Many other methods were tried to maintain mushroom quality, i.e., modified atmosphere packaging, y-irradiation, and chemical washing (reviewed in Burton and Noble, 1993).

Based on all these studies, Kerry Burton (in HDC research review, 2002) concluded that the most important environmental factors affecting bruising sensitivity were water potential of casing and humidity in the growing room. Calcium chloride irrigation and casing depth are in turn more important than casing composition and compost type.

Genetics and breeding of button mushrooms

The productivity of button mushrooms has been increased considerably during the last 30 years. This increase was obtained mainly by improving cultivation techniques, i.e., composting, casing, growing conditions, and mechanical spawning & harvesting. Breeding research for advanced cultivars was conducted meanwhile for some quality and agronomic traits, e.g. cap color(Callac et al., 1998), resistance to pathogens (Moquet et al., 1998b, Foulongne-Oriol et al., 2011b), yield (Foulongne-Oriol et al., 2012b), earliness (Foulongne-Oriol et al., 2012a), etc. This knowledge has, however, not yet been used to generate a commercial cultivar that is substantially superior to the first hybrid strain Horst U1 (Fritsche 1982), which was released in the 1980s on the spawn market. The new cultivars released afterwards were either genetically identical or very similar to the first hybrid (Sonnenberg et al., 2011). The impeded progress of breeding can be largely attributed to genetic characteristic of *A. bisporus* var. *bisporus*, i.e., the atypical life cycle and sexuality, which leads to difficulties of obtaining homokaryons as breeding materials, hampers outcrossing, and limits breeding success (Kerrigan, 1994, Moquetet al., 1998a).Thus, understanding the life cycle of the button mushroom is the primary requirement for mushroom breeding in terms of a recognizable sexual interaction between mated strains, fruiting competence, meiosis, and viability of basidiospores.

Sexuality in the life cycle of *A. bisporus* var. *bisporus*

Life cycle defines the order and relative durations of successive developmental stages including nuclear and morphological events, and sexuality is the force of nature leading to plasmogamy, karyogamy, meiosis, and progeny production (Chang & Hayes, 1978). Two types of life cycles commonly exist in most basidiomycetes. The more frequent one is heterothallism where successful fusion of gametes must occur between haploids carrying different mating type alleles to allow fruiting. Haploids can be represented by

monokaryons (one haploid nucleus per cell) or homokaryons (more than one copy of otherwise identical haploid nuclei per cell). Approximately one quarter of the basidiomycetes have a homothallic life cycle with a distinction being made between primary homothallism and secondary (or pseudo) homothallism. The former either allows mating with all possible haploid partners (no mating type selection) or no mating is needed to proceed to fructification. The secondary homothallic life cycle involves the paring of nuclei from a single meiosis, but with different mating types, into a single spore leading to dikaryotic or heterokaryotic mycelia capable of fruiting. *A. bisporus* var. *bisporus* is an example of secondary homothallic sexuality.

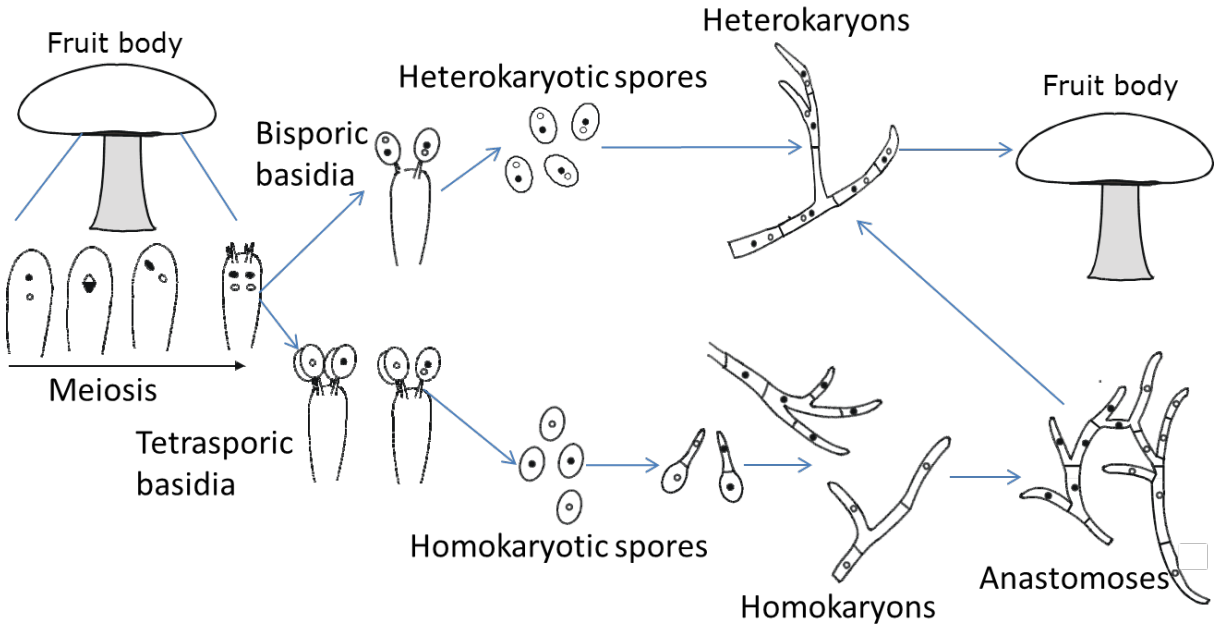

Figure 2. Life cycle and sexuality of *A. bisporus var. bisporus* (adapted from Sonnenberg et al., 2011).

Similar to most basidiomycetes, in *A. bisporus* var. *bisporus* two types of nuclei exist side by side in the fertile heterokaryotic mycelium and karyogamy only takes place in the basidium just before meiosis (Figure 2). Approximately 80%-90% of the basidia produce two fertile spores, each carrying two nuclei with different mating types. Ten to twenty percent of the basidia produce three or four spores where most spores receive one nucleus (Callac, 1993, Callac et al., 1996). Among randomly selected single spores, ca. 10% germinate into homokaryons (Kerrigan et al., 1992). Homokaryons bearing different mating types are able to mate through hyphal fusion generating fertile heterokaryons. The whole life cycle of var. *bisporus* can thus be divided into three successive phases, i.e., a common heterokaryotic phase, a rare homokaryotic phase and a short & transient diploid phase. This mixed type of life cycle of *A. bisporus* var. *bisporus* has been designated as secondarily homothallic or amphithallic. The fertile heterokaryons are morphologically indistinguishable from the infertile homokaryons, but heterokaryons are generally growing faster than homokaryons (Kerrigan et al., 1992). Several studies have shown that the majority of the single spore isolates are heterokaryotic (carrying both mating types) and preserve the heterokaryotic state for most of the alleles. Next to the number of basidia producing 2 spores, the extent of heterozygosity is affected by the way post meiotic nuclei are paired into a spore and the frequency of recombination between homologous chromosomes in meiosis. Especially the pairing of post-meiotic nuclei in spores of the bisporic basidia has been debated in a number of papers. A simple explanation for the predominance of heterothallic spores in secondarily homothallic species by random migration of nuclei was suggested (Langton, 1980). This hypothesis was further tested and confirmed in a secondarily homothallic ink-cap fungus *Coprinus bilanatus* through segregation analyses of mating type, auxotrophy

and antimetabolite resistance (Challen & Elliott, 1989). The segregation ratios of spore progenies tested conformed to random migration hypothesis, thus it was concluded that random migration could be regarded as the primary control of secondary homothallism. Theoretically one third of progeny are S-progeny (carrying two sister post meiotic nuclei) generated by random nuclear migration (Figure 3). However, sister nucleus pairing was very rare in *A. bisporus* var. *bisporus,* i.e., less than 0.5%, and might result from a delay of the second meiotic division after nuclei migration into spores has occurred (Kerrigan et al., 1993). This finding suggested that the four post-meiotic nuclei did not randomly migrate into basidiospores but that non-sister nuclei are preferentially paired in one spore (N-progeny). Nevertheless, there is insufficient evidence to support the preferential paring of non-sister nuclei given the poor and variable germination of spores reported in previous studies.

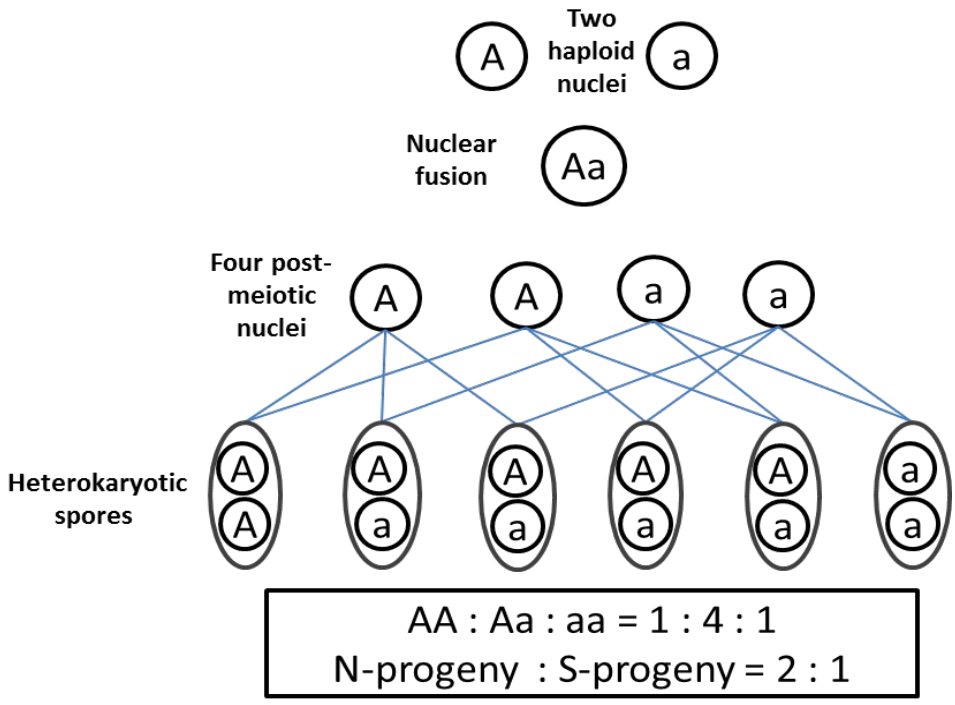

Figure 3. Heteroallelism is favored through random migration of post-meiotic nuclei (Langton F A, 1980).

The karyotype of var. *bisporus* was studied for the first time microscopically by Evans (1959) and 12 chromosomes were observed in haploids. The genome size was estimated as 34 Mb using reassociation kinetics (Arthur *et al.*, 1982). Further studies by CHEF (clamped homogeneous electric field) analysis confirmed the chromosome number as 13 and a comparable genome size of 31 Mb was found (Royer et al., 1992, Lodder et al., 1993, Sonnenberg et al., 1996). The first genetic linkage map that was generated of this variety contained 11 linkage groups with a total map length of 543.8 cM (Kerrigan et al., 1993). The recombination frequency (RF) was observed to be low (<0.2 crossover per linkage group), but all tested haploid post-meiotic nuclei had at least one crossover within the mapped genome. Compared to haploid progeny, the RF of three post-meiotic nuclei recovered from heterokaryons tended to be higher. Kerrigan (1993) found that markers in distal regions of the linkage groups often showed a tendency toward greater interval lengths indicating higher recombination frequency in distal regions. He believed that the presence of non-recombining groups of markers indicated that either crossing over is infrequent in certain large regions of the genome, or that such recombinants are generally unviable since the germination rate of basidial spores was about 0.9% in his study. The reduction of crossing over may be an evolutionary result of heterozygosity (Kerrigan et al., 1993). Meanwhile in the same study, homokaryon vigor (growth rate) was linked to a marker on chromosome 3. The single mating type locus (MAT) was mapped on chromosome 1 with the same set of haploids (Xu, 1993). The low recombination frequency in

the proximal centromere regions, in combination with preferential segregation of non-sister nuclear pairs into spores (favored heteroallelic for mating type), explains the tendency for heteroallelism to be retained in viable progeny.

The discovery of *A. bisporus* var. *burnetti* compared tovar. *bisporus*

With the discovery of the variety *A. bisporus* var. *burnetti* by Mr. Jerry Burnettein 1978, a predominant heterothallic life cycle and sexuality pattern was described and studied (Callac, 1993, Kerrigan, 1994). A series of comparisons was made between var. *burnetti* and var. *bisporus* in terms of nuclear behavior in basidia, basidiospore number, size of basidia, allele polymorphism, genome structure, *etc*. A number of genetic studies were conducted in the intervarietal hybrids since the two varieties are interfertile. Incompatibility of the two varieties is very uncommon, indicating a large genetic distance between the two varieties. The sexuality pattern is still amphithallic but with predominant heterothallism and, as in the *bisporic* variety a unifactorial incompatibility (Callac, 1993, Kerrigan, 1994). In contrast to var. *bisporus*, var. *burnetti* were mainly tetrasporic, i.e., each basidium was carrying four basidiospores after meiosis. Among all the single spore isolates, approximately 90% are homokaryons and 10% are heterokaryons. The basidia of var. *burnetti* are larger than those reported for the bisporic strain, but spores are smaller than those from bisporic strain. Genotypic characterization showed that var. *burnetti* and var. *bisporus* shared alleles up to 92% (Callac, 1993). A genome comparison between var. *bisporus* and var. *burnetti* was made with the availability of the genome sequences of bisporic stain H97 and tetrasporic strain JB137-S8 (Morinet al., 2012). On average orthologs between the two strains H97 and JB137-S8 show 96.4% amino acid identity and are arranged into large syntenic blocks. Over 390,000 SNPs were detected between the two strains with 40% in protein coding regions. Within the 74 pairs of scaffold blocks identified in the two strains, 12 were completely syntenic without inversions or translocations, and the other pairs of blocks contained inversions or translocations, or both inversions and translocations. The number of inversions and translocations between the two sequenced strains were significantly higher than those found between closely related strains within other species (Morin et al., 2012), and these differences are in line with the differences between these two varieties in morphology, sexuality and habitat.

A correlation was found between basidial spore number and ploidy of single spore offspring, i.e., the proportions of tetrasporic (and trisporic) basidia correlated with the proportions of the homokaryotic offspring in both bisporic and tetrasporic varieties (Kerrigan, 1994). The predominant inheritance of the tetrasporic trait was observed by Kerrigan et al.,(1994) in 91 first generation intervarietal hybrids. However, it was incompletely expressed in the hybrids since the mean percentage of tetra-sporic basidia (TSC) for parent JB3 (90.7%) was significantly greater than the mean of the intervarietal hybrids ranging from 69-75%, and as a consequence the number of three-sporic basidia varying from 24-26%. The penetrance of the tetrasporic trait elevated thus the basidial spore number and the genetic effect of the tetrasporic variety might be more clearly seen as a reduction of the proportion of bisporic basidia to 1.2-3.6% on average. The basidial spore number (BSN) was mapped as a predominant locus and linked with the mating type locus on chromosome 1 (Imbernon *et al.*, 1996) with a recombination frequency between BSN and MAT of 18.4% (19 recombinants among 103 offspring). According to the morphological, genetic and interfertility analyses, the sexuality of tetrasporic var. *burnetti* was also regarded as amphithallic but with heterothallism dominating.

Meiotic recombination in intervarietal hybrids

Meiotic recombination was studied in intervarietal hybrids using segregation analysis. These studies suggested that greater recombination rate occurs in the variety burnetti and the intervarietal hybrids (Callac et al., 1997, Moquet et al., 1999, Foulongne-Oriol et al., 2010, Foulongne-Oriol et al., 2011a). A total

of 324 molecular markers including AFLP (amplified fragment length polymorphism), SSR (microsatellite) and CAPs (cleaved amplified polymorphism sequence) were mapped on 13 linkage groups in the offspring intervarietal hybrid JB3-83 (homokaryons of var. *burnettii* strain JB3) × U1-7 (one of the constituent homokaryons of var. *bisporus* strain Horst U1 recovered by protoplasting) (Foulongne-Oriol et al., 2010). The map covers a total length of 1156 cM with 16% co-segregating markers, i.e., no crossovers found between co-segregating markers on the same chromosome. The largest gaps between markers tended to be on distal portions of chromosomes (Kerrigan et al., 1993) indicating an elevated recombination frequency at chromosome ends. The average number of crossovers per linkage group (0.86) of the intervarietal progeny was considerably higher than those in bisporic progeny (<0.2) observed by Kerrigan (1993). Comparative genetic linkage mapping was done in the progeny of a second generation hybrid (the hybrid of a tetrasporic offspring of an intervarietal heterokaryon crossed with a bisporic homokaryon) (Foulongne-Oriol et al., 2011a). The recombination rate was on average 0.67-fold smaller than that of first generation hybrid, which indicated the high recombination rate had an incomplete expression in the next generation or it was diminished by the bisporic genetic background. The polygenic feature of this trait, i.e., recombination frequency can thus be predicted. Distorted segregation of markers was observed in the genetic linkage maps mentioned above (Kerrigan et al., 1993, Foulongne-Oriol et al., 2010, Foulongne-Oriol et al., 2011a). A biased selection during spore isolation was suggested to explain the distortion, but meanwhile considered unlikely by Foulongne-Oriol (2011) since the spore germination rate was observed as normal. Accurate measurements for spore germination were difficult to be made because the mycelium from the early germinated spores started to overgrow the late germinating spores (Horgenet al., 1989). The germination rate of var. *bisporus* in that study was observed to exceed 50%. Germination rate of basidial spores of var. bisporus was about 0.9% in another study (Kerriganet al., 1992), and that of the intervarietal hybrids ranged from 3% to 44% (Callac, 1993), while that of var. burnetti strain JB3 was estimated at 26% (Kerrigan, 1994). The uncertainty of spore germination rate may need to be further studied.

Which is the ancestor of button mushroom?

The discovery of the predominant heterothallic life cycle in var. *burnetti* and the introduction of the tetrasporic trait into the bisporic variety can overcome the hurdles of obtaining homokaryotic breeding material and allows the generation of more crossover recombinants to be the source of variations. However, the tetrasporic variety performs poorly under commercial conditions in terms of pinning, scaling, cap shape, mushroom distribution and yield. For instance, the primordia are often wedge-shaped or quasi- two dimensional, and fuse during enlargement. The mushroom cap (pileus) is usually brown and of smaller irregular shape. The surface layer of the pileus is relatively loosely interwoven and traps fine soil debris (Callac, 1993). But especially the early maturation of the fruiting bodies causes a low quality. From an evolutionary point of view, var. *burnetti* is considered as ancestral and var. *bisporus* is the descendant through natural selection (Callac, 1993). Lethal and deleterious recessive alleles can be unmasked in haploid offspring (homokaryons), thus var. *bisporus* eliminates the haploid stage in a secondary homothallism life cycle. Consequently recessive alleles can be accumulated. Crossovers can lead to homozygosity and unmask deleterious recessive alleles, leading to lower reproductive fitness. Therefore a mechanism which reduces crossover may be selected for if the benefits of this reduction outweighs the costs (Kerrigan, 1994). Together with the evidence that all the other species of *Agaricus* and most species in related genera are tetrasporic, the tetrasporic variety is likely to have been ancestral in the genus and family (Callac, 1993), and the bisporic variety is the outcome of natural evolution. This might be sufficient to explain the poor performance of var. *burnetti.* Although the secondary homothallic life cycle (var. *bisporus*) has evolutionary advantages, it is unfavorable and inconvenient for mushroom breeding practices. At present, a pre-screening on growth rate and a test on homozygozity using molecular markers, and homokaryon isolation of var. *bisporus* is not such a hurdle any more. The low meiotic recombination frequency, however, is still impeding the generation of enough genetic variation and of a detailed genetic linkage map.

Outline of this thesis

One of the main cost factors of the Dutch mushroom industry can be reduced if mushrooms can be harvested mechanically for the fresh market. The present-day hybrids are, however, too sensitive to bruising (discoloration after mechanical damage and post-harvest handling). As indicated above, a number of studies have been done on the discoloration of mushroom caps but none of these has used the phenotypic variation within the culture collections to study the genetic determinants explaining these differences and thus generating knowledge for breeding superior strains. The objective of this thesis is to fill this knowledge gap at least to some extent and generate breeding stocks that can be used to construct commercial varieties suitable for mechanical harvest for the fresh market. In addition, segregating populations generated for this purpose were used to study in more detail the life cycle of the *bisporic* variety. Since the whole genome sequence for the button mushroom is available, SNP markers distributed evenly over the whole genome were used allowing a more detailed study of recombination frequencies generating knowledge invaluable for breeders.

In Chapter 2, the influence on bruising sensitivity was studied of three environmental factors (relative humidity (RH), water content of casing (CW), and casing depth (CD)) and the genotype (G) of strains in a factorial design. Two levels were set for each of the three environmental factors, and four different strains (two resistant and two sensitive to bruising) were grown in different factor combinations. The main purpose of this chapter is to test different environmental factors based on available knowledge that will maximize the difference between bruising resistant strains and bruising sensitive strains. The results showed that the factor combination that generated the largest difference in bruising sensitivity between the resistant strains and sensitive strains were used currently for commercial mushroom production.

In Chapter 3, the genetic variation in bruising sensitivity (BS) of *Agaricus bisporus* was studied through an incomplete set of diallel crosses. This allows estimating the heritability of BS, the combining ability of the parental lines and the estimation of the breeding value. To this end nineteen homokaryotic lines recovered from wild strains and cultivars were inter-crossed in a diallel scheme. Fifty-one successful hybrids were grown under controlled conditions, and the BS of these hybrids was assessed. BS was shown to be a trait with a very high heritability. In the population segregating for cap color, bruising insensitivity was linked to the brown offspring. The diallel scheme allowed to estimate the general combining ability (GCA) for each homokaryotic parental line and to estimate the specific combining ability (SCA) of each hybrid. One line had a low GCA (low for BS) and is seen as an attractive donor for improving resistance to bruising. The study of SCA indicates that heterosis might play a role in resistance to bruising. This chapter demonstrated for the first time how to estimating breeding value of parental homokaryons in mushroom breeding. This can be used to further study the genetic factors underlying bruising sensitivity and other quality-related traits, and to select potential parental lines for further heterosis breeding.

In Chapter 4, new details were generated by next generation sequencing technologies on meiotic behavior in *Agaricus bisporus* var. *bisporus*. Using SNP markers on a segregating homokaryotic population derived from the bisporic variety Horst U1 it was demonstrated that meiotic recombination in the var. *bisporus* is infrequent and restricted to the ends of chromosomes. By recovering constituent nuclei of heterokaryotic offspring of Horst U1, it was shown that non-sister nuclei are paired and combined with the low recombination frequency leads to the preservation of the heterozygous state. The same analysis on the present-day commercial varieties reveals that it is very likely that these are directly derived from the first commercial hybrid released in 1980 through the isolation of fertile single spore cultures. This chapter allows updating the life cycle of *A. bisporus* var. *bisporus* and provides fundamental knowledge for further meiosis study and button mushroom breeding.

In Chapter 5, the identification of quantitative trait loci (QTL) is described as a start to unravel the genetic basis for mushroom bruising sensitivity. Due to the low recombination frequencies, QTL were in most cases assigned to whole chromosomes. Since variation in cap color and discoloration by bruising are the same phenotype, QTL mapping was done for bruising sensitivity (BS) and cap color (CC). Two segregating populations for QTL mapping were generated in this chapter, where the first population was crossed with two different tester lines and the second with one tester line. CC and BS of the resulting three sets of heterokaryons were measured and analyzed at 60 min and 24 h after bruising (flush 1 & 2), and the segregation of BS and CC was observed. QTL were found for BS in the three sets of heterokaryons. The major QTL for BS and CC were consistently found in all three sets. QTL identified in this chapter will provide a basis for the breeding of advanced mushroom cultivars that are less sensitive to mechanical bruising. Since QTL analyses for edible fungi are scarce, this chapter contributes considerably to our knowledge on understanding complex traits in edible fungi.

Chapter 6 describes the analysis of multiple traits of *Agaricus bisporus* and the genetic correlations between traits. One of the main problems in generating new varieties is the restoration of quality using outbreeding with wild lines. Agronomic and quality traits are often correlated and should be evaluated in combination with each other, rather than as single traits. Significant phenotypic correlations were observed among different traits. Genetic correlations were the main cause for phenotypic correlations, which were confirmed by pleiotropic QTL shared by different traits. The two major pleiotropic QTL on chromosome 10 and chromosome 6 were found to be controlling almost all the tested traits. This chapter presents the first multi-trait QTL analysis of a mushroom species incorporating multiple agronomic and quality traits, and it will provide a primary genetic architecture for understanding the nature of trait correlations and a genetic basis for marker-assisted mushroom breeding of multiple agronomic and quality traits.

In Chapter 7, the results obtained in the experimental chapters and insights into button mushroom breeding and genetic studies are discussed.

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Chapter 2

Influence of environmental factors on bruising sensitivity and related traits in button mushrooms

Wei Gao[1, 2], Johan JP Baars[1], Oene Dolstra[1], Richard GF Visser[1]& Anton SM Sonnenberg[1]

[1] Plant Breeding, Wageningen University and Research center, Wageningen, the Netherlands

[2] Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing, PR China

To be submitted

Abstract

White button mushrooms are fragile and discolor soon after mechanical bruising. This hampers the development of mechanical harvesting systems for button mushrooms and causes loss of postharvest quality. Previous research indicated that the extent of discoloration could be influenced by environmental conditions. A factorial design analysis was conducted to screen the effect of factors influencing the bruising sensitivity of button mushrooms, and to find out a combination of environmental factors generating the largest variation among the four strains. In this way a maximal variation of the trait 'bruising sensitivity' can be induced in segregating populations intended to map QTL for this trait. Factors investigated were strain (four different strains), thickness of casing layer (2.5 cm and 5 cm), wetness of casing (dry and normal), and relative humidity in the growing room (80% and 87%). Analysis of variance (ANOVA) showed that strain was a significant factor influencing bruising sensitivity as well as thickness of casing. Significant factor interaction effect was observed. The factor combination generating the largest variation among the four strains was obtained, i.e., 5 cm, normal casing, and 87% for relative humidity in the growing room.

Introduction

All commercial button mushroom varieties are sensitive to bruising. During mechanical harvesting, packaging and transport, mushrooms collide with the conveying belt, the base and side of the package and with each other. This causes mechanical damage, especially to the mushroom caps, leading to discoloration and reduction in quality. The present mechanical harvesting system used by the Dutch mushroom industry is, therefore, not suitable for the supply of the fresh market for which high-quality standards are demanded. Mushroom quality is judged by the consumer as depending on a number of factors, e.g., color, texture, cleanliness, maturity and flavor (Burton, 2004). The main factors which contribute to loss in quality after harvest are mushroom discoloration and maturation, losses in weight and changes in texture (Burton & Noble, 1993). Since consumers select products based on visual inspection, discoloration of mushrooms is the main factor in quality. Mushroom discoloration is supposed to develop as a result of biochemical degradative processes occurring upon senescence or mechanical bruising, which leads to oxidation of natural phenols catalyzed by tyrosinase. This results in products, i.e. quinones, which chemically react with themselves and with other constituents of the cell to form melanin; brown cap color is due to the dark melanin (Jolivet et al., 1998, Burton, 2004). Burton (1986) suggested that tyrosinase and the phenols are located in different sub-cellular compartments of mushroom cells similar to higher plants, i.e., cytoplasm and vacuoles. Both physiological ageing and bruising would cause disintegration of intracellular membranes of compartments but in different timescales, i.e., postharvest storage and during harvesting. However, intracellular membrane integrity is not lost during senescence (Braaksma et al., 1994), and the rate of discoloration by postharvest aging is not affected by bruising-induced discoloration (Burton & Noble, 1993). The lack of correspondence between bruising-induced discoloration and postharvest-induced discoloration may indicate different mechanisms in stimulation of tyrosinase activity. This might be due to the fact that postharvest discoloration occurs in the whole mushroom including flesh tissue,whereas bruising only concerns the outer layer of cells of the skin(Burton, 1988).

Bruising sensitivity (discoloration after mechanical damage) was quantified with different bruising devices and methods in different studies. In a study of Noble (1997), a polystyrene shaking-box was used to bruise mushroom, which oscillated horizontally over a distance of 40 mm at a frequency of 2 Hz for 10 seconds. Burton (2004) developed a bruise-ometer delivering slip-shear treatment on the surface of mushrooms. Weijn et al., (2012) stated that the bruise-ometer developed by Burton (2004) damaged mushrooms too much and did not allow a comparison between the bruised and non-bruised area on the same mushroom. They, therefore, developed a different bruising device and an image analysis system was developed to quantify bruising sensitivity(Weijn et al., 2012). The bruising sensitivity in the Noble and

Burton studies was assessed by quantifying discoloration with the CIE L*a*b* system (Robertson, 1990). They used a Minolta meter (spectrophotometer) that produces three values: L, a, and b. Nobleet al., 1997 used the b value as an indicator of bruising sensitivity; the larger the b value the higher bruising sensitivity. Burton (2004) on the other hand used the L value (or $\log_e(100\text{-}L)$) as an indicator; the higher the value the lower bruising sensitivity. Weijn et al. (2012) used an image analysis system to quantify cap color based on whiteness index, which is equal to L minus 3*b. Whiteness index was claimed to show a closer resemblance to the perception of the human eye of the white color than the L value. The bruising sensitivity was defined as the difference in whiteness index between the non-bruised area and bruised area.

The variation of bruising sensitivity is not only determined by genetic factors, but also environmental factors (Burton, 2004). A number of studies were conducted to test the influence of cultivation conditions on bruising sensitivity and/or postharvest discoloration. Several environmental factors were tested, e.g., the climate conditions in the growing room, the casing depth, the water potential of casing, the depth of compost etc. These factors all have impact on the appearance of mushrooms on the growing beds. A balanced airflow can regulate the climate condition in the growing room, e.g., the temperature, the moisture content and the carbon dioxide concentration. The variation in air flow in different area of the growing room is a common occurrence in commercial mushroom farms (Bowman, 1987). Mushrooms (5 out of 12 crops) from areas of reduced airflow were significantly whiter than those exposed to higher airflow (Bartley, 1991). In addition, mushrooms of flushes 1 & 2 grown in high humidity (92%) were less sensitive to bruising than those grown at low humidity (85%) (Burton, 2004). Bartley (1991) suggested that high airflows probably induce surface discoloration of mushrooms on growing beds due to skin tissue damage and release of polyphenoloxidase enzymes. However, it was also found that flush 2 mushrooms were significantly less discolored after bruising than those from flushes 1 and 3 (Burton & Noble, 1993). Compost is the substrate of mushroom production, and it contains all nutrition needed for mushroom growth. Growers indicate that substrate quality affects also the quality of mushrooms although no clear definitions are available of what determines the quality of the substrate. Experiments of the influence of substrate on mushroom quality are scares. Mushrooms grown on less degraded, straw-like compost were slightly but significantly less discolored (high L value) after bruising than those grown on more degraded compost (Burton, 2004). Mushroom number and dry matter content were greater in deeper compost, but difference in compost depth had no influence on mushroom susceptibility to bruising (Noble et al., 1997). No significant relationship was observed between the susceptibility of whole mushrooms to bruising and the degree of discoloration of blocks of mushroom tissue (Noble et al., 1997). Casing layer supplies water for the growth and development of mycelium and fruit bodies. Two casing compositions, varying in content of sugar beet lime and peat, were tested at three water potential levels. With casing composition of 30% sugar beet lime (70% peat), mushrooms grown in dry casing were significantly less sensitive to bruising than those grown in medium and wet casing; while the water potential of casing with composition of 9% sugar beet lime (91% peat) did not significantly influence the bruising sensitivity (Burton, 2004). In addition, first flush mushrooms grown in wet casing discolored significantly less (higher L value) than mushrooms grown in medium and dry casing, but those grown in medium and dry casing did not show significant difference in sensitivity to bruising. Second flush mushroom did not show a response to different levels of casing water potential, and flush 3 mushrooms grown on dry casing discolored less than those from medium casing, which in turn was less discolored than those from wet casing (Burton, 2004). Bruising sensitivity was also influenced by casing depth (Noble et al., 1997).

According to these aforementioned studies, bruising sensitivity of button mushroom can be influenced by multiple environmental factors. A main objective of this study was to find for breeding purposes a suitable combination of environmental conditions for BS screening. Among all those factors mentioned above, the most important factors influencing mushroom bruising are water potential of casing and humidity in growing room (Burton, HDC research review, 2002). Casing depth is in turn more important

than casing composition and compost type. Thus, in general there is a clear picture of the factors that influence bruising sensitivity. On the other hand the impact of the interactions between factors on this trait still is largely unknown. Therefore we did a multi-factorial study to get a better insight in the relevance of interactions between factors affecting BS and to find a better environmental condition generating larger BS variation. Three environmental factors, i.e., wetness of casing, casing depth and relative humidity in the growing room were tested on four different genotypes (two resistant to bruising and the other two sensitive to bruising).

Materials and methods

Mushroom strains

In this study four white button mushroom strains (bisp 051, Le lion X20, Darmycel 21/2 and Sinden A61) were used. They were selected, based on previous research in which bruising sensitivity of a number of strains was studied (Weijn et al., 2012). Bisp051 and Le lion X20 were selected as representatives of strains with low bruising sensitivity (resistant) and Darmycel 21/2 & Sinden A61 as representatives of strains with high bruising sensitivity (sensitive). All four strains were obtained from the strain collection of Wageningen UR Plant Breeding.

Experimental design

All four selected strains were used in a cultivation test to test the effects of three environmental factors on bruising sensitivity (BS) in four replicates. Three environmental factors tested in this study were relative humidity (RH) in the growing room, casing depth (CD), and wetness of casing (CW). Each environmental factor had two levels (Table 1). The two levels of CD are 2.5 cm and 5.0 cm. The dry and normal casing soils were both ordered in CNC, which were different in wetness (either relatively dry or normal). The water content of casing was measured as 77% (w/w) and 79% (w/w) for the dry casing and the normal casing, respectively. The RH in the growing rooms during the production phase was set at 80% and 87%, respectively. Thus, 2^3 environmental factor combinations were tested on four strains (genotypes). A total of 32 treatments [strain (4) * RH (2) * casing depth (2) * types of casing(2)] were tested, and each combination of treatments was replicated four times (trays). This implies that a total of 128 trays were divided over two growing rooms that differed in RH. The trays within each room were randomized.

Table 1. Levels of environmental factors

	Factor		
Level	Relative Humidity	Casing Depth	Wetness of casing*
1	0.80	2.5 cm	Dry (77%)
2	0.87	5.0 cm	Normal (79%)

**: In brackets water content of casing soil*

Spawn preparation and mushroom cultivation

Spawn of the four strains used in this study was prepared in polypropylene boxes (280 mL, OS60+ODS60, Combiness, Eke, Belgium). To fill the 128 spawn boxes, 6 kg sorghum grains were boiled for 20 min in 10 L tap water. After draining of excess water and cooling down in the open air, the cooked sorghum grains were mixed with 2.4% (w/w) gypsum and 0.7% (w/w) lime. Each box was filled with 80 grams cooked sorghum and sterilized at 121 °C for 20 min. A piece of mycelial inoculate (3 cm^2 per box) of one of the

four strains, pre-grown on MMP (1% (w/w) malt extract, 0.5% mycological peptone, 10mM MOPS, and 1.75% agar, pH7.0) was inoculated in each spawn box. Spawn was colonized within two weeks. To achieve even colonization, the spawn was shaken one week after inoculation and a second time three days later.

Commercial phase II mushroom compost (16 kg, CNC, Milsbeek) was filled in 128 trays (56 × 36 × 20 cm) and each tray was subsequently inoculated with a single box of sorghum spawn (around 110 mL). Two subsets of trays were distributed randomly over growing room having either a RH of 80% or 87%. After 16 days of spawn run (air temperature 21-23°C; RH 95%; 3500 ppm CO_2), the difference between trays in casing type (different in water content) and in depth of casing soil (CNC, Milsbeek) were established. Casing colonization was performed under the same climate conditions as used for spawn run. Casing soil was ruffled. After 3 days of recovery growth, the room was vented at a rate of 0.075 °C/h until the air temperature reached 18°C. Meanwhile, the level of CO_2 was lowered to 1000 ppm at 35 ppm/h. The RH of one room was set at 80%, and that of the other room was set at 87%. Since different depths and types of casing need different time lengths for mycelium colonization and different amount of irrigation water, casing application and irrigation before the first flush were scheduled (Table 2). Casing of 2.5 cm was applied one week later than the 5 cm casing in order to have a coherent cropping time. The total amount of water for irrigation before the first cropping flush was also different for different treatments, i.e., 7 L/m^2 for dry 5 cm casing, 11.25 L/m^2 for normal 5 cm casing, 1.5 L/m^2 for normal 2.5 cm casing, and 0.25 L/m^2 for dry 2.5 cm casing.

Table 2. The schedule of casing and irrigation before flush 1

	Irrigation by Date									
Treatment	07-05	10-05	11-05	12-05	14-05	16-05	17-05	18-05		Total irrigation (L/m^2)
dry casing 5 cm	casing	2.50	1.00	1.00	2.50			ruffling		7.00
dry casing 5 cm	casing	2.50	1.00	1.00	2.50			ruffling		7.00
dry casing 5 cm	casing	2.50	1.00	1.00	2.50			ruffling		7.00
dry casing 5 cm	casing	2.50	1.00	1.00	2.50			ruffling		7.00
normal casing 5 cm	casing	2.50	2.00	2.50	2.00	1.25	1.00	ruffling		11.25
normal casing 5 cm	casing	2.50	2.00	2.50	2.00	1.25	1.00	ruffling		11.25
normal casing 5 cm	casing	2.50	2.00	2.50	2.00	1.25	1.00	ruffling		11.25
normal casing 5 cm	casing	2.50	2.00	2.50	2.00	1.25	1.00	ruffling		11.25
dry casing 2.5 cm					casing			0.25	ruffling	0.25
dry casing 2.5 cm					casing			0.25	ruffling	0.25
dry casing 2.5 cm					casing			0.25	ruffling	0.25
dry casing 2.5 cm					casing			0.25	ruffling	0.25
normal casing 2.5 cm					casing		1.00	0.50	ruffling	1.50
normal casing 2.5 cm					casing		1.00	0.50	ruffling	1.50
normal casing 2.5 cm					casing		1.00	0.50	ruffling	1.50
normal casing 2.5 cm					casing		1.00	0.50	ruffling	1.50

BS evaluation and analysis

As described in the study of Weijn (2012), mushrooms with a suitable size for testing (diameter: 4-5 cm) were picked and bruised with three strokes of the bruise device. Pictures were taken 60 min after bruising and analysed with a computer image analysis system (Weijn et al., 2012). The difference in whiteness index between the bruised area and non-bruised area of ten mushrooms per tray was determined to get a measurement of bruising sensitivity (BS). A higher BS value represents a higher level of sensitivity to bruising. The BS measurements were performed in the same way for mushrooms of flush 1 and flush 2. Statistical analysis was performed with Genstat version 15 using data of flush 1 and flush 2,

separately. Since the factor RH was confounded with compartment it was used in the analyses of variance (ANOVA) as a block factor.

Results

Performance of strains

According to the analysis of variance (ANOVA) the influence of the factors Strain and casing depth (CD) on bruising sensitivity was highly significant (Table 3). The wetness of casing (CW) on the other hand was insignificant in flush 1 as well as in flush 2. The three two-factor interactions were significant for BS of flush 1, but only Strain × CW were significant for BS of flush 2. The three-factor interactions were not significant for BS of flush 1 and flush 2.

The strains Bisp051 and Le lion X20 were significantly less sensitive to bruising than the other two strains Darmycel 21/2 and Sinden A61 in both flush 1 and flush 2 (Table 4). This is in agreement with the expectation. The resistant strains Bisp051 and Le Lion X20 did not differ significantly in bruising sensitivity, while Darmycel 21/2 showed to be the most sensitive strain.

Table 3. Analyses of variance for bruising sensitivity of mushrooms from flushes 1 and 2.

	Flush 1			Flush 2		
Source of variation	Df	Mean square	F probability	Df	Mean square	F probability
Cmpt stratum (RH)	1	133.77		1	26.85	
Strain	3	753.25	<.001	3	386.33	<.001
CD	1	462.26	<.001	1	43.20	0.049
CW	1	18.31	0.168	1	5.60	0.475
Strain × CD	3	54.60	0.001	3	17.41	0.195
Strain × CW	3	29.95	0.028	3	36.72	0.021
CD × CW	1	44.71	0.032	1	13.56	0.267
Strain × CD × CW	3	3.62	0.767	3	13.16	0.311
Residual	111	9.53		110	10.91	
Total	127			126		

Impact of environmental conditions

Thickness of casing layer (casing depth) had highly significant influence on BS ratings in flush 1 (Table 3). Flush 1 mushroom grown on 5 cm casing soil were significantly more sensitive to bruising than those grown on a casing depth of 2.5 cm. The flush 2 mushrooms showed an opposite response to casing depth, although the differences in BS between the two casing depths were small (Table 4). The mushrooms grown on dry casing soil tend to be less sensitive to BS than those produced on normal soil, but the differences were not significant.

Nevertheless, the interaction of wetness and depth of casing (CW × CD) had significant effect on BS of flush 1 mushrooms. The water content of the thin casing layer (2.5 cm) did not make a significant difference in BS-flush 1, but of the content in the 5 cm casing layer did make a difference, i.e., mushrooms produced on a dry layer of 5 cm casing soil were less sensitive to bruising than those from the 5 cm casing layer with a normal water content. Mushrooms from the room of RH of 87% were generally more sensitive to bruising than those produced at RH of 80%. However, the setup of our experiment does not allow far reaching conclusions, since variation in RH were tested only once in two different rooms (one

room with high and one with low RH). Influences of possible variation in climate conditions other than RH between the rooms cannot be ruled out. In general, mushrooms from flush 2 were less sensitive to bruising than those from flush 1 under most tested environmental conditions (Table 4).

Table 4. Means of bruising sensitivity for each level of the factor (Strain and three environmental factors) set in flushes 1 and 2 of the bruising test.

Factor	Flush	Factor level				SEM*
Strain		*Bisp051*	*Darmycel 21/2*	*Le Lion X20*	*Sinden A61*	
	1	14.49	23.44	13.27	20.52	0.78
	2	13.86	20.19	13.09	18.44	0.83
Casing depth (CD)		*2.5 cm*	*5 cm*			
	1	16.03	19.83			0.55
	2	16.96	15.82			0.58
Relative humidity (block) (RH)		*80%*	*87%*			
	1	16.91	18.95			-
	2	15.95	16.84			-
Wetness of casing (CW)		*dry*	*normal*			
	1	17.55	18.31			0.55
	2	16.17	16.62			0.58

*: SEM represents standard error of means.

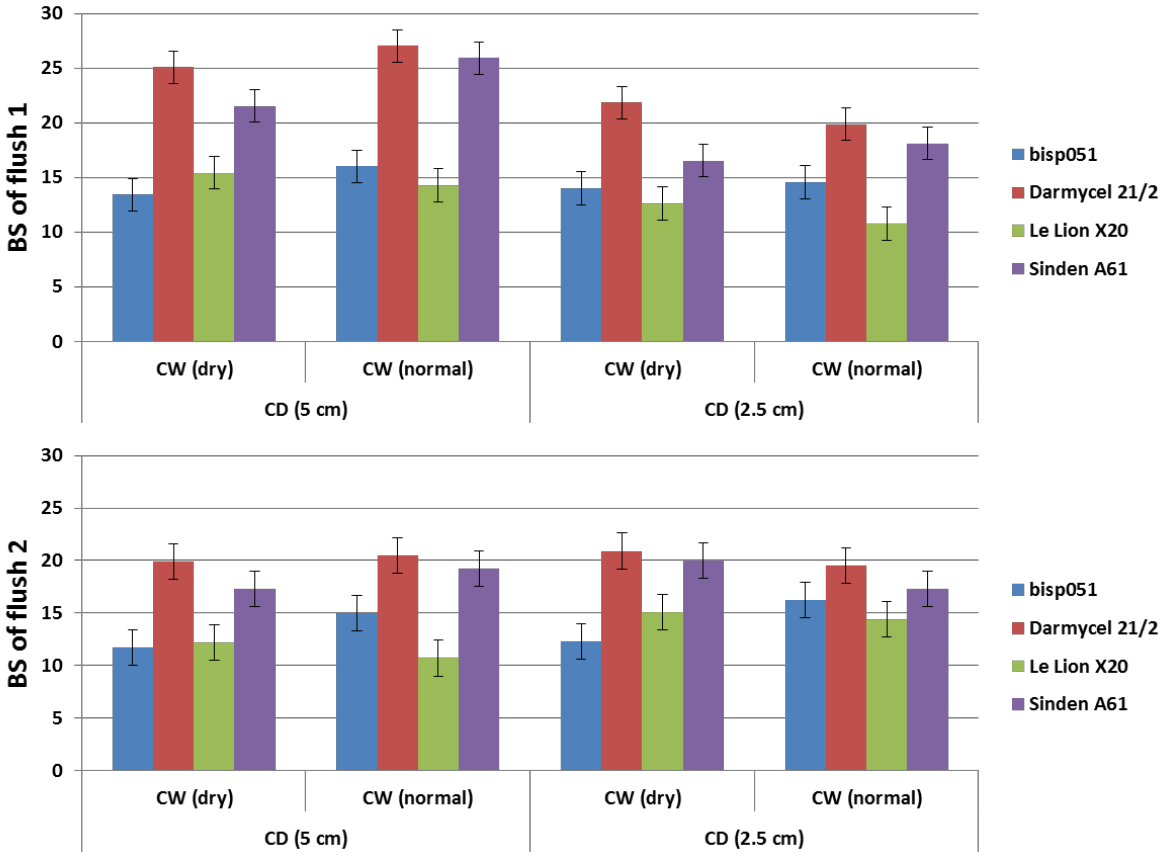

Figure 1. Mean performance of strains for bruising sensitivity of mushrooms (from flushes 1 and 2) produced under four different growing conditions. Error bars indicate standard error of means.

The interaction effects of strain by environments

The interaction between strain and CD was shown to have a significant influence on bruising sensitivity of mushrooms from flush 1, but not on those from flush 2 (Table 3). In flush 1, mushrooms of three strains (Darmycel 21/2, Le lion X20 and Sinden A61) produced on a layer of 2.5 cm casing soil, were less sensitive to bruising than those from a 5 cm layer (Figure 1). Caps of Bisp051 produced on a thin or normal casing layer did not show a clear and significant difference in BS.

Sinden A61 in flush 1 showed significantly higher sensitivity to bruising when grown on normal casing (5 cm) than dry casing (5 cm). On both dry and normal casing Darmycel 21/2 grown on 2.5 cm casing was significantly less sensitive than those grown on 5 cm casing. Bisp051 grown on dry casing was significantly less sensitive to bruising than that grown on normal casing soil in flush 2, but the difference was not significant in flush 1. The other three strains did not show significant difference in BS of flush 2 when they were grown on casing of different water content (Figure 1). Le lion X20 in flush 1 was generally less sensitive on 2.5 cm casing but less sensitive on 5 cm casing in flush 2.

Differences in bruising sensitivity depending growing conditions

The datasets on bruising sensitivity of flush 1 and flush 2 were analyzed again to get statistics for each of the eight possible combinations of testing condition enabling comparison between conditions with respect to their suitability to discriminate strains for bruising sensitivity. Factor combination C4 generated the largest difference in bruising sensitivity among the four strains in both flushes, i.e., a wider range of BS values (Min-Max) generated by the factor combination of C4 than the other combinations. According to the BS value of the four strains (Table 5), this larger variation was mainly generated by the fact that C4 increased the bruising sensitivity of the two sensitive strains by a larger extent than the other 7 condition combinations. The high coefficient of variance (CV%) in flush 2 (23%) under the condition of C4 was due to the large differences between replicates of the two sensitive strains (Darmycel 21/2 and Sinden A61). In contrast, factor combination C6 generated the smallest difference in BS among the four tested strains.

Table 5. Statistics for BS of the four strains under all eight combinations environmental conditions applied.

Code	Factor combination	Flush	bisp051	Le Lion X20	Darmycel 21/2	Sinden A61	SEM	Min-Max	CV%
C1	dry/5 cm/RH 0.8	1	12.85	15.61	25.59	19.68	1.09	10.90-28.46	11.80
		2	10.09	13.78	18.58	14.4	1.02	7.51-20.14	14.30
C2	normal/ 5 cm/ RH 0.8	1	16.65	11.55	23.77	25.82	1.56	9.41-28.32	16.00
		2	15.60	11.09	18.26	17.94	1.99	9.65-23.72	23.50
C3	dry/5 cm/RH 0.87	1	13.97	15.24	24.57	23.39	1.50	10.86-27.59	15.60
		2	13.32	10.57	21.20	20.19	1.61	9.24-25.21	19.80
C4	normal/5 cm/RH 0.87	1	15.37	17.01	30.24	25.99	1.68	12.34-36.28	15.20
		2	14.61	10.32	22.65	20.5	1.96	5.80-30.16	23.00
C5	dry/5 cm/RH 0.8	1	15.41	12.59	20.18	14.16	1.38	9.08-21.09	17.70
		2	8.44	15.91	21.84	21.13	0.75	7.81-23.08	8.90
C6	normal/ 5 cm/ RH 0.8	1	13.89	10.52	16.72	15.56	0.92	8.88-19.08	13.00
		2	16.19	15.69	18.49	17.63	1.11	13.69-22.05	13.10
C7	dry/5 cm/RH 0.87	1	12.6	12.63	23.48	18.9	1.77	9.35-26.4	20.90
		2	16.19	14.3	19.96	18.87	1.89	11.75-26.11	21.80
C8	normal/5 cm/RH 0.87	1	15.21	11.02	22.99	20.63	1.41	9.82-25.46	16.10
		2	16.28	13.07	20.54	16.88	1.57	10.52-26.77	18.80

Discussion

In this study, the main effects of genotype and environmental factors on bruising sensitivity of button mushroom were analyzed in a factorial design. Three environmental factors, i.e., relative humidity in the growing room (RH) (although confounded with the difference of growing rooms), casing depth (CD) and wetness of casing (CW) were tested on four genotypes. It turned out to be that bruising sensitivity of the four strains was significantly influenced by the tested environmental factors, i.e., bruising sensitivity was sensitive to environmental change. Thus, it is necessary to find a suitable environmental condition to generate sufficient phenotypic variations for genetic studies of this trait.

Genotype (strain) was a highly significant factor generating phenotypic variation of bruising sensitivity. In ANOVA it generated larger variance for BS than the environmental factors. This indicates that genotype is the main source of phenotypic variation of bruising sensitivity, and difference in bruising sensitivity is mainly controlled by genetic factors. As expected, the two preselected resistant strains showed less sensitivity to bruising than the two preselected sensitive strains in all the environmental conditions. Environmental condition of RH 87%, CD 5 cm, CW normal is used currently by most of the mushroom growers, apparently because most agronomic traits are optimal under these conditions. Since all commercial mushroom strains are genetically similar (Sonnenberg *et al.*, 2011), this is routinely used as an optimal condition by almost all mushroom growers. However, it is not always an optimal condition for the low bruising sensitivity of strains, as shown for the commercial variety Le lion X20. Mushrooms on 5 cm casing were more sensitive to bruising than those on 2.5 cm casing in flush 1. On average 5 cm casing allowed the four strains to produce earlier by more than 4 days than 2.5 cm casing (data not shown). This earlier production might be caused by the fact that the 5 cm casing was applied by one week earlier than the 2.5 cm casing because it was expected that colonization of a thicker layer would take more time than the thinner casing layer. The thick casing layer (5 cm) improved several agronomic traits, e.g., firmness, casing colonization, maturation, stipe shape and size of mushroom compared to 2.5 cm casing (data not shown). Although RH 87% (confounded with compartment) was beneficial for mushroom distribution, firmness and maturation (data not shown), it turned out to be a factor level generating higher bruising sensitivity in flush 1 compared to that of RH 80%. Moisture content of casing in this study as an independent environmental factor did not show significant influence on bruising sensitivity. This might be due to the small difference in water content between the two types of casing, which is insufficient to generate phenotypic variation. Besides, different strain favored different environmental condition for lower bruising sensitivity, e.g., in flush 1, Bisp051 favored dry casing no matter the depth of casing, and Darmycel 21/2 favored 2.5 cm casing no matter the levels of water content. Thus, the environmental condition used for commercial mushroom production might not be applicable to all new released cultivars, especially strains that are genetically more distant from the present-day hybrids. The significant interaction effects of genotype (strain) by environmental factors (G × E) on bruising sensitivity (BS) also indicated that different strains had different responses to environmental changes. For instance, the two levels of relative humidity in the growing room only gave significant differences in BS for bisp051 in flush 1 but not the other three strains. However, the two levels of casing depth generated significant difference in BS for all the other three strains but not bisp051 in flush 1. The significant G × E effects suggest that a particular variety might need a special package of environmental conditions to meet the superior quality requirements.

The direction of the effects of environmental factors on bruising sensitivity obtained in this study is in several aspects different from that reported in previous studies. For instance, Burton (2004) found that first flush mushrooms grown in wet casing discolored significantly less (higher L value) than mushrooms grown in medium and dry casing, and second flush mushroom did not show a response to different level of casing water potentials. Based on the findings in his study, Burton (2004) suggested that to reduce bruising, mushrooms should be grown wet (casing water and humidity) and allowed to dry out somewhat towards the end of the crop period. No significant effect of water content of casing on bruising sensitivity was observed in our study and mushrooms grown on 5 cm casing were significantly more sensitive to bruising than those grown on 2.5 cm casing. However, Noble (1997) observed that mushrooms grown on

25 mm casing (shallow) showed a greater sensitivity to bruising than mushrooms grown on 40 or 55 mm casing which had similar (b value) ($P<0.001$). This significant difference, however, was not observed using $\log_e(100\text{-}L)$ value(Noble et al., 1997). Burton (2004) reported that first and second flush mushrooms grown in high humidity (92%) were less sensitive to bruising less than those grown in low humidity (85%). In contrast, we found that first flush mushrooms grown in high humidity (87%) were significantly more bruised than those grown in low humidity (80%). There are a number of reasons to explain the discrepancies between the previous and our studies. Since the most significant effect on differences in BS is caused by the genotype of strains used and since there is a significant interaction between strain and environment, the primary reason for the deviation of results in different studies is likely due to different strains used in these studies. Four distinct strains were tested in our study, of which Le lion X20 behaved quite differently with the other three strains, e.g., in flush 2 it had lower bruising sensitivity in 5 cm casing than 2.5 cm casing. This indicates that one genotype is insufficient to give a general conclusion about the effect of environmental factors on bruising sensitivity. A commercial strain Hauser A12 was used in the study of Nobel (1997), and Burton (2004) did not mention which strain he used to test the bruising sensitivity. A second reason might lie on the different casing and watering schedule. In the study of Burton (2004), the water potential of casing was monitored weekly for the water content and water release curves and adjusted by watering as required. The casing was kept moist by regular light watering between each flush in the study of Noble (1997). The watering was scheduled in our study as to keep the different levels of casing factors until picking flush 1. Trays in flush 2 were watered at the same time, and thinner casing was watered less than the 5 cm casing. Thus, the difference of factor level might be minimized during flush 2. Additionally, different bruise method and the way of measurement might also attribute the opposite results. For instance, the bruising applied to mushrooms in the study of Burton (2004) was considered to give too much damage to mushrooms by Weijn (2012). In this study background color (the non-bruised area) was included in the calculation, but it was not included in the measurement of bruising sensitivity in previous studies. Besides, the difference in whiteness index between the bruised area and non-bruised area was used in this study for the measurement of bruising sensitivity, and L value or b value was mainly used in previous studies of Burton (2004) and Noble (1997). In addition, the compost and casing materials in our study were bought from commercial suppliers, but these materials were self-prepared in the two previous studies. In conclusion, the results of our study were not comparable with those reported in previous studies. The opposite direction of environmental effects may be attributed to the differences in materials and methods used in previous studies and this study.

If casing was treated as one factor of four levels (Table 2) according to the watering schedule. The normal 5 cm casing significantly resulted in the highest bruising sensitivity and normal 2.5 cm casing resulted in the lowest ($P<0.05$). The bruising sensitivity of flush 2 mushrooms on these four types of casing was not significantly different. The disappearance of any difference in bruising sensitivity of flush 2 mushrooms might result from the watering during the break between flush 1 and flush 2. Apart from bruising sensitivity, these four types of casing generated significant differences also in earliness, distribution, casing colonization, maturation and size for flush 1 mushroom, and scaling for flush 2 mushrooms ($P<0.05$). Compared to the other three types of casing in this study, dry 5 cm casing appeared to be a better condition to generate earlier mushroom production, more even distribution, better casing colonization, and bigger mushrooms in flush 1, and smoother mushrooms in flush 2. Mushrooms of flush 1 matured later in normal 5 cm casing.

The combination of three environmental factors, i.e., RH 87%, normal, 5 cm casing generated the greatest BS variation among the four strains in both flush 1 and flush 2 (Table 5) mainly due to an increase of BS of the two sensitive strains. Since this is the environmental condition that is widely used in commercial mushroom farms where modern hybrid strains are grown, and genotype is the main factor determining bruising sensitivity, breeding for new resistant cultivar might be the only way to improve the bruising sensitivity of current fresh mushrooms. Later on, this selected commercial condition will be used to

cultivate the segregating populations for a large phenotypic variation to map the genetic determinants of mushroom bruising sensitivity.

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Chapter 3

Genetic Variation and Combining Ability Analysis of Bruising Sensitivity in *Agaricus bisporus*

Wei Gao[1, 2], Johan JP Baars[1], Oene Dolstra[1], Richard GF Visser[1]& Anton SM Sonnenberg[1]

[1] Plant Breeding, Wageningen University and Research center, Wageningen, the Netherlands

[2] Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing, PR China

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Abstract

Advanced button mushroom cultivars that are less sensitive to mechanical bruising are required by the mushroom industry, where automated harvesting still cannot be used for the fresh mushroom market. The genetic variation in bruising sensitivity (BS) of *Agaricus bisporus* was studiedthrough an incomplete set of diallel crosses to get insight in the heritability of BS and the combining ability of the parental lines used and, in this way, to estimate their breeding value. To this end nineteen homokaryotic lines recovered from wild strains and cultivars were inter-crossed in a diallel scheme. Fifty-one successful hybrids were grown under controlled conditions, and the BS of these hybrids was assessed. BS was shown to be a trait with a very high heritability. The results also showed that brown hybrids were generally less sensitive to bruising than white hybrids. The diallel scheme allowed to estimate the general combining ability (GCA) for each homokaryotic parental line and to estimate the specific combining ability (SCA) of each hybrid. The line with the lowest GCA is seen as the most attractive donor for improving resistance to bruising. The line gave rise to hybrids sensitive to bruising having the highest GCA value. The highest negative SCA possibly indicates heterosis effects for resistance to bruising. This study provides a foundation for estimating breeding value of parental lines to further study the genetic factors underlying bruising sensitivity and other quality-related traits, and to select potential parental lines for further heterosis breeding. The approach of studying combining ability in a diallel scheme was used for the first time in button mushroom breeding.

Introduction

Agaricus bisporus (button mushroom) is one of the most widely cultivated mushrooms in the world. The Netherlands is one of the largest button mushroom producers (230,000 tons in 2009) (Sonnenberg *et al.*, 2011), and mechanical harvesting is widely used in the Dutch mushroom industry in order to reduce the high labor costs. So far, mechanical harvesting is mostly used for industrially processed mushrooms and not for the fresh market. Mushrooms for the fresh market are now handpicked and this represents a large cost factor. However, mechanical harvesting causes bruising and discoloration leading to a lower mushroom quality, a shorter shelf life and thus a lower price. Fully automated harvesting of button mushrooms for the fresh market requires strains that are less sensitive to mechanical damage. In order to develop such varieties, a better understanding of the genetic basis for cap discoloration after mechanical damage is needed. Mushroom discoloration caused by mechanical damage is a consequence of enzyme-catalyzed oxidation of phenols into quinones. These slightly colored quinones undergo further reactions forming dark melanins (Jolivet *et al.*, 1998, Weijn *et al.*, 2012a). It is assumed that the enzymes and substrates are physically separated in different cellular compartments. Visible-near-infrared spectroscopy was used to confirm the release of vesicular contents during cytoplasm breakdown and the activation of tyrosinase which catalyzes the oxidation of phenolics to quinones (Esquerre *et al.*, 2009). Several factors can thus affect discoloration after bruising, e.g. substrates, enzymes and intracellular membranes. Genetic analysis for bruising sensitivity is a tool for unraveling the basis of different mechanisms involved in mushroom bruising and discoloration, which is the prerequisite for mushroom breeding.

Mushroom breeding with homokaryons shows striking similarities with hybrid maize breeding making use of inbred lines. The combining ability analysis approach worked out in maize by Sprague and Tatum (1942) is of direct use to determine the breeding value of parental lines (Griffing, 1956). This approach allows separation of the combining ability among lines into a general and a specific part, usually referred to as general and specific combining ability (GCA and SCA, respectively). The first refers to the mean contribution of lines to the performance of hybrids, and the latter refers to deviation from the expected hybrid performance using GCA estimates of the parental lines. Different methods have been used to estimate combining ability of lines (Griffing, 1956). The GCA of a line is commonly used in plant breeding as a measure of its breeding value. The GCA of inbred lines is determined and used in maize breeding to

predict promising hybrids (Naspolini *et al.*, 1981, Viana & Matta, 2003). This approach was also successfully applied in the development of sunflower hybrids with improved resistance to *Phomopsis*(Deglene *et al.*, 1999). Interesting combining ability analyses were recently performed in watermelon (Bahari *et al.*, 2012) and oil palm (Noh *et al.*, 2012). A GCA estimate is considered to be a good indicator of the relative value of a parental line in terms of frequency of favorable genes and of its genetic divergence, which allows the selection of superior parental lines. The differences between lines in GCA are mainly due to the additive and additive × additive gene interactions, whereas the differences in the SCA of lines are attributable to non-additive, often dominant epistatic interactions (Deglene et al., 1999). GCA and SCA estimates can depend strongly on environmental factors as shown for grain yield in maize (Rojas & Sprague, 1952, Walejko & Russell, 1977). The combining ability of lines usually has a polygenic base and is trait-dependent as shown in a study in rice to elucidate heterosis for ten agronomic traits (Qu *et al.*, 2012). The estimates for GCA and SCA facilitate heterosis breeding and enable a dedicated choice of parents to create segregating populations for genetic analysis.

The method of diallel crosses was first applied to fungi by Simchen and Jinks (1964) in *Schizophyllum commune*, where the variation in mycelium growth rate within a set of crosses between monokaryons was studied. The variation was shown to be due to both additive and dominant gene action (Simchen & Jinks, 1964). Later on, this method was used in genetic and breeding studies in this species (Simchen, 1966a, Simchen, 1966b, Williams *et al.*, 1976). The analysis of combining ability was used also in genetic studies of growth rate and mushroom production in oyster mushroom, *Pleurotus sapidus*. In that study, a random monokaryon was selected for each of the eight wild dikaryons, and the eight monokaryons were crossed in a diallel scheme (Wang & Anderson, 1972).

Breeding value can also be estimated in button mushroom (*A. bisporus*). *A. bisporus* is a member of the Homobasidiomycetes. Homobasidiomycets are characterized by the fact that they contain two types of haploid nuclei with different mating types that stay apart in each cell. 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. 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 favorable. 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 (Casselton & Olesnicky, 1998). The majority of Homobasidiomycetes show this heterothallic life cycle. The button mushroom *A. 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 (Elliot, 1972, Summerbell *et al.*, 1989). 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-tetrad mating, a form of selfing where mating occurs among the products of a single meiosis. It is rare that basidia 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 (Callac, 1993). This variety produces predominantly four spored basidia and each spore germinates into homokaryotic mycelia. The two varieties are designated as *A. bisporus* var. *bisporus* and *A. bisporus* var. *burnetti,* respectively. Since var. *burnetti* is poor in various agronomic and quality traits, all commercially cultivated strains are *Agaricus bisporus* var. *bisporus*. The ability of mating homokaryons with different mating types enables the evaluation of the general performance of a particular homokaryon under different genetic backgrounds and the estimation of the breeding value for the homokaryon in mushroom breeding programs. Homokaryotic lines with contrasting performance are commonly used to generate segregating

populations and map genomic regions involved in traits and identify candidate genes (Foulongne-Oriol, 2012).

A collection of wild strains and traditional cultivars of the button mushroom was screened for bruising sensitivity (BS). The results indicated that some wild strains showed less BS than commercial lines (Weijn *et al.*, 2012b). A very common activity in breeding is the transfer of a new desirable trait derived from a wild line (donor strain) into existing commercial lines (recipient strains) through recurrent backcross selection (Sonnenberg *et al.*, 2005). In this study, the homokaryotic parental lines of some selected heterokaryotic lines were recovered through protoplasting. These lines were used to generate a diallel set of crosses in order to study the combining ability for BS and the breeding value of the parental lines. This has never been done before in button mushroom breeding. This study intends to examine whether this approach provides a good basis for the selection of parental lines for breeding advanced cultivars that are less sensitive to mechanical bruising and the choice of parental lines for genetic studies to elucidate genes involved in BS and other agronomic traits.

Materials and methods

Selection of heterokaryotic parental strains

A broad selection of varieties from the culture collection of Wageningen UR-Plant Breeding was recently studied for sensitivity to bruising (Weijn et al., 2012b). The selection included wild isolates originating from different geographic regions, traditional strains generated by single spore or multi-spore selection that were used before 1980, and present-day hybrids used worldwide since 1981. Strain CH1, CH2, TW6, TW7 and TW8 are known to be genetically related from previous studies. These strains showed a large variation in bruising sensitivity and cap color. Eighteen bruising resistant and sensitive strains were selected (Table 1) and used to recover the constituent homokaryotic parental lines by protoplasting the vegetative mycelium.

Table 1. Bruising resistant and sensitive strains used to generate homokaryons.

No.	CODE	sensitivity	color	No.	CODE	sensitivity	color
1	WB18	resistant	brown	10	TW8	resistant	white
2	WB2	resistant	brown	11	WW2	resistant	white
3	WB4	resistant	brown	12	WB15	sensitive	brown
4	WB5	resistant	brown	13	WB16	sensitive	brown
5	WW1	resistant	off-white	14	WB17	sensitive	brown
6	CH1	resistant	white	15	TW6	sensitive	white
7	CH2	resistant	white	16	TW7	sensitive	white
8	TO7	resistant	white	17	WW7	sensitive	white
9	TW1	resistant	white	18	WW8	sensitive	white

* TW=traditional white; CH= commercial hybrid; TO= traditional off-white; WW= wild white; WB=wild brown;

Protoplasting and protoclone isolation

Vegetative mycelium of 18 selected strains was protoplasted according to the method described by Sonnenberg *et al.* (1988). Suspensions containing protoplasts were serially diluted and plated onto MMP (1% malt extract, 0.5% mycological peptone, 10 mM MOPS, and 1.75% agar, pH7.0) + 0.6 M Sucrose for regeneration. After incubation at 24 °C for 5-7 days around 200regenerated single protoplasts (protoclones) were isolated for each strain under a stereo microscope and transferred to a new MMP plate. Protoclones were sub-cultured onto MMP plates covered with cellophane. After 10-14 days

incubation at 24 °C, the mycelium from the cellophane was put into a micro-centrifuge tube for DNA extraction.

Identification of homokaryons

Initial selection of putative homokaryons was based on colony morphology and growth rate that differed from the original heterokaryons. Homokaryons were identified using PCR methods. Primers (Table 2) designed based on known gene sequences were tested for their ability to screen putative homokaryotic protoclones. The absence of one band compared to that of heterokaryons was taken as an indication that a particular protoclone was homokaryotic. Alternatively, ISSR–PCR was used. Each PCR (15μL) with primers G-6-PD, 39Tr 2/5-2/4, PIN150 and P33N10 contained 20 ng DNA template, 1× incubation buffer, 300μM each dNTP, 15 pmol primer, 0.3U Taq DNA polymerase; each PCR (15μL) with ISSR primers contained 2 ng DNA template, 1× incubation buffer, 300μM each dNTP, 2 pmol primer, 0.3 U Taq DNA polymerase. Amplifications were performed as follows: after an initial denaturing step at 94 °C for 5 min, the samples were processed through 35 cycles, each consisting of 45 s at 94 °C, 45 s at annealing temperature 58 or 55 °C and 90 s at 72 °C, and a final extension step at 72 °C during 5 min. PCR products were separated on 1% agarose gels. The genetic diversity present in the set of recovered parental lines was studied with the KASPar SNP genotyping system (KBiosciences Competitive Allele Specific PCR SNP genotyping system) using 535 SNP markers. The genotyping was done by Dr. Van Haeringen Laboratorium B.V., Wageningen. These markers showed sequence polymorphisms between CH2B (http://genome.jgi-psf.org/Agabi_varbisH97_2/Agabi_varbisH97_2.home.html) and CH2A (resequenced by ServiceXs, Leiden, the Netherlands). The similarity (Squared Euclidean distance) between strains was calculated with SPSS (IBM, 19th edition) with the method of between-group linkage. A dendrogram was made to present the degree of similarity between the recovered homokaryotic lines.

Table 2. Molecular markers used for identification of homokaryons.

Marker		Sequence (5'-3')	Ta (°C)	Polymorphic Strains	Reference
G-6-PD	Forward	GTAATGTACACGGAGAC	58	TW8, WB2,TW7, WW2	This article
	Reverse	ACTCTGAAGGAACTTGG			
39Tr 2/5-2/4	Forward	CCTCGCGCAAGCAGATACAA	58	TW1, WB5, TW5	This article
	Reverse	TTGTCCGAGACTTACTCACG			
PIN 150	Forward	AGGTGACATGTCAGAAGCGC	58	CH1, CH2, TW6	(Kerrigan *et al.*, 1993, Sonnenberg *et al.*, 1996)
	Reverse	CAATCTCAAGCTTGCCTGG			
P33N10	Forward	ACTATAAAGCGTGAGCTATACG	58	WW7	(Kerrigan et al., 1993, Sonnenberg et al., 1996)
	Reverse	TATCTTCTGCGCTGTGTTGCT			
ISSR A2		HVV(GTT)5	55	TO7, WW1, WB18	This article
ISSR A7		VVH(TTG)5	55	WB4	This article
ISSR B		NDV(CT)8	55	WW8, WB16	This article
ISSR A		NDB(CA)7-C	55	WB17	This article

Ta: annealing temperature

Crossing between homokaryons

Homokaryons obtained through protoplasting were inter-crossed on compost agar plates (75 g dried milled phase II compost of 0.1 mm particle size was suspended in 1 L of tap water together with 17.5 g of agar. The medium was sterilized for 1 hour at 121°C and poured into petri dishes). Crossings were identified based on the morphology of mycelium interactions and confirmed using PCR. Mycelium of the contact zone was isolated and transferred onto a new MMP medium plate to confirm that it was

homogenous, after which the cross was confirmed with the same PCR primers that were used for homokaryon identification.

Fruiting test

Crossings that were identified as hybrids were put in a climate controlled fruiting test. Hybrid strains were grown in trays (56 × 36 ×20 cm) filled with 16 kg commercial phase II compost (CNC). Each hybrid had two replicates (trays). The cultivation conditions were the same as described in a previous study (Weijn et al., 2012b). Trays were distributed randomly on five shelf-layers in the growing room.

Quantification of bruising sensitivity (BS)

Mushrooms of each hybrid were picked at their respective peaks of the first and second flush for screening of bruising sensitivity (BS). BS measurements were performed following the protocol described by Weijn *et al* (2012). In short, mushrooms were bruised mechanically and pictures were taken of the mushrooms after incubation for 60 minutes at room temperature in a humid chamber. BS was quantified from the pictures with computer image software, using the CIE $L^*a^*b^*$ color system (Weijn et al., 2012b). The bruising parameters used in this research are the whiteness index (WI) and the whiteness index difference (WI_DIFF). WI is calculated as $L-$ (3 × b), as defined by Hunter (Hunterlab application note, 2008). The WI difference (BS) is the difference between the average WI of the bruised area and the average WI of the control (non-bruised) area, and the values for WI_DIFF differ thus from 0 (no visible bruising) to higher values with increasing bruising sensitivity. For each hybrid 20 mushrooms from two replicates (ten per tray) were analyzed. In addition, the pictures were ranked by eye in order to check the correlation between the data scored using the computer and visual scoring.

Combining ability of homokaryotic parental lines

Because of incompatibility of some parental lines, only a partial diallel design was available for the combining ability study envisaged. The performance data of the resulting F1 hybrids were analyzed in a way derived from Griffing's method, i.e. only including one set of F_1 hybrids but neither parents nor reciprocal F_1 hybrids (Griffing, 1956, Acquaah, 2012). Since each homokaryotic parental line contributes half of the genetic information to the hybrid, the mean performance (MP) of a particular homokaryotic parent is half of the mean BS of all crossings having this line as one parent. The general combining ability (GCA) of a particular line was calculated as the deviation of the mean performance of the line from half of the overall mean of all crosses in the diallel matrix. The deviation of BS of a particular cross from its expectation was used as an estimate of the specific combining ability (SCA) of the hybrid. The representation of the mathematical relationship of each cross is:

$$MP_A = T_A / 2n_A; MP_B = T_B / 2n_B$$
$$G_A = MP_A - \left[\Sigma T / 2N\right]$$
$$G_B = MP_B - \left[\Sigma T / 2N\right]$$
$$E\left(X_{AB}\right) = \left[\Sigma T / N\right] + G_A + G_B = MP_A + MPb_B$$
$$S_{AB} = X_{AB} - E\left(X_{AB}\right)$$

$\sum T$ is the total BS value of all the crosses in the diallel design, and N is the total number of crosses. T_A and n_A are the sum of BS values and number of crosses having line A as a parent. $T_B$and $n_B$is the sum of BS values and number of crosses having line B as a parent. G_A and G_B represent the GCA of line A and B,

respectively, and S_{AB} the SCA of hybrid A × B. X_{AB} is the BS value of hybrid A × B, and E(X_{AB}) is the expected BS value of hybrid A × B.

Analyses of variance (ANOVAs) were done using the model $Y = \mu + G + \varepsilon$ to test the presence of significant differences between hybrids (G) within flushes, and the model $Y = \mu + G + F + G \times F + \varepsilon$ to test also for the presence of significant flush (F), genotype (G) and genotype by flush interaction effects (G×F); Y represents the bruising sensitivity of hybrids, G the genotypic effect, F is the flush effect, $G \times F$ is the genotype by flush interaction effect, and ε represents the error effect. The broad-sense heritability (H^2) for hybrid means was calculated for each flush by $H^2=\sigma^2_G/(\sigma^2_G+\sigma^2_e/n)$, and across flushes by $H^2=\sigma^2_G/[\sigma^2_G+(\sigma^2_{GF}/r)+(\sigma^2_e/nr)]$, where σ^2_G represents the genetic variance, σ^2_e is the error variance, $\sigma^2_{GF}$was theinteraction variance of genotype by flush, n was the number of replicates (n=2), and r was the flush number (r=2). Multiple comparison for all hybrids was based on Fisher's unprotected LSD test ($P<0.05$).

Results

Recovery of homokaryons

All 18 strains mentioned in Table 1 were used to recover homokaryons. Previous research has shown that homokaryotic and heterokaryotic protoclones show in general considerable differences in colony morphology and growth rate. Mycelia of homokaryons grow generally slower than that of heterokaryons and show fewer branches when inspected by microscope. Putative homokaryons were selected using these criteria. The presence of only one single nuclear type in such protoclones was confirmed by the absence of size polymorphisms upon PCR with primers that generate DNA fragments of different sizes in heterokaryons (results not shown). Protoplasting succeeded for all the 18 strains, but not all constituent nuclei of each selected strain were recovered as homokaryons. Out of seven strains (TW1, CH1, WW1, WW7, TW7, CH2, and WW2) both homokaryotic parental types were recovered and of six strains (TW8, WB2, WB4, WB5, TW6 and TW5) only one homokaryotic parental type were recovered (Table 3). The constituent nuclei of the remaining five strains were not recovered. The recovery of homokaryotic parental lines is likely influenced by the regeneration ability of protoclones, i.e., either a nuclear type is not viable without its counterpart or its regeneration is so slow that these protoclones are overgrown by protoclones from the counterpart. Homokaryons of the same nuclear type showed generally homogeneous growth, and only one was selected and used for the diallel crosses.

Table 3. List of strains from which homokaryons were recovered.

No.	Name	sensitivity	Cap color	Homokaryon I	Homokaryon II
1	TW8	resistant	white	M31	
2	TW1	resistant	white	K2	K20
3	CH1	resistant	white	O1	O13
4	WW1	resistant	off-white	Mes09199	Mes09200
5	WB2	resistant	brown	Mes09143	
6	WB4	resistant	brown	Mes01557P8	
7	WB5	resistant	brown	S3	
8	WW7	sensitive	off-white	Z6	Z8
9	TW7	sensitive	white	Q1	Q26
10	TW6	sensitive	white	N8	
11	WB15	sensitive	brown	Mes09119	
12	CH2	resistant	white	CH2A	CH2B
13	WW2	unknown	white	Mes09206	Mes09208

The genetic diversity among homokaryotic lines was estimated with 535 SNP markers developed from the sequences of CH2B (JGI) and CH2A (Service Xs, Leiden). Although these SNP markers were selected on

differences of only two lines, they give some indication of genetic relationship between the homokaryons used (Figure 1). The genetic distance between homokaryons recovered from the same commercial line was large and due to the fact that these commercial lines (CH lines) result from a cross between haploid single spore cultures of two genetically distinct traditional lines. Mes09199 and O1 were not included in the dendrogram because of insufficient marker data, and K20 was not included because of a very slow mycelium growth. Although SNP data derived from only two lines cannot be used to show pedigree relationship of the lines used, the data show at least that there is considerable genetic variation in the homokaryons used.

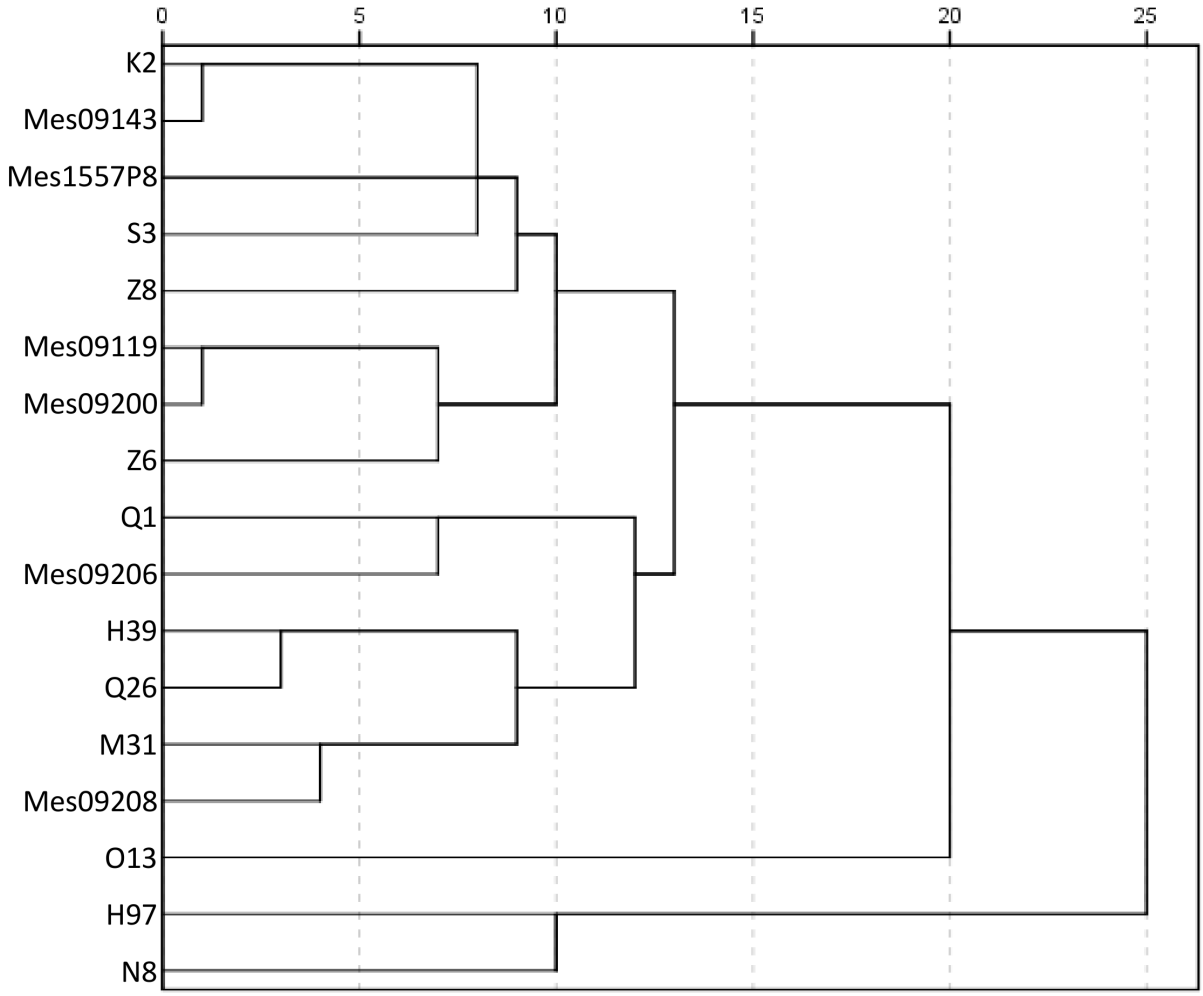

Figure 1. Genetic similarity of recovered homokaryons.
The dendrogram wasgenerated in SPSS with method of average linkage between groups. The scale bar at the top of the dendrogram shows the genetic distance between strains, the shorter the distance the higher the similarity.

Crossing and fruiting test

Nineteen recovered homokaryotic lines were intercrossed using a diallel crossing scheme (Table 4). The homokaryon K20 recovered from TW1 was not used for crossing because of its very slow growth. Not all combinations of the 19 lines were compatible. For instance, Mes01557P8 and Mes09200 were only compatible with Mes09143 and Z6. In contrast, Mes09143 was compatible with most parental lines apart from S3, Mes09199, Q1 and K2. The mating system of most basidiomycetes allows successful crossings among more than 90% of non-related individuals within a basidiomycete species (Raper, 1966, Kothe, 2001). In this study, however, the success rate of the crossings (less than 30%) was much lower, which

might indicate a high frequency of one or more mating type alleles in the set of homokaryons recovered from genetically related heterokaryons. Fifty-one confirmed hybrids were finally obtained for fruiting and the assessment of bruising sensitivity. Forty-nine out of 51 hybrids produced mushrooms (Table 5). Hybrid 41 (O13 × N8) and 43 (Q26 × N8) failed to produce mushrooms. Some hybrids had a very low productivity in one tray or both trays (flush 2), which did not produce enough mushrooms to allow BS analysis.

Table 4. Compatibility between homokaryons used in a diallel crossing scheme.

9143	S3	1557P8	9200	9208	9199	9119	Q1	CH2A	N8	K2	Z6	CH2B	Q26	O13	9206	O1	M31	Z8	
*	I	c	c	c	i	c	i	c	c	i	c	c	c	c	c	c	c	C	9143
	*	i	i	i	i	i	i	i	i	i	i	c	i	i	i	c	i	I	S3
		*	i	i	i	i	i	i	i	i	c	i	i	i	i	i	i	I	1557P8
			*	i	*	i	i	i	i	i	c	i	i	i	i	i	i	I	9200
				*	i	i	i	i	i	i	c	i	i	i	*	i	i	I	9208
					*	i	i	i	i	c	c	i	i	i	i	c	i	C	9199
						*	i	i	i	i	i	c	i	i	i	i	i	I	9119
							*	i	i	i	i	c	*	c	i	i	i	I	Q1
								*	c	i	i	*	c	i	i	i	i	C	CH2A
									*	i	c	i	c	c	i	c	c	I	N8
										*	c	i	i	i	i	i	i	I	K2
											*	c	c	c	i	i	i	*	Z6
												*	c	i	i	c	i	C	CH2B
													*	c	i	c	c	I	Q26
														*	i	*	c	C	O13
															*	c	c	I	9206
																*	i	C	O1
																	*	C	M31
																		*	Z8

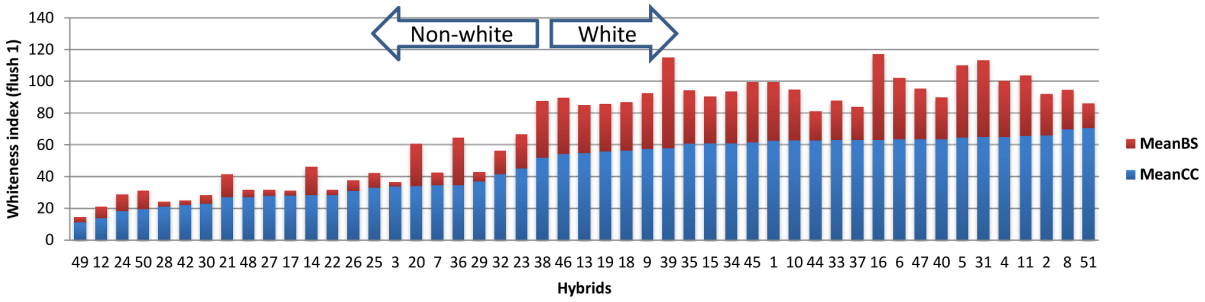

Figure 2. Bruising sensitivity (BS) and cap color (CC) of hybrids in flush 1.
The hybrids were sorted by the mean CC (whiteness index of cap color). The BS and CC values are plotted on top of each other for each hybrid.

Variation in bruising sensitivity (BS) among hybrids

The test for BS (expressed as whiteness index) showed a large variation among hybrids, which ranged from 2.85 to 57.01 in flush 1 and from 2.63 to 45.71 in flush 2 (Table 5). The higher the value the more the level of discoloration is. A good correlation was found between data of computer software and visual scoring. The BS of hybrids in flush 1 was highly correlated with that in flush 2 (r = 0.82; Pearson, P<0.001). ANOVA showed that genotype and flush were both significant factors for BS, and interaction effect of genotype by flush was significant as well (P<0.05). Mushrooms of flush 2 were significantly less sensitive than mushrooms of flush 1 (P<0.05). The cap color (CC) was also measured with the same imaging system, which ranged from 11.29 to 70.56 in flush 1 and from 3.55 to 71.70 in flush 2 (Table 5). The higher the value the whiter the mushroom is. The overall mean of CC-flush 2 was significantly higher than the overall mean of CC-flush 1 (P<0.05). Non-white hybrids (including off-white, light brown and brown hybrids)

were generally less sensitive than white hybrids in both flushes, which is consistent with the findings of a previous study (Weijn et al., 2012b). For instance, the bar chart in Figure 2 shows that white mushrooms of flush 1 have generally higher BS than non-white hybrids.

Bruising sensitivity is a quantitative trait and shows continuous variation among strains. Broad-sense heritability (H^2) of BS-flush 1 and BS-flush 2 were 0.99 and 0.97 respectively, and also high across flushes (H^2 = 0.93). This indicates that the genotypic variation for BS is highly inheritable.

General combining ability

The diallel crossing scheme among homokaryons allowed testing each homokaryon for BS in different genetic backgrounds. The mean performance of parental lines in flush 1, flush 2 and the two flushes combined are shown in Table 6. It turned out that not all homokaryotic lines derived from resistant strains gave rise to bruising resistant hybrids. This holds true, for instance of crosses with M31, CH2B, O1 and O13, homokaryons derived from resistance strains. Among all these tested parental lines Mes09143 was the most resistant line, and Z8 the most sensitive one.

Table 5. Summary of bruising sensitivity, cap color of hybrids in two successive flushes.

Hybrid	Parent 1	Parent 2	BS-flush 1 Mean (SD)	BS-flush 2 Mean (SD)	CC-flush 1 Mean (SD)	CC-flush 2 Mean (SD)
1	CH2A	Z8	37.23(8.57)	32.63(6.69)	62.58(3.58)	63.26(4.21)
2	CH2A	N8	26.35(4.66)	16.58(4.1)	66.01(3.38)	66.95(3.87)
3	CH2A	Mes09143	2.85(3.02)	*	34.06(5.21)	*
4	CH2A	Q26	35.13(6.33)	23.87(4.14)	65.13(3.11)	71.70(1.66)
5	CH2B	Z8	45.70(3.96)	43.42(7.87)	64.73(2.38)	64.53(3.91)
6	CH2B	O1	38.69(1.82)	*	63.49(2.25)	*
7	CH2B	Mes09143	8.21(3.85)	7.48(5.14)	34.61(6.72)	36.6(8.76)
8	CH2B	Q1	24.60(4.97)	25.93(3.84)	70.03(2.67)	66.74(4.04)
9	CH2B	Z6	34.88(4.83)	29.94(6.98)	57.75(3.80)	61.11(3.7)
10	CH2B	Q26	32.13(3.14)	26.46(6.39)	62.89(4.42)	69.08(2.67)
11	CH2B	Mes09119	37.97(4.23)	35.23(5.14)	65.86(3.47)	67.02(3.91)
12	CH2B	S3	6.84(3.26)	9.39(4.85)	14.37(5.66)	14.00(5.52)
13	K2	Z6	30.57(4.70)	33.38(4.01)	54.74(4.91)	57.91(3.30)
14	K2	Mes09199	17.65(6.34)	14.56(4.44)	28.71(5.90)	43.45(6.62)
15	M31	O13	29.48(6.02)	30.66(5.79)	61.13(4.62)	67.97(2.87)
16	M31	Z8	53.81(4.58)	45.71(6.33)	63.46(3.04)	61.81(3.69)
17	M31	Mes09143	3.01(3.88)	6.25(4.63)	28.44(7.78)	32.6(8.72)
18	M31	Q26	30.59(7.09)	*	56.49(2.57)	*
19	M31	Mes09206	30.12(3.85)	35.68(5.85)	55.90(3.04)	59.99(5.22)
20	Mes09199	Z8	26.71(6.75)	13.26(3.93)	34.29(6.31)	44.38(4.25)
21	Mes09199	Z6	14.64(6.53)	12.37(5.29)	27.27(5.68)	44.03(8.26)
22	Mes09200	Mes09143	3.09(3.24)	6.75(4.07)	28.87(4.86)	38.77(7.57)
23	Mes09200	Z6	21.40(4.20)	16.92(3.11)	45.46(5.46)	50.55(5.19)
24	Mes09143	Z6	10.84(4.27)	11.44(5.26)	18.30(4.56)	34.86(6.89)
25	Mes09143	Z8	9.07(4.43)	2.92(3.66)	33.39(6.56)	31.01(10.91)
26	Mes09143	Q26	6.62(4.31)	2.63(2.57)	31.18(5.23)	29.27(4.94)
27	Mes09143	Mes09119	3.78(3.85)	6.11(5.36)	28.14(9.56)	29.1(7.79)
28	Mes09143	Mes09206	3.28(3.86)	8.46(5.04)	21.28(7.00)	31.71(6.89)
29	Mes09143	Mes09208	5.99(3.83)	3.65(3.90)	37.05(12.58)	54.92(7.25)
30	O1	S3	5.82(5.09)	8.6(4.18)	22.90(10.08)	3.55(3.97)
31	O1	Z8	48.66(4.66)	36.29(5.28)	64.92(2.87)	58.98(4.58)
32	O1	Mes09143	14.81(5.23)	*	41.74(5.14)	*
33	O1	Q26	24.73(4.55)	*	63.23(3.44)	*

34	O1	N8	32.51(8.12)	20.3(4.30)	61.28(2.88)	59.37(3.16)
35	O1	Mes09206	33.48(4.55)	22.08(3.70)	60.95(3.29)	63.69(3.49)
36	O1	Mes09199	29.93(6.76)	16.62(3.84)	34.75(4.54)	47.17(5.21)
37	O13	Q1	20.70(5.10)	12.27(4.38)	63.40(5.19)	60.06(3.15)
38	O13	Z6	35.54(5.71)	23.56(5.82)	52.02(8.19)	60.79(3.33)
39	O13	Z8	57.01(6.72)	29.29(4.01)	58.08(3.93)	59.4(3.75)
40	O13	Q26	26.33(3.37)	16.85(3.10)	63.79(5.09)	67.73(2.84)
41	O13	N8	-	-	-	-
42	O13	Mes09143	2.95(2.72)	3.65(4.05)	22.31(4.99)	33.06(7.67)
42	Q26	N8	-	-	-	-
44	Z6	Mes09208	18.23(4.19)	27.89(1.97)	63.02(1.69)	58.81(1.83)
45	Z6	Q26	38.01(6.57)	27.77(7.21)	61.55(3.73)	65.23(2.28)
46	Z6	N8	35.42(3.64)	25.78(4.81)	54.51(4.57)	59.64(3.67)
47	M31	N8	31.76(3.75)	27.94(5.66)	63.77(1.47)	64.03(2.65)
48	Mes09143	N8	4.41(3.62)	5.11(3.59)	27.37(3.62)	39.05(4.30)
49	Mes01557P8	Mes09143	3.61(4.08)	7.51(4.62)	11.29(6.59)	16.16(9.39)
50	Mes01557P8	Z6	11.7(5.26)	13.62(6.97)	19.96(10.07)	21.08(9.40)
51	CH2A	CH2B	15.58(3.46)	14.59(5.01)	70.56(2.70)	69.03(3.25)

"*" indicates hybrids did not produce enough mushrooms to allow BS and CC analysis; "-" indicates hybrids did not produce mushrooms at all. The higher BS value indicates higher bruising sensitivity, and the higher CC value indicates whiter mushroom cap. SD indicates the standard deviation.

The expected and observed performance of hybrids for BS in flushes 1 and 2 are depicted in Figure 3. The actual performance showed a clear positive correlation with the expectation. The correlation coefficients for the respective flushes were 0.88 and 0.86 (P<0.001) indicating that the variation in BS among hybrids has an additive, possibly polygenic nature. Under polygenic inheritance with additive gene action offspring resemble more closely the average of their parents (the mid parent) than either one of the individual parents (Karlin *et al.*, 1979).

Table 6. Mean performance of parental lines in crosses for BS and the corresponding GCA estimates based on data from flush 1 & 2 and combined analyses.

	Number of hybrids			Mean performance			GCA		
Parent	Flush 1	Flush 2	Combined	Flush 1	Flush 2	across flush	Flush 1	Flush 2	across flush
Mes09143	27	23	27	2.89	3.07	3.08	-8.57	-6.49	-7.5
S3	3	4	4	3.25	4.5	4.07	-8.21	-5.06	-6.51
Mes01557P8	4	4	4	3.81	5.25	4.53	-7.65	-4.31	-6.05
Mes09200	4	4	4	6.13	5.92	6.02	-5.33	-3.64	-4.56
Mes09208	3	3	3	5.04	5.86	5.45	-6.42	-3.7	-5.13
Mes09199	8	8	8	11.16	7.11	9.14	-0.3	-2.45	-1.44
Mes09119	4	4	4	10.44	10.34	10.39	-1.02	0.78	-0.19
Q1	4	4	4	11.33	9.55	10.44	-0.13	-0.01	-0.14
CH2A	9	8	10	11.08	10.95	10.11	-0.38	1.39	-0.47
N8	10	10	10	13.06	9.57	11.32	1.6	0.01	0.74
K2	4	4	4	12.03	12	12.01	0.57	2.44	1.43
Z6	19	19	19	12.73	10.98	11.86	1.27	1.42	1.28
CH2B	17	16	17	13.26	12.02	12.86	1.8	2.46	2.28
Q26	13	10	14	13.56	9.75	12.18	2.1	0.19	1.6
O13	11	12	12	14.29	9.66	12.03	2.83	0.1	1.45
Mes09206	6	6	6	11.12	11.09	11.11	-0.34	1.53	0.53
O1	14	10	15	14.79	10.4	12.49	3.33	0.84	1.91
M31	11	10	12	14.92	14.64	14.87	3.46	5.08	4.29
Z8	13	13	13	21.05	15.53	18.29	9.59	5.97	7.71

N is the number of trays having the homokaryon as a parent (two replicates per hybrid), odd number indicates one tray of the hybrid did not produce enough mushrooms; MP is the mean performance of individual homokaryons (the

mean BS of hybrids); GCA is the general combining ability; negative GCA indicates lower bruising sensitivity positive GCA indicates higher bruising sensitivity compare to the general mean BS of all hybrids in the diallel matrix.

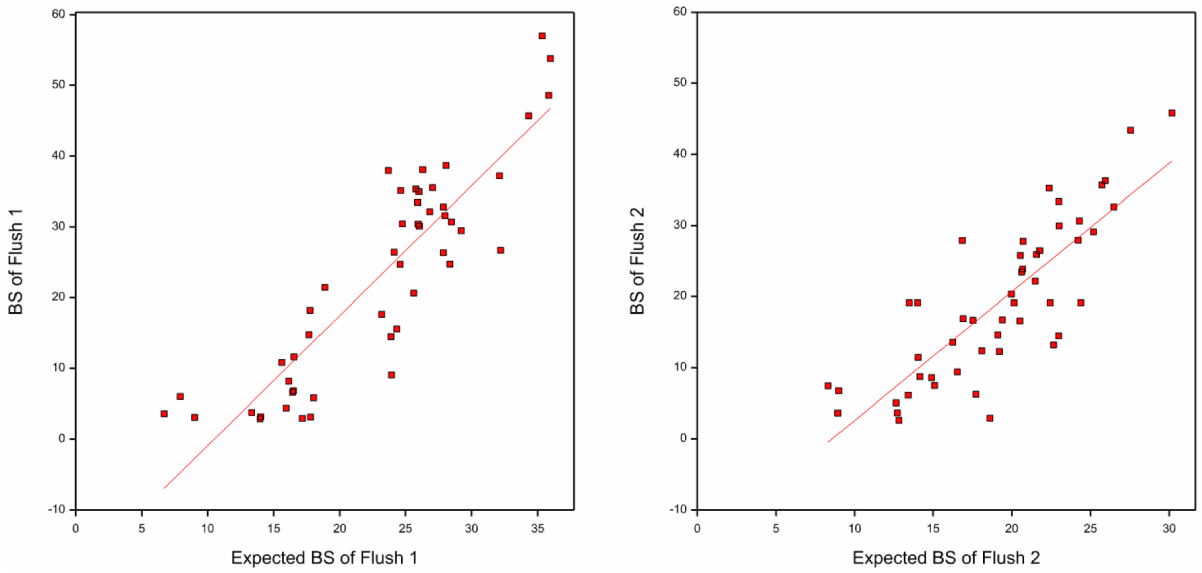

Figure 3. Correlation of observed BS of hybrids and their expected value (flushes 1 and 2).

The GCA of a homokaryon for BS is equivalent to its breeding value (Lynch & Walsh, 1998). BS is defined as the discoloration relative to the non-bruised area and is zero with no visible bruising damage and its value increases with increasing discoloration. A positive value of GCA indicates thus a higher BS than the average and a negative value the opposite, i.e. a lower BS than the average. The GCA analysis shows that the parental homokaryons Mes09143, S3, Mes01557P8, Mes09200 and Mes09208 have a high negative GCA, and are thus good donors for bruising resistance (BR) (Table 6). Especially Mes09143 is of interest with negative GCA values of -8.57 in flush 1 and -6.49 in flush 2. Its GCA estimate was based on the highest number of crosses due to its rare mating type and is thus rather reliable. The best performing homokaryon Mes09143, derived from a wild resistant brown strain, showed no or hardly any discoloration of mushrooms in all crosses (Figure 4). The mean performance of crosses with Mes09143 was 3.08 across flushes, and the individual hybrids with this line in their pedigree varied in BS between 2.63 and 14.81. This indicates that Mes09143 is carrying dominant alleles for BR and an attractive donor for BR.

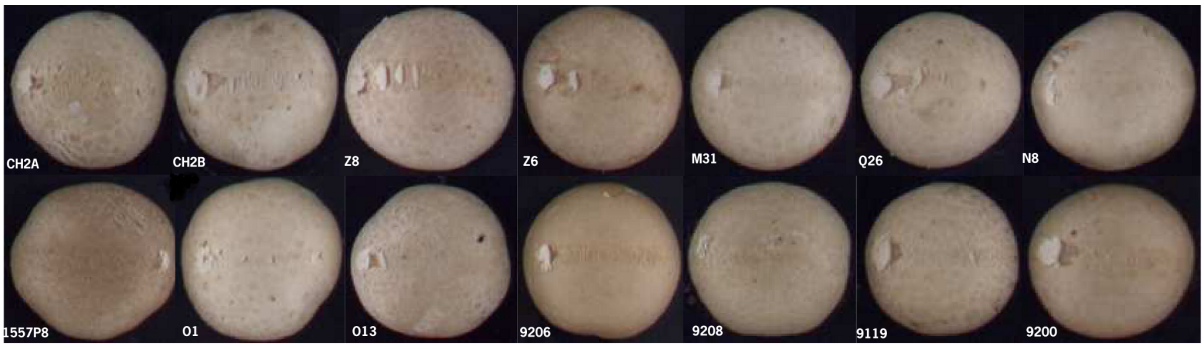

Figure 4. Bruised mushrooms of hybrids with Mes09143 as one of the parents (flush 1, 60 min after bruising).

At the opposite end of the spectrum, Z8 is a good donor for BS, as Z8 showed in the two successive flushes GCA values of 9.59 and 5.97 respectively. Z8 and Z6 are the two constituent homokaryons of a white wild

strain sensitive to bruising (WW7). All hybrids with Z8 as a parental line were sensitive to bruising except for the one with Mes09143 (the strain that was dominant for BR) (Figure 5). The mean performance of Z8 across flushes was 18.29, and the individual hybrids with this line as a parent varied in BS across flushes between 5.99 and 49.87. This indicates that Z8 presents a dominant pattern for bruising sensitivity (BS). Z6 did not show a clear pattern in the variation of BS between hybrids.

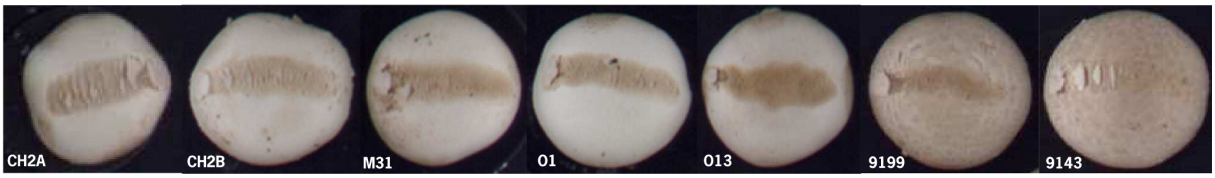

Figure 5. Bruised mushrooms of hybrids with Z8 as one of the parents (flush 1, 60 min after bruising).

Some lines did show intermediate GCA estimates, e.g. Mes09206, Q1, CH2A, N8, K2, and Z6, CH2B, nevertheless, derived from insensitive strains. For instance, hybrids with either CH2A or CH2B as a parent varied considerably in sensitivity, depending on the partner in the hybrid (Figure 6 & 7). The mean performance of CH2A in crosses was 10.11 across flushes, and the BS of its individual hybrids across flushes varied between 2.85 and 34.93; the mean performance of CH2B across flushes was 12.86, and the BS of its individual hybrids across flushes varied between 7.84 and 44.53. The cross between CH2A and CH2B represents the most bruising-resistant commercial white hybrid (CH2) currently on the market. This suggested that its parental homokaryons complement each other genetically resulting in a hybrid with a relative resistance to bruising, but genes involved are not dominant.

Figure 6. Bruised mushrooms of hybrids with CH2A as a parental line (flush 1, 60 min after bruising).

Specific combining ability

The deviation of BS from the expected value was used as a measure of SCA. A positive value for SCA indicates that the BS of the cross is higher than expected (more sensitive), and negative SCA indicates the BS value of the cross is lower than expected (more resistant). The SCA values of all hybrids in flushes 1 and 2 are presented in Table 7. All brown hybrids had negative SCA except for the cross Mes09200×Z6. As mentioned previously, Mes09200 was a homokaryon recovered from an off-white stain but expressed a brown cap color in all crosses and was thus an exceptional crossing partner. All except one, homokaryons derived from brown varieties gave rise to bruising resistant hybrids and homokaryons from white varieties resulted in either sensitive or resistant crosses. It is remarkable that most crosses among 'white' homokaryons were more sensitive than expected. Nevertheless, there is still substantial variation in bruising sensitivity among white crosses. The white crosses of CH2A×CH2B, O13×Q1 and O13×Q26 were more resistant than expected (more negative SCA than expected), in which the cross of CH2A×CH2B was the most insensitive one among white hybrids. The homokaryons (parental lines) of crosses mentioned above were all derived from white commercial varieties, and these varieties were genetically related. Combinations between these homokaryons are thus a special case and our data indicate that these lines complement each other genetically resulting in a relatively low BS.

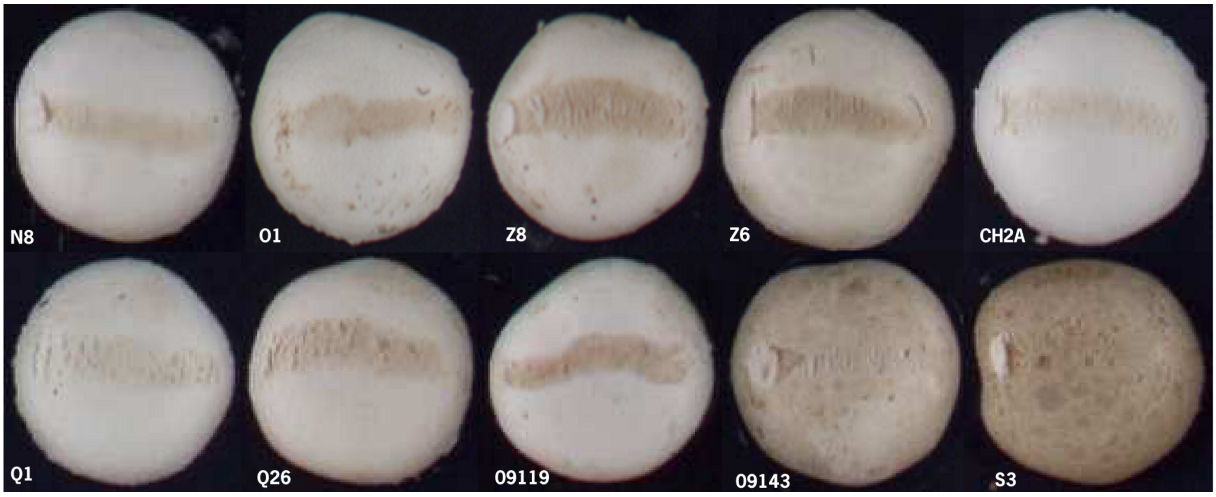

Figure 7. Bruised mushrooms of hybrids with CH2B as a parental line (flush1, 60 min after bruising).

Table 7. SCA of hybrids in two successive flushes.

Hybrid	Parent 1	Parent 2	SCA (flush 1)	SCA (flush 2)	Hybrid	Parent 1	Parent 2	SCA (flush 1)	SCA (flush 2)
1	CH2A	Z8	5.10	6.15	26	Mes09143	Q26	-9.83	-10.19
2	CH2A	N8	2.21	-3.94	27	Mes09143	Mes09119	-9.55	-7.30
3	CH2A	Mes09143	-11.12	-	28	Mes09143	Mes09206	-10.73	-5.70
4	CH2A	Q26	10.49	3.17	29	Mes09143	Mes09208	-1.94	-5.28
5	CH2B	Z8	11.39	15.87	30	O1	S3	-12.22	-6.30
6	CH2B	O1	10.64	-	31	O1	Z8	12.82	10.36
7	CH2B	Mes09143	-7.94	-7.61	32	O1	Mes09143	-2.87	-
8	CH2B	Q1	0.01	4.36	33	O1	Q26	-3.62	-
9	CH2B	Z6	8.89	6.94	34	O1	N8	4.66	0.33
10	CH2B	Q26	5.31	4.69	35	O1	Mes09206	7.57	0.59
11	CH2B	Mes09119	14.27	12.87	36	O1	Mes09199	3.98	-0.89
12	CH2B	S3	-9.67	-7.13	37	O13	Q1	-3.89	-6.94
13	K2	Z6	5.81	10.40	38	O13	Z6	9.55	2.92
14	K2	Mes09199	-5.54	-4.55	39	O13	Z8	22.70	4.10
15	M31	O13	1.30	6.36	40	O13	Q26	-0.49	-2.56
16	M31	Z8	17.84	15.54	42	O13	Mes09143	-13.20	-9.08
17	M31	Mes09143	-14.80	-11.46	44	Z6	Mes09208	0.46	11.05
18	M31	Q26	2.11	-	45	Z6	Q26	11.72	7.04
19	M31	Mes09206	4.08	9.95	46	Z6	N8	9.63	5.23
20	Mes09199	Z8	-5.50	-9.38	47	M31	N8	3.78	3.73
21	Mes09199	Z6	-9.25	-5.72	48	Mes09143	N8	-11.54	-7.53
22	Mes09200	Mes09143	-5.93	-2.25	49	Mes01557P8	Mes09143	-3.09	-0.81
23	Mes09200	Z6	2.54	0.02	50	Mes01557P8	Z6	-4.84	-2.61
24	Mes09143	Z6	-4.78	-2.61	51	CH2A	CH2B	-8.76	-8.38
25	Mes09143	Z8	-14.87	-15.68					

A positive SCA indicates that the BS of the cross is higher than expected (more sensitive), and negative SCA indicates the BS value of the cross is lower than expected (more resistant). "-" indicates missing data because of no mushrooms or not enough mushrooms.

Discussion

In this study, the GCA of a particular line is calculated as the deviation of the mean performance of a particular line from half of the overall mean of all crosses in the diallel scheme. GCA is equivalent to the breeding value of an individual (Lynch & Walsh, 1998), and the GCA of a line is a reflection of the number of loci with positive alleles for the trait of interest and their effect (Adebambo, 2011). The high negative

GCA of Mes09143 indicates that this strain has a high number of loci with alleles having a favorable effect on the level of bruising resistance (BR). Therefore such a strain can be an interesting source (donor) for genetic improvement of bruising sensitive mushrooms. The opposite accounts for Z8, probably having a low frequency for genes with a beneficial effect on BR and thus a good combiner for BS in the segregation analysis. The ones with intermediate GCA are neither donors for BR nor donors for BS, because the trait expression in their crosses is always determined by the counter parents. They might be good recipients in breeding programs if they carry other favorable agronomic traits. GCA analysis is thus useful to select parental lines for different purposes. Parental lines with a favorable GCA can be used to stack positive alleles of genes involved in the trait of interest through recombination and selection, whereas parental lines with opposite GCA values are good parents for the creation of a dedicated mapping population to study the genetics of traits of interest.

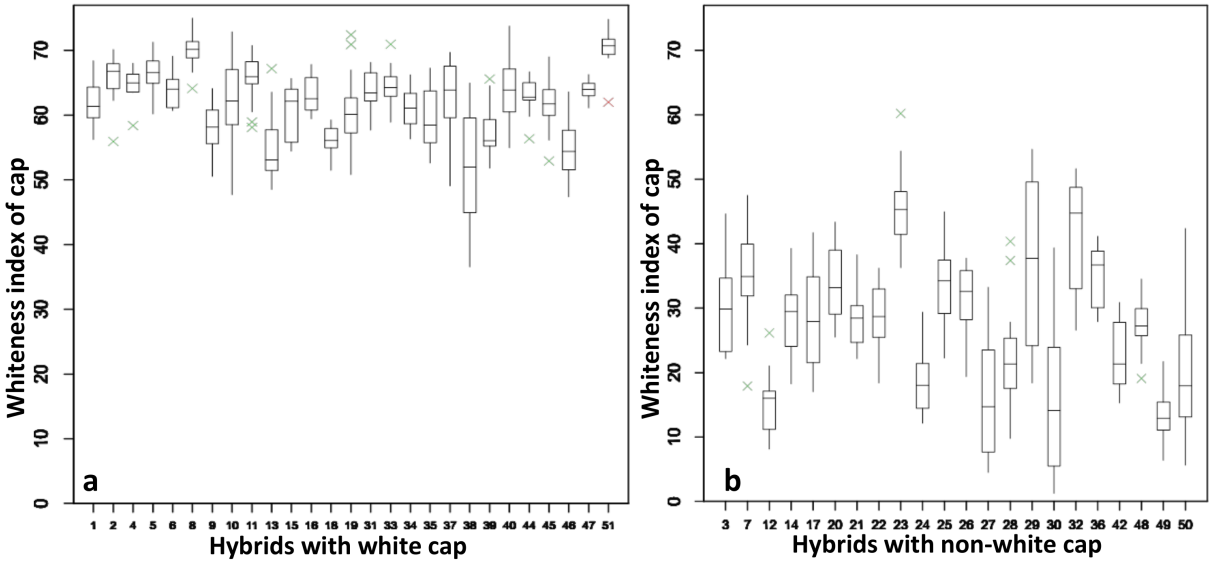

Figure 8a. Boxplots describing variation in cap whiteness for each hybrid classified as white (flush 1). The box spans the interquartile range of the values of cap whiteness of each hybrid, so that the middle 50% of the data lie within the box, with a line indicating the median. Whiskers extend beyond the ends of the box as far as the minimum and maximum values. The crosses indicate outliers. The cap whiteness of these hybrids were measured based on 20 individual mushrooms of flush 1 per hybrid. Long boxes indicate large variation of cap whiteness, and short boxes indicate small variation of cap color. By comparing Figure 8a and Figure 8b individual mushrooms of non-white hybrids show larger difference of cap color than that of white hybrids.

Figure 8b. Boxplots describing variation in cap whiteness for each hybrid classified as non-white (flush 1). The legend is the same as described for Figure 8a.

Negative SCA estimates were observed in brown hybrids and positive SCA estimates in most white hybrids. This might indicate that the cap color interferes with bruising resistance. Whether the major gene for cap color has a pleiotropic effect and thus also influences BS or other genes linked to this gene are involved is not known, which can be seen from a segregation analysis. This also indicates that breeders should not only use white lines in further breeding programs to improve bruising resistance, but also brown lines. Not only the sign of SCA value, also the magnitude of the value is meaningful. It was suggested that the high magnitude means high genetic divergence and high heterosis in the hybrids (da Cruz *et al.*, 2010). In addition, hybrids displaying heterosis are said to have favorable combining ability (Qu et al., 2012). The lowest negative SCA estimate of hybrid strain CH2 among all the white hybrids indicates that heterosis due to genetic complementation is an important cause for its relatively low sensitivity to bruising. Similarly, heterosis might be an important factor explaining the bruising resistance of hybrids between the most bruising resistant homokaryon Mes09143 and the most bruising sensitive

homokaryon Z8. The difference between the observed performance of an F1 hybrid and the expected value based on the GCA of its parents indicate gene interactions (Deglene et al., 1999). The better than expected performance of the hybrid between Mes09143 and Z8 cannot be explained by additive effects alone but indicate interference between alleles (dominance or even over-dominance). These lines widely differing in the breeding value for sensitivity to bruising, can be used to generate a segregating population for mapping purposes. In a successive project, we will generate a segregating population from this hybrid to map QTLs and find candidate genes involved in bruising sensitivity.

Cap color of button mushrooms is controlled by a major QTL on chromosome 8 with a recessive allele for white (Foulongne-Oriol *et al.*, 2012). Therefore it was not surprising to see two distinct groups of hybrids for cap color, i.e. white and non-white. The ones depicted in Figure 8a have a more or less white cap color with a mean whiteness index ranging from 56.29 (off-white) to 69.81 (white) in flush 1 (values are in Table 5). The box plot for cap color of these hybrids based on 20 individual mushrooms of flush 1 per hybrid shows little variation in cap color. The group of hybrids with a non-white cap color had in their pedigree at least one homokaryon (Mes09143, Mes01557P8, S3) from wild accessions with a brown cap color (Figure 8b). All these crosses produced mushrooms with cap colors varying from light brown to brown (and this range is designated as non-white) with a range in whiteness index of cap color from 13.66 (brown) to 48.07 (light brown) in flush 1. The larger variation of non-white cap color compared to white cap color indicates a more complex genetic base for cap color than just one gene. The variation might be due to the dosage variation of brown cap color QTL and the involvement of modifier genes. The modifier loci for cap color have been found in a previous study (Foulongne-Oriol et al., 2012). Hybrid 27 (Mes09143 × Mes09119), for example, with both parental lines recovered from brown heterokaryons produced mushrooms with brown caps. Hybrid 12 (CH2A/derived from a white hybrid × S3/derived from a brown hybrid) produced brown mushrooms as well. However, a cross (CH2B/derived from a white hybrid × Mes09119/derived from a brown hybrid) produced white mushrooms although Mes09119 was derived from a brown strain and CH2B from a white strain. This indicates that WB15, from which Mes09119 was obtained, is heterozygous for the major locus for cap color. Another exceptional strain was WW1, an off-white strain identified in a previous study. Both homokaryotic parental lines apparently have the brown QTL allele for cap color since all hybrids having one of them as parent were brown or light brown.

In addition to the variation in cap color between the non-white hybrids, also individual mushrooms of the same non-white hybrids show large differences with respect to cap color. Especially for crosses between homokaryons recovered from white and brown varieties, mushrooms are produced with high variation in cap color, varying from brown to light brown, i.e. hybrids 27, 29, 30, 32 (Figure 8b). Hybrids among brown strains (Figure 8b, hybrid 49) and among white strains (Figure 8a, hybrids 2, 4, 6 etc.) produced mushrooms with much less variation in color (thus either brown or white). We can therefore designate the crosses as brown, light brown, off-white, and white. This might indicate the incomplete expression of brown alleles.

The WI of control area for white hybrids is always higher owing to its light color than that of the bruised area; even very light discoloration can be seen. All brown hybrids used in this study were clearly less brown than present-day brown commercial strains and allowed good assessment of discoloration upon bruising. Nevertheless, a very light discoloration might be partly obscured by the background color despite the fact that the discoloration is assessed relatively to the non-bruised background color of the same mushroom. This might also be the reason why brown strains generally show less discoloration than white strains. Next to this, strong scaling of some non-white caps might also influence the BS measurement. The WI of the control area was sometimes lower than that of the bruised area, which gave a negative BS value. Occasionally the cap skin was damaged so severely that white tissue underneath the skin was visible. Such white tissue might interfere with the assessment of BS, although care was taken to select only areas with intact skin for the measurements. Nevertheless white spots maybe in some cases

the reason for negative values of discoloration. Negative values were corrected into zero if there was really no discoloration.

Mushroom breeding is similar to plant breeding but differs in a number of aspects. Mushrooms have a relatively short life cycle with a haploid phase (homokaryon) that can be easily propagated and maintained as infertile vegetative mycelium. Homokaryons can be considered equivalent to double haploids or inbred lines used nowadays in breeding of many plant species. The nuclei of fertile diploid mycelium (heterokaryon) stay apart (n + n) and do not fuse as in diploid plants (2n). The constituent nuclei can thus be recovered from the heterokaryons as haploid homokaryons, and the intact and original genome combination can be maintained forever. This is a practical advantage of mushroom breeding over plant breeding. A complication is that in button mushrooms the mating type of homokaryons (haploids) limits the production of heterokaryons. It is thus wise to start heterosis breeding with a broad selection of compatible lines in order to have sufficient lines to complete a full diallel scheme. The fact that this paper reports for the first time the estimation of breeding value by using diallel crossings underlines that mushroom breeding lacks behind plant breeding. However, it also shows that the same techniques and strategies used in plant breeding can be used in mushroom breeding.

This study was the first attempt to analyze the combining ability of the parental homokaryons of a set of button mushrooms hybrids. It has shown that in *A. bisporus* bruising sensitivity is a highly inheritable trait. The study also showed that this approach is an excellent way to estimate the breeding value of homokaryons of button mushroom, and facilitates the selection of parental lines for heterosis breeding. In a follow-up study, hybrids of lines differing strongly in bruising sensitivity will be used to generate segregating populations to enhance our knowledge of genetics of bruising sensitivity.

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Chapter 4

Meiotic Recombination in the Life Cycle of *Agaricus bisporus* var. *bisporus*

Wei Gao[1,2], Patrick Hendrickx[1], Johan JP Baars[1], Narges Sedaghat Tellgerd[1], Brian Lavrijssen[1], Richard GF Visser[1], Anton SM Sonnenberg[1]

[1] Plant Breeding, Wageningen University and Research center, Wageningen, the Netherlands.

[2] Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing, PR China.

To be submitted

Abstract

Agaricus bisporus (button mushroom) is an economically important edible fungus with a long history of cultivation. Nevertheless, breeding for this mushroom is lagging behind other crops, and only a few new varieties have been commercially released in the past 30 years. This is mainly caused by the atypical sexuality of *A. bisporus* var. *bisporus*, which represents all commercial varieties and most wild collected lines. Most basidia produce only two spores containing non-sister nuclei with compatible mating types that germinate into fertile mycelia. This is unfavourable for generating homokaryotic breeding materials. Only a small percentage of basidia produce four spores that each germinate into homokaryons suitable for mating and thus breeding. Three decades ago, a new variety was discovered that produces mainly four spored basidia and hence be more suited for cross breeding. This variety has, however, low quality and is not yet used to generate superior new strains. This study first reviews the details known about the life cycle of var. *bisporus* and presents new details on meiotic behaviour generated by next generation sequencing technologies in the var. *bisporus*. Then it demonstrates that meiotic recombination in the var. *bisporus* is infrequent and restricted to the ends of chromosomes through segregation analysis and resequencing. In combination with frequent pairing of non-sister nuclei this leads to the preservation of the heterozygous state in heterokaryotic offspring. Genetic analysis of present-day commercial varieties reveals that these are directly derived from the first commercial hybrid released in 1980 through the isolation of fertile single spore cultures. This study allows updating the life cycle of *A. bisporus* var. *bisporus* and provides fundamental knowledge for further meiosis study and button mushroom breeding.

Introduction

Agaricus bisporus (button mushroom) has a long history of cultivation of more than 300 years, and the first commercial cultivation was attempted in France in the 18th century (Pardo *et al.*, 2010, Tournefort, 1707). Button mushrooms are grown worldwide with a product volume of 3.9 million tonnes in 2009, mainly produced in China, the USA, Poland, the Netherlands, South Korea and France (Sonnenberg *et al.*, 2011). The productivity of the crop has been improved considerably during the last 30 years mainly by improving cultivation techniques, i.e., composting, casing, growing conditions, and mechanical spawning & harvesting. Genetic studies and breeding trials for advanced cultivars were conducted meanwhile for cap color (Callac *et al.*, 1998), resistance to pathogens (Moquet *et al.*, 1998b, Foulongne-Oriol *et al.*, 2011b), yield (Foulongne-Oriol *et al.*, 2012b), earliness (Foulongne-Oriol *et al.*, 2012a), resistance to mechanical bruising (Gao et al, Chapter 5) and some other agronomic and quality traits (Gao et al, to be published). This has, however, not yet generated a commercial cultivar that is substantially superior to the first hybrid strain Horst U1 (Fritsche, 1981), which was released in the 1980s onto the market. The new cultivars released afterwards were either genetically identical or very similar to the first hybrid (Sonnenberg et al., 2011). The impeded progress of breeding can be largely attributed to the atypical life cycle and sexuality of *A. bisporus* var. *bisporus*, which leads to difficulties of obtaining homokaryons as breeding materials, hampers outcrossing, and limits breeding success (Kerrigan, 1994, Moquet *et al.*, 1998a).Thus, understanding the life cycle is a primary requirement for mushroom breeding in terms of a recognizable sexual interaction between mated strains, fruiting competence, meiosis, and viability of basidiospores.

Life cycle defines the order and relative durations of successive developmental stages including nuclear and morphological events, leading to plasmogamy, karyogamy, meiosis, and progeny production (Chang & Hayes, 1978). Two types of life cycle are commonly found in basidiomycetes. The more frequent one is heterothallism where successful fusion of gametes must occur between haploids carrying different mating type alleles to allow fruiting. Haploids can be represented by monokaryons (one haploid nucleus per cell) or homokaryons (more than one copy of otherwise identical haploid nuclei per cell). Approximately one quarter of the basidiomycetes have a homothallic life cycle with a distinction being made between primary homothalism and secondary (or pseudo) homothalism. The former allows mating

with all possible haploid partners (no mating type selection) or no mating is needed to complete the life cycle, i.e., fructification. The secondary homothallic life cycle involves paring of nuclei from a single meiosis, but with different mating types, into a single spore leading to dikaryotic or heterokaryotic mycelium capable of fruiting. *A. bisporus* var. *bisporus* is an example of a basidiomycete with a secondary homothallic life cycle.

Similar to most basidiomycetes, in *A. bisporus* var. *bisporus* two types of nuclei exist side by side in the fertile heterokaryotic mycelium and karyogamy only takes place in the basidium just before meiosis (Figure 1). Approximately 80%-90% of the basidia produce two fertile spores, each carrying two nuclei with different mating types. Ten to twenty percentages of the basidia produce three or four spores where most spores receive one nucleus (Callac, 1993, Callac et al., 1996). Among randomly selected single spores, ca. 10% germinate into homokaryons (Kerrigan et al., 1992). Homokaryons bearing different mating types are able to mate through hyphal fusion generating fertile heterokaryons. The whole life cycle of var. *bisporus* can thus be divided into three successive phases, i.e., a common heterokaryotic phase, a rare homokaryotic phase and a short & transient diploid phase. This mixed type of life cycle of *Agaricus bisporus* var. *bisporus* has been designated as secondarily homothallic or amphithallic. The fertile heterokaryons are morphologically indistinguishable from the infertile homokaryons, but heterokaryons are generally growing faster than homokaryons (Kerrigan et al., 1992). Several studies have shown that the majority of the single spore isolates are heterokaryotic (carrying both mating types) and preserve the heterokaryotic state for most of the alleles. Next to the number of basidia producing 2 spores, the extent of heterozygosity is affected by the way post meiotic nuclei are paired into a spore and the frequency of recombination between homologous chromosomes in meiosis. Especially the pairing of post-meiotic nuclei in spores of the bisporic basidia has been debated in a number of papers. A simple explanation for the predominance of heterothallic spores in secondarily homothallic species by random migration of nuclei was suggested (Langton F A, 1980). This hypothesis was further tested and confirmed in a secondarily homothallic ink-cap fungus *Coprinus bilanatus* through segregation analyses of mating type, auxotrophy and antimetabolite resistance (Challen & Elliott, 1989). The segregation ratios of spore progenies tested conformed to random migration hypothesis, thus it was concluded that random migration could be regarded as the primary control of secondary homothallism. Theoretically one third of progeny are S-progeny (carrying two sister post meiotic nuclei) generated by random nuclear migration. However, sister nuclear pairing was found to be very rare in *A. bisporus* var. *bisporus*, i.e., less than 0.5%, and might result from a delay of the second meiotic division until after nuclei migration into spores has occurred (Kerrigan et al., 1993). This finding suggested that the four post-meiotic nuclei did not randomly migrate into basidiospores but that non-sister nuclei are preferentially paired in one spore (N-progeny). Nevertheless, there is insufficient evidence to support the preferential paring of non-sister nuclei given the poor and variable germination of spores reported in above-mentioned previous studies.

The karyotype of var. *bisporus* was studied for the first time microscopically by Evans (1959) and 12 chromosomes were observed in haploids. The genome size was estimated to be 34 Mb using re-association kinetics (Arthur et al., 1982). Further studies by CHEF (clamped homogeneous electric field electrophoresis) analysis confirmed the chromosome number as 13 and a comparable genome size of 31 Mb (Royer et al., 1992, Lodder et al., 1993, Sonnenberg et al., 1996). The first genetic linkage map of this variety was generated comprising of 11 linkage groups with a total map length of 543.8 cM (Kerrigan et al., 1993). The recombination frequency (RF) was observed to be low (<0.2 crossover per linkage group), but all tested haploid post-meiotic nuclei had at least one crossover within the mapped genome. Compared to haploid progeny, the RF of three post-meiotic nuclei recovered from heterokaryons tended to be higher. In addition, the single mating type locus (MAT) was mapped on chromosome 1 with the same set of haploids (Xu, 1993). Kerrigan (1993) found that markers in terminal regions often showed a tendency toward greater interval lengths indicating higher recombination frequency in terminal regions. He believed that the presence of non-recombining groups of markers indicated that either crossing over is infrequent in certain large regions of the genome, or that such recombinants are generally unviable

since the germination rate of basidial spores was about 0.9% in his study. The reduction of crossing over may be an evolutionary result of "increasing reinforcing selection on retention of heterozygosity" (Kerrigan et al., 1993). The low recombination frequency in the proximal centromere regions, in combination with the proposed preferential pairing of non-sister nuclei into spores (favoured heteroallelic for mating type), explains the tendency for heterozygosity to be retained in the progeny of *A. bisporus* var. *bisporus*.

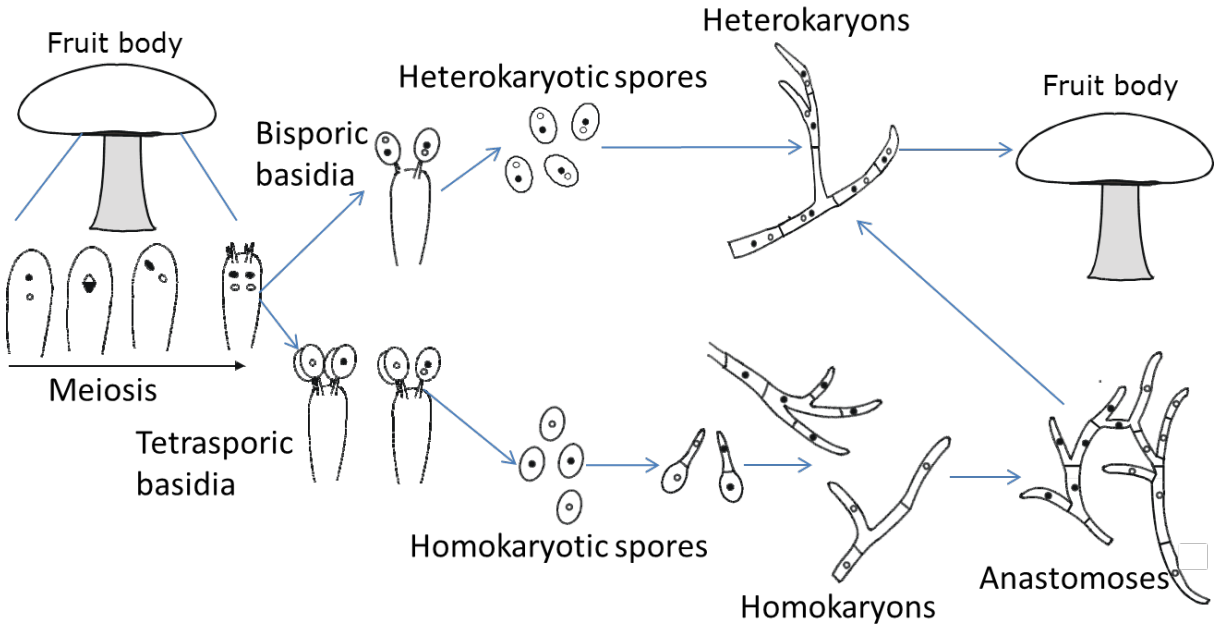


Figure 1. Life cycle and sexuality of *Agaricus bisporus var. bisporus* (adapted from Sonnenberg et al, 2011)

A new variety, i.e. *A. bisporus* var. *burnetti* was discoveredby Mr. Jerry Burnettein 1978, and later described as predominantly heterothallic (Callac, 1993, Kerrigan, 1994). A series of comparisons was made between var. *burnetti* and var. *bisporus* in terms of nuclear behaviour in basidia, basidial spore number, size of basidia, allele polymorphism, genome structure, etc. The main difference with the variety *bisporus* is that most basidia produce four spores. The sexual pattern of *A. bisporus* var. *burnettii* is also amphithallic but predominantly heterothallic and with a unifactorial mating system (Callac, 1993, Kerrigan, 1994). In contrast to var. *bisporic*, the tetrasporic variety produces approximately 90% homokaryons and 10% heterokaryons. Genetic studies were conducted in the intervarietal hybrids since the two varieties are interfertile and incompatibility of the two varieties is very uncommon, indicating a large genetic distance between the two varieties. The predominant inheritance of the tetrasporic trait was observed by Kerrigan et al. (1994) in 91 first generation intervarietal hybrids. BSN was mapped as the predominant locus for basidial spore number and linked with the mating type locus on chromosome 1 (Imbernon et al., 1996). The recombination frequency between BSN and MAT is 18.4% (19 recombinants among 103 offspring). BSN & MAT were predicted to be associated with fertility (fruiting ability) observed in the mating and fruiting tests. In a fruiting test 74% of the bisporic second-generation hybrids fruited, while only 28% of tetrasporic second generation hybrids fruited. According to the morphological, genetic and interfertility analyses, the sexuality of tetrasporic var. burnetti was also regarded as amphithallic but with heterothallism dominating. Meiotic recombination was studied in the intervarietal hybrids through segregation analysis (Callac et al., 1997, Moquet et al., 1999, Foulongne-Oriol et al., 2010, Foulongne-Oriol et al., 2011a). Substantial greater recombination rates were observed compared to *bisporus* varieties suggesting that the meiotic recombination in the tertra-sporic variety is normal and dominant in the intervarietal hybrids. A total of 324 molecular markers including AFLP (amplified fragment length polymorphism), SSR (microsatellite) and CAPs (cleaved amplified polymorphism

sequence) were mapped on 13 linkage groups in the offspring of the intervarietal hybrid JB3-83 (a homokaryon of var. *burnettii* strain JB3) × U1-7 (a homokaryon of var. *bisporus* strain U1) (Foulongne-Oriol et al., 2010). The map covers a total length of 1156 cM with 16% co-segregating markers, i.e., no crossovers found between co-segregating markers on the same chromosome. The largest gaps between markers tended to be on distal portions of chromosomes as observed by Kerrigan (1993). An average of 11.1 crossovers per individual was observed over the whole mapped genome. The average number of crossovers per linkage group (0.86) of the intervarietal progeny was considerably higher than those in bisporic progeny (<0.2) observed by Kerrigan (1993). A comparative genetic linkage mapping was done in the progeny of a second-generation hybrid (a homokaryon of a intervarietal hybrid, carrying alleles of normal recombination frequency linked to BSN and MAT of the tetra-sporic variety, crossed with a bisporic homokaryon) (Foulongne-Oriol et al., 2011a). The recombination rate was on average 0.67-fold smaller than that of first generation intervarietal hybrid. This indicates that the recombination frequency is a complex trait influenced by more than one gene, not all linked to BSN and/or MAT.

A. bisporus is not only an economically important fungus but also a genetically interesting organism. It is a species representing two different life cycles with the most interesting phenomenon being the difference in meiotic recombination. Its short life cycle, small genome and availability of whole genome sequences makes this fungus an ideal model to study genes involved in meiotic recombination, next to genes responsible for BSN. Understanding the genetics behind these different life styles might generate tools to manipulate meiosis towards reducing linkage drag (high recombination frequency) or preserve favourable genome regions (low recombination frequency) and even substitute whole chromosomes. Although a number of genetic studies of *A. bisporus* have been published in the last decades, the most relevant questions are not yet fully answered. These questions are 1) do post-meiotic nuclei migrate to basidiospores randomly on two-spored basidia, 2) is the low germination rate of spores due to lethal alleles and, if so, how much does this affect the accuracy of genetic linkage mapping, e.g., segregation distortion, 3) where does recombination take place in low recombining bisporic varieties. Additional data are needed to have a better understanding of meiosis in the *bisporic* variety. This chapter describes the generation of some of these data. The frequency and location of meiotic recombination in *A. bisporus* var. *bisporus* was studied through segregation analysis in three homokaryotic populations using Single Nucleotide Polymorphism (SNP) markers. With these SNPs, the genetic diversity of some heterokaryotic strains was investigated including commercial lines, pre-hybrids, and wild lines. The constituent nuclei of heterokaryotic offspring of Horst U1 and present-day commercial varieties were genotyped by selected SNPs distributed over the genome. This was done to demonstrate that the commercial lines are likely derived from Horst U1 as a fertile single spore culture. A commercial present-day variety (Sylvan A15) was also resequenced to demonstrate that if such a variety was obtained via a fertile single spore culture of Horst U1 and to see if resequencing can be used for a precise location of meiotic recombination.

Materials and Methods

Genome wide segregation analyses

All strains and spores used in this study were obtained from the fungal collection of Wageningen UR Plant Breeding. Three heterokaryons were used in this study, i.e., Horst U1 (a commercial line), WB2 (a brown wild line), and WW7 (an white wild line), all of the var. *bisporus* type. The constituent homokaryons of U1 (H97 and H39) were available from the original breeding program of this line. Mes09143 and Z8 were recovered through protoplasting from WB2 and WW7, respectively. The pedigree of homokaryotic offspring for segregation analyses is shown in Figure 2.

Cultivation trials were carried out in the growing facility of Unifarm (Wageningen UR) as described previously (Weijn et al., 2012). Basidial spores of Horst U1 and the other two crosses were collected at room temperature and used to generate three segregating populations. The first population consisted of

144 homokaryotic offspring of Horst U1; the second population consisted of 191 homokaryotic offspring of the hybrid H97×Mes09143; the third population consisted of 186 homokaryotic offspring of the hybrid H39×Z8.

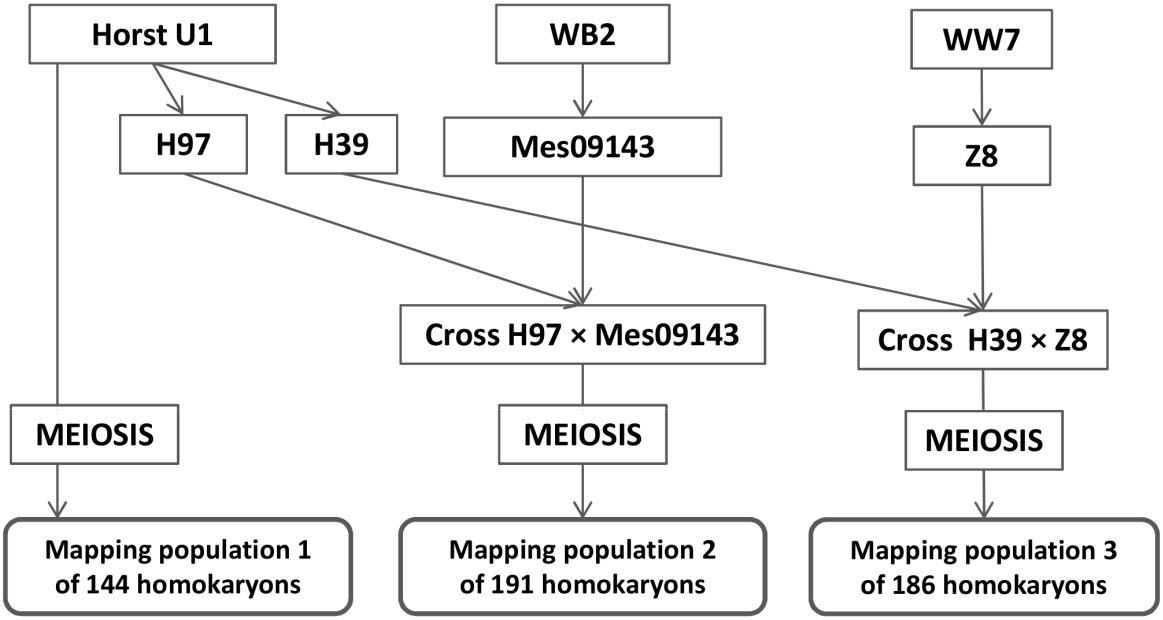


Figure 2. The pedigree presentation of segregating populations.

With the availability of whole genome sequence of H97 (Morin *et al.*, 2012), the other three homokaryons (H39, Mes09143, and Z8) were resequenced using the Illumina technology (Service Xs, Leiden). SNPs were detected by aligning sequences of the three lines with the reference genome H97, version 3 (Foulongne-Oriol et al., 2013). A total of 654 SNP markers was selected from the sequence alignment of H97 and H39 and used for the genotyping of population 1. Segregation analysis was first conducted for population 1. Due to the low recombination frequency of this population, most SNP markers co-segregated and clustered per linkage group. Since similar low recombination frequencies were expected for the other populations, less than 10 markers per chromosome were selected for population 2 and 3. SNP markers were selected in such a way that they were evenly distributed on each chromosome. DNA of three populations was extracted from freeze-dried mycelium with the DNA extraction kit (Promega, 96-wells plate Magnabot). The SNP analysis was performed by a biotech company (Dr. Van Haeringen Laboratorium B.V., Wageningen) with KARSP assay, and the SNP scores were checked using the program SNP viewer (LGC KBiosciences) to avoid scoring mistakes. Data of failed and doubtful markers were discarded.

The genetic linkage mapping was performed using Joinmap 4.1 (Van Ooijen, 2011) with a haploid model (HAP) by default parameter setting (independence LOD score; significance levels from 2.0 LOD to 10.0 LOD). In all three populations, the groups at the level of LOD ≥ 5 were used to calculate maps. During mapping in Joinmap 4.1, skewed segregation of markers was tested with chi-square ($P<0.05$), and the crossover frequency was calculated as the crossover number per individual per chromosome.

Genotyping heterokaryotic strains

A selection of 534 SNP markers, used to genotype population 1, was used to study genetic relationship of *A. bisporus* heterokaryoticstrains in the Wageningen UR Plant Breeding collection. A total of 35

heterokaryotic strains (11 present-day commercial strains, 5 traditional or pre-hybrid strains and 17 wild strains) were genotyped. Previous studies showed that most of the present-day commercial lines were genetically identical or very similar to the first commercial hybrid Horst U1 (unpublished data) indicating that most of the present-day commercial lines are copies or derivatives from Horst U1 as fertile single spore cultures. Most traditional commercial lines (used before 1980) originate from multi-spore cultures or fertile single spore cultures.

To demonstrate that present-day commercial lines are derived from Horst U1 as a fertile single spore culture, the constituent nuclei of 8 commercial lines and 19 heterokaryotic SSIs of U1 were recovered through protoplasting. Since recombination frequency is very low, only 3 to 6 markers per chromosome were used to genotype these recovered homokaryons.

Re-sequencing Horst U1 and Sylvan A15

With the availability of the whole genome sequence of H97 as a reference (Morin et al., 2012), the heterokaryotic genomes of Horst U1 and Sylvan A15 were re-sequenced with Illumina technology and aligned with the reference genome (H97) for SNP detection. SNP numbers were counted in intervals of 1Kb along each chromosome. The genotype differences between U1 and A15 were demonstrated as the differences in number of SNPs detected in U1 and A15 aligned with the reference genome of H97.

Isolation of single spore cultures and identification of homokaryons

Spores of 16 different spore prints were suspended in sterilized tap water and plated in serial dilutions on malt-mycological pepton (MMP) agar medium (Sonnenberg et al., 1988). These spore prints were labelled with three capital letters as shown in the Results section. Petri dishes filled with commercial spawn (Sylvan A15) were used to stimulate the spore germination, and placed together with spore plates in a covered foam box in an incubation room (25°C). Single spore isolation (SSI) was started with the first spore germination for each spore prints. Homokaryotic and heterokaryotic SSIs and the mating type of homokaryons were identified with PCR primers flanking the P1N150 locus. Previous research has shown that this locus is linked to the mating type and the H97 allele contains a DNA transposon (Sonnenberg et al., 1999). This locus is thus polymorphic for mating type of H39 and H97.

Results

Segregation analysis

In order to study meiotic recombination in detail in the *bisporic* variety, we used a large population of offspring and a high number of markers in a population of homokaryons derived from the first commercial hybrid Horst U1. To verify the meiotic behaviour in *bisporic* varieties, we studied meiotic recombination in two other populations (Figure 2) where two genetically non-related *bisporic* homokaryons were involved and where we used only a limited number of SNP markers.

Population 1 (homokaryotic offspring of U1)

A comparison of the whole genome sequence of haploid H39 with the haploid H97 reference genome revealed an average of one SNP per 110 bp. A total of 654 SNP markers and 142 individuals were used for the segregation analysis of population 1. Markers were selected in such a way that they were evenly distributed over the genome, i.e. varying from 86 (the longest CHR1) to 32 (the shortest CHR13) (Table 1). The total genetic map length is 512 cM and consists of 13 linkage groups corresponding to the chromosome number. Linkage group 10 is the longest one (70 cM) and linkage group 9 is the shortest (18 cM; Figure 3). The analysis of segregation ratios showed that 205 markers (31%) deviate from the

expected 1:1 ratio (chi-square test, *P*<0.05) including all the markers from CHR3, CHR4, CHR5, half of the markers from CHR7, and one or two markers from some other chromosomes. There were more H39 than H97 alleles on CHR3 (generally 1.5:1, P<0.05) and CHR4 (generally 1.9:1, P<0.01), and more H97 than H39 alleles on CHR5 (generally 1.5:1, P<0.05). Co-segregating markers counted for 55% of all markers. The largest interval is 37.48 cM, which is on the distal position of CHR11.

The low recombination frequency is amply demonstrated by the large percentage of parental type (non-recombinant) chromosomes found in the offspring (Table 1). For instance, a total of 118 progeny (83%) have inherited the unchanged parental type of CHR1 (52 individuals inherited CHR1 of H39 and 66 individuals inherited CHR1 of H97). In total, 74% of all chromosomes do not show any recombination for the 654 markers used. The recombinations for the whole population are divided into 412 single crossovers, 61 double crossovers and 9 triple crossovers. The crossover frequency per individual per chromosome varied from 0.13 to 0.53. The average crossover frequency is 0.3 per chromosome per individual, i.e., 3.9 crossovers per individual over the genome. The number of crossover events per individual varied from zero to 14 with most individuals having 2 to 5 crossover events, and there were 11 individuals having no crossovers at all (Figure 4). It is remarkable that, on average, 50% of the crossovers were scored by markers at the end of chromosomes, resulting in the largest intervals at the ends of the linkage groups (Figure 3, highlighted in red; Supplementary file 1)

Table 1. Overview of the segregation analysis of population 1 (Horst U1)

			marker range (Kb)							crossover				
LG	length (cM)	CHR size (Kb)	top	bottom	No. of markers	distorted markers	0H39	0H97	% parental type	1CO	2CO	3CO	frequency	Ratio of end CO
I	29.19	3350.7	5.6	3343.2	86	0	52	66	83%	24	0	0	0.17	0.21
II	35.02	3489.8	60.1	3468.3	74	1	63	48	78%	27	4	0	0.25	0.83
III	21.47	3131.9	23.1	3090.3	54	53	71	46	82%	23	2	0	0.19	0.74
IV	18.39	3112.8	123.1	3056.3	64	64	77	46	87%	14	4	1	0.18	0.40
V	26.72	2550.7	58.8	2511.1	55	54	43	74	82%	20	4	1	0.22	0.32
VI	40.66	2329.8	21.5	2291.9	46	0	43	59	72%	32	7	1	0.35	0.65
VII	44.71	2323.2	49.8	2299.9	60	27	45	53	69%	37	6	1	0.37	0.35
VIII	32.03	1953.2	12.8	1865.3	41	1	54	53	75%	35	0	0	0.25	0.20
IX	17.23	1688.4	59.5	1602.3	33	0	56	69	88%	13	3	0	0.13	0.63
X	70.24	1762.8	17.3	1742.8	37	2	43	37	56%	50	11	1	0.53	0.63
XI	62.41	1708.5	18.3	1689.2	40	0	44	44	62%	49	4	1	0.42	0.32
XII	62.22	1692.0	241.8	1681.5	32	2	41	48	63%	38	12	3	0.50	0.49
XIII	51.51	1334.1	14.5	1323.2	32	1	58	30	62%	50	4	0	0.41	0.74
Total	511.79	30427.8			654	205			74%	412	61	9		
Average	39.37												0.30	0.50

The first row shows the headings of the contents in the columns of this table. LG represents linkage groups corresponding to chromosome numbers; length (cM) is the map length of each linkage group; CHR size (Kb) represents the physical size of each chromosome; top and bottom shows the physical positions of the most top marker and the most bottom marker on each chromosome; the column "number of markers" shows the number of SNP markers mapped on each linkage group, and the column "distorted markers" shows the number of markers significantly deviating from 1:1 segregation in population 1; "0H39" or "0H97" representing number of chromosomes do not have crossover at all (zero crossover) but inherited the entire parental chromosome from either H39 or H97; the column "% parental type" shows the percentage of progeny having that parental chromosome out the whole population; "1CO", "2CO", and "3CO" represents the number of individuals having a single crossover, double crossover and triple crossover; "frequency" represents the crossover frequency (number of crossovers per chromosome per individual); "ratio of end CO" represents the percentage of crossovers happened at the end of each chromosome.

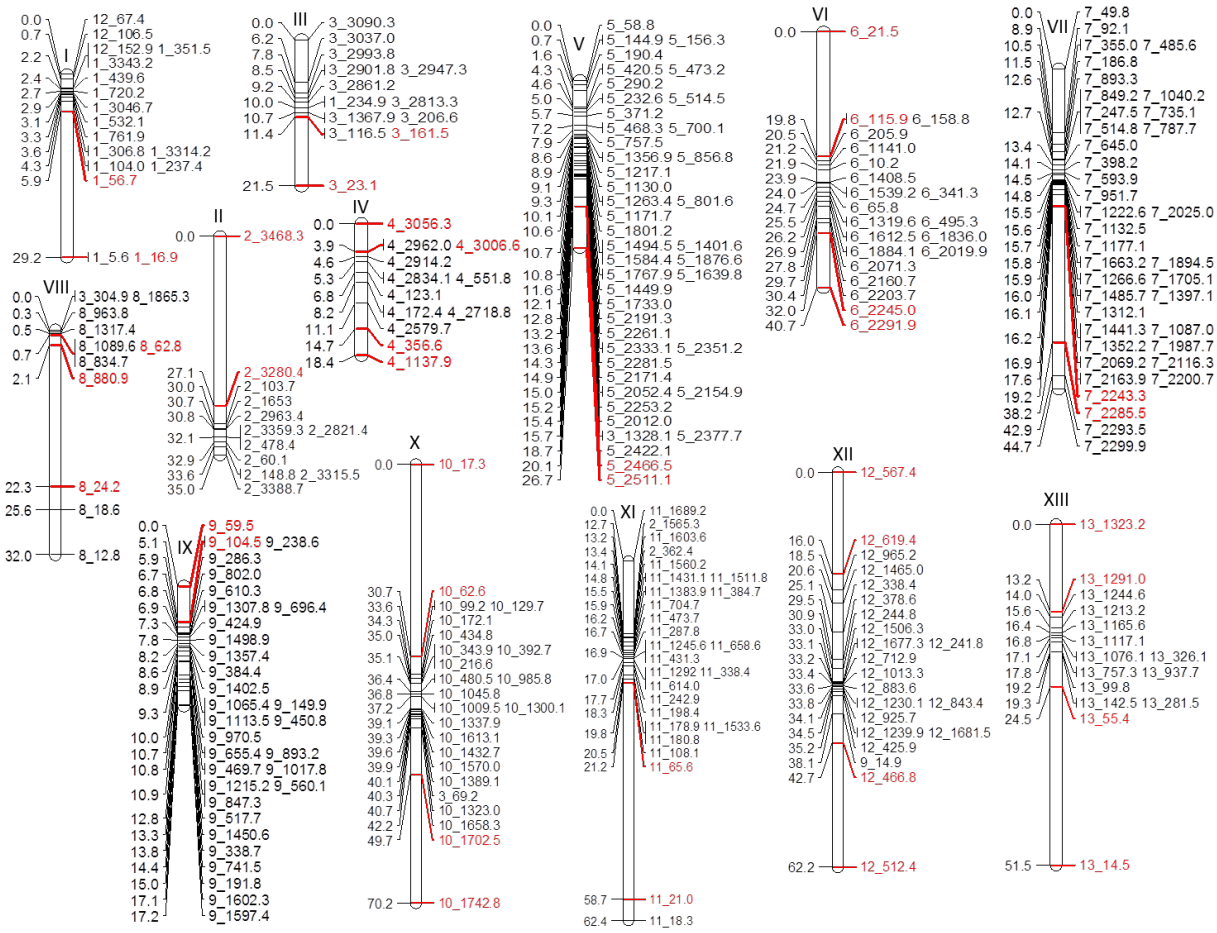


Figure 3. Genetic Linkage map of population 1.

The markers were labelled as scaffold number and their physical positions. The markers highlighted in red indicate the largest intervals of respective linkage groups. Co-segregating markers are not presented on the map chart except for the border markers (makers on the same map positions). All of the marker information can be seen from Supplementary file 1.

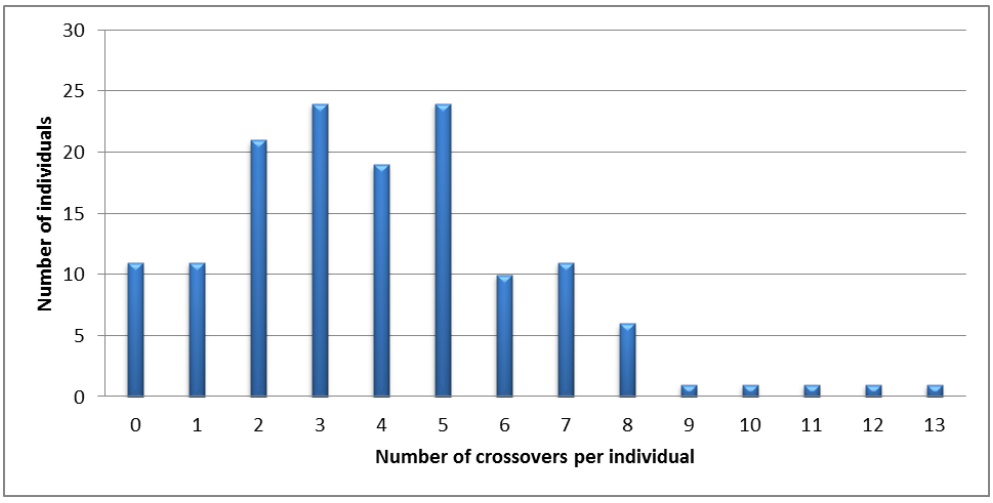


Figure 4. Distribution of crossover number per individual

Population 2 (homokaryotic offspring of Mes09143 × H97)

A comparison of the whole genome sequence of haploid Mes09143 with the haploid H97 reference genome detected an average SNP call of one per 50 nucleotides. Since most markers co-segregated due to the low crossover frequency in population 1, a limited number of markers were selected for the segregation analysis of population 2 & 3. Ninety-five SNP markers were successfully used for the genotyping of population 2 (191 individuals) and mapped on 13 linkage groups, and all markers developed for the same chromosome mapped on one linkage group as expected. The map showed colinearity with the map of population 1. The total map length is as short as 164 cM. Linkage group 8 is the longest one with a length of 77 cM with the largest map interval of 40 cM indicating higher recombination frequency. Twenty-eight markers (29%) showed a skewed segregation. All markers of CHR 1, 6, 7 and 10 deviated from the 1:1 ratio ($P<0.05$), and the segregation ratio of markers of two genotypes was around 2:3 (Mes09143: H97). Besides, 2 markers of CHR 9, and 2 markers of CHR 13 showed skewed segregation ratios. The linkage group was numbered after the chromosome number. The map positions and physical positions of markers for population 2 are listed in Supplementary file 2.

The number and frequency of crossover events for each linkage group are shown in Table 2. Over the whole population, a total of 255 crossovers were detected including 210 single crossovers, 12 double crossovers and 1 triple crossover. As in population 1, the low meiotic recombination frequency is demonstrated by the number of individuals with no recombination at all (41 individuals) or only 1 recombination over the whole genome (85 individuals). In the complete population, more than 90% of the chromosomes are of the parental type. For example, of 191 individuals examined, 108 individuals had all alleles for chromosome 1 of H97, and 70 individuals had all alleles for chromosome 1 of Mes09143. The total number of crossovers per chromosome varied with different chromosomes, e.g. only 1 crossover on CHR11 and a total of 102 crossovers on CHR8. Every chromosome showed co-segregating markers except for chromosome 8 (Table 3). The crossover frequency varied from 0.01 to 0.55, and the average frequency was 0.10 per chromosome per individual.

Table 2. Overview of the segregation analysis in population 2.

Linkage Group	Map length	No. of markers	No. of co-segregating markers	Largest interval	No of crossovers					crossover frequency
					0 Mes09143	0 H97	1	2	3	
1	7.09	6	2	4.88	70	108	13	0	0	0.07
2	5.88	7	1	2.15	90	91	9	1	0	0.06
3	4.36	8	5	3.27	91	92	8	0	0	0.04
4	1.05	8	2	0.53	106	82	2	0	0	0.01
5	8.69	8	6	8.14	93	83	15	0	0	0.08
6	30.10	7	1	17.35	58	88	41	4	0	0.26
7	13.04	8	2	10.28	64	105	22	0	0	0.12
8	77.16	7	0	40.33	46	59	69	16	1	0.54
9	2.76	7	2	1.16	103	82	5	0	0	0.03
10	0.62	5	3	0.61	73	117	1	0	0	0.01
11	3.82	8	3	2.20	96	87	7	0	0	0.04
12	4.82	8	2	1.61	87	95	9	0	0	0.05
13	5.02	8	5	3.97	79	103	9	0	0	0.05
Total	164.41	95	34				210	21	1	
Average	12.65	7.23								0.10

No. of crossovers was presented as number of individuals with single crossover (1), double crossovers (2), and triple crossovers (3)

Population 3 (offspring of H39×Z8)

A comparison of the whole genome sequence of haploid H39 with the haploid Z8 genome detected an average SNP call of one per 77 nucleotides. One hundred and eighty homokaryotic offspring of population 3 were successfully genotyped with 76 SNP markers. A total of 37 markers (49%) showed skewed segregations in population 3, which were all the markers on chromosome 3, 5, 6, 11 and 13. Five more markers showed skewed segregations, two on chromosome 2, one on chromosome 8, one on 10, and one on 12. Originally eight SNP markers per chromosome were developed for population 3, but some SNP markers failed for genotyping. For example, only 2 markers were successful for chromosome 6. There were 13 markers with more than 10% missing values over the 180 genotypes and 22 genotypes with more than 10% missing values over the 76 markers. Seventy-six markers were mapped in 13 linkage groups as expected and showed colinearity with the map for population 1. The total map length was 86 cM and the map length varied from 0 (chromosome 6) to 27.10 cM (chromosome 10). The map positions and physical positions of markers for population 3 are listed in Supplementary file 3.

The number and frequency of crossover events for each linkage group of population 3 are shown in Table 3. Similar to population 1, most of the progeny (93) did not have recombination within the mapped genome region; a total of 55 progeny were detected as having one crossover; 27 progeny were detected as having two crossovers; 5 progeny were found having three crossover. As for the other populations, most chromosomes (>90%) in the whole population are of parental type. For instance, 103 individuals inherited all markers for chromosome 2 of H39, and 77 individuals inherited all markers for chromosome 2 of Z8. There were a total of 40 (53%) co-segregating markers. No recombination was observed on chromosome 6, probably due to the low coverage of only 2 markers available for that chromosome. Chromosome 10 had the highest crossover frequency (0.17). Not all the selected SNPs were successful, thus not enough markers covered the whole genome. This might be a reason for the lower crossover frequency and the shorter map length for population 3 compared to population 1 & 2.

The segregation analysis of these three homokaryotic populations proved that the recombination frequency of var. *bisporus* is indeed low, and an average of 0.3 crossover per chromosome (4 crossover per individual) was observed with sufficient markers applied for genotyping. The map position of markers in three populations revealed that large map intervals exist between markers at the distal chromosome regions indicating higher RF at the ends of chromosomes.

Table 3 Overview of segregation analysis of population 3

Linkage Group	Map length (cM)	Number of markers	No. of co-segregating markers	Largest interval	No of crossovers				crossover frequency
					0 H39	0 Z8	1	2	
1	2.44	7	4	1.86	84	92	4	0	0.02
2	0.00	7	6	0.00	103	77			0.00
3	2.92	7	4	2.15	70	105	5		0.03
4	9.97	7	4	8.79	77	88	13	1	0.08
5	14.70	5	0	10.40	57	102	20	1	0.12
6	0.00	2	1	0.00	100	71			0.00
7	2.32	7	5	2.32	79	97	4		0.02
8	5.57	6	3	4.78	84	88	8		0.04
9	9.30	5	4	4.79	70	91	12	1	0.08
10	23.32	6	3	22.74	86	63	30		0.17
11	2.40	6	0	1.23	103	73	4		0.02
12	2.59	4	1	1.84	88	88	4		0.02
13	10.02	7	5	10.02	107	59	14		0.08
Total	85.55	76	40						
Average	6.58	5.85							0.05

Genotyping wild lines and commercial lines

With 534 SNP markers used for population 1 (H97/H39 SNPs), a total of 35 strains were genotyped: 17 wild strains, 7 pre-hybrids or traditional strains and 11 present-day commercial strains. The genotyping profile (Figure 5) showed clearly that, the 11 commercial lines are nearly identical and contain almost all alleles present in Horst U1. The traditional commercial lines separated into two groups, i.e. Somycel 9.2, Darlington 745 and Somycel X135 on the one hand and Sinden A61 and Darmycel 21/2 on the other hand. This agrees with the common knowledge of the two types of varieties used in mushroom production before 1980, i.e. the off-white and white varieties. Previous research showed that strains within the off-white and white groups are genetically related (unpublished data). The two constituent homokaryons of Horst U1 are derived from Somycel 9.2 (H97, off-white type) and Somycel 53 (H39, white type). Although Somycel 53 was not included in this study, it was known from previous research that Somycel 53 was genetically closely related to Sinden A61 (unpublished data). The genotyping profile (Figure 5) shows indeed that, next to the heterozygous regions, the off-white traditional lines (Somycel 9.2, Darlington 745 and Somycel X135) have mainly H97 alleles whereas the white traditional varieties (Sinden A61 and Darmycel 21/2) have mainly H39 alleles. The genetic variation within the wild lines is high and only bisp210, bisp221 and, bisp005 are similar showing small differences on several chromosomes, and bisp51 and bisp60 are similar (Figure 5).

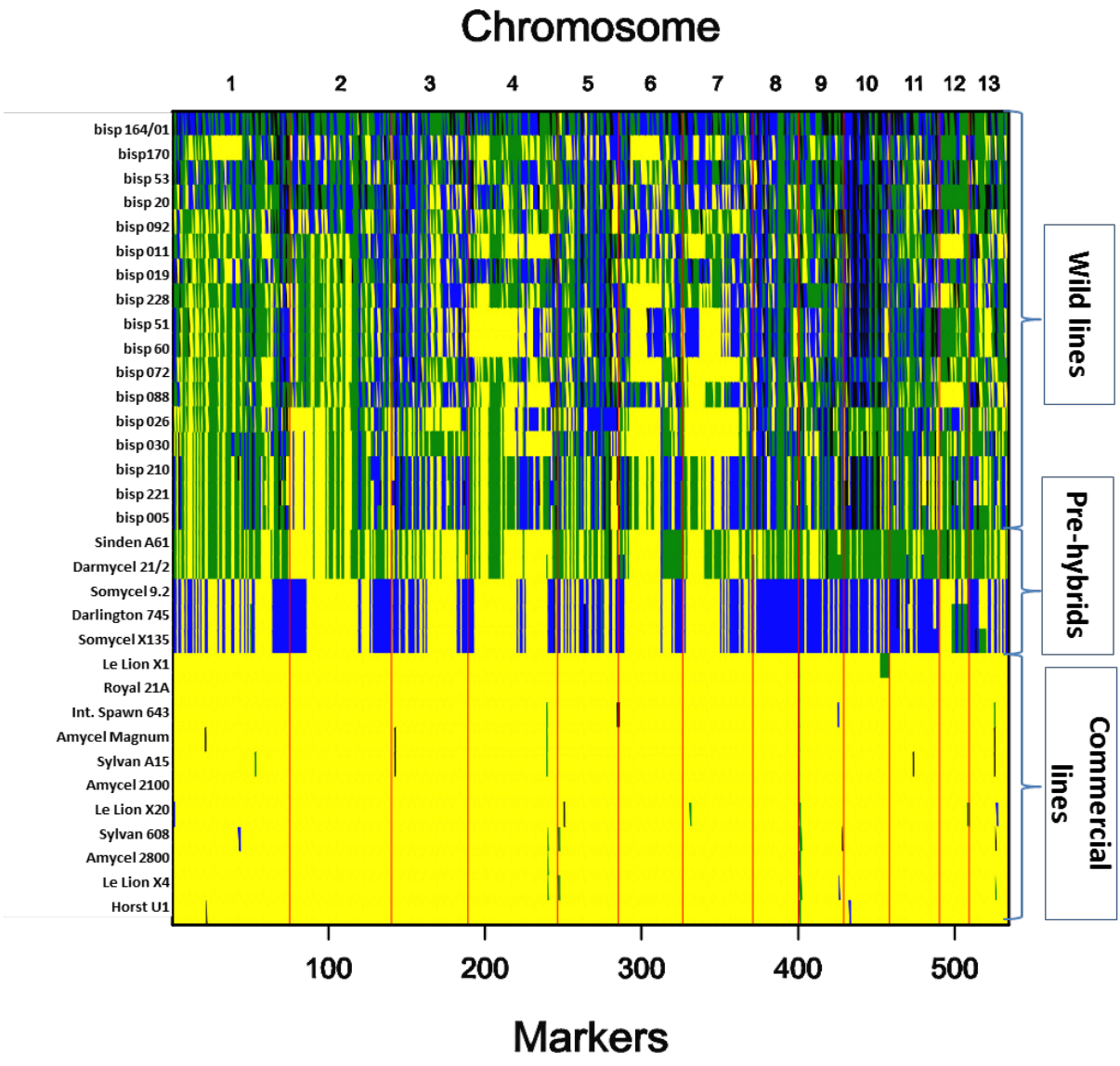


Figure 5. SNP genotypes of wild strains, pre-hybrids and commercial lines

The names of 35 strains are listed vertically at the left side of the picture, and 534 SNP markers of 13 chromosomes are plotted horizontally and sorted with chromosome positions. Yellow indicates heterozygous genotype, i.e. both alleles of Horst U1 are present (H39 and H97); blue indicates the detection of only the H97 allele and green the detection of only the H39 allele; black indicates missing values or no SNP call.

Genotyping constituent nuclei of heterokaryons

To assess meiotic recombination in fertile heterokaryons, the constituent nuclei of 20 heterokaryotic offspring (heterokaryons) of Horst U1 were recovered through protoplasting and genotyped with 56 SNP markers (Table 4). These heterokaryons were named as "BKRhet" followed with a number. For this diad genotyping markers were chosen near the ends and the middle of each chromosome. Both of the two constituent nuclei of 18 heterokaryons were recovered as homokaryons, while only one of the nuclear types was recovered from the remaining 2 heterokaryons (BKRhet23 and BKRhet06). As a control, also the 20 original heterokaryons of Horst U1 were genotyped using the same SNP markers. That allowed the derivation of the missing markers and genotypes of homokaryons not recovered from BKRhet23 and BKRhet06. Crossover recombination was found on chromosome (CHR) 1, 3, 4, 5, 6, 7, 8, 13 (Figure 7). Ten out of 20 heterokaryons were observed to be crossover recombinants. CHR5 and CHR6 had more crossovers (4 for each) than the other chromosomes. Both reciprocal crossover and non-reciprocal crossovers were found across constituent nuclei, which in combination with chromosome reshuffling over the genome indicates a random but otherwise balanced segregation of homologues in meiosis I (Figure 6). Although the number of individuals and markers are limited, the data did not indicate a substantial difference in meiotic recombination frequency in heterokaryons compared to homokaryons.

Table 4. Markers used for genotyping heterokaryons of Horst U1

CHR	marker	No. of marker	position (Kb)	CHR	marker	No. of marker	position (Kb)
1	ChrI_T1	1	104.08	7	ChrVII_B1	4	2069.17
1	ChrI_T2	2	306.82	7	CHR7B1	5	2163.99
1	ChrI_M1	3	1687.71	8	ChrVIII_T1	1	62.81
1	ChrI_B1	4	3314.30	8	CHR8A1	2	286.42
2	CHR2B1	1	478.46	8	ChrVIII_B1	3	1865.36
2	ChrII_B1	2	610.24	9	ChrIX_T1	1	238.64
2	ChrII_M1	3	1834.39	9	ChrIX_B3	2	1357.51
2	CHR2A1	4	3012.59	9	CHR9B1	3	1602.37
2	ChrII_T3	5	3315.53	10	CHR10A1	1	99.27
3	ChrIII_T3	1	1078.57	10	ChrX_T3	2	552.27
3	ChrIII_M1	2	1922.83	10	ChrX_B1	3	1337.94
3	ChrIII_B1	3	2993.89	10	CHR10B1	4	1570.07
3	CHR3B1	4	3037.10	11	CHR11B1	1	180.89
4	CHR4B1	1	123.13	11	ChrXI_T2	2	431.37
4	ChrIV_B1	2	172.49	11	ChrXI_T3	3	614.00
4	ChrIV_M1	3	1185.99	11	ChrXI_B2	4	1292.04
4	ChrIV_T3	4	2314.32	11	ChrXI_M1	5	1603.64
4	ChrIV_T2	5	2673.81	12	CHR12A1	1	244.81
5	ChrV_T1	1	58.90	12	ChrXII_B1	2	292.51
5	ChrV_T2	2	144.91	12	ChrXII_M1	3	619.35
5	ChrV_M1	3	1310.07	12	CHR12B1	4	925.79
5	ChrV_B2	4	2171.49	12	ChrXII_T2	5	1239.97
6	ChrVI_T1	1	65.82	13	CHR13A1	1	55.45
6	ChrVI_B3	2	673.02	13	ChrXIII_T1	2	185.87
6	ChrVI_B1	3	2245.08	13	CHR13m1	3	576.18
7	ChrVII_T2	1	465.02	13	ChrXIII_M1	4	662.55

7	ChrVII_T3	2	542.45	13	ChrXIII_B2	5	1076.17
7	ChrVII_M1	3	1352.23	13	CHR13B1	6	1117.19

No. of markers were used in Figure 6 (at the top beneath the chromosome number).

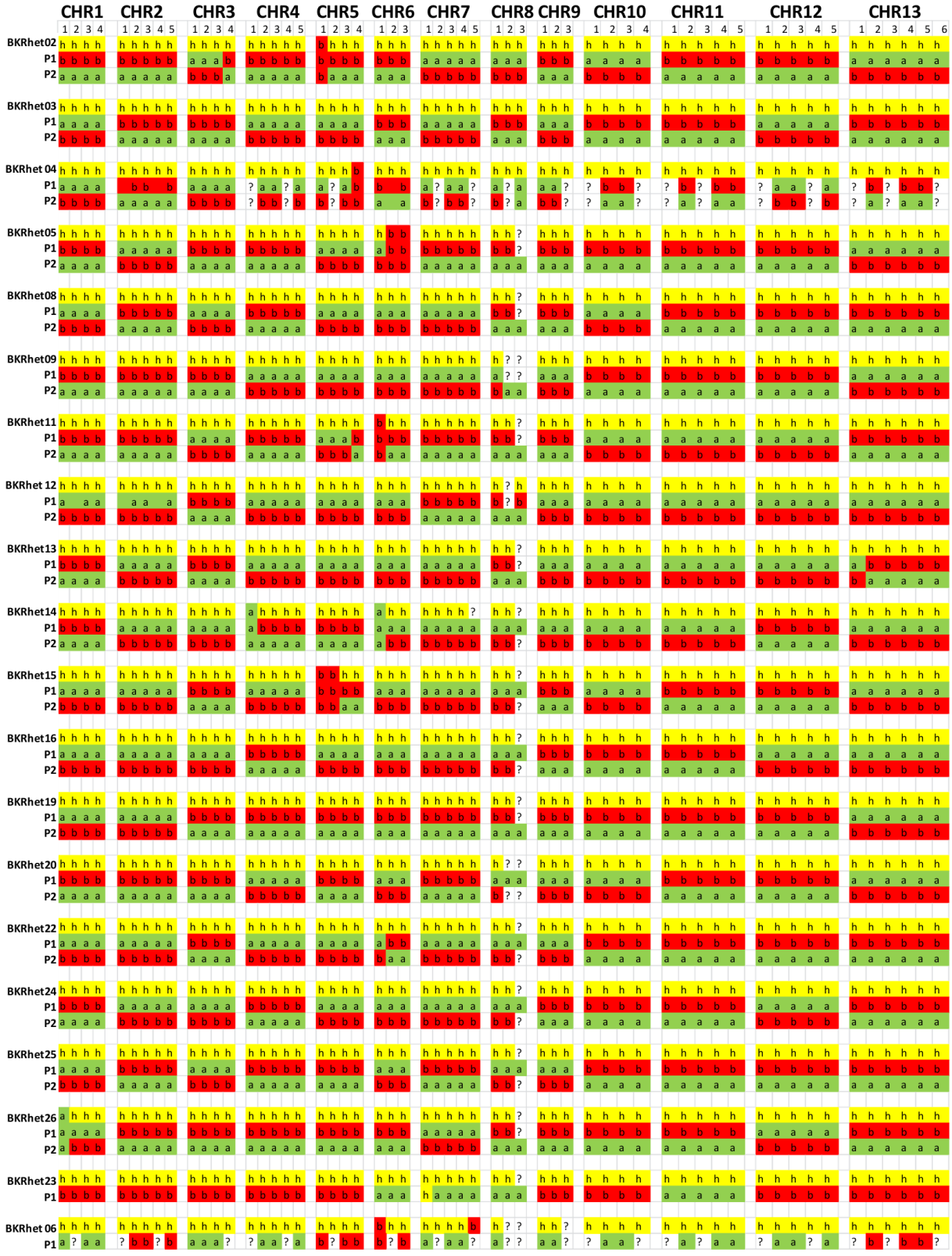


Figure 6. Genotyping heterokaryotic offspring of Horst U1 and their recovered homokaryons.

Yellow "h" indicates heterozygous (alleles of H39 and H97); green "a" indicates the H39 alleles and red "b" is the H97 allele. "BKRhet" followed with numbers indicate heterokaryotic offspring of U1; "P1" is the first recovered constituent nucleus; and "P2" is the second recovered constituent nucleus.

The constituent nuclei of 9 present-day commercial strains were also recovered as homokaryons through protoplasting. The genotypes of each pair of nuclei are similar to those found in the constituent nuclei of Horst U1, i.e. most alleles present in Horst U1 are found in these lines and no new alleles are present, reciprocal and non-reciprocal recombination are found and otherwise perfect complementary sets of parental Horst U1 chromosomes (Figure 7). This indicates that the constituent nuclei of present-day commercial varieties are likely to have resulted from pairing of non-sister post-meiotic nuclei of Horst U1 and are thus generated from fertile single spore cultures of Horst U1.

Figure 7. Genotyping commercial lines and their constituent nuclei recovered as homokaryons.
Yellow "h" indicates heterozygous (H39 and H97 alleles); green "a" indicates the H39 allele and red "b" the H97 allele. "P1" is the first recovered constituent nucleus; and "P2" is the second recovered constituent nucleus.

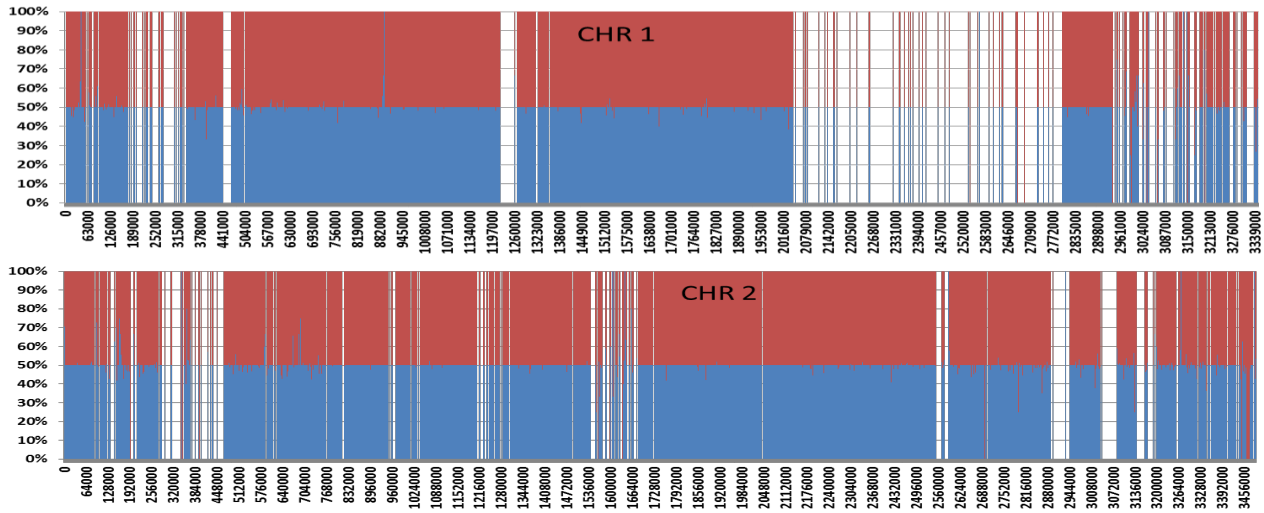
Re-sequencing of Sylvan A15 (SNP comparison)

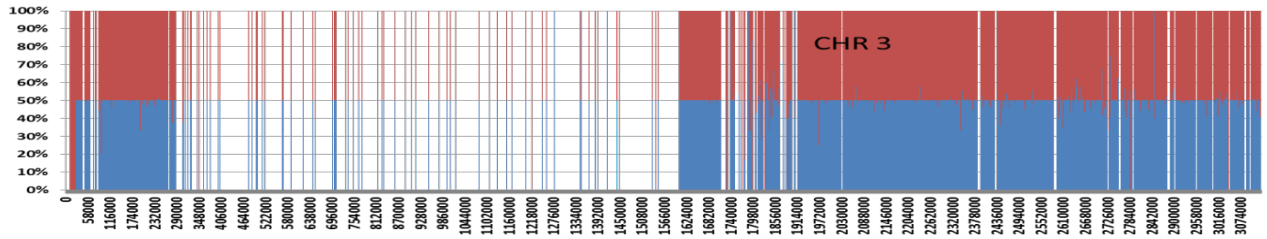
The resequencing of H39, one of the constituent nuclei in Horst U1, revealed a high genetic difference with H97, the other constituent nucleus of Horst U1. More than 300,000 SNPs were detected, an average of one SNP per 110 nucleotides. The SNPs were, however, not evenly distributed over the whole genome. In some areas SNP density dropped to less than 1 in 10,000 nucleotides. The reason for these homologues regions is unknown but might be due to the fact that both lines share common ancestry. In most areas, however, the SNP density is high enough for a precise location of recombination events when resequencing offspring nuclei. To test if recombination can be located in this way in heterokaryons, both Horst U1 and the present-day commercial line Sylvan A15 were resequenced since it is suspected that Sylvan A15 originated from a recombined fertile single spore culture of Horst U1. SNP frequencies were plotted in intervals of 1 kb along all chromosomes. All sequence reads were compared with the reference genome H97 and the presence or absence of a SNP was reported. Since Horst U1 is heterozygous for its

whole genome it reproduced the SNP frequency distribution between H97 and H39. As previously mentioned, Sylvan A15 is expected to be derived as a heterokaryotic single spore culture of Horst U1. In such a culture four possible genotypes can be expected as illustrated in Figure 8. If no recombination has occurred between homologous chromosomes or the recombination is reciprocal, the region will be heterozygous as in Horst U1 and no difference in SNP frequency with Horst U1 will be detected. If recombination is not reciprocal and leads to homozygosity for H39, the SNP frequency will also be identical to a heterozygous region since this region reproduces the SNP frequency between H97 and H39. If a recombination is non-reciprocal and leads to homozygosity for H97, the SNP will drop to zero since that region will be identical to the reference genome (H97). The latter type of recombination can thus be detected when resequencing whole genomes of heterokaryotic offspring. A comparison in SNP frequency between Horst U1 and Sylvan A15 revealed indeed regions homozygous for H97 (Figure 9), e.g. chromosomes 2, 3, 5, 6, 7, 8, 10 and 11. All these regions were located at the very ends of chromosomes, i.e. the last 50 kb regions, which were comparable with the chromosome positions of the largest map intervals observed in the U1 population. These homologous chromosome regions were probably generated through reciprocal recombination, i.e., crossovers. A zooming in on these locations also revealed that some of the regions homozygous for H97 resulted from a double crossover not further apart than 20-40 kb (Supplementary file 4).

Figure 8. Presentation of recombination types in the offspring of U1

Additionally, some small homozygous regions for H97 are located in the middle part of chromosomes in Sylvan A15 (100% red bars in Figure 9). Examples are regions on chromosome 3 around 348 Kb, 1218 Kb, 1566 Kb, 1740-1914 Kb. This indicates that recombination can occur within small chromosome regions, i.e., 1 kb. On the other hand, also regions were detected with SNP in the Sylvan A15 genome where no SNPs were present in the Horst U1 genome (100% blue bars in Figure 9). Examples are around 45 Kb of CHR1, 1600 Kb of CHR2, 1276 Kb of CHR3, 2494 Kb of CHR4. That indicates new alleles in narrow regions (< 1 kb), which might be due to sequence errors but also to mutations.

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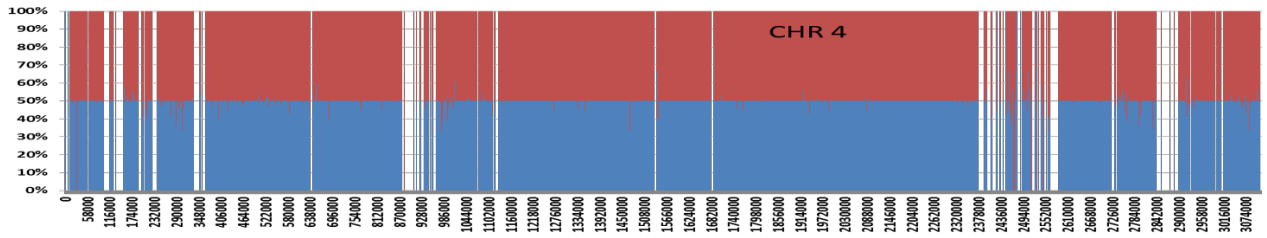
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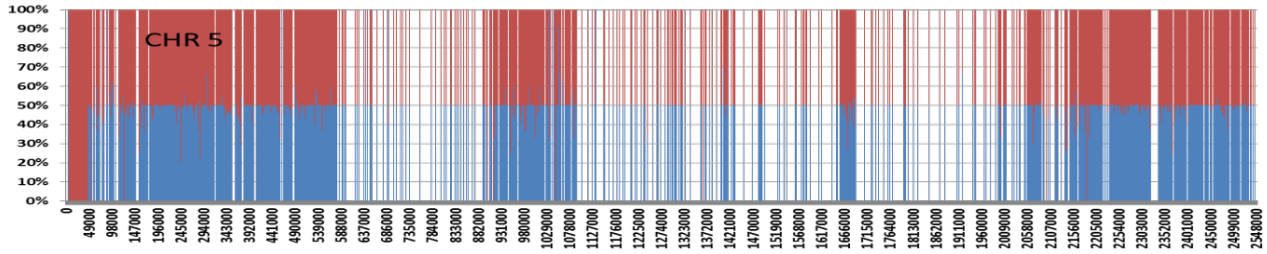
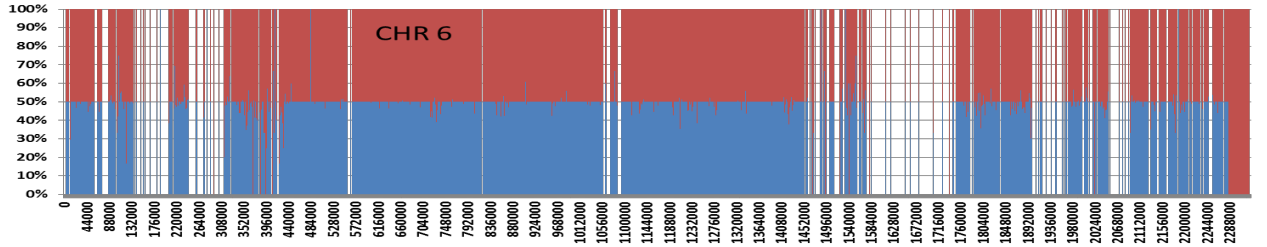

CHR 5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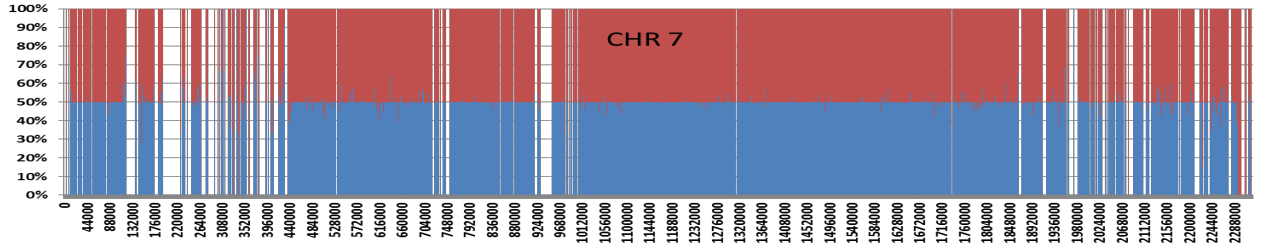
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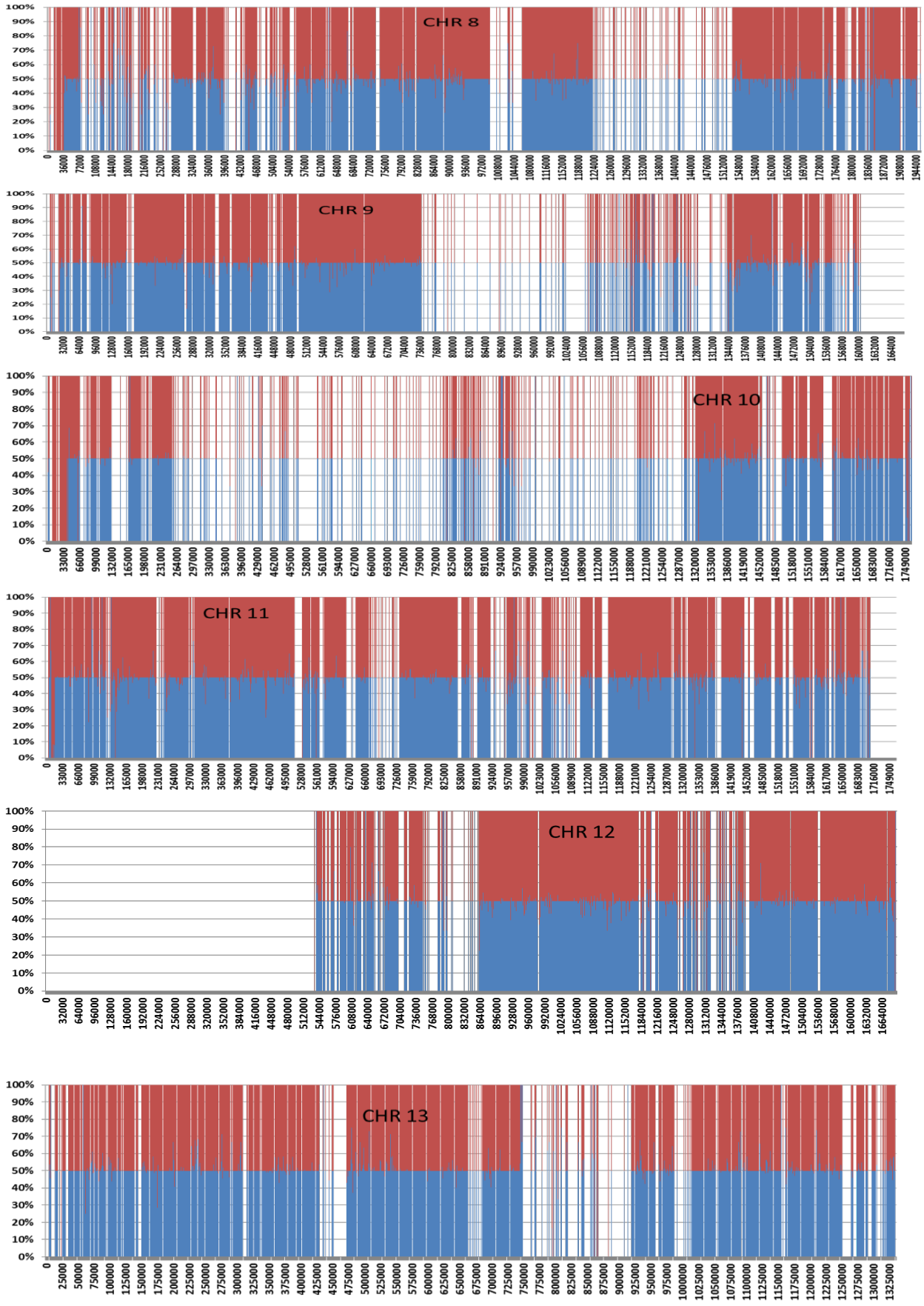

Figure 9. Stacked bars for comparison of SNP numbers between U1 and A15 aligned with H97.

SNP numbers were counted within 1Kb intervals along each chromosome. Red bars represent SNP numbers of Horst U1 aligned with H97, and blue bars represent SNP numbers of Sylvan A15 aligned with H97. Equal length of blue bar (0-50%) and red bar (51-100%) means that equal numbers of SNP were detected in Sylvan A15 and Horst U1 when aligning sequence reads with H97. A 100% red bar indicates no SNPs were detected within that genome region of Sylvan A15 due to homozygosity for H97. A 100% blue bar indicates SNPs were detected in Sylvan A15 but not in Horst U1. Unequal length of red bars and blue bars indicate either more SNPs detected in Horst U1 or more SNPs detected in Sylvan A15. The figure also shows clearly regions where the SNP frequency is very low (white regions).

Spore germination and ploidy levels of single spore cultures

Previous research has shown that spore germination and percentage of homokaryons among random selected spores can vary considerable from spore print to spore print even within the same variety. Since this can cause bias in the offspring analyzed we collected spore prints from different mushrooms of Horst U1 at different developmental stages and different environmental temperatures during spore production. For the convenience of description, the 16 spore prints were named with three capital letters (Table 5), e.g. BNA, BNB, etc. Most spores started germinating on the 5th or 6th day after spore spreading except for spores collected from prints BMZ and BMU. Spores of BMZ (flat cap, 19 °C) started germination on the 36th day after spreading, and BMU (flat, 22 °C) started germination on the 20th day after spreading. The time span in which the first 100 SSIs were picked varied also between spore prints. For instance, the 100 SSIs of BNB or BNG were picked within 2 days, and those of BNC or BNZ were picked within 3 days, while the first 100 SSIs of BMT were picked within 33 days. This shows that spores of some spore prints germinate almost synchronously while other germinate more sequential over longer periods. The first 96 SSI were scored for ploidy with marker P1N150 linked to the mating type. Although some of them failed in PCR, most spores could be successfully assessed for their ploidy level and mating type. From all spore prints less homokaryons than heterokaryons were isolated except for BNF (52% homokaryons). The percentage of homokaryons varied from 11% (BMZ) to 52% (BNF, broken, 25 °C). There was a tendency for more homokaryons of mating type H39 at 16 °C and more H97 at 19 °C. Developmental stage or temperature during spore production was not observed to be correlated with the ratio of homokaryons, the first day of germination or time needed to pick up 100 SSI. Homokaryons did not germinate later than heterokaryons. Because of the overgrowth of the earlier SSIs, the germination rate is an underestimation and can go up to at least 52%.

Table 5. Germination of spores collected at different developmental stages of the mushroom and different temperatures

				Days after spore spreading								
No.	Spore print	Stage	°C	start picking (1)	end picking (100)	spores over grown	mating type scoring	Hom-H97	Hom-H39	Heter-	Hom-rate	Germination rate
1	BNA	closed	16	5	33	35	91	22	14	55	0.40	0.40
2	BNB	broken	16	6	7	15	95	16	6	73	0.23	0.48
3	BNC	open	16	5	7	9	92	10	8	74	0.20	0.47
4	BND	Flat	16	6	33	33	91	16	6	69	0.24	0.39
5	BMW	closed	19	6	38	38	92	5	22	65	0.29	0.41
6	BMX	broken	19	6	38	38	92	5	11	76	0.17	0.37
7	BMY	open	19	5	36	36	62	11	13	38	0.39	0.42
8	BMZ	Flat	19	36	38	38	95	7	3	85	0.11	0.44
9	BMV	closed	22	5	33	39	92	8	17	67	0.27	0.40
10	BMS	broken	22	5	33	39	95	9	21	65	0.32	0.39
11	BMT	open	22	5	38	38	93	9	8	76	0.18	0.37
12	BMU	Flat	22	20	38	38	82	13	9	60	0.27	0.38
13	BNE	closed	25	6	38	38	64	7	18	39	0.39	0.38
14	BNF	broken	25	5	14	18	60	5	26	29	0.52	0.45
15	BNG	open	25	5	6	12	92	7	9	76	0.17	0.50

16	BNH	Flat	25	5	36	36	78	7	24	47	0.40	0.40

All the spores were spread on plates and incubated on the same day. The isolation period of the first 100 spores was recorded as the start picking day (days after spore spreading) and the end-picking day (days after spore spreading). Afterwards spores were counted until the earlier ones overgrown each other.

Discussion

This is the first study using SNP markers after the release of the whole genome sequence (WGS) of the button mushroom. The WGS allows the selection of markers evenly distributed over the whole genome and thus an accurate estimation of meiotic recombination. The marker order in the linkage maps of population 1, 2 and 3 generally agrees with the physical map. The differences seen are mainly in regions with strong linkage that are usually prone to errors and can be explained by the fact that loci mapping close to each other are interchangeable, whereas loci further apart are 100% fixed at their estimated positions (Joinmap 4.0 manual). Additionally, the missing values of genotyping can result in the order of markers in the genetic linkage map deviating from the corresponding physical positions. The occasional difference found between marker order in the linkage map and physical map might also be due in part to incorrect assembly of the physical map (Foulongne-Oriol et al., 2013). All three maps are otherwise colinear. Distorted segregation of markers was observed in all three populations, i.e., 31%, 29%, and 49% of the markers showing skewed segregation in population 1, 2 and 3, respectively. These numbers are higher than those found by Foulongne-Oriol (2010) in the intervarietal map (9%) but comparable to those found by Kerrigan (1993) in the map of var. *bisporus* (33%). Although some of the homokaryons used to generate these populations overlap with those used for our populations, no consistency was found for particular chromosomes involved in distorted segregation.

Population 1 and 2, and population 1 and 3 share one parent, whereas population 2 and 3 do not share parental lines. The 4 different parental lines used for these populations are genetically not related and we can thus consider the data generated as representative for the variety *bisporus*. Especially data from population 1 are useful since as many as 645 SNP markers were used to genotype 142 homokaryotic individuals in the offspring. Compared to the linkage map generated from an intervarietal hybrid (Foulongne-Oriol et al., 2010), the recombination frequency between homologous chromosomes of var. *bisporus* is low. This is especially illustrated by the high number of parental (non-recombined) chromosomes found in this population, i.e. 74%. The total map length of population 1 was 512 cM and the mean crossover frequency was 0.3 per individual per chromosome. A previous intra-variety (offspring of *var. bisporic × var. burnettii*) map showed linkage group lengths ranging from 33 cM to 136 cM with a total map length 1156 cM, and the average crossover frequency is 0.86 per individual per chromosome (Foulongne-Oriol et al., 2010). The linkage map of population 1 showed that recombination mainly occurs at the chromosome ends. This underlined that a larger part of all chromosomes show an extremely low crossover frequency in var*. bisporus.* Although we used less SNP markers in population 2 and 3, the low recombination frequencies in these populations were amply illustrated by the high number of parental type of chromosomes found of 91% and 94% for population 2 and 3, respectively. Since the markers used in population 2 and 3 were not located at the very ends of chromosomes, the number of parental types appeared higher than in population 1 (74%) where also markers were used near the ends of chromosomes.

The biological meaning of the low recombination frequency in the var. *bisporus* is unclear. Previous researchers have suggested that the preservation of heterozygosity prevents the generation of homozygosity for putative lethal alleles in offspring of var. *bisporus*(Kerrigan et al., 1993, Callac et al., 1997) and a low recombination frequency combined with pairing of non-sister nuclei is indeed an excellent mechanism to preserve heterozygosity and maintain deleterious alleles as was reported for *Microbotryum violaceum*(Hood & Antonovics, 2000). A low germination percentage of spores and skewed segregation of alleles in homokaryotic offspring might indicate the existence of such alleles. The

germination frequencies reported for var. *bisporus*, however, vary enormously from 0.9% (Kerrigan et al., 1993) up to 66% (Elliot, 1972). We used 16 different spore prints of Horst U1 and found a germination rate varying from 37% up to 52% with indications that the real percentages can be higher. This variation might indicate the presence of deleterious alleles. However, in the 142 individuals of population 1 we did not find a completely skewed segregation of parental type of chromosomes and certainly none of the parental types were absent. Despite the apparently normal recombination frequency observed in the population of a intervarietal hybrid where bisporic homokaryon H97 (one of the constituent nuclei of Horst U1) was used to generate the hybrid, a substantial number of chromosomes in the individuals (48%) were parental types and showed no recombination for the markers used (Foulongne-Oriol et al., 2010). There was no evidence for the absence of any of the parental type H97 chromosomes in homokaryotic offspring. Although in the intervarietal offspring also some skewed segregation in recombined chromosomes was seen, no genomic regions derived from H97 were reported to be absent. These data indicate that, if present, the occurrence of deleterious alleles is rare and does not offer a good explanation for the preservation of heterozygosity in heterokaryotic offspring of the variety *bisporus*.

In order to have an impression of recombination over the entire chromosome we resequenced the whole genome of the heterokaryotic present-day commercial variety Sylvan A15 and Horst U1. We wanted to examine the whole genome sequence (WGS) of this variety anyway to show that this most used present-day variety is likely generated by selecting a heterokaryotic single spore culture from Horst U1. SNP lists generated after comparing the WGS with the reference strain H97 can be used to visualize regions homologous for H97 since that regions will be devoid of SNPs. If SNP density is high enough, even very small regions of recombination can be assessed since each SNP allele will be confirmed by its neighbouring SNP. SNP detection by other methods is less reliable since SNP density is much lower and an individual SNP allele change will not always be confirmed by a nearby SNP. The WGS of Sylvan A15 clearly showed regions homologous for H97 at one end of chromosomes 2, 3, 4, 6, 8, 10 & 11. Except for chromosome 4, these are also the chromosomes for which telomere sequences are available (Foulongne-Oriol et al., 2013) and we could, therefore, locate these recombinations in the last 50 kb of these chromosomes. The absence of homologous regions for H97 in other chromosome ends are likely due to the fact that either these regions are homologous for H39 or no sequences for these ends are available. Some homologous regions resulted from double crossovers not more than 25-40 kb apart. These crossovers would not have been detected with our regular SNP detection method (KASPar SNP genotyping). Occasionally we also see very small H97 homologous regions far from chromosome ends. These might, however, also be due to sequencing errors or mutations in Sylvan A15. We have compared the resequenced Sylvan A15 only with the reference genome H97. The resequenced H39 genome is not of sufficient quality to compare the Sylvan A15 sequence with H39 in order to detect homologous regions for H39. A higher recombination frequency near ends of chromosomes is not uncommon, and reported previously for yeast (Barton et al., 2008), nematode (Villeneuve, 1994), mouse (Jensen-Seaman et al., 2004) and human (Barlow & Hulten, 1998). Specifically, crossover rates were on average greater in euchromatic regions near chromosome ends exclusive of the subtelomeres (Barton et al., 2008, Suet al., 2000). However, as far as we know *A. bisporus* var. *bisporus* is the first organism reported with almost exclusively recombination at chromosome ends.

While our data sheds some light on meiotic recombination in var. *bisporus* it does not provide us with sufficient data to conclude how nuclei are paired in 2-spored basidia, i.e. by a preferential non-sister nuclei pairing or a random pairing. Several papers have been published on this subject and can be divided by those that advocate a preferential pairing of non-sister nuclei and those that state that a random pairing of nuclei is the most likely explanation. Summerbell et al., (1989) genotyped 367 single spore cultures derived from different heterokaryons with 4 RFLP markers. They also observed a very low recombination percentage and 95.6% of the single spore cultures examined had preserved their heterozygosity (Summerbell et al., 1989). Previous studies done by Spear et al., (1983) using isozymes as markers were also interpreted as a preferential pairing of non-sister nuclei in bisporic basidia (Spear et

al., 1983). Elliot (1972) isolated spores from bisporic basidia via micromanipulation and tested 20 diads (spore pairs coming from the same bisporic basidium) for fruiting. Eleven diads produced mushrooms and must thus contain non-sister nuclei (N-progeny) and 5 did not fruit and might thus contain sister nuclei (S-progeny). Of each of the 4 remaining diads, one was infertile thus probably carrying only one post meiotic nucleus (i.e. a homokaryon), and the other fertile and might thus carry three post meiotic nuclei. This is a segregation ratio of fertile: infertile of 2 : 1 and suggests a random distribution of 4 post-meiotic nuclei over 2 spores as proposed by (Langton F A, 1980) for secondarily homothallic basidiomycetes (Figure 10a). The hypothesis of random nuclear migration was tested further in a secondarily homothallic ink-cap fungus *Coprinus bilanatus* through segregation analyses of mating type, auxotrophy and antimetabolite resistance (Challen, 1989). In this case spores were sampled randomly and are thus derived from different meiotic events. The segregation ratios of spore progenies tested confirmed the random migration hypothesis and it was concluded that this could be regarded as the primary control of secondary homothallism. The studies of Elliott (1972), Langdon (1980) and Challen et al., (1989) did not use genetic markers or only a very limited number of markers and could thus not discriminate between S-progeny and true homokaryons. S-progeny (pairing of sister nuclei in one spore) is identical to a second division restitution and results in genetically identical nuclei per spore in the absence of recombination or very similar nuclei when recombination frequency is very low and occurs only at the end of chromosomes, as in var. *bisporus*. Since *A. bisporus* cells contain a variable number of nuclei in homo- and heterokaryons, S-progeny can also not be discriminated from true homokaryons by counting nuclei. For breeding purposes, usually a single marker linked to the mating type locus (MAT) is used to discriminate homokaryons from heterokaryons. If no crossover occurs between the MAT locus and the centromere, the S-progeny (A1A1 or A2A2) cannot be discriminated from true homokaryons (Figure 10a). If there is a crossover between the MAT locus and the centromere, the S-progeny is scored as a heterokaryon based on the presence of both mating type and discarded when screening for homokaryons (Figure 10b). Our study indicates that recombination in var. *bisporus* occurs preferentially at the end of chromosomes. We, therefore, looked again carefully at the genotypes of the offspring in population 1, since for that population a high number of markers were used and for some chromosomes also markers near the end. Initially we discarded these markers since markers at the end of chromosomes tend to be unreliable. A total of 53 homokaryons were identified that were heterozygous for 1 to 3 (or more) SNP markers at the ends of chromosomes (Figure 11). This high frequency of heterozygous markers in these regions might indicate that a substantial proportion of what we considered as true homokaryons might in fact be S-progenies. The best way to test this is to resequence these strains and confirm heterozygosity at the ends of chromosomes. A subsequent recovering of both nuclear types by protoplasting can be subjected again to resequencing and confirm in this way whether these lines were true S-progenies. Visual inspection of spores in *bisporus* varieties show that approximately 10% of the basidia bear 4 spores. One would thus expect ca. 10% of the single spores to be homokaryons having one mating type. We have estimated germination percentages of different spore prints and genotyped these with a marker linked to the mating type. Although germination rate was not 100% (varying between 37 and 52%) the percentage of homozygosity of the mating type was higher than expected, i.e. varying from 11% up to 50%. This might also indicate that a substantial number of these single spore cultures homozygous for the mating type are in fact S-progenies. A definitive answer to how spores are distributed over spores on each type of basidium (2, 3 or 4-spored) can only be given if sufficient spores are isolated from different basidia via micromanipulations and sufficient complete sets of spores from individual basidia are germinated allowing genotyping. A WGS of these cultures will discriminate true homokaryons and S-progeny and will give a final conclusion on how nuclei are distributed over two spores.

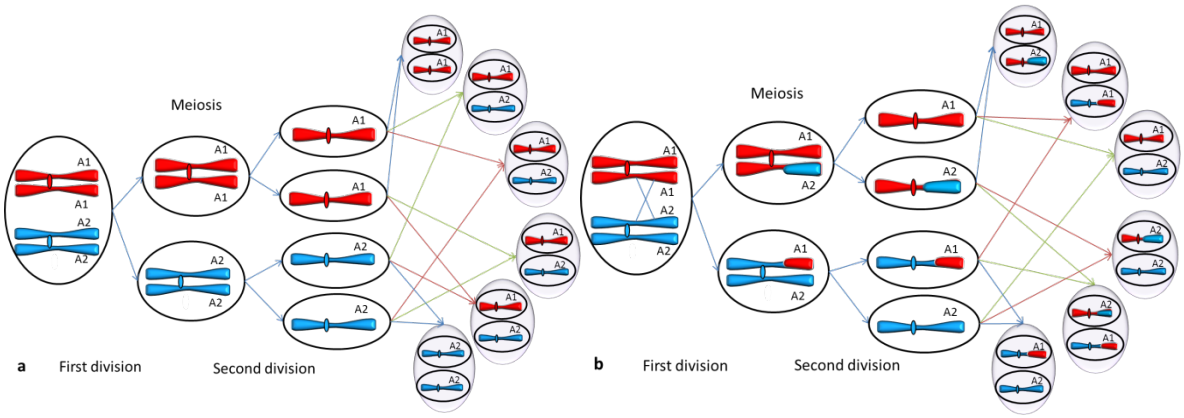

Figure 10a. A schematic presentation of possible outcomes in case of random pairing of nuclei on 2-sporic basidia of var. *bisporus* without a crossover between centromere and MAT locus.
For the case of simplicity only one chromosome is presented harbouring the mating type locus. In this case, S-progeny are indistinguishable from homokaryons produced by tetrasporic or trisporic basidia if only a marker for the mating type is used to identify homokaryons.
Figure 10b. A model of meiotic sporulation through random nuclei migration with a crossover between centromere and MAT locus.
In this case, S-progeny are indistinguishable from heterokaryons if only one MAT-marker is used for distinguishing heterokaryons from homokaryons.

In this study we have genotyped the constituent nuclei of heterokaryotic offspring of Horst U1 and a number of present-day commercial varieties. The two nuclei in these diads showed a perfect complementary set of chromosomes and originate from pairing of non-sister nuclei. Recombination frequency was low in both sets and both types of recombination were seen, i.e. reciprocal and non-reciprocal, the latter leading to homozygous regions. This indicates that present-day commercial lines are likely derived from the first hybrid (Horst U1) introduced on the market in 1980. Horst U1 has been generated from a cross between a homokaryotic single spore culture (H39, obtained from the traditional commercial line Somycel 53) and a homokaryotic single spore culture (H97, from Somycel 9.2) (Fritsche, 1981). An independent breeding program, using the same traditional lines, would never produce a hybrid with the same genetic make-up as Horst U1. It is extremely unlikely that an independent isolation of a single spore culture from both traditional lines will generate homokaryons identical to H39 or H97 because of an independent segregation of chromosomes in meiosis I. It is possible, however, that some of the commercial lines are derived from another variety that was obtained as a fertile single spore culture of Horst U1. Horst U1 was the first button mushroom variety that obtained protection by European community plant variety rights in 1980. Within three years after introduction on the market, a number of new varieties appear that were phenotypically identical or very similar to Horst U1. Later research and this study show that these varieties are also genetically very similar to Horst U1. At that time the International Union for the protection of new varieties of plants (UPOV) had not been involved in essentially derived varieties (EDV). In 1991 UPOV published the definition of EDV and according to that definition fertile single spore cultures of button mushrooms should be considered as EDVs since 1) they are mainly derived from the original variety; 2) they are discernible from the original variety and 3) they are identical (except for the above differences) to the original variety for those characteristics that stem from the genotype or the combination of genotypes from the original variety (1991 Act of the UPOV convention). The generation of true new hybrids using genetically different lines or wild varieties takes 5 to 10 years and is thus a large investment. Once on the market, it is relatively easy to generate an EDV from such a variety within a year as has been seen in the early eighties where shortly after the introduction of Horst U1 very similar varieties appear on the market with different names. These “new” varieties can easily outcompete an original variety and minimize return of investment done for the first variety. This is now also considered as an important reason for the absence of real new varieties of button

mushrooms worldwide. This paper will contribute to the understanding of the typical life cycle of the bisporic variety and how EDVs can be generated and might thus contribute to a consensus within the mushroom breeding industry to come to a better protection of new varieties and thus attract new investors.

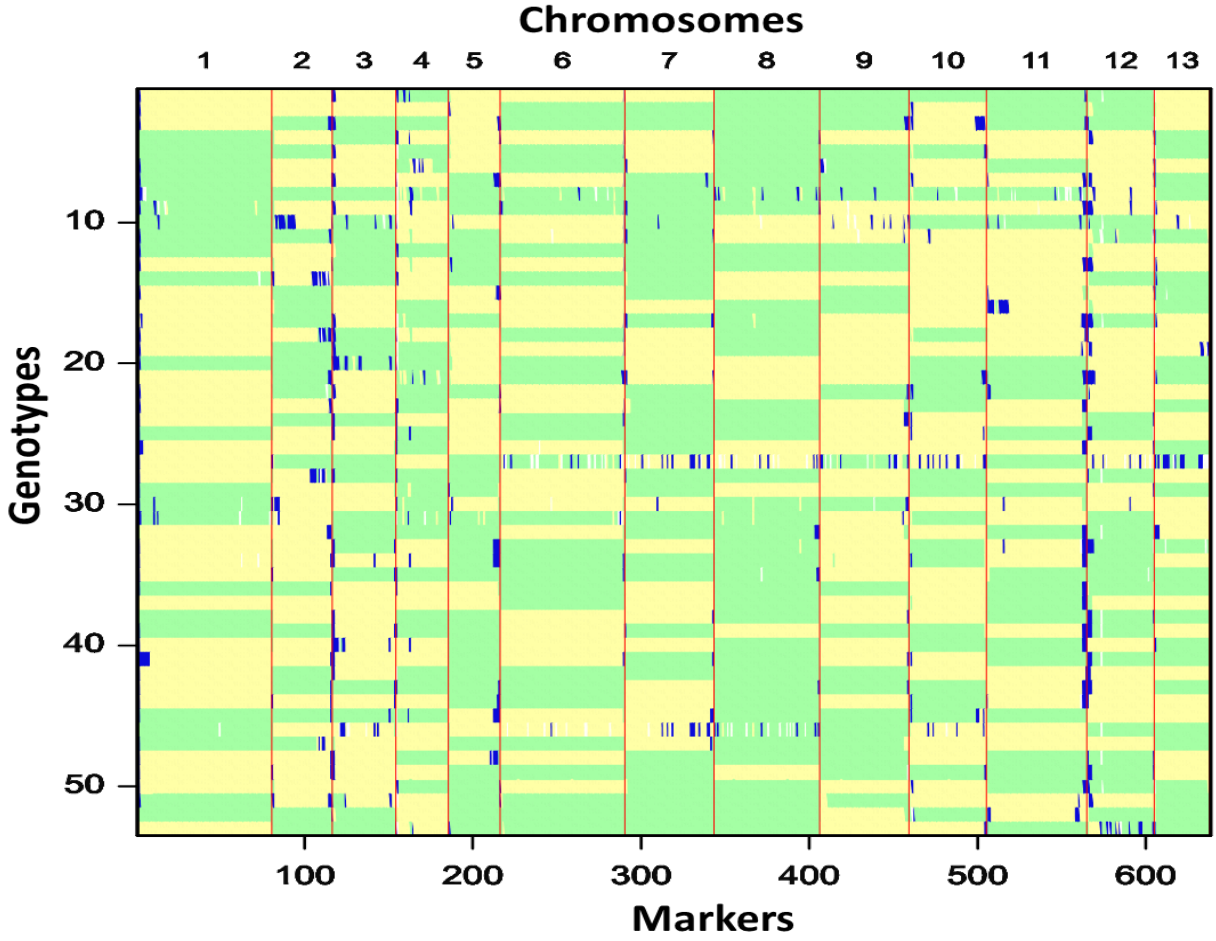

Figure 11. A genotype plot for possible S-progeny.
Yellow indicates homozygous for H97; green indicates homozygous for H39, blue indicates heterozygous as Horst U1, most frequently in distal regions of chromosomes; black indicates missing values.

The availability of two fully compatible varieties within the species *A. bisporus* with a different type of life cycle offers the opportunity to study genes involved in the different meiotic behavior, i.e. recombination over the entire chromosome and recombination restricted to chromosome ends. Also genes involved in the generation of predominant bi-sporic basidia or tetrasporic basidia can be studied in this way. Both traits are dominant in the intervarietal hybrids and allow the generation of segregating populations for these traits. It is many breeders' dream to be able to choose between reducing linkage drag when introducing new traits from wild sources or retain favorable allele combinations when using elite lines. Button mushroom offers a unique model system to study those genes and see if homologs with similar function are present in plants. At present, a reverse breeding approach offers a way to eliminate recombination by knocking out the DMC1 gene (Dirks et al., 2009). The disadvantage of a complete elimination of recombination is a disturbance of a balanced segregation of chromosomes in meiosis I leading to aneuploidy. *A. bisporus* var. *bisporus* seems to restrict recombination to the last 50 kb of each chromosome ends and meets in this way the obligate need for at least one crossover and thus balanced chromosome segregation and at the same time leaves most of the chromosomes unchanged. It also offers the possibility to substitute whole chromosomes and do "breeding by chromosome".

Supplementary file 1

SNP list used for segregation analysis of population 1

Nr	Linkage group	Locus Name	Scaffold	Physical position	Map Position
1	1	12_67438	12	67438	0.00
2	1	12_106540	12	106540	0.73
3	1	12_152906	12	152906	2.21
4	1	1_351523	1	351523	2.21
5	1	1_579542	1	579542	2.21
6	1	1_804015	1	804015	2.21
7	1	1_896267	1	896267	2.21
8	1	1_917503	1	917503	2.21
9	1	1_931823	1	931823	2.21
10	1	1_976120	1	976120	2.21
11	1	1_1076528	1	1076528	2.21
12	1	1_1110702	1	1110702	2.21
13	1	1_1154470	1	1154470	2.21
14	1	1_2165150	1	2165150	2.21
15	1	1_2449578	1	2449578	2.21
16	1	1_2879945	1	2879945	2.21
17	1	1_2907561	1	2907561	2.21
18	1	1_3212728	1	3212728	2.21
19	1	1_3236907	1	3236907	2.21
20	1	1_3259313	1	3259313	2.21
21	1	1_3343165	1	3343165	2.21
22	1	1_439624	1	439624	2.44
23	1	1_720171	1	720171	2.69
24	1	1_3046668	1	3046668	2.92
25	1	1_532094	1	532094	3.15
26	1	1_761872	1	761872	3.34
27	1	1_306820	1	306820	3.62
28	1	1_397969	1	397969	3.62
29	1	1_485912	1	485912	3.62
30	1	1_626389	1	626389	3.62
31	1	1_676466	1	676466	3.62
32	1	1_836996	1	836996	3.62
33	1	1_848698	1	848698	3.62
34	1	8_1008376	8	1008376	3.62
35	1	1_1021607	1	1021607	3.62
36	1	1_1065818	1	1065818	3.62
37	1	1_1079563	1	1079563	3.62
38	1	1_1199726	1	1199726	3.62
39	1	1_1268264	1	1268264	3.62
40	1	1_1296280	1	1296280	3.62
41	1	1_1314004	1	1314004	3.62
42	1	1_1362592	1	1362592	3.62
43	1	1_1408024	1	1408024	3.62
44	1	1_1453197	1	1453197	3.62
45	1	1_1501674	1	1501674	3.62
46	1	1_1550478	1	1550478	3.62
47	1	1_1596463	1	1596463	3.62
48	1	1_1640654	1	1640654	3.62
49	1	1_1687705	1	1687705	3.62

50	1	1_1732544	1	1732544	3.62
51	1	1_1778558	1	1778558	3.62
52	1	1_1825071	1	1825071	3.62
53	1	1_1863350	1	1863350	3.62
54	1	1_1908165	1	1908165	3.62
55	1	1_1951620	1	1951620	3.62
56	1	1_1994998	1	1994998	3.62
57	1	1_2039652	1	2039652	3.62
58	1	1_2084789	1	2084789	3.62
59	1	1_2132849	1	2132849	3.62
60	1	1_2255285	1	2255285	3.62
61	1	1_2324610	1	2324610	3.62
62	1	1_2368564	1	2368564	3.62
63	1	1_2406180	1	2406180	3.62
64	1	1_2534608	1	2534608	3.62
65	1	1_2592176	1	2592176	3.62
66	1	1_2670199	1	2670199	3.62
67	1	1_2732699	1	2732699	3.62
68	1	1_2798242	1	2798242	3.62
69	1	1_2821405	1	2821405	3.62
70	1	1_2837686	1	2837686	3.62
71	1	1_2860276	1	2860276	3.62
72	1	1_2927300	1	2927300	3.62
73	1	1_3058788	1	3058788	3.62
74	1	2_3087688	2	3087688	3.62
75	1	2_3120131	2	3120131	3.62
76	1	1_3191119	1	3191119	3.62
77	1	1_3278981	1	3278981	3.62
78	1	1_3306199	1	3306199	3.62
79	1	1_3314244	1	3314244	3.62
80	1	1_104017	1	104017	4.33
81	1	1_149428	1	149428	4.33
82	1	1_194746	1	194746	4.33
83	1	1_237433	1	237433	4.33
84	1	1_56660	1	56660	5.89
85	1	1_5616	1	5616	29.17
86	1	1_16897	1	16897	29.19
87	2	2_3468345	2	3468345	0.00
88	2	2_3280441	2	3280441	27.11
89	2	2_103709	2	103709	30.01
90	2	2_1652991	2	1652991	30.72
91	2	2_2963442	2	2963442	30.79
92	2	2_3359277	2	3359277	32.05
93	2	2_478401	2	478401	32.14
94	2	2_523087	2	523087	32.14
95	2	2_711615	2	711615	32.14
96	2	2_759270	2	759270	32.14
97	2	2_844482	2	844482	32.14
98	2	2_1163256	2	1163256	32.14
99	2	2_1246122	2	1246122	32.14
100	2	2_1337817	2	1337817	32.14
101	2	2_1363272	2	1363272	32.14
102	2	2_1432042	2	1432042	32.14
103	2	2_1743338	2	1743338	32.14
104	2	2_1791100	2	1791100	32.14

105	2	2_2109827	2	2109827	32.14
106	2	2_2290473	2	2290473	32.14
107	2	2_2514313	2	2514313	32.14
108	2	2_2594667	2	2594667	32.14
109	2	2_2821386	2	2821386	32.14
110	2	2_60102	2	60102	32.85
111	2	2_148767	2	148767	33.56
112	2	2_184294	2	184294	33.56
113	2	2_280387	2	280387	33.56
114	2	2_398823	2	398823	33.56
115	2	2_433572	2	433572	33.56
116	2	2_569698	2	569698	33.56
117	2	2_610178	2	610178	33.56
118	2	2_624790	2	624790	33.56
119	2	2_625761	2	625761	33.56
120	2	2_670099	2	670099	33.56
121	2	2_804327	2	804327	33.56
122	2	2_893181	2	893181	33.56
123	2	2_933809	2	933809	33.56
124	2	2_978820	2	978820	33.56
125	2	2_1023914	2	1023914	33.56
126	2	2_1072770	2	1072770	33.56
127	2	2_1117651	2	1117651	33.56
128	2	2_1205643	2	1205643	33.56
129	2	2_1291861	2	1291861	33.56
130	2	2_1387162	2	1387162	33.56
131	2	2_1473075	2	1473075	33.56
132	2	2_1518138	2	1518138	33.56
133	2	2_1608299	2	1608299	33.56
134	2	2_1697780	2	1697780	33.56
135	2	2_1834325	2	1834325	33.56
136	2	2_1879618	2	1879618	33.56
137	2	2_1924230	2	1924230	33.56
138	2	2_1967265	2	1967265	33.56
139	2	2_2015931	2	2015931	33.56
140	2	2_2063873	2	2063873	33.56
141	2	2_2153820	2	2153820	33.56
142	2	2_2198477	2	2198477	33.56
143	2	2_2243498	2	2243498	33.56
144	2	2_2334467	2	2334467	33.56
145	2	2_2379301	2	2379301	33.56
146	2	2_2423839	2	2423839	33.56
147	2	2_2473127	2	2473127	33.56
148	2	2_2549003	2	2549003	33.56
149	2	2_2645798	2	2645798	33.56
150	2	2_2691297	2	2691297	33.56
151	2	2_2735132	2	2735132	33.56
152	2	2_2777725	2	2777725	33.56
153	2	2_2855793	2	2855793	33.56
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155	2	2_3212903	2	3212903	33.56
156	2	2_3254091	2	3254091	33.56
157	2	2_3288604	2	3288604	33.56
158	2	2_3298739	2	3298739	33.56
159	2	2_3315474	2	3315474	33.56

160	2	2_3388702	2	3388702	35.02
161	3	3_3090272	3	3090272	0.00
162	3	3_3037038	3	3037038	6.20
163	3	3_2993825	3	2993825	7.83
164	3	3_2901765	3	2901765	8.54
165	3	3_2947254	3	2947254	8.54
166	3	3_2861171	3	2861171	9.25
167	3	3_2813307	3	2813307	9.96
168	3	3_2766094	3	2766094	9.96
169	3	3_2674275	3	2674275	9.96
170	3	3_2636752	3	2636752	9.96
171	3	3_2599944	3	2599944	9.96
172	3	3_2552089	3	2552089	9.96
173	3	3_2506697	3	2506697	9.96
174	3	3_2461430	3	2461430	9.96
175	3	3_2419694	3	2419694	9.96
176	3	3_2370081	3	2370081	9.96
177	3	3_2327251	3	2327251	9.96
178	3	3_2280731	3	2280731	9.96
179	3	3_2236575	3	2236575	9.96
180	3	3_2194830	3	2194830	9.96
181	3	3_2150204	3	2150204	9.96
182	3	3_2102174	3	2102174	9.96
183	3	3_2058431	3	2058431	9.96
184	3	3_2026818	3	2026818	9.96
185	3	3_2010945	3	2010945	9.96
186	3	3_1964553	3	1964553	9.96
187	3	3_1922769	3	1922769	9.96
188	3	3_1691443	3	1691443	9.96
189	3	3_1652191	3	1652191	9.96
190	3	3_1602763	3	1602763	9.96
191	3	3_1532459	3	1532459	9.96
192	3	3_1438415	3	1438415	9.96
193	3	1_234868	1	234868	9.96
194	3	3_1367926	3	1367926	10.67
195	3	3_1245861	3	1245861	10.67
196	3	3_1201114	3	1201114	10.67
197	3	3_1164300	3	1164300	10.67
198	3	3_1078512	3	1078512	10.67
199	3	3_1017475	3	1017475	10.67
200	3	3_994277	3	994277	10.67
201	3	3_947109	3	947109	10.67
202	3	3_885801	3	885801	10.67
203	3	3_814149	3	814149	10.67
204	3	3_772377	3	772377	10.67
205	3	3_704552	3	704552	10.67
206	3	3_565708	3	565708	10.67
207	3	3_512351	3	512351	10.67
208	3	3_484253	3	484253	10.67
209	3	3_475375	3	475375	10.67
210	3	3_358418	3	358418	10.67
211	3	3_206594	3	206594	10.67
212	3	3_116488	3	116488	11.37
213	3	3_161496	3	161496	11.37
214	3	3_23132	3	23132	21.47

215	4	4_3056283	4	3056283	0.00
216	4	4_2961966	4	2961966	3.91
217	4	4_3006562	4	3006562	3.91
218	4	4_2914231	4	2914231	4.62
219	4	4_551837	4	551837	5.33
220	4	4_740304	4	740304	5.33
221	4	4_782310	4	782310	5.33
222	4	4_826983	4	826983	5.33
223	4	4_948084	4	948084	5.33
224	4	4_991182	4	991182	5.33
225	4	4_1050279	4	1050279	5.33
226	4	4_1094090	4	1094090	5.33
227	4	4_1235816	4	1235816	5.33
228	4	9_1260535	9	1260535	5.33
229	4	4_1414709	4	1414709	5.33
230	4	4_1460605	4	1460605	5.33
231	4	4_1550224	4	1550224	5.33
232	4	4_1591224	4	1591224	5.33
233	4	4_1723769	4	1723769	5.33
234	4	4_1769109	4	1769109	5.33
235	4	4_1814348	4	1814348	5.33
236	4	4_1908493	4	1908493	5.33
237	4	4_1996751	4	1996751	5.33
238	4	4_2040072	4	2040072	5.33
239	4	4_2133276	4	2133276	5.33
240	4	4_2226432	4	2226432	5.33
241	4	4_2262875	4	2262875	5.33
242	4	4_2270292	4	2270292	5.33
243	4	4_2314323	4	2314323	5.33
244	4	4_2358593	4	2358593	5.33
245	4	4_2673814	4	2673814	5.33
246	4	4_2761154	4	2761154	5.33
247	4	4_2786915	4	2786915	5.33
248	4	4_2810209	4	2810209	5.33
249	4	4_2834141	4	2834141	5.33
250	4	4_123071	4	123071	6.76
251	4	4_172432	4	172432	8.19
252	4	4_218860	4	218860	8.19
253	4	4_261906	4	261906	8.19
254	4	4_308834	4	308834	8.19
255	4	4_375151	4	375151	8.19
256	4	4_422575	4	422575	8.19
257	4	4_464557	4	464557	8.19
258	4	4_507087	4	507087	8.19
259	4	4_600826	4	600826	8.19
260	4	4_646766	4	646766	8.19
261	4	4_692296	4	692296	8.19
262	4	4_871960	4	871960	8.19
263	4	4_874369	4	874369	8.19
264	4	4_1185933	4	1185933	8.19
265	4	4_1287513	4	1287513	8.19
266	4	4_1324429	4	1324429	8.19
267	4	4_1368883	4	1368883	8.19
268	4	4_1505607	4	1505607	8.19
269	4	4_1631032	4	1631032	8.19

270	4	4_1679416	4	1679416	8.19
271	4	4_1863115	4	1863115	8.19
272	4	4_1952158	4	1952158	8.19
273	4	4_2179873	4	2179873	8.19
274	4	4_2627119	4	2627119	8.19
275	4	4_2718810	4	2718810	8.19
276	4	4_2579709	4	2579709	11.09
277	4	4_356558	4	356558	14.74
278	4	4_1137901	4	1137901	18.39
279	5	5_58839	5	58839	0.00
280	5	5_144905	5	144905	0.73
281	5	5_156334	5	156334	0.73
282	5	5_190350	5	190350	1.60
283	5	5_420524	5	420524	4.33
284	5	5_473164	5	473164	4.33
285	5	5_290159	5	290159	4.63
286	5	5_232557	5	232557	5.04
287	5	5_274542	5	274542	5.04
288	5	5_319273	5	319273	5.04
289	5	5_386847	5	386847	5.04
290	5	5_514536	5	514536	5.04
291	5	5_371232	5	371232	5.75
292	5	5_468329	5	468329	7.18
293	5	5_649252	5	649252	7.18
294	5	5_700083	5	700083	7.18
295	5	5_757456	5	757456	7.89
296	5	5_856795	5	856795	8.60
297	5	10_861637	10	861637	8.60
298	5	5_1075564	5	1075564	8.60
299	5	5_1310008	5	1310008	8.60
300	5	5_1356943	5	1356943	8.60
301	5	5_1217115	5	1217115	8.85
302	5	5_1129976	5	1129976	9.10
303	5	5_801577	5	801577	9.30
304	5	5_949337	5	949337	9.30
305	5	5_1263405	5	1263405	9.30
306	5	5_1171733	5	1171733	10.08
307	5	5_1801152	5	1801152	10.64
308	5	5_1494488	5	1494488	10.67
309	5	5_1401572	5	1401572	10.68
310	5	5_1584353	5	1584353	10.68
311	5	5_1876550	5	1876550	10.72
312	5	5_1767857	5	1767857	10.77
313	5	5_1639805	5	1639805	10.80
314	5	5_1449872	5	1449872	11.55
315	5	5_1733025	5	1733025	12.14
316	5	5_2191348	5	2191348	12.85
317	5	5_2261147	5	2261147	13.21
318	5	5_2333052	5	2333052	13.55
319	5	5_2351150	5	2351150	13.55
320	5	5_2281539	5	2281539	14.32
321	5	5_2171434	5	2171434	14.95
322	5	5_2052435	5	2052435	14.97
323	5	5_2095477	5	2095477	14.97
324	5	5_2154902	5	2154902	14.97

325	5	5_2253242	5	2253242	15.18
326	5	5_2012047	5	2012047	15.42
327	5	3_1328102	3	1328102	15.68
328	5	5_1977351	5	1977351	15.68
329	5	5_2233549	5	2233549	15.68
330	5	5_2377744	5	2377744	15.68
331	5	5_2422062	5	2422062	18.66
332	5	5_2466532	5	2466532	20.12
333	5	5_2511052	5	2511052	26.72
334	6	6_21479	6	21479	0.00
335	6	6_115852	6	115852	19.76
336	6	6_158762	6	158762	19.76
337	6	6_205905	6	205905	20.47
338	6	6_1141044	6	1141044	21.25
339	6	6_10240	6	10240	21.88
340	6	6_1408481	6	1408481	23.89
341	6	6_341276	6	341276	24.04
342	6	6_387122	6	387122	24.04
343	6	6_450172	6	450172	24.04
344	6	6_629099	6	629099	24.04
345	6	6_673016	6	673016	24.04
346	6	6_808426	6	808426	24.04
347	6	6_853783	6	853783	24.04
348	6	6_1034164	6	1034164	24.04
349	6	6_1095507	6	1095507	24.04
350	6	6_1272598	6	1272598	24.04
351	6	6_1361872	6	1361872	24.04
352	6	6_1449286	6	1449286	24.04
353	6	6_1539225	6	1539225	24.04
354	6	6_65819	6	65819	24.75
355	6	6_495312	6	495312	25.46
356	6	6_539363	6	539363	25.46
357	6	6_717353	6	717353	25.46
358	6	6_899311	6	899311	25.46
359	6	6_943781	6	943781	25.46
360	6	6_989820	6	989820	25.46
361	6	6_1061653	6	1061653	25.46
362	6	6_1187436	6	1187436	25.46
363	6	6_1228362	6	1228362	25.46
364	6	6_1319555	6	1319555	25.46
365	6	6_1612510	6	1612510	26.17
366	6	6_1660240	6	1660240	26.17
367	6	6_1704273	6	1704273	26.17
368	6	6_1750828	6	1750828	26.17
369	6	6_1792474	6	1792474	26.17
370	6	6_1836040	6	1836040	26.17
371	6	6_1884121	6	1884121	26.87
372	6	6_1928573	6	1928573	26.87
373	6	6_1972631	6	1972631	26.87
374	6	6_2019906	6	2019906	26.87
375	6	6_2071256	6	2071256	27.78
376	6	6_2160748	6	2160748	29.74
377	6	6_2203699	6	2203699	30.45
378	6	6_2245015	6	2245015	31.95
379	6	6_2291865	6	2291865	40.66

380	7	7_49812	7	49812	0.00
381	7	7_92100	7	92100	8.90
382	7	7_485571	7	485571	10.53
383	7	7_354974	7	354974	10.53
384	7	7_186788	7	186788	11.52
385	7	7_893262	7	893262	12.64
386	7	7_849157	7	849157	12.66
387	7	7_996572	7	996572	12.67
388	7	7_527374	7	527374	12.67
389	7	7_247488	7	247488	12.67
390	7	7_714511	7	714511	12.67
391	7	7_271668	7	271668	12.67
392	7	7_962514	7	962514	12.67
393	7	7_666920	7	666920	12.67
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396	7	7_855225	7	855225	12.67
397	7	7_805905	7	805905	12.67
398	7	7_619796	7	619796	12.67
399	7	7_542386	7	542386	12.67
400	7	7_686578	7	686578	12.67
401	7	7_573909	7	573909	12.67
402	7	7_1040242	7	1040242	12.67
403	7	7_759033	7	759033	12.67
404	7	7_735052	7	735052	12.68
405	7	7_514777	7	514777	12.69
406	7	7_787698	7	787698	12.69
407	7	7_645033	7	645033	13.38
408	7	7_398150	7	398150	14.09
409	7	7_593915	7	593915	14.47
410	7	7_951704	7	951704	14.79
411	7	7_1529499	7	1529499	15.50
412	7	7_1938604	7	1938604	15.50
413	7	7_2024957	7	2024957	15.50
414	7	7_1753843	7	1753843	15.50
415	7	7_1572719	7	1572719	15.50
416	7	7_1222612	7	1222612	15.50
417	7	7_1801517	7	1801517	15.50
418	7	7_1846556	7	1846556	15.50
419	7	7_1132455	7	1132455	15.62
420	7	7_1177123	7	1177123	15.73
421	7	7_1663209	7	1663209	15.79
422	7	7_1894513	7	1894513	15.82
423	7	7_1266563	7	1266563	15.85
424	7	7_1705098	7	1705098	15.91
425	7	7_1485728	7	1485728	15.96
426	7	7_1397072	7	1397072	16.03
427	7	7_1312073	7	1312073	16.10
428	7	7_1441346	7	1441346	16.16
429	7	7_1086990	7	1086990	16.18
430	7	7_1987654	7	1987654	16.21
431	7	7_1352225	7	1352225	16.21
432	7	7_2116295	7	2116295	16.92
433	7	7_2069170	7	2069170	16.92
434	7	7_2163929	7	2163929	17.63

435	7	7_2200662	7	2200662	17.64
436	7	7_2243288	7	2243288	19.16
437	7	7_2285499	7	2285499	38.15
438	7	7_2293489	7	2293489	42.86
439	7	7_2299925	7	2299925	44.71
440	8	8_1865304	8	1865304	0.00
441	8	8_1818423	8	1818423	0.00
442	8	8_1772885	8	1772885	0.00
443	8	8_1731060	8	1731060	0.00
444	8	8_1694681	8	1694681	0.00
445	8	8_1646174	8	1646174	0.00
446	8	8_1598613	8	1598613	0.00
447	8	8_1554916	8	1554916	0.00
448	8	8_1508285	8	1508285	0.00
449	8	8_1464686	8	1464686	0.00
450	8	8_1414864	8	1414864	0.00
451	8	8_1388359	8	1388359	0.00
452	8	8_1337571	8	1337571	0.00
453	8	8_1271792	8	1271792	0.00
454	8	8_1227750	8	1227750	0.00
455	8	8_1180566	8	1180566	0.00
456	8	8_1138528	8	1138528	0.00
457	8	8_921627	8	921627	0.00
458	8	8_790289	8	790289	0.00
459	8	8_750079	8	750079	0.00
460	8	8_661199	8	661199	0.00
461	8	8_624452	8	624452	0.00
462	8	8_604453	8	604453	0.00
463	8	8_557782	8	557782	0.00
464	8	8_508133	8	508133	0.00
465	8	8_459995	8	459995	0.00
466	8	8_419517	8	419517	0.00
467	8	3_304903	3	304903	0.00
468	8	8_9637771	8	9637771	0.28
469	8	8_1317400	8	1317400	0.53
470	8	8_62806	8	62806	0.71
471	8	8_286357	8	286357	0.71
472	8	8_326729	8	326729	0.71
473	8	8_370806	8	370806	0.71
474	8	11_933288	11	933288	0.71
475	8	8_1089645	8	1089645	0.71
476	8	8_834732	8	834732	0.75
477	8	8_880856	8	880856	2.13
478	8	8_24193	8	24193	22.28
479	8	8_18616	8	18616	25.58
480	8	8_12814	8	12814	32.03
481	9	9_59481	9	59481	0.00
482	9	9_238578	9	238578	5.07
483	9	9_104542	9	104542	5.07
484	9	9_286348	9	286348	5.90
485	9	9_802044	9	802044	6.73
486	9	9_610306	9	610306	6.82
487	9	9_1307827	9	1307827	6.87
488	9	9_696404	9	696404	6.94
489	9	9_424892	9	424892	7.34

490	9	9_1498887	9	1498887	7.75
491	9	9_1357449	9	1357449	8.20
492	9	9_384370	9	384370	8.62
493	9	9_1402534	9	1402534	8.94
494	9	9_1065383	9	1065383	9.31
495	9	9_149864	9	149864	9.31
496	9	9_450786	9	450786	9.32
497	9	9_915411	9	915411	9.32
498	9	9_1113504	9	1113504	9.32
499	9	9_970472	9	970472	10.03
500	9	9_655419	9	655419	10.74
501	9	9_893208	9	893208	10.74
502	9	9_469748	9	469748	10.77
503	9	9_1017812	9	1017812	10.80
504	9	9_1215196	9	1215196	10.86
505	9	9_560053	9	560053	10.90
506	9	9_847333	9	847333	10.94
507	9	9_517714	9	517714	12.82
508	9	9_1450649	9	1450649	13.33
509	9	9_338714	9	338714	13.77
510	9	9_741527	9	741527	14.45
511	9	9_191752	9	191752	15.02
512	9	9_1602312	9	1602312	17.08
513	9	9_1597404	9	1597404	17.23
514	10	10_17280	10	17280	0.00
515	10	10_62648	10	62648	30.71
516	10	10_99213	10	99213	33.59
517	10	10_129671	10	129671	33.60
518	10	10_172121	10	172121	34.31
519	10	10_434818	10	434818	35.02
520	10	10_343857	10	343857	35.05
521	10	10_392663	10	392663	35.09
522	10	10_216571	10	216571	35.09
523	10	10_693899	10	693899	36.45
524	10	10_552213	10	552213	36.45
525	10	10_630188	10	630188	36.45
526	10	10_779350	10	779350	36.45
527	10	10_820864	10	820864	36.45
528	10	10_511475	10	511475	36.45
529	10	10_732662	10	732662	36.45
530	10	10_600691	10	600691	36.45
531	10	10_985777	10	985777	36.45
532	10	10_480504	10	480504	36.45
533	10	10_1045849	10	1045849	36.79
534	10	10_1300050	10	1300050	37.16
535	10	10_1071055	10	1071055	37.16
536	10	10_1218996	10	1218996	37.16
537	10	10_1009533	10	1009533	37.16
538	10	10_1172800	10	1172800	37.16
539	10	10_1255658	10	1255658	37.16
540	10	10_1124983	10	1124983	37.16
541	10	10_1337879	10	1337879	39.09
542	10	10_1613061	10	1613061	39.31
543	10	10_1432745	10	1432745	39.59
544	10	10_1570012	10	1570012	39.85

545	10	10_1389075	10	1389075	40.09
546	10	3_69210	3	69210	40.35
547	10	10_1323043	10	1323043	40.72
548	10	10_1658349	10	1658349	42.25
549	10	10_1702467	10	1702467	49.66
550	10	10_1742770	10	1742770	70.24
551	11	11_1689159	11	1689159	0.00
552	11	2_1565313	2	1565313	12.66
553	11	11_1603577	11	1603577	13.19
554	11	2_362435	2	362435	13.41
555	11	11_1560236	11	1560236	14.11
556	11	11_1431112	11	1431112	14.82
557	11	11_1469708	11	1469708	14.82
558	11	11_1511779	11	1511779	14.82
559	11	11_384683	11	384683	15.53
560	11	11_564262	11	564262	15.53
561	11	11_726086	11	726086	15.53
562	11	11_799297	11	799297	15.53
563	11	11_824197	11	824197	15.53
564	11	11_843455	11	843455	15.53
565	11	11_868889	11	868889	15.53
566	11	11_1150716	11	1150716	15.53
567	11	11_1205341	11	1205341	15.53
568	11	11_1338354	11	1338354	15.53
569	11	11_1383901	11	1383901	15.53
570	11	11_704655	11	704655	15.94
571	11	11_473665	11	473665	16.24
572	11	11_287831	11	287831	16.66
573	11	11_658620	11	658620	16.95
574	11	11_754430	11	754430	16.95
575	11	11_776563	11	776563	16.95
576	11	11_907914	11	907914	16.95
577	11	11_1245553	11	1245553	16.95
578	11	11_431306	11	431306	16.95
579	11	11_1291976	11	1291976	16.95
580	11	11_338351	11	338351	16.96
581	11	11_613999	11	613999	16.96
582	11	11_242932	11	242932	17.74
583	11	11_198373	11	198373	18.35
584	11	11_178901	11	178901	19.78
585	11	11_1533611	11	1533611	19.79
586	11	11_180827	11	180827	19.79
587	11	11_108133	11	108133	20.52
588	11	11_65604	11	65604	21.24
589	11	11_21030	11	21030	58.72
590	11	11_18310	11	18310	62.41
591	12	12_567414	12	567414	0.00
592	12	12_619354	12	619354	16.03
593	12	12_965192	12	965192	18.48
594	12	12_1465036	12	1465036	20.64
595	12	12_338364	12	338364	25.06
596	12	12_378613	12	378613	29.48
597	12	12_244814	12	244814	30.91
598	12	12_1506287	12	1506287	32.96
599	12	12_1419295	12	1419295	33.06

600	12	12_1282942	12	1282942	33.06
601	12	12_1677269	12	1677269	33.06
602	12	12_1592363	12	1592363	33.06
603	12	12_1546477	12	1546477	33.06
604	12	12_292508	12	292508	33.06
605	12	12_1636136	12	1636136	33.06
606	12	12_241848	12	241848	33.06
607	12	12_712902	12	712902	33.25
608	12	12_1013269	12	1013269	33.42
609	12	12_883642	12	883642	33.57
610	12	12_1196550	12	1196550	33.77
611	12	12_1155639	12	1155639	33.77
612	12	12_843390	12	843390	33.77
613	12	12_1230116	12	1230116	33.77
614	12	12_1064920	12	1064920	33.77
615	12	12_1108948	12	1108948	33.77
616	12	12_925727	12	925727	34.07
617	12	12_1681512	12	1681512	34.47
618	12	12_1239908	12	1239908	34.47
619	12	12_425861	12	425861	35.18
620	12	9_14945	9	14945	38.08
621	12	12_466808	12	466808	42.74
622	12	12_512356	12	512356	62.22
623	13	13_1323179	13	1323179	0.00
624	13	13_1290995	13	1290995	13.22
625	13	13_1244610	13	1244610	14.04
626	13	13_1213215	13	1213215	15.58
627	13	13_1165554	13	1165554	16.38
628	13	13_1117134	13	1117134	16.77
629	13	13_617354	13	617354	17.13
630	13	13_490125	13	490125	17.13
631	13	13_804874	13	804874	17.13
632	13	12_757423	12	757423	17.13
633	13	13_1076107	13	1076107	17.13
634	13	13_892081	13	892081	17.13
635	13	13_409813	13	409813	17.13
636	13	13_367045	13	367045	17.13
637	13	13_1025969	13	1025969	17.13
638	13	13_576123	13	576123	17.13
639	13	13_981652	13	981652	17.13
640	13	13_710006	13	710006	17.13
641	13	13_326097	13	326097	17.13
642	13	13_531661	13	531661	17.13
643	13	13_662489	13	662489	17.13
644	13	13_937712	13	937712	17.84
645	13	13_757299	13	757299	17.84
646	13	13_99834	13	99834	19.23
647	13	13_142518	13	142518	19.27
648	13	13_231744	13	231744	19.27
649	13	13_185807	13	185807	19.27
650	13	13_214301	13	214301	19.27
651	13	13_275246	13	275246	19.27
652	13	13_281488	13	281488	19.27
653	13	13_55392	13	55392	24.47
654	13	13_14469	13	14469	51.51

Supplementary file 2

Marker information for population 2.

marker	chromo-some	map position	physical position (H97 version 3)	marker	chromo-some	map position	physical position (H97 version 3)
ChrI_B1	1	0.00	3314304	ChrVII_B1	7	11.35	2069230
MHchrI_05	1	1.60	2703648	MHchrVII_04	7	11.36	1588639
MHchrI_04	1	1.60	2036488	MHchrVII_03	7	11.37	1220326
ChrI_M1	1	1.60	1687705	MHchrVII_05	7	13.04	2185387
MHchrI_01	1	2.21	273193	MHchrVIII_05	8	0.00	1922859
ChrI_T1	1	7.09	104077	ChrVIII_B1	8	26.07	1865364
MHchrII_05	2	0.00	3398925	MHchrVIII_04	8	29.50	1725237
ChrII_T3	2	1.08	3315534	MHchrVIII_03	8	29.50	1391402
MHchrII_03	2	3.22	2163613	MHchrVIII_02	8	30.07	580089
MHchrII_04	2	4.65	2823373	ChrVIII_T1	8	36.82	62806
MHchrII_01	2	5.86	446501	MHchrVIII_01	8	77.16	19963
ChrII_B1	2	5.88	610238	MHchrIX_01	9	0.00	1475763
ChrII_M1	2	5.88	1834385	MHchrIX_02	9	1.16	988642
ChrIII_B1	3	0.00	2993885	ChrIX_T1	9	1.17	238638
MHchrIII_05	3	1.09	2964588	MHchrIX_04	9	2.23	151013
ChrIII_M1	3	4.36	1922829	MHchrIX_03	9	2.23	794722
MHchrIII_04	3	4.36	1595557	ChrIX_B3	9	2.76	1357509
MHchrIII_03	3	4.36	1354697	MHchrIX_05	9	2.76	478908
ChrIII_T3	3	4.36	1078572	MHchrX_01	10	0.00	792482
MHchrIII_02	3	4.36	727754	MHchrX_02	10	0.62	228187
MHchrIII_01	3	4.36	383086	ChrX_T3	10	0.62	552273
MHchrIV_05	4	0.00	2973860	MHchrX_03	10	0.62	579798
ChrIV_T2	4	0.00	2673814	ChrX_B1	10	0.62	1337939
MHchrIV_04	4	0.00	2180612	ChrXI_M1	11	0.00	1603637
ChrIV_M1	4	0.20	1185993	MHchrXI_05	11	2.20	1560210
MHchrIV_02	4	0.40	825678	ChrXI_B2	11	3.27	1292036
MHchrIV_03	4	0.53	1260523	MHchrXI_02	11	3.27	1291679
MHchrIV_01	4	1.05	262496	MHchrXI_03	11	3.27	783568
ChrIV_B1	4	1.05	172492	ChrXI_T2	11	3.27	431366
ChrV_T1	5	0.00	58899	MHchrXI_04	11	3.56	463590
MHchrV_02	5	8.14	704374	MHchrXI_01	11	3.82	265973
ChrV_M1	5	8.14	1310068	ChrXII_B1	12	0.00	292509
MHchrV_03	5	8.14	1454844	MHchrXII_05	12	0.31	1664214
MHchrV_04	5	8.14	1945729/1949268	MHchrXII_03	12	0.54	595333
ChrV_B2	5	8.14	2171494	MHchrXII_04	12	0.54	1215930
MHchrV_05	5	8.14	2206121	ChrXII_T2	12	0.54	1239968
MHchrV_01	5	8.69	245921/249795	MHchrXII_02	12	1.61	733480
MHchrVI_05	6	0.00	2301415	ChrXII_M1	12	3.21	619414
ChrVI_B1	6	17.35	2245075	MHchrXII_01	12	4.82	1043497
MHchrVI_04	6	22.37	1874194	MHchrXIII_05	13	0.00	147203
MHchrVI_03	6	22.90	1633178	MHchrXIII_04	13	3.97	1278581
MHchrVI_02	6	22.90	575593	ChrXIII_B2	13	3.97	1076167
MHchrVI_01	6	23.96	207974	MHchrXIII_03	13	3.97	685943
ChrVI_T1	6	30.10	65819	MHchrXIII_02	13	3.97	994994
MHchrVII_01	7	0.00	48668	ChrXIII_M1	13	3.97	662549
ChrVII_T2	7	10.28	465022	ChrXIII_T1	13	5.02	185867

MHchrVII_02	7	10.81	884696	MHchrXIII_01	13	5.02	363261
ChrVII_M1	7	11.35	1352285				

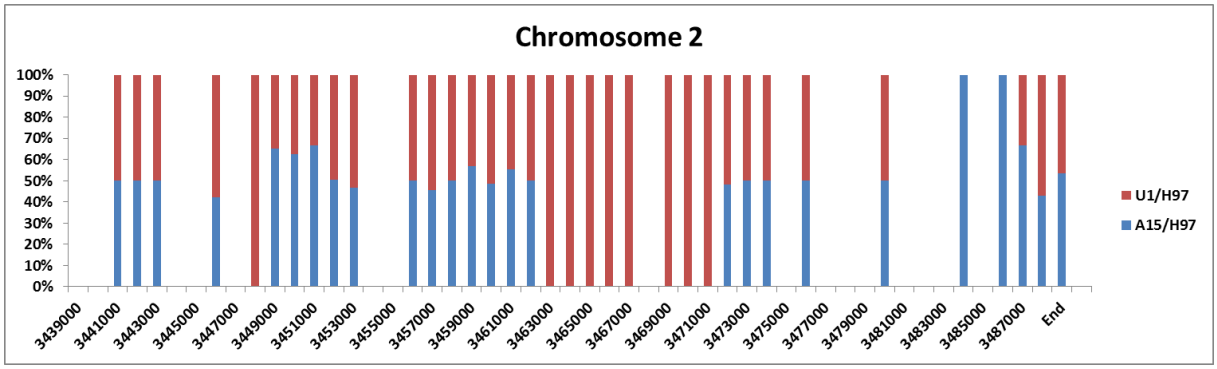
Supplementary file 3

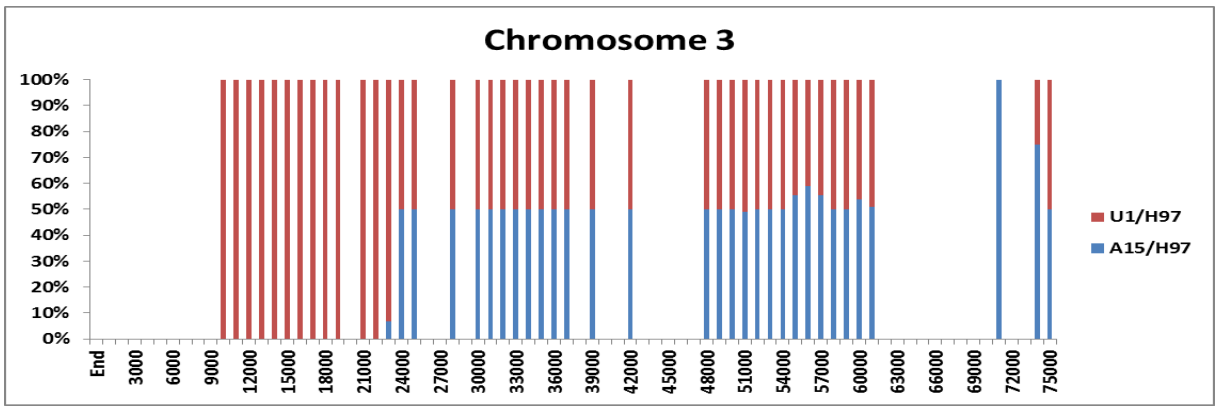
Marker information for population 3.

Locus	chromo-some	map Position	physical position (H97 version 3)	Locus	chromo-some	map position	physical position (H97-version 3)
HZchrI_01	1	0.00	56261	HZchrVII_03	7	2.32	1334483
ChrI_T2	1	1.86	306880	HZchrVII_02	7	2.32	1219329
HZchrI_02	1	1.86	408022	ChrVII_T3	7	2.32	542446
HZchrI_03	1	1.86	528619	HZchrVII_01	7	2.32	468917
HZchrI_04	1	1.86	668634	ChrVIII_B1	8	0.00	1865364
HZchrI_05	1	1.86	968651	HZchrVIII_04	8	4.78	1630046
CHR1B1	1	2.44	3236967	HZchrVIII_03	8	4.78	1160533
CHR2B1	2	0.00	478461	HZchrVIII_02	8	4.78	1110462
HZchrII_01	2	0.00	529952	HZchrVIII_01	8	4.78	797640
HZchrII_02	2	0.00	1388551	CHR8A1	8	5.57	286417
HZchrII_03	2	0.00	1505090	CHR9B1	9	0.00	1602372
HZchrII_05	2	0.00	2401839	HZchrIX_03	9	4.51	289880
HZchrII_06	2	0.00	2999949	ChrIX_T1	9	4.51	238638
CHR2A1	2	0.00	3012593	HZchrIX_02	9	4.52	289223
HZchrIII_05	3	0.00	3061812	HZchrIX_01	9	9.30	60091
CHR3B1	3	0.77	3037098	HZchrX_01	10	0.00	26878
HZchrIII_04	3	2.92	2817563	CHR10A1	10	22.74	99273
HZchrIII_03	3	2.92	2804192	HZchrX_02	10	22.88	228187
HZchrIII_02	3	2.92	2779415	HZchrX_03	10	22.99	1345631
ChrIII_T2	3	2.92	206654	HZchrX_04	10	23.10	1371062
HZchrIII_01	3	2.92	102381	CHR10B1	10	23.32	1570072
HZchrIV_06	4	0.00	3037822	HZchrXI_05	11	0.00	870820
HZchrIV_05	4	8.79	2370603	CHR11B1	11	0.23	180887
ChrIV_T3	4	8.79	2314383	ChrXI_T3	11	0.36	614059
HZchrIV_04	4	8.79	2132337	HZchrXI_03	11	0.57	444245
HZchrIV_03	4	8.79	1490686	HZchrXI_02	11	1.17	214583
HZchrIV_01	4	8.79	608195	HZchrXI_01	11	2.40	101596
CHR4B1	4	9.97	123131	HZchrXII_01	12	0.00	681754
HZchrV_03	5	0.00	2477711	HZchrXII_02	12	1.84	900073
ChrV_M1	5	10.40	1310068	CHR12B1	12	1.84	925787
ChrV_B2	5	10.96	2171494	CHR12A1	12	2.59	244814
HZchrV_01	5	11.65	239605	CHR13B1	13	0.00	1117194
ChrV_T2	5	14.70	144965	HZchrXIII_05	13	0.00	1055643
HZchrVI_02	6	0.00	986714	HZchrXIII_03	13	0.00	1024717
HZchrVI_03	6	0.00	1451562	HZchrXIII_04	13	0.00	632731
CHR7B1	7	0.00	2163989	CHR13m1	13	0.00	576183
HZchrVII_05	7	2.32	1887367	HZchrXIII_01	13	0.00	419543
HZchrVII_04	7	2.32	1761749	CHR13A1	13	10.02	55452

Supplementary file 4

Stacked bars for zooming in on the recombination locations on chromosomes of Sylvan A15. The legend is the same as that of Figure 9.


Chromosome 2
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
3439000
3441000
3443000
3445000
3447000
3449000
3451000
3453000
3455000
3457000
3459000
3461000
3463000
3465000
3467000
3469000
3471000
3473000
3475000
3477000
3479000
3481000
3483000
3485000
3487000
End
U1/H97
A15/H97


Chromosome 3
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80%
70%
60%
50%
40%
30%
20%
10%
0%
End
3000
6000
9000
12000
15000
18000
21000
24000
27000
30000
33000
36000
39000
42000
45000
48000
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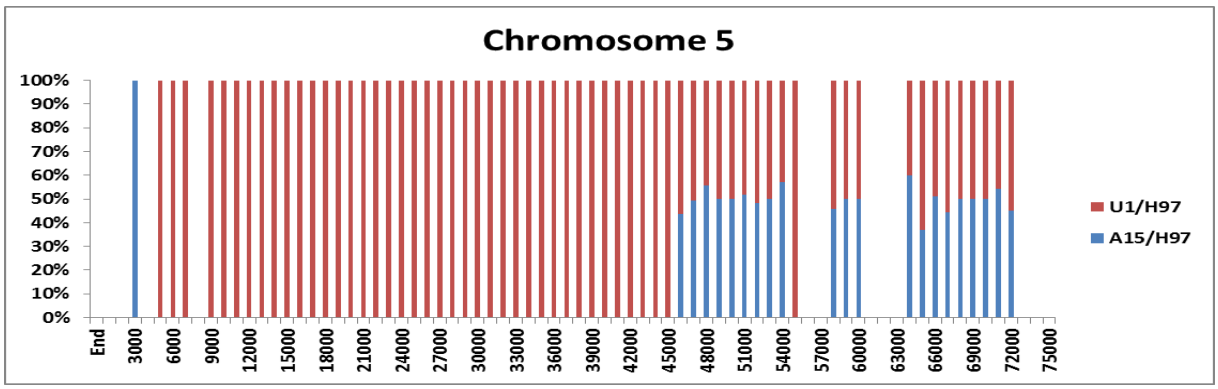
66000
69000
72000
75000
U1/H97
A15/H97


Chromosome 5
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90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
End
3000
6000
9000
12000
15000
18000
21000
24000
27000
30000
33000
36000
39000
42000
45000
48000
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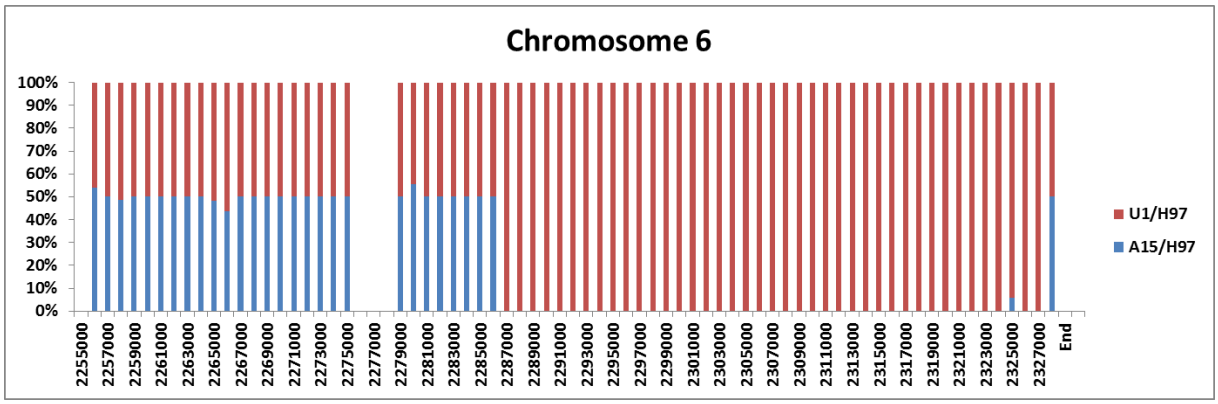
66000
69000
72000
75000
U1/H97
A15/H97


Chromosome 6
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90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
2255000
2257000
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2261000
2263000
2265000
2267000
2269000
2271000
2273000
2275000
2277000
2279000
2281000
2283000
2285000
2287000
2289000
2291000
2293000
2295000
2297000
2299000
2301000
2303000
2305000
2307000
2309000
2311000
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2317000
2319000
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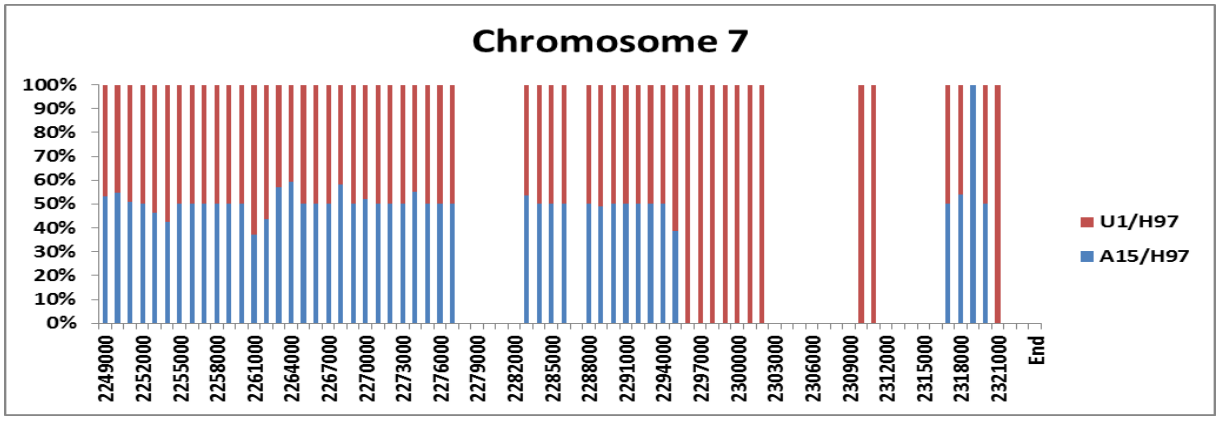
2325000
2327000
End
U1/H97
A15/H97


Chromosome 7
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80%
70%
60%
50%
40%
30%
20%
10%
0%
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2252000
2255000
2258000
2261000
2264000
2267000
2270000
2273000
2276000
2279000
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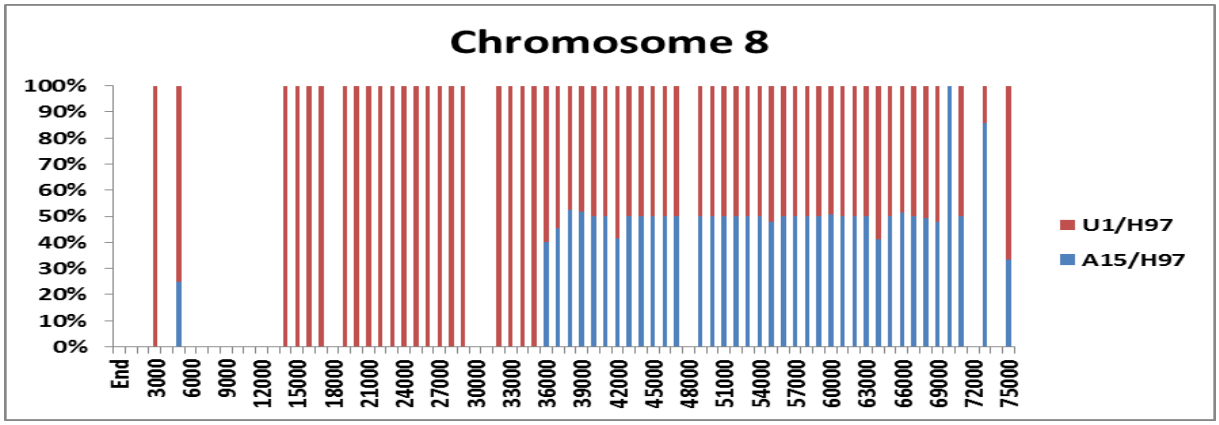
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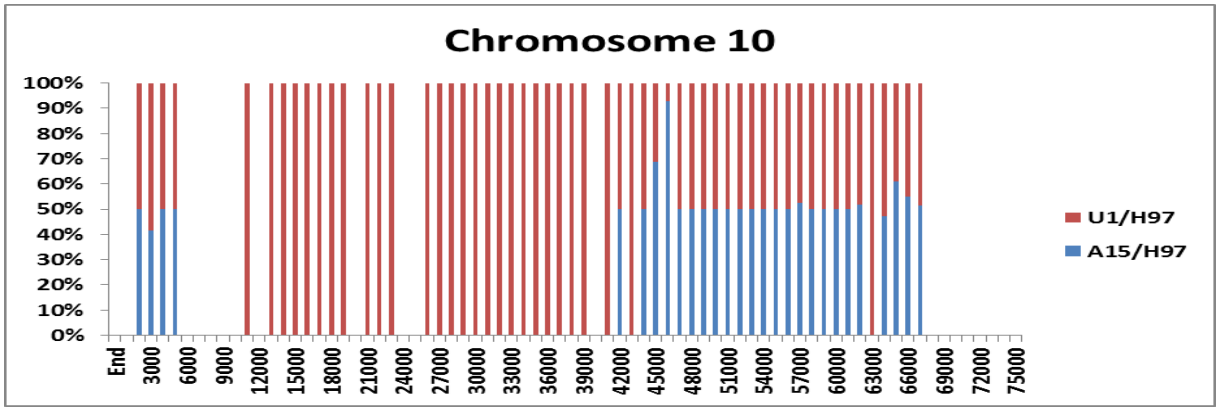
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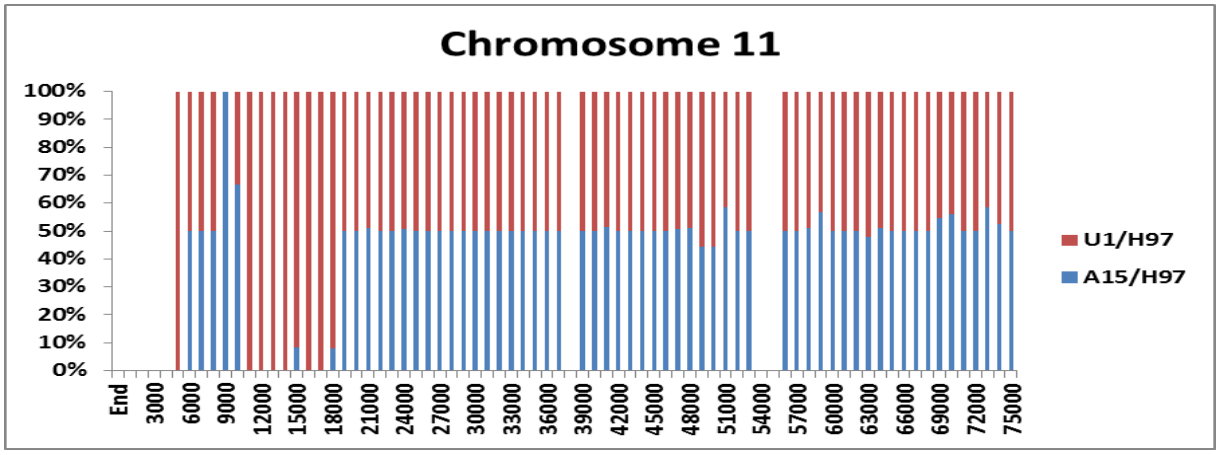
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Chapter 5

Quantitative Trait Locus Mapping for Bruising Sensitivity and Cap Color of *Agaricus bisporus* (button mushrooms)

Wei Gao[1,3], Amrah Weijn[2], Johan JP Baars[1], Jurriaan Mes[2],Richard GF Visser[1] and Anton SM Sonnenberg[1]

1. Plant Breeding, Wageningen University & Research Centre, Wageningen, The Netherlands

2. Food & Biobased Research, Wageningen University & Research Centre, Wageningen, The Netherlands

3. Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing, PR China

Submitted

Abstract

New cultivars that are resistant to mechanical damage allow a fully automated harvesting of mushrooms and in this way can reduce the production costs considerably. In order to unravel the genetic basis for mushroom bruising sensitivity and to generate lines for breeding programs, quantitative trait loci (QTL) were identified in two segregating populations. Population 1 consists of homokaryotic single spore cultures (homokaryons) of a cross between a white resistant line (CH2B) and a brown resistant line (Mes09143), and population 2 consists of homokaryons of across between a resistant line (CH2A) and a sensitive line (Z8). Population 1 was crossed with two different tester lines creating two sets of heterokaryons that allowed the phenotyping of mushrooms in different genetic backgrounds, and a third tester line was used for population 2. The resulting three sets of heterokaryons were used for QTL detection. Since cap color has the same phenotype as discoloration, QTL mapping was done for both bruising sensitivity (BS) and for cap color (CC). The phenotypes were assessed at 60 min and 24 h after bruising in flush 1 and flush 2. QTLs of BS and CC were assigned to chromosomes, and beneficial alleles were detected in all three resistant parental lines. Co-segregation of the BS-QTL and CC-QTL was observed in both population 1 and population 2. QTLs identified in this study will provide a basis for the breeding of advanced mushroom cultivars that are less sensitive to mechanical bruising.

Introduction

The homobasidiomycete *Agaricus bisporus* (button mushroom) is one of the most widely cultivated mushrooms in the world. Homobasidiomycetes are characterized by the fact that they contain two types of haploid nuclei with different mating types that stay apart in each cell. Fusion of nuclei only takes place in basidial cells just before meiosis. Each diploid nucleus produces four haploid post-meiotic nuclei and these are distributed over four spores formed by each basidial cell. The spores germinate into infertile haploid mycelia, i.e., homokaryons (or monokaryons). Homokaryons with different mating type can anastomose and subsequent exchange of nuclei leads to the formation of fertile heterokaryotic (dikaryotic) mycelium. The presence of both mating types within one mycelial cell triggers a developmental process leading to reproductive fruiting bodies provided environmental conditions are favourable. This non-self compatibility or heterothallism is controlled by one or two unlinked loci. The majority of homobasidiomycetes show this heterothallic life cycle. The button mushroom *A. bisporus* var. *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 (Elliot, 1972, Summerbell et al., 1989). 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-tetrad mating, a form of selfing where mating occurs among the products of a single meiosis. It is rare that basidia 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 (Callac, 1993). This variety produces predominantly four spored basidia and each spore germinates into a homokaryotic mycelium. The two varieties are designated as *A. bisporus* var. *bisporus* and *A. bisporus* var. *burnetti*, respectively. Since var. *burnetti* is poor in various agronomic and quality traits, all commercially cultivated lines are *A. bisporus* var. *bisporus.*

As in plants, fungal lines with contrasting performance are commonly crossed to generate segregating populations and used to map genomic regions involved in traits and identify candidate genes (Foulongne-Oriol, 2012). This is usually done by protoplasting the heterokaryotic parental lines and recovering the constituent nuclei as homokaryons which are subsequently used for outcrossing. In the button mushroom, mating between homokaryons is controlled by one mating type locus (MAT) (Xu, 1993). Homokaryotic offspring can be used directly as a segregating population for genetic linkage mapping (haplotyping). Since homokaryons are infertile they have to be crossed with a compatible homokaryotic tester line to

produce mushrooms for QTL mapping. Genetic linkage maps have been generated so far in both bisporic populations of var. *bisporus* and intervarietal populations (Foulongne-Oriol et al., 2010, Kerrigan et al., 1993). The haploid genome size of *A. bisporus* is 30.4 Mb, and it contains 13 chromosomes and has been sequenced completely (Morin et al., 2012).

A very common strategy for breeding is the introduction of a new trait derived from a wild donor strain into an existing commercial line (Sonnenberg, 2005). Some trait related genes or genome loci have been identified to facilitate marker assisted introgression breeding, e.g., a major locus for cap color (Callac et al., 1998), QTLs for the disease resistance, e.g. *Pseudomonas tolaasii* and *Lecancillium fungicola*(Moquet et al., 1999, Sonnenberg, 2005, Foulongne-Oriol et al., 2012b), and QTLs for yield-related traits (Foulongne-Oriol et al., 2012a). Despite these genetic studies and findings, hardly any new varieties for button mushrooms have been released since the first hybrids were produced in 1980, i.e. Horst U1 and U3 (Fritsche, 1981). Many new cultivars came out afterwards to the market with various new names, but most of them are genetically similar to the first hybrid and are likely obtained by selecting heterokaryotic offspring of Horst U1 (Sonnenberg et al., 1999). This indicates that the breeding of *A. bisporus* still lags behind its possibilities, and the large amount of wild germplasm resources bearing superior traits has not been exploited to its full potential.

The Netherlands is one of the largest button mushroom producers in the world. Nearly 60% of the production is harvested mechanically, and around40% are handpicked (Straatsma et al., 2007); The capacity of the mechanical harvesting system (Havatec BV, Noordwijkerhout) is up to 12,000Kg/h. Mushrooms are mechanically harvested in a later developmental stage compared to handpicked mushrooms and have therefore a lower quality and price. During mechanical harvest and handlingmost mushrooms are bruised and consequently discolor (browning) and are, therefore, mainly used for canning and processing. Mushrooms for the fresh market are mainly handpicked. A fully automated mechanical harvesting system for the fresh market might be feasible, but requires the availability of strains that are less sensitive to mechanical bruising. Mushroom browning is a complex process, which can be induced and influenced by a number of environmental factors such as postharvest storage, mechanical bruising and the attack of pathogens (Jolivet et al., 1998, Soler-Rivas et al., 1997, Stoop, 1999). The final product that determines the brown discoloration of mushrooms is melanin; many enzymes are involved in the synthesis of melanin; polyphenoloxidase (PPO or tyrosinase), involved in the last steps of the melanin synthesis as been studied in detail (Burton et al., 1993, Gerritsen et al., 1994, Van Leeuwen & Wichers, 1999). A total of 42 relevant genes involved in the melanin synthesis pathway have been identified in the genome of *A. bisporus* (Weijn et al., 2013a). Although PPO related genes are considered as potentially the most important candidates involved in mushroom browning it is unknown so far which key genes control the bruise-induced discoloration and which genes are responsible for the observed variation in bruising sensitivity among mushroom strains (Weijn et al., 2012). A study of breeding value of parental lines showed the polygenic feature of bruising sensitivity (Gao et al., 2013). In order to unravel the crucial genes involved in bruising sensitivity (BS), QTL analysis was conducted in this study. To our knowledge, no QTL related to bruising sensitivity (or resistance) have been reported before.

In a previous study, a large scale screening on bruising sensitivity of a collection of wild strains and commercial cultivars has been done. A wide variation of BS was found, and some wild strains were much less sensitive than commercial strains (Weijn et al., 2012). Strains representing the extremes of BS were selected and protoplasted to recover the constituent nuclei as homokaryotic parental lines. To estimate the breeding value of these homokaryons, they were crossed in all possible combinations (diallel matrix) and the general combining ability (GCA) was determined by assessing BS of mushrooms of each heterokaryon (Gao et al., 2013). Four representative homokaryotic lines from that study were selected and crossed to generate two heterokaryons. Homokaryotic offspring of these two heterokaryons were used in this study for QTL analysis on BS. Like BS, cap color is also determined by the presence or absence of melanin in the outer layer of the mushroom cap. Although discoloration was measured relative to the

non-bruised area and thus corrected for cap color variation, the latter might interfere. We assessed, therefore, also QTL for cap color. This study will provide a genetic basis for breeding button mushroom strains that are less sensitive to mechanical bruising.

Materials and methods

A. bisporus strains and segregating populations

All strains used in this study were obtained from the fungal collection of Plant Breeding, Wageningen UR. The pedigree of strains is listed in Figure 1. Three original heterokaryotic lines were involved, i.e., WB2 a brown wild line resistant to bruising, Horst U1 a white commercial line resistant to bruising, and a white wild line WW7 sensitive to bruising. One of the constituent nuclei of line WB2 (i.e. Mes09143) and both constituent nuclei of line WW7 (i.e. Z6 & Z8) were recovered through protoplasting the vegetative mycelia. The constituent nuclei of Horst U1 (H97 and H39) were available from the original breeding program. A previous study has shown that Mes09143 is a good donor for bruising resistance (BR) having the lowest negative general combining ability (GCA); H39 & H97 are good recipients for either BR or BS having intermediate GCA; Z8 is a good donor for BS having the highest positive GCA, while Z6 had intermediate GCA (Gao et al., chapter 3).

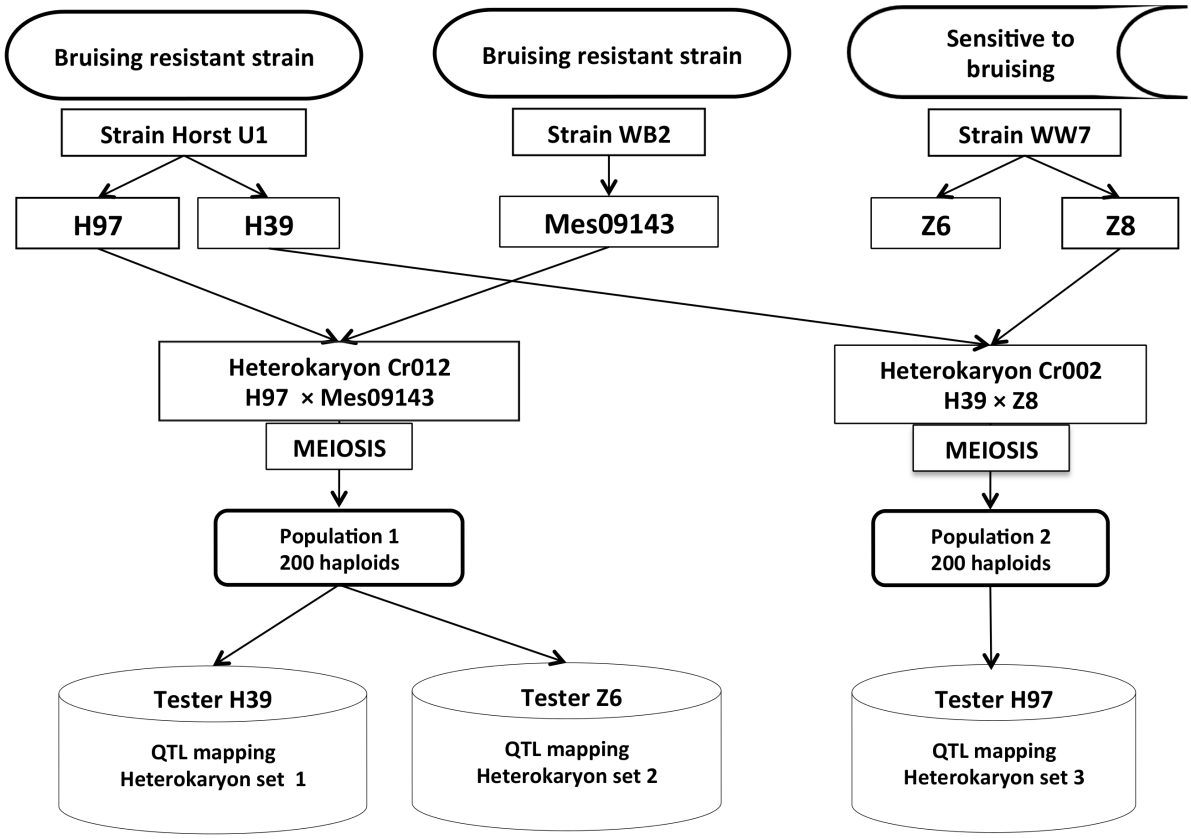


Figure 1. Flow diagram to illustrate the generation of the three segregating populations used in this study

Four homokaryons recovered from the original heterokaryotic lines were crossed to generate two heterokaryons (Figure 1), which were subsequently used to generate two segregating populations. Population 1 represents the homokaryotic offspring of a heterokaryon constructed by crossing a parental homokaryon of Horst U1 (H97) and a parental homokaryon of a wild line (Mes09143). Population 2 represents the homokaryotic offspring of heterokaryon formed by crossing a parental homokaryon of

Horst U1 (H39) and a parental homokaryon of a wild line WW7 (Z8). Both homokaryotic populations were used to generate two linkage maps. For phenotyping, homokaryons must be mated first with a compatible homokaryon. Heterokaryons can be constructed by intercrossing homokaryons within one population. For *A. bisporus* it is known that this kind of selfing causes inbreeding depression (Xu, 1995). Mushroom breeding uses, therefore, a tester homokaryon that is compatible with both mating types present in the segregating population. Siblings in a segregating population can thus be mated with one and the same tester homokaryon, i.e., adding to each individual the same genetic background. In this way, variation in phenotype is likely due to the variation in genetic constitution of the segregating population. The tester line, however, can also have a major influence on the performance of traits. It is, therefore, informative to test the segregation of phenotypes in different backgrounds by using different tester lines. The two segregating populations of this study were crossed with three different tester lines to generate 3 sets of heterokaryons. Population 1 was either crossed with tester line H39 (the other parental homokaryon of Horst U1) or with tester line Z6 (parental homokaryon of wild line WW7). In this way we could study the segregation of bruising sensitivity and cap color in a mainly Horst U1 background (heterokaryon Set 1) or a mainly wild (WW7) background (heterokaryon Set 2). Horst U1 represents the present standard for agronomic and quality traits for button mushroom and in this way we can see how the introduction of wild germplasm affects its important traits. Population 2 was crossed with tester line H97 (the other parental homokaryon of Horst U1). This allows the study of another wild germplasm in the genetic background of Horst U1 (heterokaryon Set 3). The three sets of heterokaryons were cultivated and used to analyse QTLs for bruising sensitivity and cap color. The three original heterokaryotic lines (Horst U1, WB2, and WW7), and the two F1 hybrids (Cr012 & Cr002) were also included in the cultivation trials.

Cultivation tests and phenotypic measurements

Three independent cultivation trials were carried out for the three sets of heterokaryons at the mushroom farm of Unifarm in Wageningen UR with controlled climate. Mushrooms were grown on commercial compost (CNC Substrates), spawned in 0.1 m^2 boxes (40 × 30 × 21 cm) filled with 8 kg of compost. Each individual was grown once in one box. Mushrooms were taken for analysis at the production peaks of flush 1 and 2. For all three sets of heterokaryons, 20 mushrooms of each individual were measured for bruising sensitivity at 60 min after bruising as described in a previous study (Weijn et al., 2012b). The mushrooms were subsequently stored in a cold room (4°C, 93% for relative humidity) for 24 h and assessed again for BS. In this way, four different data sets were generated, i.e., 60 min flush 1, 24 h flush 1, 60 min flush 2, and 24 h flush 2. Bruising sensitivity (BS) and cap color (CC) were measured with the same computer image system (Weijn et al., 2012b). BS was measured as the difference of whiteness index between the control (non-bruised) area and the bruised area on the same mushroom, and CC was the whiteness index of the non-bruised area. In order to make the description clear, data of different time points were coded. BS value of flush 1 mushrooms at 60 min after bruising was coded as BS160; BS value of flush 1 mushrooms at 24 h of after bruising was coded as BS124; that of flush 2 mushrooms at 60 min after bruising was coded as BS260; that of flush 2 mushrooms at 24 h after bruising was coded as BS224. Similarly, CC data at different time points were coded as CC160, CC124, CC260, and CC224. The three sets of heterokaryons were named as Set 1, 2 and 3.

Statistical analysis

Statistical analysis was performed in SPSS (IBM statistics 19) and Genstat (Version 15). Untransformed data were used for summary statistics. Before applying analysis of variance (ANOVA), data were transformed using the square root (SQRT) function to fit a normal distribution. ANOVA was performed with data at each time point for the genotypic variation independently, according to the following model: $Y = \mu + G + \varepsilon$, where μ is the mean value, G is the genotypic effect, and ε is the residual effect. Mushrooms of flush 1 were also sampled for other purposes than assessment of BS and CC. That led to a shortage of

mushrooms for some genotypes and, therefore, only data of flush 1 and 2, 60 minutes after bruising and flush 2 data, 24 hours after bruising were used for QTL analysis. For that reason, we also used different samples in flush 1 of most of the genotypes for the assessment of BS and CC at 60min and 24h in flush 1, whereas for flush 2 the same mushrooms were used for the assessment after 60 min and 24 h. Data of 60 min (flush 1 & 2) were used for ANOVA to estimate the flush effect with the following model $Y= \mu + G + F + G\times F + \varepsilon$, and data of flush 2 (60min & 24h) were used for ANOVA to estimate the time (after bruising) effect with the following model $Y= \mu + G + T + G\times T + \varepsilon$, which were predicted and combined as data of flush 2 (BSflush2 or CCflush2). Flush 1 mushrooms at 60min after bruising of Set 1 and Set 2 were used for ANOVA to estimate the effect of tester lines with a model $Y= \mu + G + C + G\times C + \varepsilon$. In these ANOVA models, G represents genotypic effect; F represents flush effect, T the time (after bruising) and C the tester line; $G\times F$ represents the genotype and flush interaction, $G\times T$ the genotype and time (after bruising) interaction and $G\times C$ the genotype and tester line interaction; ε is the residual. The multiple comparison was performed according to the Student-Newman-Keuls (SNK) test. Transgression of BS and CC were determined by comparing the three sets of heterokaryons to their respective original heterokaryons.

Broad-sense heritability (H^2) was calculated for samples of each time point independently with a model $H^2=\sigma^2_G/ [\sigma^2_G +(\sigma^2_e/\mathrm{r})]$, for samples across flush (flush 1 and flush 2) with a model $H^2=\sigma^2_G/ [\sigma^2_G + (\sigma^2_{G\times F}/nr) + (\sigma^2_e/r)]$, and for samples across time after bruising (60 min and 24 h) with a model $H^2=\sigma^2_G/ [\sigma^2_G + (\sigma^2_{G\times T}/nr) + (\sigma^2_e/r)]$, and for data across tester lines (Set 1 and Set 2) with a model $H^2=\sigma^2_G/ [\sigma^2_G + (\sigma^2_{G\times C}/nr) + (\sigma^2_e/r)]$, where σ^2_G represented the genetic variance, σ^2_e was the error variance (mean square of residual), $\sigma^2_{G\times F}$ was the variance of genotype and flush interaction, $\sigma^2_{G\times T}$ was the variance of genotype by time (after bruising) interaction, $\sigma^2_{G\times C}$ was the variance of genotype by tester line interaction. In this study, n was the number of flushes (n=2) or time points after bruising (n=2) or tester lines (n=2), r was the number of replicates within the experiment (r=1).

Genetic linkage map construction

The segregation analysis and genetic linkage maps of the two homokaryotic populations were reported in another study (Gao *et al*, chapter 4). Briefly, SNP markers were selected to be evenly distributed based on the genome sequences of the reference homokaryon H97 (Morin et al., 2012). The total map length of population 1 (heterokaryon Set 1 and Set 2) was 164 cM generated with 95 SNP markers. It has an average crossover frequency of 0.1 per individual per chromosome. The total map length of population 2 (Heterokaron Set 3) was 86 cM generated with 76 SNP markers, and it has an average crossover frequency of 0.05 per individual per chromosome. Because of the low recombination frequency, the linkage maps are very short and most regions have a low resolution. The QTL mapping is thus restricted in most cases to assigning QTLs to chromosomes.

QTL detection

QTL detection with simple interval mapping (SIM) and composite interval mapping (CIM) were done in Genstat (Genstat 15th edition) with the model of the single trait (single environment) QTL. After the initial scan (SIM), the loci having a test statistic larger than the threshold (*-log10(P)*) were detected, and the ones having the largest *–log10(P)* on each chromosome were selected as co-factors for CIM. Several rounds of CIM were done until no new QTLs were detected. QTL mapping for BS was done with data from flush 1, 60 minutes after bruising and flush 2, 60 minutes and 24 hours after bruising (BS160, BS260 and BS224). The same set was used to map QTL for CC (CC160, CC260 and CC224). Combined data (60 min and 1 hour) of flush 2 (CCflush2 and BSflush2) were used for QTL detection as well in order to increase the power to find consistent QTL. Set 1 and Set 2 were divided into two groups according to the cap color, i.e., a non-white group (off-white, light brown and brown) and a white group. QTL mapping for BS was also done for both non-white and white groups separately in order to avoid possible interference from cap color. Only results from the composite interval mapping are shown in this chapter. Because of the low

resolution of the genetic linkage map QTLs on the same chromosome were considered as one QTL, which were named after the chromosome (CHR) number.

Results

Statistical analysis of BS and CC of Population 1 (Heterokaryon Set 1 & 2)

Heterokaryon Set 1 and 2 were generated from homokaryotic population 1 crossed with two different tester lines. A summary statistics of BS and CC was done for the original heterokaryotic lines Horst U1 and WB2, the heterokaryon Cr012 used to generate offspring 1 and heterokaryons of Set 1 and Set 2 (Table 1). Data at four different time points were analyzed independently. Heterokaryotic lines WB2 and Horst U1 were both less sensitive to bruising than the sensitive line WW7 as found in previous tests (Weijn et al., 2012b), and WB2 was significantly less sensitive to bruising than Horst U1 at all the four different time points (*P<0.05*). The BS of the heterokaryon Cr012 (Mes09143 x H97) was close to the mid-parent (mean of the two original heterokaryons). Cr012 showed a higher BS124 than both original heterokaryons in the first a cultivation test (Set 1), and no clear explanation was found for this deviation. Since WB2 is brown and Horst U1 is white the CC value (whiteness index of cap) of WB2 is much lower than Horst U1. The standard deviation of CC-WB2 was higher than that of CC-Horst U1 indicating a large variation in cap color of this brown strain. The cap color of the hybrid (Cr012) was on average close to its brown parent WB2, which indicated the existence of the dominant brown allele as found in previous studies (Callac et al., 1998).

The average BS value of heterokaryon Set 2 was significantly higher (more sensitive) than that of heterokaryon set 1, and the average CC value of heterokaryons of Set 2 is lower (darker) than that of heterokaryons of Set 1 (P<0.05). This indicates the significant influence of different genetic backgrounds (tester lines) on the phenotypic expression of BS and CC.

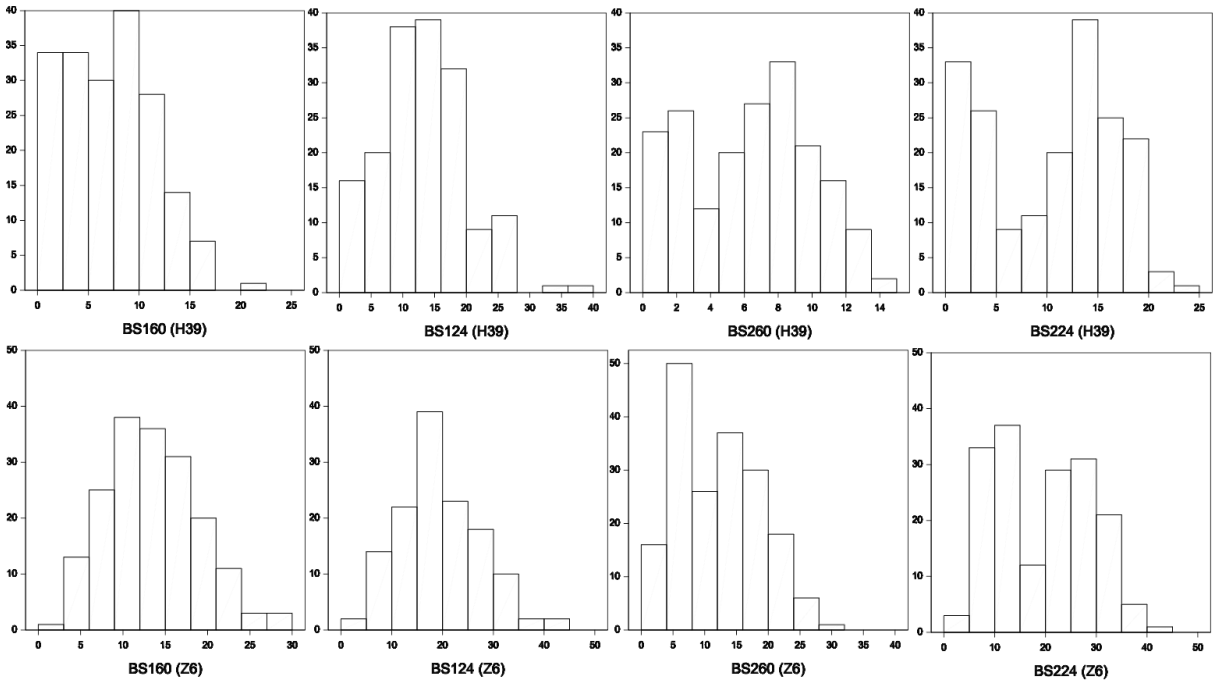


Figure 2. Frequency distribution of bruising sensitivity of population 1.
BS of heterokaryons of Set 1 and Set 2 is distinguished with their respective tester lines, i.e., H39 for Set 1 and Z6 for Set 2. The horizontal axis indicates value range of BS, and the vertical axis indicates the frequency of individuals.

The BS and CC distribution of population 1 (Set 1 & 2) is shown in Figure 2 and Figure 3 respectively. The continuous distribution of BS indicates its polygenic feature. A typical bimodal distribution was observed for CC in Figure 4, which was consistent with the fact that cap color, i.e., white or non-white, was indeed mainly controlled by one locus. Clearly two groups are present, i.e. non-white group with large variation of CC, and white-group with smaller variation. To a lesser extent, also a bimodal distribution of BS was found for flush 2 only (Figure 2). Specifically, the non-white individuals of Set 1 and Set 2 were less sensitive to bruising than the white individuals indicating the possible co-segregation of loci for BS and CC.

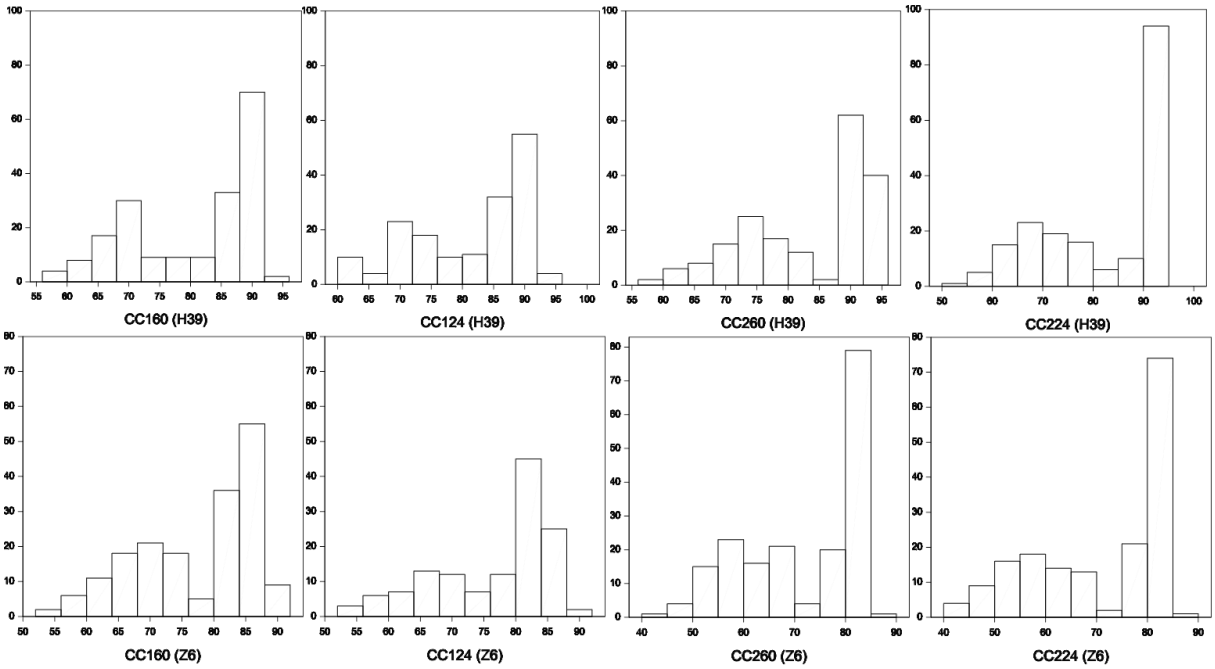


Figure 3. Frequency distribution of cap color of population 1.
CC of heterokaryons of Set 1 and Set 2 is distinguished with their respective tester lines, i.e., H39 for Set 1 and Z6 for Set 2. The horizontal axis indicates value range of BS, and the vertical axis indicates the frequency of individuals.

Transgression (individuals more sensitive than the original heterokaryotic lines) was found for both BS and CC in Set 1 & Set 2 using multiple comparison (SNK test, $p<0.05$). Transgression for BS was found in both Set 1 and Set 2 at all the measured time points, but transgression for bruising resistance (BR, individuals more resistant than WB2) was found only with data of BS124 and BS260 of Set 1. No transgression for BR was found in Set 2, which might be due to the high BS level of the tester line Z6. Besides, the mushroom quality of Set 2 was very bad showing early maturation, low firmness, and irregular appearance (data not shown). In both Set 1 and Set 2 transgression for brown cap (color) was found with data at almost all the time points (apart from data of 24h flush 1), i.e., some progeny had a significantly darker cap color than the brown heterokaryotic line WB2. Transgression for white cap (color) was only found with data of 60 min (CC160 & CC260) in Set 1 and data of flush 1 (CC124 & CC160) in Set 2, i.e., some individuals of Set 1 and Set 2 were whiter than the white heterokaryotic line Horst U1 at certain time points.

Set 1 and Set 2 were split up into two groups (non-white and white) based on the observation of flush 1 of Set 1 (Table 1). The mean BS of the non-white group was significantly lower than that of the white group. As expected, the mean CC value of the non-white group was significantly lower than that of the white group ($P<0.001$). The standard deviation (SD) of the CC-non-white was larger than that of CC-white, indicating the larger variance of cap color in non-white mushrooms compared to white mushrooms (SD is shown in parentheses in Table 1). The average whiteness index for Set 1 & Set 2 also differed, obviously

caused by the different testers (H39 & Z6) used (Table 1). That resulted in a shift for some individuals from white in Set 1 to off-white in Set 2.

Table 1. Summary statistics for BS and CC of population 1 (Heterokaryon Set 1 & 2)

		Original and hybrid heterokaryons			Progeny heterokaryons			
	Trait	Horst U1	WB2	Cr012	Whole set	Non-white	White	Min-Max
BS Set 1	BS160	9.54 (2.25)	1.07 (1.37)	6.42 (4.50)	7.22 (4.94)	3.39 (2.13)	10.30 (2.84)	0-20.04
	BS124	13.36 (2.99)	4.37 (3.16)	15.42 (5.34)	13.07 (7.83)	7.92 (4.59)	17.24 (5.56)	0.40-37.97
	BS260	7.61 (2.09)	4.09 (2.90)	4.53 (3.78)	6.35 (4.54)	3.30 (2.53)	8.84 (2.26)	0.21-14.94
	BS224	13.49 (2.97)	5.71 (2.69)	8.12 (4.76)	10.11 (7.16)	4.23 (3.53)	15.24 (3.05)	0.10-24.30
BS Set 2	BS160	6.80 (2.88)	2.96 (3.29)	4.53 (2.30)	13.22 (6.96)	9.54 (3.76)	16.58 (4.58)	2.24-29.14
	BS124	11.78 (4.63)	3.81 (3.16)	5.41 (2.14)	19.15 (9.15)	13.45 (6.48)	22.98 (6.48)	3.39-40.17
	BS260	6.46 (1.96)	2.42 (2.75)	4.64 (2.75)	11.45 (7.14)	6.14 (2.43)	16.81 (4.46)	1.06-31.56
	BS224	9.84 (3.02)	3.81 (3.16)	5.44 (2.66)	18.38 (10.06)	9.83 (2.90)	26.39 (5.43)	3.05-41.85
CC Set 1	CC160	89.58 (1.04)	66.74 (3.74)	64.43 (3.80)	80.73 (10.56)	70.29 (6.06)	88.67 (1.63)	56.95-92.28
	CC124	92.73 (1.49)	66.08 (3.21)	64.95 (2.75)	80.71 (9.87)	71.82 (6.00)	88.28 (2.33)	60.38-92.54
	CC260	90.64 (1.55)	77.46 (2.92)	66.79 (3.62)	83.35 (10.68)	72.99 (5.90)	91.54 (1.28)	57.36-93.92
	CC224	93.00 (1.19)	76.16 (3.35)	65.18 (4.41)	81.67 (12.69)	69.48 (6.55)	91.78 (1.47)	53.23-93.94
CC Set 2	CC160	86.13 (2.49)	64.27 (7.89)	62.51 (8.35)	76.88 (10.62)	67.2 (5.94)	84.77 (2.43)	44.92-90.44
	CC124	84.94 (1.83)	57.76 (4.14)	56.46 (4.26)	76.00 (9.90)	66.67 (6.61)	82.86 (2.49)	53.49-88.78
	CC260	80.34 (1.13)	60.41 (4.06)	71.73 (3.17)	71.06 (12.09)	59.67 (6.58)	81.11 (2.04)	44.92-85.07
	CC224	86.51 (1.21)	57.76 (4.14)	68.76 (3.29)	69.84 (13.60)	56.85 (7.10)	80.98 (2.79)	40.25-85.31

Mean values and standard deviations (in parentheses) of heterokaryons are shown in this table. The data range of the heterokaryons set is shown in the last column.

Two-way ANOVA was performed with SQRT of BS & CC across time after bruising (data of flush 2, Set 1) and across flushes (data of 60min, Set 1). Untransformed data were used for ANOVA to test the effect of tester lines. Results of ANOVA showed that the genotype was a significant factor for both BS and CC (α =0.001) (Table 2). BS and CC were also significantly influenced by time (after bruising), flushes, and tester lines (across Set 1 & Set 2) (α=0.001). In average, the discoloration level of heterokaryons of Set 1 and Set 2 at 24 h after bruising (BS224) was significantly higher than that at 60 min after bruising (BS260); BS of flush 2 (BS260) was significantly lower than that of BS of flush 1 (BS160); the mean BS of Set 1 (population 1 crossed with tester H39) was significantly lower than that of Set 2 (population 1 crossed with tester Z6). The interactions of genotype by time, genotype by flushes and genotype by tester lines were all significant (α=0.001) indicating that each genotype reacts differently with respect to these factors and the interaction effects should be taken into account when assessing BS and CC.

Broad-sense heritability (H^2) was high for both BS and CC at all the individual time points and also across time, flush and tester lines (Table 2), which varied from 0.86 to 0.99. The high heritability indicated that BS and CC are both highly inheritable traits, and genotypic effect was the main effect to generate phenotypic variations for BS and CC.

Table 2. ANOVA, heritability of BS and CC across time, flush, and tester lines (Population 1)

Source of variation (BS)	d.f.	m.s.	F	Source of variation (CC)	d.f.	m.s.	F
	SQRT of BS260 & BS224 (Set 1)				SQRT of CC260 & CC224 (Set 1)		
G	197	19.45	55.83	*G*	197	10.46	190.03
T	1	706.78	2028.69	*T*	1	5.3	96.31
G × T	194	1.51	4.34	*G × T*	194	0.14	2.57
error	6017	0.35		*error*	6017	0.06	
H^2		**0.93**		H^2		**0.99**	
	SQRT of BS160 & BS2260(Pop 1)				SQRT of CC160 & CC260 (Pop 1)		

G	197	13.72	44.61	*G*	197	8.72	181.9
F	1	39.97	129.94	*F*	1	38.42	800.95
G × F	193	2.22	7.21	*G × F*	193	0.6	12.46
error	6643	0.31		*error*	6643	0.05	
H²		**0.88**		***H²***		**0.96**	
	BS160-H39 & BS160-Z6				CC160-H39 & CC160-Z6		
G	191	848.94	55.96	*G*	191	4129.55	247.56
C	1	75919.9	4927.95	*C*	1	30595.98	1791.95
G × C	182	180.62	10.06	*G × C*	182	318.74	21.31
error	8909	14.37		*error*	8909	15.52	
H²		**0.86**		***H²***		**0.95**	

G: genotype, T: time after bruising, F: flush, C: tester line G × T: genotype and time interaction, G × F: genotype and flush interaction, G × C: genotype and tester line interaction, H^2: heritability.

Correlation of data across time points (60 min & 24 h), flushes (flush 1 & flush 2) was tested with spearman's rank correlation. For both BS and CC, data across time (after bruising) and flushes were highly correlated. The correlation coefficients of CC across time and flushes were higher compared to those of BS (Table 3). The correlation of BS or CC across two tester lines (H39 and Z6) was tested based on the mean value of BS160 and CC160 (sample size: 180). BS160-Set 1 and BS160-Set 2 highly correlated (r=0.70, α=0.001), and CC160-Set 1 and CC160-Set 2 also highly correlated (r=0.77, α=0.001). The high correlation of different data points underlines the consistency in phenotyping BS and CC in this experimental setup.

Table 3. Correlation coefficient of BC and CC in Set 1 (sample size: 172) and Set 2 (sample size: 133)

Bruising sensitivity					Cap color				
Set 1	BS124	BS160	BS224	BS260	**Set 1**	CC124	CC160	CC224	CC260
BS124	1				CC124	1			
BS160	0.855	1			CC160	0.916	1		
BS224	0.685	0.803	1		CC224	0.818	0.827	1	
BS260	0.634	0.765	0.927	1	CC260	0.817	0.847	0.956	1
Set 2	BS124	BS160	BS224	BS260	**Set 2**	CC124	CC160	CC224	CC260
BS124	1				CC124	1			
BS160	0.862	1			CC160	0.818	1		
BS224	0.668	0.694	1		CC224	0.795	0.800	1	
BS260	0.673	0.721	0.942	1	CC260	0.773	0.791	0.973	1

Tested with spearman's rank correlation, all correlations are significant at α=0.001 level.

BS and CC of Population 2 (Heterokaryon Set 3)

The summary statistics for BS and CC of heterokaryon set 3 is presented in Table 4. The two original heterokaryotic lines (Horst U1 and WW7) showed significant difference in both BS and CC (P<0.001). WW7 was much more sensitive to bruising than Horst U1 as expected, and WW7 was less white than Horst U1. The BS of heterokaryon Cr002 was intermediate compared to its two original heterokaryotic lines (Horst U1 & WW7), and CC of the heterokaryon Cr002 was generally close to the mid-parent as well. No transgression was found in population 3 for BS, while transgression was found for CC, i.e., some individuals were significantly whiter than Horst U1 (P<0.05). The continuous distribution for both BS and CC of Set 3 indicates the polygenic features for both BS and CC (Figure 4).

Table 4. Summary statistic for BS and CC of population 2 (heterokaryon Set 3)

	Original heterokaryons and hybrids			Progeny heterokayrons	
Trait	U1	WW7	Cr002	Mean	Min-Max

BS160	11.60 (3.64)	36.22 (4.85)	22.68 (5.30)	20.67 (6.15)	11.20-33.68
BS124	20.48 (3.10)	47.85 (4.41)	27.90 (4.67)	29.25 (8.13)	15.28-44.18
BS260	6.20 (1.81)	29.14 (6.23)	17.86 (4.08)	20.02 (6.36)	9.97-31.58
BS224	11.01 (3.07)	39.72 (6.78)	24.06 (4.50)	25.95 (7.56)	14.13-45.00
CC160	88.83 (1.63)	81.68 (3.73)	84.73 (2.22)	87.69 (2.40)	80.82-90.66
CC124	89.43 (1.90)	83.85 (3.37)	82.61 (1.75)	88.36 (2.72)	79.41-97.82
CC260	91.04 (1.94)	82.75 (2.13)	86.98 (2.20)	89.20 (2.58)	84.12-93.01
CC224	91.85 (1.47)	82.68 (3.10)	88.30 (2.20)	89.53 (2.86)	82.48-94.57

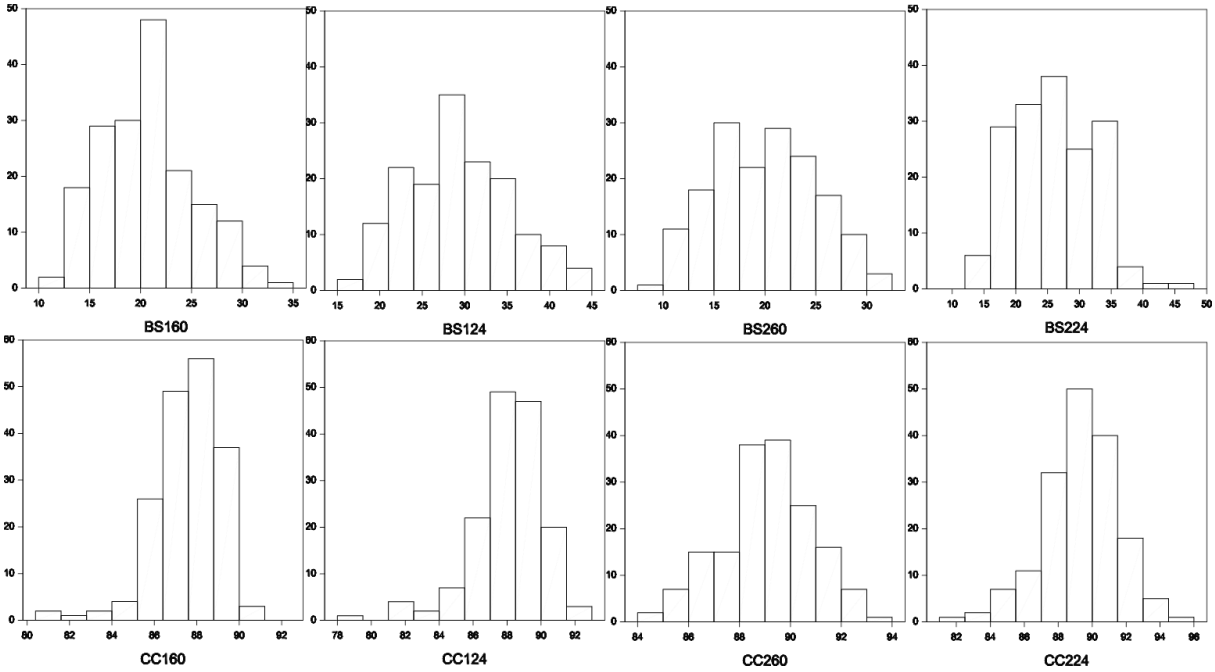


Figure 4. Frequency distribution of BS and CC of population 2 (Heterokaryon Set 3)

One-way ANOVA was performed with BS and CC of the four different time points respectively. Two-way ANOVA was performed across time (after bruising) and flushes. Significant genotypic effects were observed for both BS and CC (α=0.001). BS and CC were significantly influenced by time (after bruising) and flushes (α=0.001); the interactions of genotype by time, genotype by flushes were both significant (α=0.001).

Broad-sense heritability (H^2) was high for both BS and CC at all the time points (Table 4) and across time, flush and tester lines (Table 5), which varied from 0.80 to 0.97. The high heritability in Set 3 indicates again that BS and CC are highly inheritable traits, and the genotypic effects are the main effects to generate phenotypic variations for BS and CC. The BS and CC of different time point was highly correlated (Table 6). The correlation coefficient (r) of BS across time and flushes was generally higher compared to that of CC.

Table 5. Analysis of variance, heritability of BS and CC across time, flush, and tester lines (Population 2)

Source of variation	d.f.	m.s.	F	Source of variation	d.f.	m.s.	F
	BS260 & BS224				CC260 & CC224		
G	177	1083.05	64.74	*G*	177	121.11	31.24
T	1	52985.91	3167.12	*T*	1	147.11	37.94
G × *T*	174	30.57	1.83	*G* × *T*	174	6.58	1.7

error	5608	16.73		*error*	5608	3.88	
H²	**0.96**			***H²***	**0.93**		
	BS160& BS260				CC160 & CC260		
G	193	916.58	53.76	*G*	193	91.14	23.97
F	1	766.27	44.94	*F*	1	4553.77	1197.41
G × F	173	137.7	8.08	*G × F*	173	22.47	5.91
error	8635	17.05		*error*	8635	3.8	
H²	**0.89**			***H²***	**0.80**		

G: genotype, *T*: time after bruising, *F*: flush, *G × T*: genotype and time interaction, *G × F*: genotype and flush interaction, H^2: heritability.

Table 6. Correlation coefficients of BC and CC of heterokaryon Set 3, sample size: 152

	BS124	BS160	BS224	BS260		CC124	CC160	CC224	CC260
BS124	1				CC124	1			
BS160	0.87	1			CC160	0.76	1		
BS224	0.60	0.70	1		CC224	0.55	0.55	1	
BS260	0.61	0.72	0.94	1	CC260	0.54	0.60	0.90	1

Tested with spearman's rank correlation, all correlations are significant (α=0.001).

QTL detection in Population 1 (Heterokaryon Set 1 and 2)

Bruising sensitivity

Since heterokaryon Set 1 and 2 were generated from population 1 crossed with two different tester lines, results of Set 1 and Set 2 are presented together. With the availability of the genome sequence and the physical positions of SNP markers, the linkage group of the genetic map in this study is equivalent to chromosomes, i.e., the linkage group is numbered after the chromosome number. Because of the short genetic linkage map (due to the low recombination frequency), the upper and lower bounds of QTLs almost cover whole chromosomes apart from chromosome 8. Across data of all time points, common QTLs for BS were detected on CHR 3, 8 and 10 in Set 1 (Table 7) and on CHR 7, 8 and 10 in Set 2 (Table 8). The major QTL for BS was on CHR 8 in both Set 1 and Set 2. In Set 1 it explained 64% (BS260) to 82% (BS224) BS variance, and in Set 2 it explained 44% (BS160) to 82% (BS224) BS variance. The high value (more sensitive to bruising) allele of this QTL was from H97, i.e., the beneficial allele of this QTL for low bruising sensitivity was from Mes09143. The high value allele of QTLs on CHR 7 was also from H97, and high value alleles of QTLs CHR 3 and 10 were consistently from Mes09143. The differences in QTL detected for Set 1 and Set 2 indicate the impact of tester lines on phenotype expression. QTL detected with data of flush 2 (BS160 and BS224) were generally consistent, whereas some QTL are only detected in flush 1 or flush 2. For example, apart from the three consistent QTL on CHR 3, 8 and 10 in heterokaryon set 1, three extra QTL on chromosome 1, 6 and 12 were specific for BS160 but not with data of flush 2, and one QTL on CHR 4 was specific for BS224. In addition to the three consistent QTL on CHR 7, 8, 10 detected in heterokaryon set 2 a QTL on CHR 9 was found with both BS 260 and BS224 but not with BS160. Different QTL were detected over data of different flush and time points indicating the interaction effects of QTL by flush and QTL by time points.

BS was assessed by measuring the discoloration of the bruised area relative to the background (non-bruised area on the same mushroom). Although this will corrected for the cap color of the mushroom it is possible that the cap color interferes (Figure 5). Set 1 and Set 2 were, therefore, split up into a non-white (NW) and a white (W) group based on the cap color of heterokaryon Set 1 (Flush 1). As expected, the major QTL on chromosome 8 disappeared, while the minor QTLs detected in the whole set of heterokaryons became more significant in the two groups.

Figure 5. The visible bruising sensitivity of brown heterokaryons and white heterokaryons (Set 1, 60min). A similar level of BS is less visible in the brown cap color background.

Similar to the QTL mapping with the whole set of heterokaryons, consistent QTL were detected for flush 2 data of Set 1, i.e., QTL on CHR 3 and CHR 10 were detected in the non-white group with both NW-BS260 and NW-BS224, and QTL on CHR 2, 3, and 10 were detected in the white group with both W-BS260 and W-BS224 (Table 7). Thus QTL on CHR 3 and CHR 10 were consistent in both non-white and white groups for BS of Flush 2. Different from QTL detected in flush 2, consistent QTL for non-white and white group in flush 1 are located on CHR 1 and CHR 3. In addition, QTL CHR6 was specific for NW-BS160, and CHR 9 and CHR 12 were specific for W-BS160. All QTLs detected in non-white and white groups separately had higher explained variance than those detected in the whole set of heterokaryons. The most significant QTL was on CHR 10 with the explained variance up to 34% for W-BS260. The high value alleles of CHR 3, 9, and 10 were from the parental homokaryon Mes09143, and those of CHR 1, 2, 6 and 12 were from the parental homokaryon H97. In summary, most minor QTL (all except CHR8) are found in the whole set of heterokaryons and in the white & non-white groups separately, and the explained variance was higher in the two groups.

Table 7. QTL for bruising sensitivity of heterokaryon Set 1

Traits	Locus Name	Linkage group	Position	-log(p)	%Explained Variance	Additive effects	High value allele	Lower bound	Upper bound
BS160	MHchrI_01	1	2.21	7.25	3.93	0.846	H97	0	7.09
	C3P3	3	2.72	9.74	5.48	0.998	Mes09143	0	4.357
	MHchrVI_01	6	23.96	3.30	1.50	0.522	H97	0	30.101
	MHchrVIII_03	8	29.5	59.09	71.21	3.598	H97	14.469	44.535
	ChrX_B1	10	0.62	4.37	2.21	0.634	Mes09143	0	0.62
	MHchrXII_05	12	0.31	3.09	1.39	0.502	H97	0	4.815
NW-BS160	ChrI_M1	1	1.19	5.99	17.80	0.865	H97	0	7.303
	MHchrIII_05	3	2.44	5.89	16.43	0.831	Mes09143	0	3.631
	MHchrVI_02	6	27.65	7.17	21.61	0.953	H97	0	35.103
W-BS160	ChrI_M1	1	1.92	5.45	11.62	0.967	H97	0	6.93
	MHchrIII_05	3	0.97	5.47	11.30	0.954	Mes09143	0	4.89
	ChrIX_B3	9	0	4.80	10.46	0.918	Mes09143	0	1.16
	ChrX_B1	10	0	6.42	16.52	1.154	Mes09143	0	0
	ChrXII_B1	12	5.84	5.23	10.61	0.924	H97	0	5.838
BS260	ChrIII_M1	3	4.36	7.05	4.80	0.788	Mes09143	0	4.357
	C8P28	8	27.79	47.37	64.43	2.890	H97	10.491	45.089
	ChrX_B1	10	0.62	15.66	13.38	1.317	Mes09143	0	0.62

NW-BS260	ChrIII_M1	3	0	2.91	9.02	0.776	Mes09143	0	3.631
	ChrX_B1	10	0	7.67	31.24	1.444	Mes09143	0	1.481
W-BS260	ChrII_B1	2	4.81	4.28	9.90	0.686	H97	0	4.814
	ChrIII_M1	3	4.89	3.56	7.64	0.603	Mes09143	0	4.89
	ChrX_B1	10	0	10.74	33.89	1.269	Mes09143	0	0
BS224	C3P3	3	2.72	6.95	3.44	1.166	Mes09143	0	4.357
	ChrIV_T2	4	0	2.88	1.19	0.687	Mes09143	0	1.051
	C8P28	8	27.79	65.19	81.8	5.689	H97	15.298	40.282
	ChrX_B1	10	0.62	11.11	6.05	1.547	Mes09143	0	0.62
NW-BS224	ChrIII_M1	3	0	3.19	10.72	1.179	Mes09143	0	3.631
	ChrX_B1	10	0	6.28	25.52	1.819	Mes09143	0	1.481
W-BS224	MHchrII_03	2	3.87	2.92	8.12	0.837	H97	0	4.814
	MHchrIII_05	3	0.97	2.58	6.66	0.758	Mes09143	0	4.89
	ChrX_B1	10	0	5.17	17.37	1.225	Mes09143	0	0

High value allele: the origin of the allele having higher BS. Threshold: -log(P)=2.8

In Set 2, minor (non CHR8) QTL on CHR 3 and 7 were detected with both NW-BS160 and W-BS160; QTL on CHR 4, 7 and 9 were detected with W-BS260, but no QTLs were detected with NW-BS260. No consistent QTL were detected with NW-BS224 (CHR 6 and 10) and W-BS224 (CHR 3 and 7) (Table 8). CHR7 was a consistent and the most significant minor QTL over different time points of Set 2, and the explained variance was up to 25% for W-BS260. The high value allele of CHR7 was consistently from the parental homokaryon H97. Fewer QTLs were found in the two groups of Set 2 separately compared to the whole set of heterokaryons. For instance, no QTL was detected with NW-BS260, and the one on CHR 10 detected in the whole set of heterokaryons (BS160 & BS260) was not detected in either NW or W group. This might be due to the small subpopulation size, and the phenotypic variation was not big enough to detect the marker-trait association. Nevertheless, there are still extra QTLs found in the NW & W groups but not in the whole heterokaryon Set 2. The one on CHR 4 was detected with W-BS160 but not with BS160, and the one on CHR 6 was detected with NW-BS224 but not with W-BS224. In summary, QTLs on CHR 3 and CHR 7 were generally consistent for both Set 2 heterokaryons and NW & W groups.

Table 8. QTL for bruising sensitivity of heterokaryon Set 2

Traits	Locus name	Linkage group	Position	-log(p)	%Expl. Var.	Add. eff.	High value allele	Lower bound	Upper bound
BS160	C3P2	3	1.69	9.02	9.63	1.70	Mes09143	0.00	4.49
	ChrVII_T2	7	2.83	10.42	11.44	1.85	H97	0.00	13.62
	MHchrVIII_03	8	31.26	29.51	43.79	3.63	H97	0.00	66.79
	ChrX_B1	10	0.63	3.12	2.77	0.91	Mes09143	0.00	0.63
NW-BS160	ChrIII_M1	3	3.78	3.08	11.98	1.31	Mes09143	0.00	3.78
	ChrVII_T2	7	3.76	2.77	11.00	1.26	H97	0.00	12.14
W-BS160	ChrIII_B1	3	0.00	4.40	10.98	1.49	Mes09143	0.00	5.04
	ChrIV_T2	4	0.00	2.88	6.43	1.14	H97	0.00	1.96
	ChrVII_B1	7	12.77	7.44	20.42	2.03	H97	0.00	14.83
BS260	MHchrIV_03	4	0.54	5.55	3.18	1.15	H97	0.00	1.09
	C7P7	7	7.15	6.56	4.32	1.34	H97	0.00	13.62
	C8P29	8	29.27	52.39	67.48	5.30	H97	12.32	46.22
	MHchrIX_03	9	2.37	5.14	2.86	1.09	Mes09143	0.00	2.91
	ChrX_B1	10	0.63	4.78	2.73	1.07	Mes09143	0.00	0.63

W-BS260	MHchrIV_02	4	0.32	5.28	14.32	1.73	H97	0.00	1.96
	C7P5	7	5.47	7.02	24.70	2.27	H97	0.00	14.83
	ChrIX_B3	9	0.00	2.74	6.38	1.15	Mes09143	0.00	1.17
BS224	MHchrII_03	2	2.75	4.02	1.64	1.20	Mes09143	0.00	6.07
	ChrIII_B1	3	4.49	6.65	3.01	1.62	Mes09143	0.00	4.49
	ChrVII_B1	7	1.74	3.84	1.59	1.18	H97	0.00	13.62
	C8P29	8	29.27	61.91	81.58	8.43	H97	16.20	42.34
	ChrIX_B3	9	2.91	2.83	1.07	0.97	Mes09143	0.00	2.91
	ChrX_B1	10	0.63	7.24	3.65	1.78	Mes09143	0.00	0.63
NW-BS224	ChrVI_T1	6	0.00	2.56	10.59	0.92	Mes09143	0.00	36.42
	ChrX_B1	10	0.00	3.49	15.76	1.12	Mes09143	0.00	1.55
W-BS224	ChrIII_B1	3	0.00	3.65	12.04	1.87	Mes09143	0.00	5.04
	MHchrVII_05	7	14.83	2.73	9.02	1.62	H97	0.00	14.83

Add. eff. : additive effect; %Expl. Var.: explained variance, high value allele: the origin of the allele having higher BS. Threshold: -log(P)=2.8

In conclusion, CHR 8 is the major QTL for BS for the whole heterokaryon Set 1 and Set 2. Chromosome 8 also contains the major QTL for cap color. Since the very low recombination frequency, a sorting for color also leads to sorting for this QTL and thus the absence of CHR8 in white and non-white groups separately. As a result, the significance of the other QTLs, which were minor in the whole set of heterokaryons, increased in significance. Most of the high BS alleles for all the other QTLs were consistent in Set 1 and Set 2. Exceptions are CHR 2 and 6 with a high value for H97 in Set 1 and for Mes09143 in Set 2, and CHR 4 with high value for Mes09143 in Set 1 and H97 in Set 2. This clearly shows the, possible epistatic, influence of the tester lines. In summary, CHR 3, 9 and 10 of H97 are beneficial alleles for bruising resistance, and Mes09143 has beneficial alleles on CHR 1, 7 and 8 for resistance to bruising.

Cap color

A major QTL for cap color was found on chromosome 8 in population 1 with both tester lines. It had an extremely high explained variance (R^2) for the phenotype, which varied from 77% to 85%. This is in agreement with an earlier study of Foulongne-Oriol et al., (2010). Apart from this major QTL, several minor QTL were found, but locations varied between different time points and heterokaryon sets (Table 9). Only the QTL on chromosome 5 was a consistent one in both Set 1 and Set 2. QTL on chromosome 1, 4, 5 and 8 were consistent for the second flush of Set 2 only. The –log (p) values of most minor QTL were just above the threshold, and they explained less than 3% of the CC variation. The high value (white) allele of the major QTL on chromosome 8 was from H97, while the high value alleles of the other minor QTL were generally from the brown parent Mes09143. It is known that brown is dominant over white and thus that the allele from the brown parent is the dominant one (Callac et al., 1998). The minor QTL might thus explain to a larger extent the variation in brown color seen in the non-white individuals of Set 1 and Set 2.

Table 9. QTL for cap color detected in heterokaryon Set 1 and Set 2

	Heterokaryon Set 1						Heterokaryon Set 2					
Traits	Locus name	LG	-log(p)	Explained Var.	additive effect	High value allele	Locus name	LG	-log(p)	%Expl. Var.	additive effect	High value allele
CC160	ChrII_T3	2	3.00	0.88	0.938	Mes09143						
	ChrIII_M1	3	4.73	1.50	1.228	Mes09143						
	ChrV_T1/	5	3.00	0.94	0.972	Mes09143						
							MHchrVII_05	7	3.86	1.60	1.191	Mes09143
	MHchrVIII_03	8	74.95	77.31	8.808	H97	C8P29	8	67.21	85.15	8.677	H97
	ChrX_B1	10	3.80	1.21	1.103	H97						
CC260							ChrI_B1	1	3.11	1.07	1.19	Mes09143
	MHchrII_04	2	3.15	1.10	1.053	Mes09143						

							MHchrIV_03	4	6.78	2.61	1.855	Mes09143
							MHchrV_01	5	4.50	1.57	1.439	Mes09143
	MHchrVIII_03	8	71.00	80.04	8.985	H97	MHchrVIII_03	8	74.08	84.73	10.57	H97
CC224							ChrI_B1	1	4.14	1.53	1.589	Mes09143
	MHchrII_04	2	3.19	0.91	1.141	Mes09143						
							MHchrIV_03	4	6.78	2.67	2.101	Mes09143
							MHchrV_01	5	4.12	1.46	1.555	Mes09143
	MHchrVIII_03	8	79.38	84.75	11.015	H97	MHchrVIII_03	8	70.47	85.49	11.893	H97
	ChrX_B1	10	3.25	0.94	1.159	Mes09143						
CCflush2							ChrI_M1	1	3.92	1.58	0.093	Mes09143
	MHchrII_04	2	2.98	1.15	0.064	Mes09143						
							MHchrIV_03	4	6.22	2.67	0.122	Mes09143
							MHchrV_01	5	4.72	1.92	0.103	Mes09143
	MHchrVIII_03	8	66.15	78.12	0.53	H97	MHchrVIII_03	8	66.45	83.56	0.68	H97

%Expl. Var.: explained variance, high value allele: the origin of the allele having higher BS. Significant threshold: -log(P)=2.7

Population 2 (Heterokaryon Set 3)

Bruising sensitivity

Since for the construction of Heterokaryon Set 3 only homokaryons were used derived from white lines, only white mushrooms were produced. Over all time points, a total of 7 QTL on CHR 1, 2, 3, 5, 7, 8, 10 were detected for BS (Table 10). The ones on CHR 1 and 2 were the two major and consistent QTL. The QTL on CHR1 had an explained variance varying from 12% to 16%, and that on CHR2 varying from 28% to 42%. Two minor QTL on CHR 7 and 8 were only found for flush 1, and three minor QTL on CHR 3, 5 and 10 were specific for flush 2 (Table 10). The high BS alleles of all QTL were from the sensitive parental line Z8 indicating that alleles of this strain have a considerable undesirable effect on sensitivity to bruising. In contrast, alleles of H39 on CHR 1 and 2 are major beneficial alleles for bruising resistance. In summary, there is overlap between QTL detected in the three sets of heterokaryons. Across all time points, QTL on CHR 3, 8 and 10 were found in all three sets of hetetrokaryons. QTL were also shared by the white offspring in Set 1 and Set 3 for, i.e. the major QTL on CHR 1 for W-BS160 and CHR 2 with data of flush 2 (W-BS260 and W-BS224).

Table 10. QTL for bruising sensitivity of heterokaryon Set 3

Trait	Locus Name	Linkage group	Position	-log(p)	%Expl. Var.	Add. eff.	High value allele
BS160	ChrI_T2	1	1.86	12.782	16.034	1.815	Z8
	CHR2A1	2	0	19.122	27.842	2.392	Z8
	CHR7B1	7	0	3.598	3.576	0.857	Z8
	CHR8A1	8	5.57	6.517	7.233	1.219	Z8
BS260	ChrI_T2	1	1.86	9.608	12.072	1.771	Z8
	CHR2A1	2	0	25.124	42.105	3.308	Z8
	CHR3B1\HZchrIII_04	3	1.84	4.135	4.68	1.103	Z8
	ChrV_M1	5	10.4	3.051	3.113	0.9	Z8
	CHR10A1	10	22.74	3.584	3.551	0.961	Z8
BS224	ChrI_T2	1	1.86	10.592	15.941	2.526	Z8
	CHR2A1	2	0	18.739	32.457	3.604	Z8
	CHR3B1\HZchrIII_04	3	1.84	4.346	5.853	1.53	Z8
BSflush2	ChrI_T2	1	1.86	11.17	15.523	2.208	Z8
	CHR2A1	2	0	21.639	36.069	3.366	Z8
	CHR3B1\HZchrIII_04	3	1.84	4.913	6.164	1.391	Z8

	CHR10A1	10	22.74	2.997	3.064	0.981	Z8

Cap color

A total of 4 QTL on CHR 2, 7, 10, and 12 were detected for CC in heterokaryon Set 3 (Table 11). The high value (white) alleles of the QTL on CHR 2 and 7 are both from the more white parent H39, but the other two QTL on CHR 10 and 12 have the high value alleles from the lesser white parent Z8. Consistent QTL for CC in Set 3 were located on CHR 2 and 7, and the major QTL is that on CHR7 which explains 15% to 20% CC variance. The two QTL on CHR 2 and 7 were also detected in Set 1 and Set 2, in which also a QTL was detected on CHR 2 with Set 1 data of all time points and a QTL on CHR 7 detected with CC160 in Set 2. However, the explained variance in Set 1 and Set 2 were much lower than that in Set 3. QTL for CC detected in Set 3 are relevant for retaining a white color or improving whiteness in breeding programs using only white varieties.

Table 11. QTL for cap color of population 2

Trait	Locus name	Linkage group	Position	-log(p)	%Expl. Var.	Add. eff.	High value allele
CC160	CHR2A1	2	0	3.779	5.787	0.375	H39
	CHR7B1/HZchrVII_05	7	1.16	9.018	17.041	0.644	H39
	CHR12B1	12	1.84	3.784	5.794	0.376	Z8
CC260	ChrVII_T3	7	2.32	8.588	19.654	0.793	H39
CC224	CHR2A1	2	0	3.26	5.594	0.514	H39
	ChrVII_T3	7	2.32	7.662	15.312	0.851	H39
	HZchrX_01/CHR10A1	10	9.48	4.079	9.624	0.674	Z8
CCflush2	CHR2A1	2	0	3.084	5.349	0.447	H39
	ChrVII_T3	7	2.32	8.17	16.952	0.796	H39
	HZchrX_01/CHR10A1	10	3.79	2.867	5.843	0.467	Z8

Add. eff. : additive effect; %Expl. Var. : explained variance, high value allele: the origin of the allele having higher BS

Discussion

Discoloration of white mushrooms after mechanical damage is one of the main quality issues for mushrooms harvested mechanically in the Netherlands and one of the obstacles to sell these for the fresh market. Varieties with a lower sensitivity for bruising can solve this problem. Discoloration is mainly caused by the formation of melanin and a previous study has shown that up to 42 genes are involved in its biosynthetic pathway (Weijn et al., 2012a). This shows the complexity of the trait bruising sensitivity and it might be that other, so far, unknown genes are involved too. Rather than using expression studies of candidate genes, we used segregating populations derived from parents with opposite phenotypes to map regions in the genome that can explain to a large extent resistance to bruising. In a previous study, breeding value was estimated in a selection of strains from the Plant Breeding collection (Gao et al., chapter 3). Three heterokaryotic parents, differing in bruising sensitivity, were selected to generate 2 segregating populations and map QTL for bruising sensitivity and cap color with the intention to provide a genetic basis for breeding button mushroom that is resistant to mechanical bruising. This is the first QTL analysis for bruising sensitivity of *A. bisporus* and also the first the QTL analysis for cap whiteness in a white population. Since reports on QTL analyses for edible fungi are scarce, this study contributes considerably to our knowledge on QTL analysis for edible fungi in general and to the understanding of complex traits in edible fungi.

Segregating populations and QTL mapping for bruising sensitivity

Two different segregating populations of homokaryons were generated for QTL mapping of bruising sensitivity. For mapping bruising sensitivity, fruiting bodies are required and in order to obtain these, homokaryons are mated with a compatible homokaryon (a tester homokaryon) to restore the heterokaryotic phase. For each locus, these mushrooms (F2) thus contain either the allele from parental homokaryon 1 or parental homokaryon 2 in one nucleus and all contain the same allele from the tester line in the other nucleus. If parental homokaryon 1 has allele A, parental homokaryon 2 allele B and the tester line allele C, then each heterokaryon formed from the segregating population has either the combination AC or BC at a genetic locus. In this way alleles of a segregating homokaryotic offspring are mapped in a shared genetic background of the tester line.

The two parental homokaryons (Mes09143 & H97) of population 1 both originate from resistant heterokaryons (WB2 and Horst U1). Several QTL were detected for bruising sensitivity and the beneficial alleles for resistance to bruising were indeed found in both parental lines with both tester lines. The use of different tester lines allows the study of traits in two different genetic backgrounds, i.e., a resistant background (H39) and a sensitive background (Z6). The two tester lines indeed had a large impact on the phenotype and this influenced the magnitude of QTL and to some extent the location. The bruising sensitivity of population 1 in the sensitive background (heterokaryon Set 2) was significantly higher than that in a resistant background (heterokaryon Set 1). The two parental homokaryons (H39 and Z8) of population 2 originate from a resistant heterokaryon (Horst U1) and a sensitive heterokaryon (WW7). The only tester line of this population is H97. The beneficial alleles of QTL for bruising resistance in heterokaryon Set 3 were detected only in the resistant parent H39. The tester line H97 and the parental line H39 are the two constituent homokaryons of the commercial line Horst U1. Like all present-day commercial lines, this strain has a certain resistance to bruising but the level of resistance is not enough to allow mechanical harvest for the fresh market. The intention of this study was, therefore, to map the beneficial alleles underlying the QTL in both constituent homokaryons of Horst U1 and in one of the constituent homokaryons (Mes09143) derived from a more resistant line WB2. This can be used to retain the bruising resistance in Horst U1 and add additional resistance from a more resistant strain in an introgression-breeding program. By crossing population 1 (offspring of H97 x Mes09143) with tester homokaryon H39, the beneficial alleles of the three resistant homokaryons (Mes09143, H97, and H39) can be stacked in one or more individuals of Set 1. Many heterokaryons of Set 1 were indeed more resistant to bruising than Horst U1 indicating that the beneficial QTLs of WB2 (Mes09143) were introduced in a Horst U1 background.

QTL analyses has been done so far only in an intervarietal population of *var. bisporus* × *var. burnetti* (Moquet et al., 1999, Foulongne-Oriol et al., 2012a, Foulongne-Oriol et al., 2012b) and not in an intravarietal population of *var. bisporus*. Since the recombination frequency is normal, offspring of intervarietal heterokayons generate linkage maps of high resolution. Here we used only lines from bisporic varieties because of the poor quality (and thus bruising sensitivity) of the tetra-sporic lines we have tested so far. The disadvantage of using only bisporic varieties is that linkage maps have a very low resolution and QTL can only be assigned to chromosomes. Mapping genes involved in recombination frequency and introducing this in breeding stock of bisporic varieties should be done in the near future.

Phenotypic data processing

Some non-white individuals had a negative BS, i.e., the whiteness index of control area (cap color) was lower (darker) than whiteness index of the bruised area. Inspection of the images by eye showed that some of these samples showed no discoloration at all (even not after 24 hours) but none of these showed a bruised area that was lighter than the non-bruised (control) area. This situation was observed also in a previous study (Gao et al., chapter 3). There are two likely explanations for these negative BS values. First, during bruising the thin skin of the mushroom cap can be disrupted revealing the white tissue underneath (brown mushrooms also have white tissue underneath the brown skin). Although care was

taken to avoid those areas, some images might contain bits of areas of disrupted skins. Secondly, a number of mushrooms have scales on the cap, especially visible in brown or light brown mushrooms. These patterns caused an uneven color of the mushroom cap that might explain the negative values. Since the absolute values of these negative BS are small, all negative values of these individual mushrooms were transformed to zero before calculating the means of each genotype. Some non-white genotypes showed variation in cap color or scaling for different mushrooms of the same genotype and on the same tray. Cap color and possibly scaling, are influenced by the developmental stage of the mushroom. Although we picked mushrooms at the peak of each flush and averages were taken from 20 mushrooms for each treatment for some genotypes not enough mushrooms could be picked on the same day for the same developmental stage. For some genotypes a peak of a flush is spread over more than a day and some genotypes mature rapidly. Larger trays will generate more mushrooms and a better choice for picking mushrooms all at the desired stage.

The measurements of bruising sensitivity at different time points were used for QTL mapping in this study. Some QTL were specific for one time point or one flush, which was in agreement with the fact that time after bruising, flush and tester lines all had significant effects on bruising sensitivity based on ANOVA. Different QTL detected with 60 min data and 24-hour data indicate that different mechanisms might be involved in bruise-induced discoloration and post-harvest discoloration of long-term storage. It was suggested in a previous study that the lack of interaction between bruising-induced discoloration and postharvest discoloration might indicate differences in the mechanisms of stimulating tyrosinase activity (Burton & Noble, 1993). Flush specific QTL might indicate that the different compost or casing conditions in flush 1 & 2 influence the trait and cause significant QTL by flush interaction.

Trait distribution

The bimodal distribution of cap color in heterokaryon Set 1 and Set 2 indicates that one major gene is responsible for cap color. Similar bimodal distributions for cap color were shown in previous studies (Foulongne-Oriol et al., 2012a). Although in one of the previous papers cap color was considered as a dominant trait (Callac et al, 1998), we often see in our breeding programs an incomplete dominance since a cross between homokaryons derived from a brown and a white line usually produce mushrooms with a lighter color than the brown line. The cap color of the F1 heterokayrons used to generate population 1 is close to the brown original line WB2. Since we could only recover one homokaryon (Mes09143) of this brown resistant line (WB2) we do not know the color allele of the other constituent nucleus and cannot conclude if the color close to the original brown line means complete or incomplete dominance. For BS also a bimodal distribution was observed in Set 1 and Set 2 indicating BS might also be controlled by one major locus. With cap color as a group factor, bruising sensitivity of the white group is significantly higher than the non-white group, which is in agreement with previous studies (Gao et al, 2013). The co-segregation of QTL for bruising sensitivity and cap color was observed in both population 1 and population 2. Due to the low recombination frequency it is difficult to tell if the QTL for cap color and bruising sensitivity represent the same genes. Bruising sensitivity is correlated with cap color at opposite directions within the non-white group and white group, i.e., the darker the mushroom the higher the resistance in the non-white group, and the whiter the mushroom the higher the resistance. For instance, BS160 positively correlated with CC160 in non-white ($r=0.3$, sample size 90), i.e., darker individuals had lower bruising sensitivity; BS224 negatively correlated with CC224 in the white group ($r=0.4$, sample size 105), i.e., whiter individuals had lower bruising sensitivity. That is to say, this major brown allele is for the most part responsible for the difference in bruising sensitivity between brown and white individuals, but not for the differences among white individuals. Heterokaryon Set 3 showed a continuous distribution of bruising sensitivity and cap color, which was in agreement with the polygenic feature of these two traits.

Cap color

The major QTL for cap color was found to be located on chromosome 8 in this study as well as in previous studies (Callac et al., 1998, Foulongne-Oriol et al., 2012a) and selection for this QTL separates the progeny in non-white and white. Major alleles are often accompanied with modifier alleles. Modifier alleles for cap color were found on several other chromosomes in population 1 and they are likely responsible for the cap color ranging from brown to very light brown. Based on the distribution histograms of CC160 (non-white) and CC160 (white), the large variation within the non-white group indicates that the effect of this major locus can be deflated by some modifier loci generating varied cap color. Based on data from population 1, it is difficult to conclude whether these modifier loci also determine the variation in whiteness of mushroom cap in a white population. In a previous study, the inheritance of cap color was studied in an offspring of a cream (off-white) × white cross. The cap color of progeny showed variations and was shown to be a polygenic trait instead of a single gene controlled trait (Miller *et al.*, 1974). With segregating population 2 generated from the cross of Z8 (less white)× H39 (white), QTL on CHR 2, 7, 10 and 12 for cap whiteness were detected in Set 3. Three of them were also detected in Set 1 and Set 2 apart from the one CHR 12. No QTL were detected on chromosome 8 for cap color in population 2. This implies that the brown allele on chromosome 8 is only a major determinant for non-white or white mushroom cap, and the QTL on other chromosomes are the genetic determinants for whiteness. Nevertheless, compared to the high heritability of cap color in heterokaryon Set 3, there is still a substantial part of whiteness variation that could not be explained by the detected QTL. This might indicate that there are undetected QTL for whiteness.

Gene expression and biochemical analysis in offspring

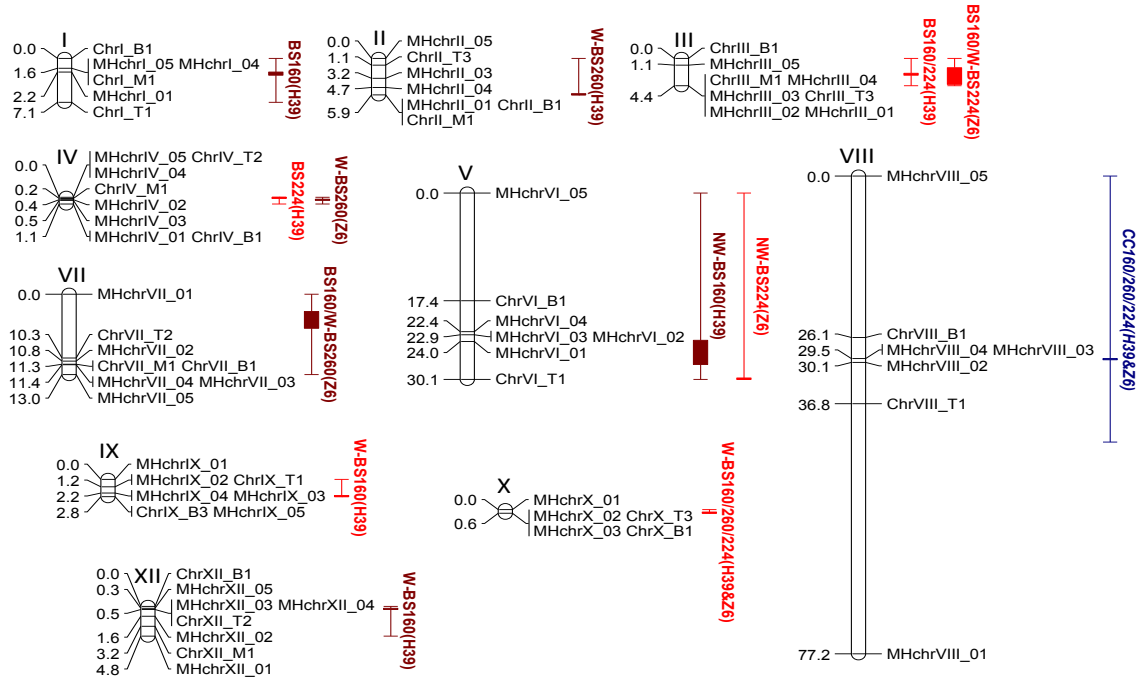
According to the melanin biosynthesis pathway, a number of enzymes and phenolic compounds might be involved in the mechanism of the bruised-induced discoloration (Weijn *et al.*, 2012a, Weijn *et al.*, 2013b). The level of gene expression and the phenolic compound are expected to associate with the degree of bruising sensitivity. Thus, next to the QTL analysis for bruising sensitivity, several individuals of heterokaryon Set 1 were selected and analyzed for gene expression (of the genes involved in the melanin biosynthesis pathway), also the phenolic compounds present in cap skin tissue of the selected individuals were determined. For most genes analyzed, no different expression was found between resistant and sensitive offspring. Only small differences in the expression level of three genes on chromosome 5, i.e., *L-chain*, *PPO_3* and *PPO_5*,were observed among several non-white individuals(Weijn, 2013). Nevertheless, chromosome 5 was not detected as a major QTL that associate with bruising sensitivity. Phenolic compound analysis was performed on the cap skin tissue of the selected individuals. A high correlation was found between the total concentration of phenolics and bruising sensitivity for the white individuals, but this correlation disappeared among non-white individuals. The lack of correlation between levels of gene expression and differences in bruising sensitivity might indicate that other, so far undetected, mechanisms are involved.

In conclusion, this study assigned QTLs for bruising sensitivity and cap color on chromosomes of button mushrooms and thus contributes to our understanding of the genetic base for those traits. However, the very low recombination frequency found in the bisporic varieties used here makes it difficult to assign QTL to chromosomal regions and thus finding candidate genes involved. It also hampers the introduction of the bruising insensitive in commercial lines since linkage drag will impede the restoration of the quality of a commercial receptor variety. This shows the need to introduce a “normal” meiotic recombination found in the burnettii variety into breeding stock.

Additional file 1

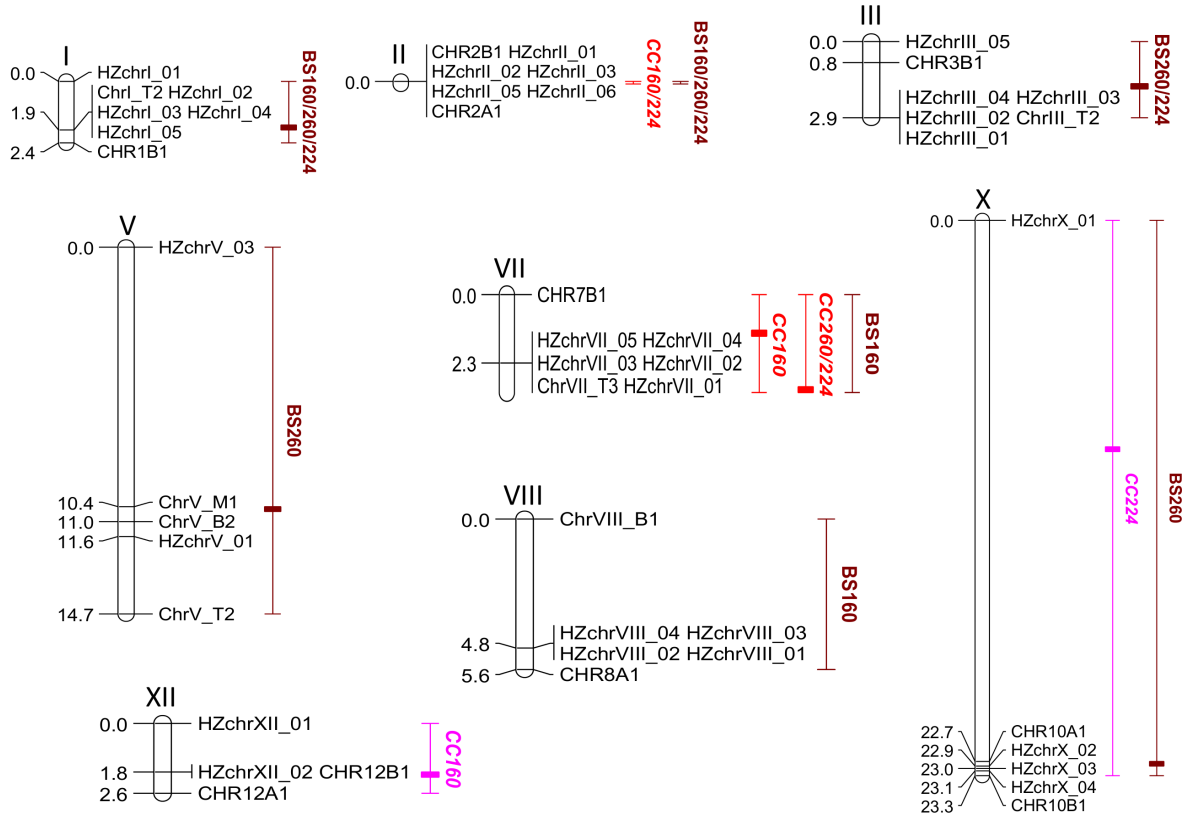
QTLs of BS and CC of population 1 (with explained variance higher than 10%) are shown in Additional file 1. Dark red indicates BS-QTLs with high value alleles from parental line H97; red indicates BS-QTLs with high value alleles from parental line Mes09143. QTLs start with BS are the ones detected in the whole population and also in both white and

non-white groups. QTLs start with W- or NW- are the ones detected only in white or in non-white groups respectively. The only major CC-QTL is presented in blue.



Additional file 2

QTLs of BS and CC of population 2 are shown in Additional file 2. Dark red indicates BS-QTLs having high value alleles from parental line Z8; red indicates CC-QTLs having high value alleles from H39; pink indicates CC-QTLs having high value alleles from Z8.



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Chapter 6

Multi-trait QTL analysis for multiple agronomic and quality characters of *Agaricus bisporus*

Wei Gao[1,2], Johan JP Baars[1], Chris Maliepaard[1], Richard GF Visser[1], Anton SM Sonnenberg[1]

1. Plant Breeding, Wageningen University & Research Centre, Wageningen, The Netherlands

2. Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing, PR China

Submitted

Abstract

The demand for button mushrooms of high quality is increasing. Superior button mushroom varieties require the combination of multiple optimal traits to maximize productivity and quality. Very often these multiple traits are correlated and should, therefore, be evaluated in combination with each other, rather than as single traits. In order to unravel the genetic basis of multiple traits of *Agaricus bisporus* and the genetic correlations among traits, multi-trait QTL analysis was conducted in three sets of heterokaryons. Significant phenotypic correlations were observed among different traits. The presence of pleiotropic QTLs shared by different traits genetically explains the phenotypic correlations. Two major pleiotropic QTLs on chromosome 10 and chromosome 6 explained most of the variations of all the tested traits. This study is the first multi-trait QTL analysis of a mushroom species incorporating multiple agronomic and quality traits, and it will provide a primary genetic architecture for understanding the nature of correlation between traits and a genetic basis for marker-assisted mushroom breeding of multiple agronomic and quality traits.

Introduction

The Homobasidiomycete *Agaricus bisporus* (button mushroom) is one of the most widely cultivated edible fungi in the world. Homobasidiomycetes are characterized by the fact that they contain two types of haploid nuclei with different mating types that stay side by side in each cell. Fusion of nuclei only takes place in basidial cells just before meiosis. Each diploid nucleus produces four haploid nuclei after meiosis and these are distributed over four spores formed by each basidial cell. The spores germinate into infertile haploid mycelia, i.e., homokaryons. Homokaryons with different mating types can anastomose and subsequent exchange of nuclei leads to the formation of fertile heterokaryotic (dikaryotic) mycelium (heterokaryons). The presence of both mating types within one mycelial cell triggers a developmental process leading to reproductive fruiting provided environmental conditions are favorable. This non-self compatibility or heterothallism is controlled by one or two unlinked loci. The majority of Homobasidiomycetes show this heterothallic life cycle. The button mushroom *A. 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 (Elliot, 1972, Summerbell et al., 1989). 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-tetrad mating, a form of selfing where mating occurs among the products of a single meiosis. It is rare that basidia 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 (Callac, 1993). This variety produces predominantly four-spored basidia and each spore germinates into homokaryotic mycelia. The two varieties are thus designated as *A. bisporus* var. *bisporus* and *A. bisporus* var. *burnetti*, respectively. Since var. *burnetti* is poor in various agronomic and quality traits, all commercially cultivated lines are *A. bisporus* var. *bisporus*. As in plants, fungal lines with contrasting performance are commonly crossed to generate segregating populations and used to map genomic regions involved in traits and identify candidate genes (Foulongne-Oriol, 2012). This is usually done by protoplasting the heterokaryotic parental lines and recovering the constituent nuclei as homokaryons which are subsequently used for outcrossing. In the button mushroom, mating between homokaryons is controlled by one mating type locus (MAT) (Xu, 1993). Homokaryotic offspring can be used directly as a segregating population for genetic linkage mapping (haplotyping). Since homokaryons are infertile they have to be crossed with a compatible homokaryotic tester line to produce mushrooms for QTL mapping. Genetic linkage maps have been generated so far in both bisporic populations of var. *bisporus* and intervarietal populations (Foulongne-Oriol et al., 2010, Kerrigan et al., 1993). The haploid genome size of *A. bisporus* is 30.4 Mb, it contains 13 chromosomes and has been sequenced completely (Morin et al., 2012).

Button mushrooms are cultivated worldwide with a product volume of 3.9 million tons and a total value of 4.7 billion dollars in 2009, mainly produced in China, the USA, Poland, the Netherlands, South Korea and France (Sonnenberg et al., 2011). During the last 30 years, mushroom productivity has increased substantially as a result of improved composting technique and optimized environmental conditions, and to a lesser extent by breeding efforts. The first hybrid cultivar Horst U1 (Fritsche, 1981) was released in the 1980s. Subsequent new varieties were identical or very similar to this first hybrid indicating that these were all varieties derived from the first hybrid line (Sonnenberg et al., 2005). Mushroom breeding has been so far an applied science and generating new varieties with sufficient qualities by outbreeding appears to be a difficult task (personal communications with spawn companies and personal experiences). No elite stocks are available for mushroom breeders, which means that for the introduction of new traits wild germplasm has to be used. Since knowledge on the genetic base of most traits is unknown, the use of wild lines leads to a long breeding program with uncertain outcomes.

New button mushroom varieties must at least meet the standards of the present varieties in order to be commercially viable. Important agronomic and quality traits are yield, earliness (first harvesting day), maturation (cap opening during mushroom development), size, color, shape, firmness, aroma, scaling (smoothness of the cap skin) and shelf life (post-harvest discoloration) (Pardo et al., 2010)(Gao et al 2013, submitted). Most of these traits are quantitative and have a complex genetic basis, associated with quantitative trait loci (QTL). In *A. bisporus* only a few genetic studies have been conducted to unravel the mechanisms of the agronomic and quality traits mentioned above (Callac et al., 1998, Foulongne-Oriol et al., 2011, Foulongne-Oriol et al., 2012a, Foulongne-Oriol et al., 2012b). Although some of these QTL studies considered multiple traits, they were analyzed separately thus neglecting possible genetic correlations between traits. Agronomic traits and quality traits often interrelated. For instance, a larger number of mushrooms were correlated with a greater overall yield (a total of three or four flushes), a smaller cap size, and an earlier first flush (Pardo et al., 2010). Similarly, a positive correlation was observed between earliness and yield in a previous study, and the earliest genotype tended to have higher yield and produce a larger number of smaller mushrooms (Foulongne-Oriol et al., 2012a). Quality traits like mushroom size, shape, firmness, scaling, maturity, and etc. change in time during the fruiting period, and their development is influenced by changing environmental factors. An integrated analysis by combining multiple traits can be more powerful than a collection of single-trait analyses and allows a more realistic analysis of the data since genetic correlations between traits can be directly modeled (Malosetti et al., 2008). Furthermore, the models of multi-trait QTL analysis allow the detection of closely linked QTLs or pleiotropic chromosomal regions that result in genetic correlations among traits (van Eeuwijk et al., 2010). The aim of multi-trait QTL analysis is to describe the genetic variation within traits, and the genetic correlations among traits in terms of the signs and magnitudes of QTL effects.

In this study, a total of 9 agronomic and quality traits of button mushroom were recorded in two segregating populations during the spawn run and fruiting period, i.e., the degree of compost colonization, mycelium recovery, earliness (time of production), cap color, firmness, maturity (openness), scales (smoothness), cap size, and stipe (stem) shape (length and thickness). The aim of this study was to identify the genetic basis of the overall quality of button mushroom with multi-trait QTL analyses and to dissect the genetic correlations among these traits. To our knowledge, this is the first study incorporating multiple traits in a multi-trait QTL analysis for mushroom quality. Our study will provide insights in the genetic architecture of agronomic and quality traits of button mushroom and will provide a basis for marker-assisted breeding for superior mushroom quality.

Materials and Methods

Lines and segregating populations

Two sets of 200 homokaryotic progeny of *A. bisporus* var. *bisporus* were developed in a previous study (Gao et al., Chapter 5) directed to detect QTL for cap discoloration after mechanical damage, i.e., bruising sensitivity (Figure 1). These lines also showed a substantial variation in agronomic and quality traits and were thus used in a separate analysis reported here to study segregation for those traits. The two sets of homokaryons (segregating populations) were crossed with three different tester lines (Figure 1) to generated heterokaryons that were cultivated and used to analyze QTL for a number of traits. The homokaryotic parental lines of a brown wild line WB2 (Mes09143) and a white wild line WW7 (Z6 & Z8) were recovered through protoplasting the vegetative mycelia. The parental homokaryons H97 and H39 were available from the original breeding program of the commercial hybrid Horst U1. Population 1 represents the offspring of a parental homokaryon of Horst U1 (H97) and a parental homokaryon of a wild line (Mes09143). The individuals were either crossed with tester line H39 (the other parental homokaryon of Horst U1) or with tester line Z6 (parental homokaryon of wild line WW7). In this way we could study the segregation in a mainly Horst U1 background or a mainly wild (WW7) background. Horst U1 represents the present standard for agronomic and quality traits for button mushroom in this way we can see how the introduction of wild germplasm affects its important traits. Population 2 represents the offspring of a parental homokaryon of Horst U1 (H39) and a parental homokaryon of a wild line (Z8). These individuals were crossed with tester line H97 (the other parental homokaryon of Horst U1). This allows the study of another wild germplasm in the genetic background of Horst U1. For the phenotyping, the three heterokaryotic parental lines (Horst U1, WB2, and WW7), and the two F1 hybrids (Cr012 & Cr002) were also included in the cultivation trials.

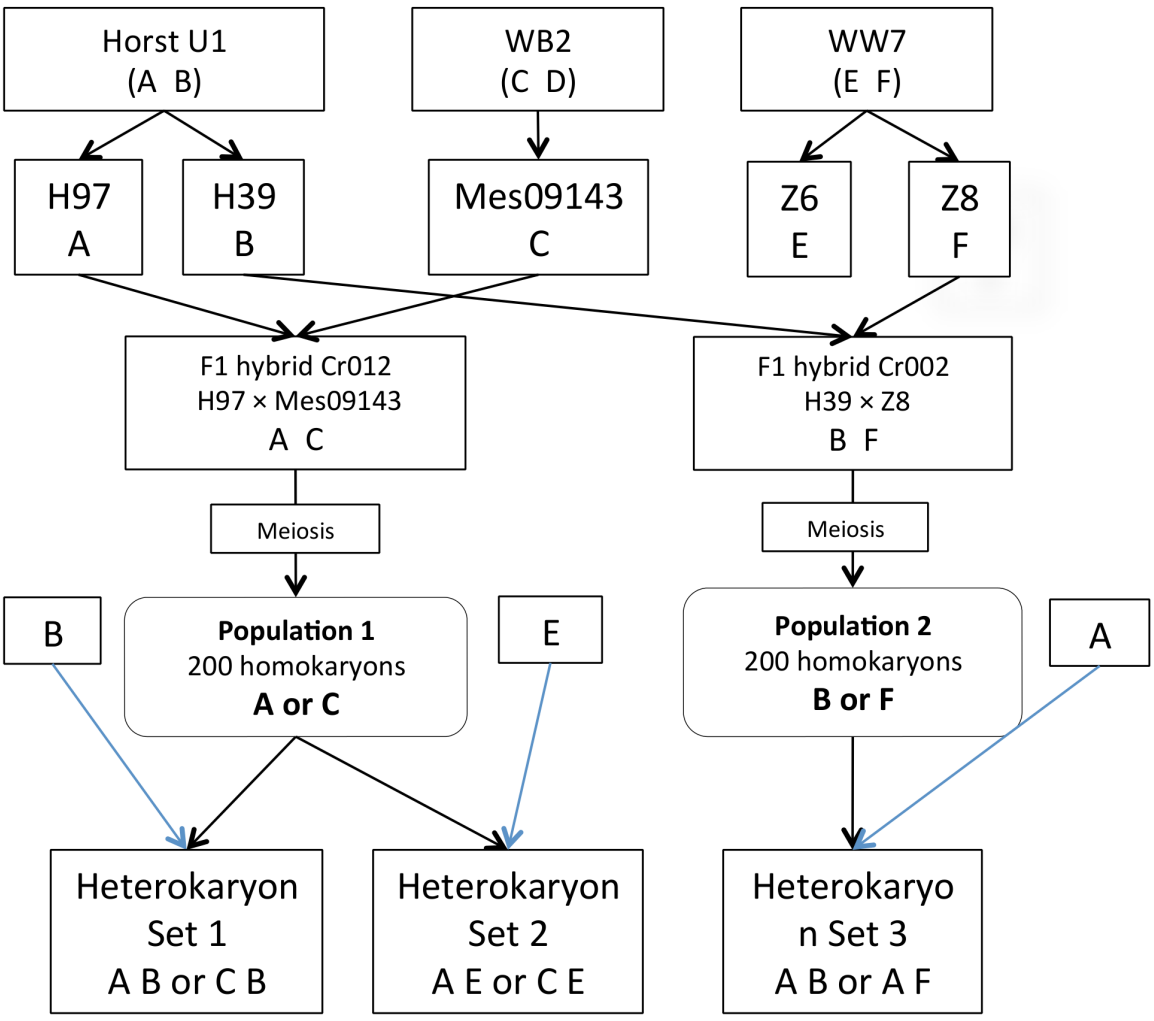

Figure 1. Pedigree of the two segregating populations and the three heterokaryon sets used in this study. It is assumed the genotypes of homokaryotic parents on one locus are A, B, C, E, and F for H97, H39, Mes09143, Z6, and Z8 respectively.

Experimental design and phenotypic evaluation

Three independent cultivation trials were carried out successively for the three sets of heterokaryons at the mushroom farm of Unifarm in Wageningen UR with controlled climate (relative humidity, temperature, and CO_2 level). Mushrooms were grown on commercial compost (CNC Substrates), spawned in 0.1 m^2 boxes (40 × 30 × 21 cm) filled with 8 Kg of compost. For all the three sets of heterokaryons each genotype has one replicate grown in one tray, and 200 trays (200 genotypes) of each heterokaryon set were randomly distributed in the growing room.

The level of compost colonization per genotype was recorded on a scale of 1 to 10 on day 14 after spawning, where a score 1 indicates very poorly colonized compost (or 10% surface colonization), and score 10 indicates very good colonization (or 100% surface colonization). All trays were covered with casing soil on the same day. Earliness (ER) was scored as time (days) between spawning (inoculation) and the first day of harvest (flush 1), and a high value for ER indicates late production. Apart from compost colonization and earliness all the other traits were scored at the peak time of two production flushes including cap color, firmness, maturity, scaling, cap size, stipe shape (flush 1 and flush 2). Cap color was scored as 1 to 5, and a high value indicates dark mushrooms. All traits were scored as an average of mushrooms of a whole tray. Firmness was estimated from 1 (very weak) to 5 (very firm) by squeezing the mushroom cap with thumb and index finger placed on opposite site of the cap. The maturation was scored visually as an association of size and developmental stage. It was scored as “1” if mushrooms were small (1 to 2 cm) and open (broken veil); mushrooms were scored as “4” when their cap diameters (size) reached to 4-5 cm and they were still closed (intact velum); mushrooms were scored as “5” when they were big (> 5 cm) and closed (intact velum). Some genotypes produce mushrooms as clusters on the growing beds that are an undesirable trait for growers. The production of all mushrooms as clusters was scored as “1”, and an even distribution of individual mushrooms was scored as “5”. Since environmental conditions of flush 1 and flush 2, e.g., the water potential of the casing soil and the nutritional content of compost, differ considerably, trait assessment for both flushes were considered as two independent traits. The type of traits measured, their range and coding are described in Table 1. The abbreviations labeled with a “1” indicate the scores for traits in flush 1, and those labeled with a “2” indicate scores for traits in flush 2.

Statistical analysis

All statistical analyses were performed with the program Genstat, version 15. Since five lines including the three heterokaryotic parental lines (Horst U1, WB2, WW7) and two F1 hybrids (Cr012, Cr002) were grown three times (in three cultivation tests) as control lines, their performances for all traits were calculated as the mean over the three cultivation tests. Lines were considered to be different if the mean values differed by more than the least significant difference (LSD) at α=0.05. For the three sets of heterokaryons phenotypic data (CC, FM, MT, SC, SZ, SS, DS) of flush 1 and flush 2 were combined and analyzed with analysis of variance (ANOVA) to test for the significance of genotype and flush effects. Flush 1 data of heterokaryon set 1 and set 2 were used to assess the significance of the tester effect. Broad-sense heritability (H^2) was calculated across flushes and tester lines (over heterokaryon set 1 and set 2) as follow: $H^2=\sigma^2_G/\ [\sigma^2_G + (\sigma^2_e/n)]$, where σ^2_G represents the genotypic variance, σ^2_e represents the residual variance, and n is the number of flushes or number of testers (indicate heterokaryon set for which this was the case) (n=2). Trait correlations were calculated using Spearman's rank correlation coefficient.

Genetic linkage map

The segregation analysis and genetic linkage maps of the two homokaryotic populations were reported in another study (Chapter 4). Briefly, the total map length of the first homokaryotic population (heterokaryon set 1 and set 2 in this study) was 164 cM generated with 95 SNP markers generally well distributed over the genome (13 chromosomes, 30.4 Mb in total). It has an average crossover frequency of 0.1 per individual per chromosome. The total map length of the second homokaryotic population (heterokaryon set 3 in this study) was 86 cM generated with 76 SNP markers, and it has an average crossover frequency of 0.05 per individual per chromosome. SNP markers were selected to be evenly distributed based on the genome sequences of the reference homokaryon H97 (Morin et al., 2012). Because of the low recombination frequency, the linkage maps are very short and have regions of low resolution. The QTL mapping is thus restricted in most cases to assigning QTLs to chromosomes.

Table 1. Quality related traits observed in the three sets of heterokaryons

Quality traits	Definition	CODE
Compost colonization	1 = very bad; 10 = very good	COCO
Earliness	time (days) from spawning to first harvest day	ER
Cap color	1 = white, 2 = off-white, 3 = light brown, 4 = brown, 5 = dark brown	CC
Firmness of caps	1 = very weak; 5 = very firm	FM
Maturation	1 = early mature; 5 = Close cap	MT
Scales on caps	1 = strongly scaled; 5 = smooth	SC
Cap size	1 = very small; 5 = large	SZ
Stipe shape	1 = short and thin; 2 = short and normal; 3 = short and thick; 4 = middle and thin; 5 = middle and normal; 6 = middle and thick; 7 = long and thin; 8 = long and normal; 9 = long and thick	SS
Mushroom distribution	1 = clustered; 5 = even	DS

QTL analysis

QTL analyses were conducted for each of the three sets of heterokaryons. It is important to realize the experimental setup here differs from plant breeding. QTL mapping is done for two homokaryotic segregating populations for which the contrasting parents are H97 and Mes09134 for population 1 and H39 and Z8 for population 2. This type of mapping is thus similar to mapping in doubled haploid (DH) population in plants. The tester lines are merely used to generate heterokaryons that allow the production of mushrooms and thus phenotyping. The tester lines are also useful to see the effect on QTL in different backgrounds. The markers used for QTL mapping are thus those of the parental lines and not those of the tester lines, i.e., the genetic linkage maps used were generated from the homokaryotic populations. For the multi-trait QTL analysis, scores of traits were all standardized by auto-scaling: subtracting the mean and then dividing by the standard deviation. Multi-trait QTL analysis was carried out with the statistical program Genstat, version 15. The variance-covariance model for QTL selection and fitting the QTL model was chosen as "unstructured", based on the Bayesian Information Criterion (BIC) (Malosetti et al., 2008, Boer et al., 2007). The threshold –log (*p-value*) for QTL detection was calculated with a genome wide significance threshold of $p<0.05$ (Li & Ji, 2005, Boer et al., 2007). Simple interval mapping was used for an initial genome wide scan to select significant candidate QTLs based on the threshold [–log (*p-value*)], and the ones having the highest –log (*p-value*) were selected as cofactors, then several rounds of composite interval mapping (CIM) were done until no new QTLs were detected. A REML (residual maximum likelihood) procedure was used iteratively to fit the final QTL model at each linkage group position. The upper bound and lower bound of QTL positions on each chromosome were also

calculated with the threshold significance. All traits were analyzed with both multi-trait and single trait analysis apart from cap color. Since it is known that cap color is controlled by a major QTL on chromosome 8 explaining more than 80% phenotypic variation (Foulongne-Oriol et al., 2012a), cap color was analysed as a single trait but not incorporated into multi-trait analysis. Results of multi-trait analyses were compared to those of single trait analyses.

Results

Performance of control lines

The original heterokaryotic lines Horst U1, WB2 and WW7 from which the parental homokaryons were derived and the F1 hybrids generated by crossing the homokaryotic parental lines (H97 x Mes09143 and H39 x Z8) are considered as control lines and were enclosed in all three cultivation trials and their mean performance across three cultivation tests was calculated (Table 2). The cap color (CC) of Horst U1, WW7 and Cr002 were consistently scored as "1" since they were white; WB2 & Cr012 were consistently scored as "3" (light brown) in all three cultivation tests. Since none of these controls show any variation in cap color, no statistical analysis was done. CC was not scored in heterokaryon set 3 since both the parental homokaryons and the tester homokaryon are derived from a white heterokaryon. The five control lines did not show significant differences in three traits: COCO, DS, and SS. The production time (ER) of the white commercial hybrid Horst U1 was significantly later (by more than 2 days) than the brown wild line WB2, but ER of Horst U1 was not significantly different from that of the white wild line WW7. ER of the F1 hybrid Cr012 resembled its brown parental heterokaryon WB2, which was also significantly earlier than the white commercial heterokaryon Horst U1; F1 hybrid Cr002 did not show significant difference in ER compared to its two original heterokaryons (Horst U1 & WW7). FM of the three original heterokaryons was not significantly different in both flush 1 and flush 2, but F1 hybrid Cr002 was significantly weaker than Horst U1 in both flushes. The two heterokaryons Horst U1 and WW7 showed a significant difference in MT and SZ for both flushes, with the commercial line Horst U1 maturing later and was smaller than the wild line WW7, and Cr002 had no significant difference with its original heterokaryon WW7 for both MT and SZ. In contrast, the wild line WW7 and the F1 hybrid Cr002 were significantly smoother (higher SC) than the commercial line U1 in flush 1 but not in flush 2. Horst U1 was significantly smoother than the F1 hybrid Cr002 in flush 2 but not in flush 1.

Table 2. Trait performance of heterokaryotic parental lines and F1 hybrids

Line	COCO	ER	DS1	DS2	FM1	FM2	MT1	MT2	SC1	SC2	SS1	SS2	SZ1	SZ2
Horst U1	9.0	44.3^{b}	3.3	3.0	4.3^{b}	4.0^{b}	4.0^{c}	4.0^{b}	2.3^{ab}	3.7^{b}	5.0	4.0	3.7^{c}	3.7^{b}
WB2	9.3	41.0^{a}	2.0	2.7	3.3^{ab}	3.3^{ab}	3.0^{abc}	3.0^{ab}	3.3^{bc}	3.0^{ab}	4.0	4.3	3.3^{bc}	4.0^{b}
WW7	9.7	43.0^{ab}	2.3	3.0	3.7^{ab}	2.3^{ab}	2.0^{a}	2.0^{a}	4.0^{c}	3.7^{b}	5.3	4.5	2.7^{ab}	3.3^{b}
Cr012	10.0	41.0^{a}	2.7	2.0	3.3^{ab}	3.3^{ab}	3.7^{bc}	3.3^{ab}	1.3^{a}	1.7^{a}	4.3	3.3	4.0^{c}	3.3^{b}
Cr002	9.3	43.3^{b}	1.3	2.0	2.7^{a}	1.7^{a}	2.3^{ab}	2.0^{a}	4.7^{c}	3.0^{ab}	5.7	4.7	2.3^{a}	2.3^{a}

Data of flush 1 and flush 2 were considered as two individual traits. Abbreviations followed with a "1" represents traits of flush 1, and those followed with a "2" represents traits of flush 2. Since COCO, MR was scored before mushroom production and ER were scored on the first harvest day of flush 1, data of these three traits do not have flush differences. Significant differences by multiple comparison (LSD) were indicated with superscript letters (α=0.05).

Statistics for three sets of heterokaryons

A total of 191 individuals of heterokaryon set (Set) 1, 185 individuals of Set 2, and 180 individuals of Set 3 produced enough mushrooms for phenotypic evaluation. The frequency distribution of the three sets of heterokaryons showed continuous variation for almost all the traits. Cap color is controlled by one major

locus on chromosome 8 and brown is partially dominant over white (Foulongne-Oriol et al., 2012a). Since the parental homokaryons used to generate population 1 are white & brown and both tester lines used to produce heterokaryons are white, approximately half of the individuals were white and the other half non-white (varying from cream, light brown to brown). Cap color shows, therefore, a bimodal distribution in these two sets of heterokaryons (Figure 2). The compost colonization (COCO) of heterokaryon Set 2 and Set 3 seem to be faster than Set 1 (Figure 3). On day 14 after spawning, around 150 individuals of Set 2 and 170 individuals of Set 3 had 100% compost colonization having the score of 10, while only 12 individuals of Set 1 had the score of 10. The COCO score on day 14 after spawning might be too late to observe the phenotypic variation of this trait in Set 2 & Set 3. The continuous distribution for all traits suggests that these are quantitative features and that there may be polygenic control of these traits. Data of two flushes were combined by ANOVA to test the effects of genotype and flush for the three heterokaryon sets independently; data of Set 1 & Set 2 (flush 1) were combined to test the effect of genetic backgrounds (tester lines). Genotype effect was significant (α=0.05) for most traits apart from DS of Set 2 and SC of Set 3. Flush effects were significant for traits DS, FM and SC of Set 1, traits FM, MT, SS, SZ of Set 2, traits FM, SC, SS of Set 3 (α=0.05). Analysis on the combined data across flush 1 of Set 1 and Set 2 revealed that genetic background (tester line) was a significant factor to influence most of traits except for CC, ER and SZ (α=0.05). Broad-sense heritability was high for all the traits of three sets of heterokaryons ranging from 0.66 for COCO across tester lines (COCO of flush 1 across Set 1 and Set 2) to 0.97 for CC of Set 2, indicating that phenotypic variation was mostly determined by genotypic effects.

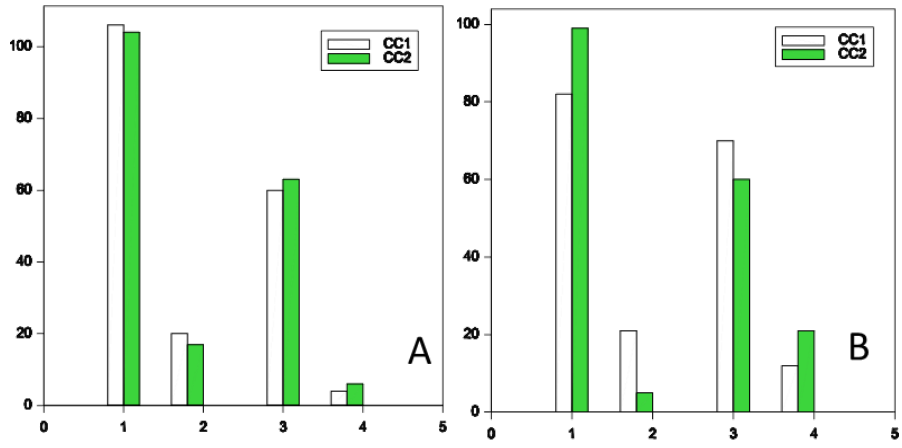

Figure 2. Frequency distribution histograms for CC of heterokaryon set 1 (A) and Set 2 (B)
CC1 represents the cap color of flush 1, and CC2 represents the cap color of flush 2. Color varied from 1 (white), 2 (off-white), 3 (light brown) to 4 (brown).

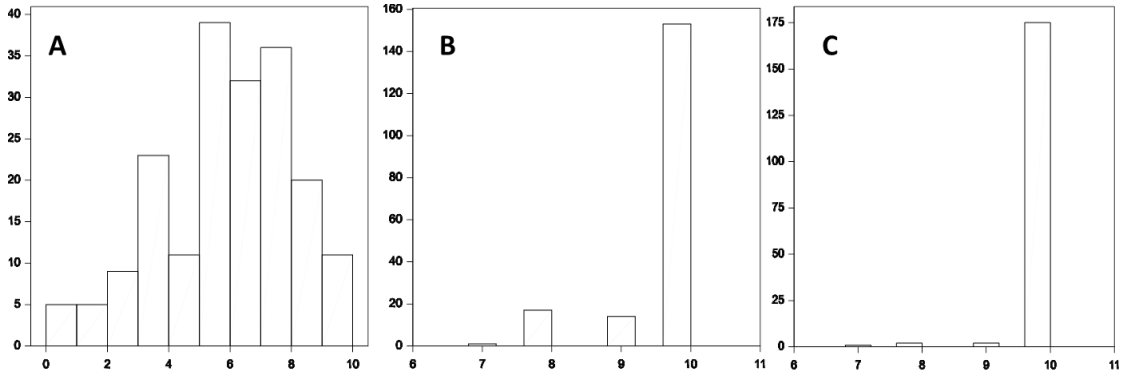

Figure 3. Frequency distribution histograms for COCO of heterokaryons Set 1 (A), Set 2 (B) and Set 3 (C).
Degree of compost colonization varies from 0 (no colonization) to 10 (fully colonized), 14 days after inoculation.

Spearman's rank correlation coefficients (*r*) among the traits were calculated per set of heterokaryons (Tables 3-5). Strong positive correlations between CC1 and CC2 were observed for both Set 1 and Set 2. A consistent negative correlation was observed between earliness (ER) and cap color (CC) in Set 1 and 2. Since high values of ER represent late individuals and high values of CC represent dark individuals, brown individuals generally produced earlier than white individuals. In a previous study the brown heterokaryotic parent JB3 also produced earlier than the white heterokaryotic parent Horst U1, but no significant correlation was observed between cap color and earliness in the segregating population (Foulongne-Oriol et al., 2012a). COCO was also negatively correlated with ER in Set 1, but there was no consistent correlation between COCO and the other traits in the Set 2 & 3. Since COCO was scored only once (14 days after inoculation), differences in colonization rate were not assessed and that might obscure correlations between COCO and other traits. For all the three sets of heterokaryons, ER was positively correlated with FM & MT but negatively correlated with SS; FM was positively correlated with MT and SZ, but negatively correlated with SS. It can generally be concluded that late individuals tended to produce firm and big mushrooms with a short stipe, and late individuals matured (opened) late. Most traits were significantly positively correlated between flush 1 and flush 2, except for SZ of Set 1, DS and SS of Set 2, and DS and SC of Set 3. The most significant correlations occurred between CC1 and CC2 of Set 1 & 2 (r=0.91). Since heterokaryon set 1 & 2 are the same set of homokaryotic progeny crossed with different tester lines, correlations for the same traits across Set 1 & 2 were also tested; apart from CC1 and CC2, significant correlations (α=0.05) were also observed between other traits (sample size 167) including ER (r=0.32), DS1 (r=0.23) and SC1 (r=0.22) and traits of flush 2 (sample size 174) including MT2 (r=0.19) and SS2 (r=0.18)

Table 3. Correlation coefficients among traits of heterokaryon set 1 (n=177). "-" indicates a non-significant correlation coefficient. All listed Spearman correlation coefficientsare significant *(α*=0.05).

CC1	*															
CC2	0.91	*														
COCO	-	-	*													
DS1	-	-	-	*												
DS2	-	-	-0.21	0.20	*											
ER	-0.22	-0.18	-0.43	-	-	*										
FM1	-0.20	-	-	-	-	-	*									
FM2	-0.17	-	-0.15	-	-	0.27	0.38	*								
MT1	-0.18	-	-0.17	0.28	0.33	-	0.23	0.16	*							
MT2	-0.28	-	-0.19	-	-	0.37	0.32	0.55	0.37	*						
SC1	-	-	-	-	-0.17	-	-0.35	-0.34	-0.20	-0.23	*					
SC2	-	-	-	-	-	-0.14	-	-	-	-	0.20	*				
SS1	-	-	-	-	-0.18	-0.35	-	-0.22	-0.29	-0.25	-	-	*			
SS2	0.13	-	-	-	-	-0.25	-	-0.34	-	-0.31	-	-	0.27	*		
SZ1	-	-	-	0.19	0.23	-	0.17	-	0.37	0.19	-	-	-0.23	-0.20	*	
SZ2	-0.19	-	-0.22	-	-	0.28	0.22	0.47	0.27	0.57	-	-	-0.23	-0.40	-	*
	CC1	CC2	COCO	DS1	DS2	ER	FM1	FM2	MT1	MT2	SC1	SC2	SS1	SS2	SZ1	SZ2

Table 4. Correlation coefficients among traits of heterokaryon set 2 (n=185). "-" indicates a non-significant correlation coefficient. All listed Spearman correlation coefficients are significant (α=0.05).

CC1	*								
CC2	0.91	*							
COCO	0.16	0.19	*						
DS1	-	-	-	*					
DS2	-	-	0.23	-	*				
ER	-0.35	-0.32	-	-	-	*			
FM1	-	-	-	-0.16	-	0.30	*		
FM2	-	-	-	-	-0.21	-	0.20	*	
MT1	-	-	-	-	0.19	0.22	0.54	-	*

MT2	-	-	-	-	-0.23	-	0.21	0.48	0.21	*						
SC1	-0.26	-0.25	-	-	-	-	-	-	-	-	*					
SC2	-	-	-	-	-0.24	-	-	-	-	-	0.19	*				
SS1	0.24	0.21	-0.16	0.27	-	-0.57	-0.46	-0.18	-0.49	-	-	-	*			
SS2	-	-	-	-	-	-	-	-0.23	-	-0.20	-	-	-	*		
SZ1	-	-	-	-	-	-	0.55	-	0.61	-	-0.17	-	-0.46	-	*	
SZ2	-	-	-	-	-0.20	-	0.21	0.43	0.18	0.47	-	-	-0.22	-0.16	0.19	*
	CC1	CC2	COCO	DS1	DS2	ER	FM1	FM2	MT1	MT2	SC1	SC2	SS1	SS2	SZ1	SZ2

QTL mapping of multiple traits

Multi-trait QTL analyses were performed for the studied traits. Because of the low recombination frequency of *Agaricus bisporus* var*. bisporus,* the genetic linkage maps of these two segregating populations are quite short. Thus, the estimated upper bound and lower bound of QTL positions are usually coveringthe whole linkage group; therefore QTLs are indicated according to the chromosome (CHR) number. The multi-trait QTL model allows the analysis of several traits simultaneously, and thus the genetic basis of the correlations between traits. The covariance between traits can be explained by the same QTLs having pleiotropic effects or QTLs being linked to the same chromosome. For simplicity, these two situations are here referred to as pleiotropic regions. Through multi-trait QTL analyses, the most significant pleiotropic region of Set 1 was CHR10 with a value of 50.1 for the –log10(*p*) (threshold=2.8); the most significant pleiotropic QTL of Set 2 was CHR6, and the –log10(p) was 12.1 (threshold=2.8); CHR10 was also the most significant pleiotropic QTL of Set 3, and the –log10(p) was 21.2 (threshold =2.7). As expected, traits having phenotypic correlations mostly shared pleiotropic regions. Different QTLs were found in Set 1 and Set 2 indicating the influence of genetic background (tester lines); different QTLs were also detected over data of the two flushes indicating either the presence of QTL by environment interactions since the environmental conditions of flush 1 and flush 2 are quite different. The differences in QTLs between flushes might also result from data errors in one flush influencing the statistical power.

Table 5. Correlation coefficients among traits of heterokaryon set 3 (n=180). "-" indicates non-significant correlation. All listed Spearman correlation coefficients are significant (α=0.05).

COCO	*														
DS1	-	*													
DS2	-	-	*												
ER	-	-	-	*											
FM1	-	-	-0.23	0.49	*										
FM2	-	-	-0.27	0.50	0.48	*									
MR	-	-	-	-0.18	-	-0.20	*								
MT1	-	-	-	0.41	0.38		-	*							
MT2	-	-	-	0.26	0.28	0.48	-	0.23	*						
SC1	-	-	-	-	-	-	0.17	-	-0.19	*					
SC2	-	-	-0.17	0.24	-	0.21	-0.23	-	-	-	*				
SS1	-	-	-	-0.51	-0.40	-0.27	-	-0.42	-	-	-0.18	*			
SS2	-	-	-	-0.30	-	-0.28	-	-0.18	-0.31	0.17	-	0.21	*		
SZ1	-	-	-0.31	0.45	0.48	0.37	-	0.50	0.35	-0.26	-	-0.39	-0.31	*	
SZ2	-	-	-	0.30	0.36	0.51	-	0.23	0.57	-	-	-0.17	-0.24	0.32	*
	COCO	DS1	DS2	ER	FM1	FM2	MR	MT1	MT2	SC1	SC2	SS1	SS2	SZ1	SZ2

Cap color (CC)

Cap color was analyzed with only single trait analysis, a single QTL was detected on CHR8 in Set 1 and Set 2, and the explained variance was up to 90% (Supplemental data 1), which was in agreement with the previous study (Foulongne-Oriol et al., 2012a).

Compost colonization (COCO)

Several QTLs were found for COCO in Set 1 & 2 (Figure 4 & Figure 5) but none for Set 3. On chromosome (CHR) 6 there was a QTL consistent over Set 1 & 2. This QTL explained 14% of the COCO variation for Set 1 (Table 6) and also was detected with single trait analysis. CHR6 only explained 2% COCO variation for Set 2. It was not detected by single trait analysis (Table 7). The high value allele for CHR6 in Set 1 was from parent H97. The QTL CHR13 explained 8% COCO variation of Set 2, and the high value allele was contributed also by H97 (Table 8).

Earliness (ER)

Earliness of different set of heterokaryons was controlled by different QTLs. For Set 1, two major QTLs for ER was detected on CHR6 and CHR10, explaining 17% and 9% of the phenotypic variation, respectively; the 'late' allele (high ER value) was contributed by Mes09143 (CHR6) and H97 (CHR10). For Set 2, a major QTL for ER was on CHR8 with 14% phenotypic variation explained, and here the allele associated with later production was from H97; For Set 3, a major QTL was also found on CHR10 explaining 15% of the phenotypic variation, and the "late" allele was from H39. With multi-trait analysis four QTLs (CHR2, 4, 6 & 10) were consistent over Set 1 and Set 3; four QTLs (CHR2, 3, 7 & 8) were consistently detected in Set 2 and Set 3; two minor QTLs were consistent (CHR2 & 5) for Set 1 and 2. These QTLs were also detected with single trait analysis apart from CHR7 (Supplementary data 1). QTL CHR7 was not significant in the single trait QTL analysis.

Distribution (DS)

Mostly minor QTLs for DS were detected in all the three sets of heterokaryons. CHR10 showed the largest amount of explained variance in Set 3 (12%) and Set 1 (7%). Apart from the minor QTL CHR11 that was consistent over the data of two flushes in Set 1, no other QTL was consistently found across DS1 and DS2 in any of the three sets heterokaryons. CHR10 was also detected in single trait analysis, but CHR11 was not significant in the single trait analysis.

Firmness (FM)

QTLs on CHR 2, 4, 5, and 10 were consistently detected across FM1 and FM2 in Set 1; of these, CHR5 and CHR10 were also found in the single trait analysis. CHR10 explained 31% FM2 variation and 14% FM1 variation, and the high value allele (firmer mushrooms) of this QTL was from H97. CHR6 was found for FM1 but not for FM2 in Set 1, but it was consistent over the three sets of heterokaryons. CHR10 also explained larger phenotypic variation of FM1 (15%) and FM2 (18%) in Set 3 than the other QTLs, and the high value allele of this QTL was from H39.

Maturation (MT)

A major QTL on CHR10 was detected with data of both flushes in Set 1, and the explained variance was 35% for MT2 and 15% for MT1. The high value allele of CHR10 was from H97. It was also detected as a minor QTL in Set 3 but not in Set 2. QTL CHR5 and CHR6 were the two consistent QTLs for the two flushes of Set 2. Minor QTLs on CHR 4, 5, 6, 7 and 9 were consistently detected across Set 1 and Set 2, and two of them (CHR 4 & 6) were consistent over all three sets of heterokaryons. QTLs for FM and MT were mainly detected on the same chromosomes, which explained the positive correlation between these two traits, i.e., correlation coefficient (r) =0.55 for MT2 and FM2 in Set 1, and r=0.48 for MT2 and FM2 in both Set 2 and Set 3. Most of the minor QTLs for MT detected in multi-trait analysis were not significant in single trait analyses, but they were mostly just below the threshold of significance in single analysis.

Scales (SC)

QTLs on CHR 4, 6 & 10 were consistently found in Set 1 and 3. CHR10 explained 30% SC1 variation in Set 1, and the high value allele (smooth mushroom, less scaling) was contributed by parent Mes09143. CHR10 also explained larger SC variation (10% for SC1 and 8% for SC2) in Set 3 than the other QTLs, but the high value alleles were different for SC1 (Z8) and SC2 (H39), and single trait analysis also gave the same results of this QTL. This inconsistency correlates with the absence of a Spearman correlation between SC1 and SC2 in Set 3. This difference in scaling between flushes is always seen in the commercial line Horst U1 where flush one is mostly scaling heavily whereas flush 2 hardly scales. Only three minor QTLs (CHR 3, 6, and 8) were detected in Set 2, of which CHR6 was not significant in single trait analysis.

Stipe shape (SS)

QTLs CHR4 & CHR6 were consistently detected for SS over the three sets of heterokaryons. CHR4 explained 20% variation of SS2 in Set 1, 15% variation of SS2 in Set 2, and 5% variation of SS1 in Set 3. The high value allele (for long and thick stipe) of this QTL was contributed by parent Mes09143 in Set 1 & 2, and that of Set 3 was contributed by parent Z8. In Set 3 there was a QTL with a larger effect, CHR10, with 13% and 6% explained variance for SS1 and SS2, respectively. For this QTL also the high value allele was from Z8.

Size (SZ)

Three consistent QTLs CHR 2, CHR4 and CHR10 were detected in Set 1 and Set 3. CHR 10 explained 14.3% SZ2 variation of Set 1 and 12% SZ1 variation of Set 3. The high value alleles (for bigger mushroom) of CHR10 were contributed by H97 for Set 1 and H39 for Set 3 respectively. CHR6 was consistent for the two flushes in Set 2, explaining 13% SZ1 variation and 15% SZ2 variation. The high value allele of CHR6 in Set 2 for bigger mushrooms was from parent Mes09143.

Pleiotropic effects of QTLs

In this study, several pleiotropic regions were detected. CHR10 was a pleiotropic region in both Set 1 and 3, affecting most traits, apart from compost colonization, and it explained a relatively larger part of phenotypic variation compared to other QTLs for all traits. Heterokaryons Set 1 and Set 2 represent the same set of homokaryotic progeny (offspring of Mes09143 x H97) crossed with two different tester lines. It is thus surprising that the pleiotropic region on CHR10 was not detected in Set 2. Since effects of genes may be influenced by their genetic backgrounds (Lynch & Walsh, 1998), effects of pleiotropic regions can be different in different genetic backgrounds (tester lines). CHR6 was a pleiotropic region in Set 2, and the high value alleles for firmer, later matured, less scaled, and bigger mushroom were from parent Mes09143 apart from that of SS1 (Figure 5). Genetic correlations and phenotypic correlations between traits normally have the same sign and the same magnitude (Roff, 1995). For pleiotropic region CHR10 QTLs of ER, FM, MT and SZ all have their high value alleles from parent H97 in Set 1 explaining the positive phenotypic correlations among these four traits, i.e., later progeny produced firmer, later maturing and bigger mushrooms. CHR10 of SC and SS in Set 1 have their high value alleles from parent Mes09143 explained the negative correlations between them and the other traits. High value alleles of QTLs for ER and COCO on chromosome 6 (Set 1) were contributed by different parental lines, i.e., parent Mes09143 for ER and parent H97 for COCO, explaining the negative correlations between ER and COCO in Set 1. However, since late individuals have the higher scores for ER, a higher level of compost colonization is associated with earlier mushroom production, which indicates that vigorous mycelium growth in the compost leads to a more efficient productivity in time.

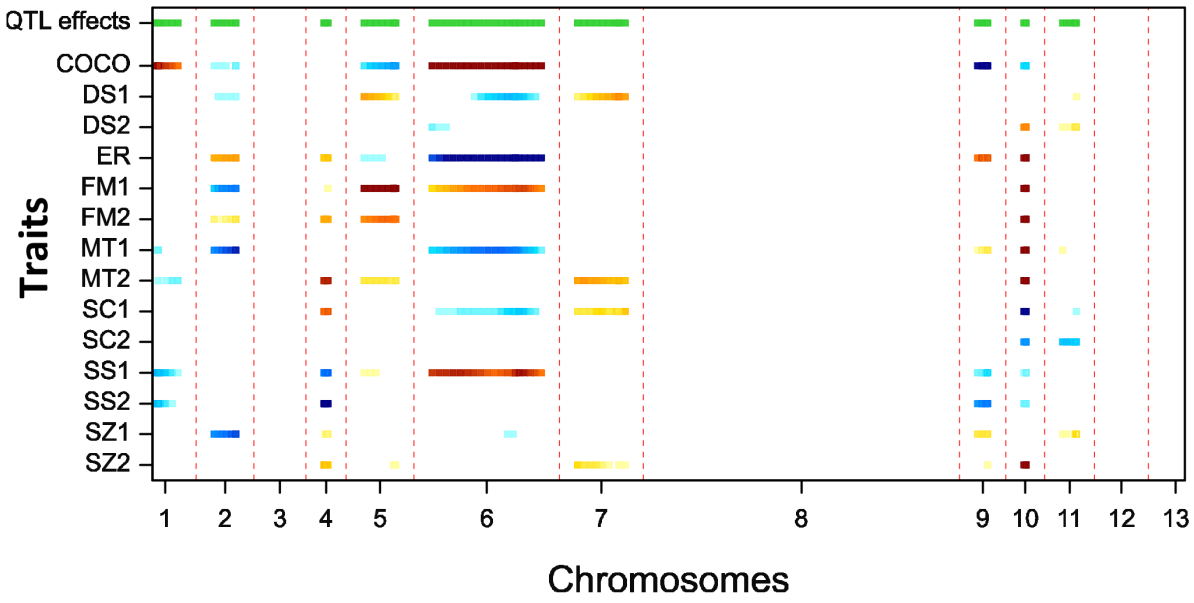

Figure 4. Multi-trait QTL analysis in heterokaryon set 1

The two color scales of the bars indicate the two parents contributing high value alleles of QTLs. The variation in blue represents alleles from Mes09143 and the darker the color the higher the effect of the QTL. Similar for the variation in red that represents the high value alleles from H97. Green bars at the top indicate chromosomes bearing QTLs with significant effects. The different widths of linkage groups reflect the different map lengths of the corresponding chromosomes and represent thus the recombination frequency of markers on each chromosome. It shows that chromosome 8 has the highest frequency of recombination of all chromosomes.

Table 6. Explained variance of QTLs detected in heterokaryon set 1

Linkage group	1	2	4	5	6	7	9	10	11
Marker name	MHchrI_05	ChrII_M1	ChrIV_B1	C5P5	MHchrVI_02	MHchrVII_05	ChrIX_T1	ChrX_B1	MHchrXI_01
Position	1.6	5.9	1.1	4.9	22.9	13.0	1.2	0.6	3.8
-log(p)	9.7	11.0	14.8	10.0	22.7	6.3	8.7	50.1	4.1
Traits									
COCO	*7.7	1.6		*3.4	*17.1		*9.6	2.3	
DS1		1.9		4.2	*4.1	5.5			1.9
DS2								*6.6	2.9
ER		4.0	3.0	1.6	*15.1		*6.3	*9.4	
FM1		*4.8	1.5	*13.3	*6.2			*13.6	
FM2		2.0	3.8	*5.7				*31.2	
MT1	1.8	*7.7			*5.3		1.8	*14.8	
MT2	1.2		*6.1	1.8		2.5		*35.0	
SC1			*5.7		2.2	2.9		*31.5	1.5
SC2								*6.9	4.1
SS1	*4.1		*6.5		*8.3		2.2	2.3	
SS2	3.8		*19.5				*5.2	2.1	
SZ1		*7.8	2.5				2.8		3.0
SZ2			*3.6			1.9		*14.3	

Asterisks indicate QTLs that were also detected by single trait analyses. The others were not significant in the single trait analyses, while they are mostly just below the threshold of significance.

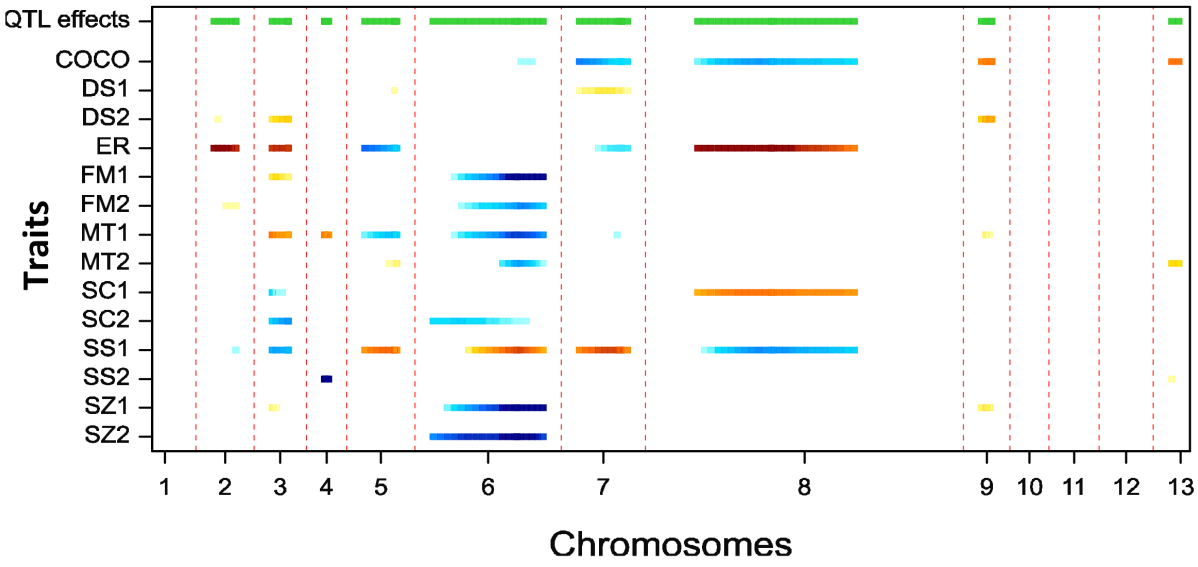

Figure 5. Multi-trait QTL analysis in heterokaryon set 2

The two color scales of the bars indicate the two parents contributing high value alleles of QTLs. The variation in blue represents alleles from Mes09143 and the darker the color the higher the effect of the QTL. Similar for the variation in red that represents the high value alleles from H97. Green bars at the top indicate chromosomes bearing QTLs with significant effects. The different widths of linkage groups reflect the different map lengths of the corresponding chromosomes and represent thus the recombination frequency of markers on each chromosome. It shows that chromosome 8 has the highest frequency of recombination of all chromosomes.

Table 7. Explained variance of QTLs detected in heterokaryon set 2

Linkage group	2	3	4	5	6	7	8	9	13
Locus name	C2P2	ChrIII_B1	MHchrIV_03	ChrV_B2	MHchrVI_01	ChrVII_T2	C8P28	ChrIX_T1	MHchrXIII_05
Position	2.2	0.0	0.5	8.1	24.0	10.3	27.8	1.2	0.0
#NAME?	4.8	7.2	6.5	9.0	12.1	5.8	8.7	3.8	4.4
Traits									
COCO					1.8	*2.8	5.1	*6.0	*7.0
DS1				2.5		3.3			
DS2		3.8						5.5	
ER	*10.9	*9.0		*4.1		2.6	*13.6		
FM1		*3.0			*12.6				
FM2					*7.0				
MT1		*7.1	*6.6	3.9	*8.0	2.0		2.2	
MT2				2.6	*5.9				3.8
SC1		3.9					*8.2		
SC2		*4.7			2.4				
SS1		4.7		*6.8	*7.6	*7.8	*5.3		
SS2			*14.7						2.3
SZ1		2.8			*12.7			2.9	
SZ2					*14.9				

Asterisks indicate QTLs that were also detected by single trait analyses. The others were not significant in the single trait analyses, while they are mostly just below the threshold of significance.

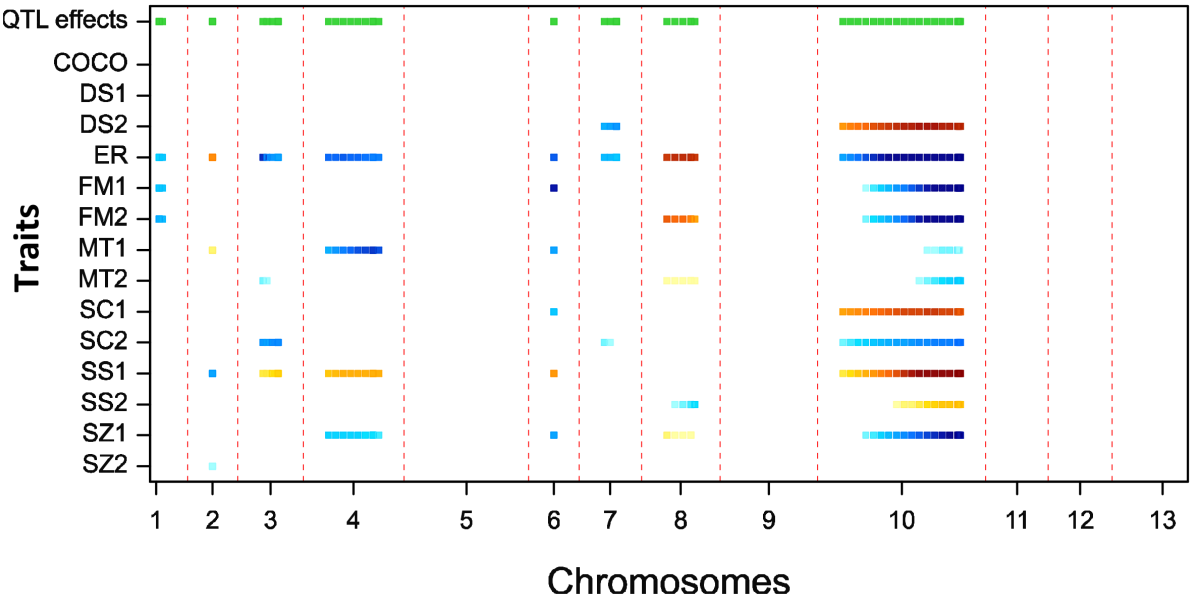

Figure 6. Multi-trait QTL analysis in heterokaryon set 3.
The two color scales of the bars indicate the two parents contributing high value alleles of QTLs. The variation in blue represents alleles from H39 and the darker the color the higher the effect of the QTL. Similar for the variation in red that represents the high value alleles from Z8. Green bars at the top indicate chromosomes bearing QTLs with significant effects. The different widths of linkage groups reflect the different map lengths of the corresponding chromosomes and represent thus the recombination frequency of markers on each chromosome.

Table 8. Explained variance of QTLs detected in heterokaryon set 3

Locus name	CHR1B1	HZchrII_06	HZchrIII_05	ChrIV_T3	HZchrVI_02	CHR7B1	ChrVIII_B1	CHR10A1
Linkage group	1	2	3	4	6	7	8	10
Position	2.44	0	0	8.79	0	0	0	22.74
-log(p)	5.105	6.379	3.626	7.806	10.045	6.231	5.445	21.246
COCO								
DS1								
DS2						*5.5		*11.6
ER	2.9	4.6	*7.2	*5	*6.1	3.6	*6.7	*14.9
FM1	3.8				*11.0			*15.0
FM2	4.5						*7.8	*17.6
MR	5.8			4.3		5.8	5.2	2.8
MT1		2.5		*9.8	5.9			2.9
MT2			3.0				2.6	4.9
SC1					4.7			*9.6
SC2			*6.8			3.0		*8.2
SS1		4.9	2.6	*4.5	5.1			*12.9
SS2								*5.6
SZ1				3.3	5.5		2.3	*12.0
SZ2		2.6						

Asterisks indicate QTLs that were also detected by single trait analyses. The others were not significant in the single trait analyses, while they are mostly just below the threshold of significance.

Discussion

Most of the present-day button mushroom varieties are genetically very similar (Moore *et al.*, 2001, Sonnenberg et al., 2011). They are all susceptible to the same diseases, have the same low biological efficiency on substrates and have very similar quality traits. New varieties with improved traits are only

commercially viable if they meet at least the quality standards of the present varieties. Previous and present breeding programs have shown that restoration of quality and agronomic traits while introducing new traits by outcrossing with wild germplasm is very difficult. One of the main reasons is the low recombination frequency in the variety *bisporus* causing substantial linkage drag. In addition, there is a lack of knowledge on the genetic basis of most agronomic and quality traits. Also the interdependency of most traits is unknown and knowledge on the influence of environmental factors on traits is limited. Recent research has generated some insight in the development of fruit bodies and crops of the button mushroom and shown that this is complex. Mushroom density on a production bed and picking regime have effect on outgrowth and thus the quality of mushrooms (Straatsma et al, 2013). This present study is a first step towards a better understanding and will contribute to a more guided breeding than what is done so far.

For a correct interpretation of QTL mapping in mushroom breeding a proper understanding of the experimental setup is needed that differs from plant breeding. First of all, source material consists of selected strains or lines from evaluation trials. Every line is heterokaryotic and has two types of haploid nuclei per cell. These lines are maintained vegetatively and can be used continuously for mushroom production. Selected lines are protoplasted to recover both constituent nuclei as haploid homokaryons (also designated as parental homokaryons). These two homokaryons thus represent the whole genetic make-up of a line and when crossed restore the original line. By recovering homokaryons in this way, the optimal allele combination present in the original nuclei might be retained. One can estimate the breeding value of each constituent nucleus of selected varieties or wild lines (Wei et al 2013; submitted). This is done by mating homokaryons in all possible combinations to generate heterokaryons those can produce mushrooms and test relevant phenotypes as done in a diallel matrix. This allows thus the selection of the most suitable homokaryon for a breeding program and resembles breeding with doubled haploid or fully inbred lines in plants. For mapping and QTL analysis, homokaryons of contrasting phenotypes (designated as parental homokaryons) are mated (corresponding to F1 hybrid of plant breeding) and fruited to generate a haploid offspring (single spore cultures). For mapping mushroom traits fruiting bodies are required and in order to obtain these, homokaryons are mated with a compatible homokaryon (a tester homokaryon) to restore the heterokaryotic phase, or more precisely to instigate the presence of two different mating types to trigger fruiting. For each locus, these mushrooms (corresponding to F2 of plant breeding) thus contain either the allele from parental homokaryon 1 or parental homokaryon 2 in one nucleus and all contain the same allele from the tester line in the other nucleus. If parental homokaryon 1 has allele A, parental homokaryon 2 allele B and the tester line allele C, then each heterokaryon formed from the segregating population has either the combination AC or BC at a genetic locus (Figure 1). In this way alleles of a segregating homokaryotic offspring are mapped in a shared genetic background of the same tester line. Segregation in the F2 therefore is due to the contrast AC vs. BC where the C allele is in common and the parental alleles A and B are contrasted. That does not imply that the choice of the tester line is irrelevant, i.e., the tester line can have a large influence on the phenotype. In previous breeding programs (Gao et al., Chapter 5) we observed that the level of phenotype expression can be influenced by the tester or that some phenotypic variation can be obscured by the tester line. Therefore, it is useful to employ different tester lines even though this increases experimental costs. Alternatively, mapping of traits can also be done using the homokaryotic offspring of heterokaryotic lines or varieties. By crossing the offspring with one and the same compatible homokaryon, also traits from the original line or variety can be mapped. This procedure is also less efficient since if one intends to introgress a trait from one variety into another variety, crossing constituent homokaryons (recovered by protoplasting) of both varieties already represents the first step of an introgression breeding program. Through introducing a trait in both constituent homokaryons of a (acceptor) variety by introgression breeding and re-joining these “upgraded” homokaryons in a cross one restores the original variety as much as possible with an improved or new trait. Mapping by selfing (intercross homokaryotic offspring) would also be a way to map traits but the button mushroom is known for its strong inbreeding depression and this would interfere with reliable assessment of the phenotype (Xu, 1995).

Correlations among traits

Agronomic and quality traits are complex and not only determined by genetic factors but also determined by environmental factors. A bad appearance of button mushroom is usually caused by ageing (senescence) during post-harvest storage or by mechanical damage during harvesting and transport (Jolivet et al., 1998, Noble et al., 1997). Quality of button mushrooms can also be influenced by a series of environmental factors during the cropping period including compost and casing depth, the formula of casing, flush number and the CO_2 level in the growing room (Noble et al., 1997, McGarry, 1994, Rama et al., 2000). Significant correlations between traits indicate that traits referring to button mushroom productivity and quality might be interrelated and influence each other. The level of compost colonization assessed 14 days after inoculation, reflecting the speed of mycelium growth, correlates with other agronomic and quality traits (e.g. earliness, maturation, size in Set 1) in this study. Cap color was found to be associated with earliness of mushroom production, i.e., brown individuals generally produced earlier than white individuals. It is unlikely that cap color is directly influencing the time of production and more likely that genomic regions involved in earliness are linked to regions determining cap color for the brown lines used here. Earliness correlated with yield and number of fruiting bodies in a previous study, but no significant correlation was found between earliness and cap color (Foulongne-Oriol et al., 2012a). That might be due to different genotypes used in both studies. In addition, firmness was found to correlate with maturation. In our study, firmness relates to the ease with which the cap can be squeezed or indented between thumb and index finger when placed on opposite site of the cap. It does not refer to tissue firmness. There is a strong correlation between cap opening and developmental stage (van Loon, 1995), although the level of cap opening can only be observed in a certain range of developmental stage. In an early developmental stage, there is no space between the cap and the stipe (Hammond & Nichols 1975). The cavity or space under the cap increases with the developmental stage and thus gives room to indention. This developmental change might mainly explain the change in firmness and thus explain the correlation between firmness and developmental stage (maturation) although changes in tissue might play some role (Rama et al., 2000). Phenotypic correlations between traits can be explained in different ways. The expression of correlated traits may be modified by the same environmental factors. Next to that, what are considered as separate traits might be different aspects of the same trait or characters directly emanating from each other (such as mentioned above, firmness and maturation). Last but not least, correlated traits may share genetic determinants. In the populations examined here, many QTLs are located on the same chromosome. The very low recombination frequency in the *bisporus* variety, however, does not allow a precise location on each chromosome and thus identification of co-localization or overlapping QTL.

The scoring of multiple traits tested for each genotype was done with mushrooms at particular moments during cropping in this study. However, quality-related traits, especially morphology traits change over time during mushroom development, and mushroom development is a complex and dynamic process. Traits need to be assessed at the right time point in order to observe genetic variation within segregating populations. For instance, the three original heterokaryons Horst U1, WB2, and WW7 did not differ in the extent of compost colonization assessed on day 14 after spawning, and no variation was found for this trait in heterokaryon set 3 at all. In order to assess variation in speed of colonization, an analysis over a time course during colonization should be conducted. Since environmental conditions also can change in time, some complex quantitative traits, e.g. compost colonization, firmness, maturation etc. might thus be better represented as a function of time. Therefore it is recommended to assess these traits on multiple time points during the cropping cycle in further studies. Flush one and two where, therefore, considered as separate time points since the moisture content of the casing soil and nutritional content of the compost differs substantially between the two flushes. A model for the study of the genetic architecture of complex and dynamic traits, so called functional mapping has been generated previously (Lin & Wu, 2006). Besides, data of time series have been incorporated into the growth model and QTL analysis in

potato (Hurtado *et al.*, 2012). Such an approach might improve the genetic analysis of quality traits for mushrooms in the future.

The multi-trait QTL analysis

Since good crop varieties combine optimal values for several traits to maximize productivity and quality, multiple traits of germplasm should be evaluated during a breeding scheme rather than single traits. The simplest approach to analyze multiple traits is to perform a series of single-trait analyses and then combine the results. However, a combined multi-trait analysis can be more powerful than a collection of single-trait analyses (Malosetti et al., 2008). In this study the major QTLs for all traits were detected in both the single- and the multi-trait QTL analyses. It seems that more consistent minor QTLs were detected in the multi-trait analyses although one has to be aware that the statistical assumptions are stronger for the multi-trait analysis, which may cause more false positive than single trait analysis. QTLs detected by single-trait analyses were mostly detected also in multi-trait analysis except for four minor ones (Supplementary data 1). All QTLs represent two or more traits. CHR 10 represents the highest number of traits for both Set 1 and Set 3, and affected almost all the traits apart from compost colonization. Chromosome 10 is thus an important chromosome for breeding for quality and some agronomic traits. As mentioned before, due to low recombination frequency QTL were assigned to chromosomes and no true linkage can be proven between QTLs in this way. The traits we have examined here are complex and it is thus not surprising that for each trait more than one QTL was found. In *Pleurotus ostreatus*, QTLs of productivity and quality appear to be scattered across the genome and were shown to have small effects on the variation of the corresponding traits (Larraya et al., 2003). Similarly, most chromosomes harbor QTLs in our study, but the three smallest chromosomes were underrepresented, and no QTLs were detected on chromosome 12 in all the three sets of heterokaryons. That might be a coincidence due to the fact that these smaller chromosomes contain the least number of genes, and the chance of being devoid of QTL is also larger for the smaller chromosomes.

Cap color is an important quality trait for traders and consumers. Button mushrooms should be either very white or dark brown. White cultivars represent still the largest part of the market (Callac et al., 1998), but the sale of brown button mushrooms is increasing. Cap color is a quantitative trait controlled by a major locus on CHR8 explaining 90% of the phenotypic variation. This locus was also reported in an intervarietal population (var. *bisporus* × var. *burnetti*) together with two minor loci on CHR 7 and CHR 13 (Foulongne-Oriol et al., 2012a). Cap color was scored for heterokaryon set 1 and set 2 in this study since one of the parental homokaryons used is obtained from a brown line. Crossed with white tester lines, half of the progeny of Cr012 are white, and half of the progeny are non-white (off white, light brown, brown). Since cap color is mainly determined by one locus (Foulongne-Oriol et al., 2012a) resulting in a white or non-white phenotype QTL mapping of this trait leads to extreme high LOD value and was, therefore, not included in the multi-trait analysis. Single-trait QTL analysis confirmed the presence of a major QTL on chromosome 8 explaining up to 94% of the phenotypic variation in cap color (additional file 1), which is in agreement with the previous studies (Foulongne-Oriol et al., 2012a). That study also detected two minors QTLs on CHR7 and CHR13. Cap color was measured more precisely with CIELAB image system in the study of Foulongne-Oriol, whereas here the color scoring ranged from 1 to 5 and that might explain the absence of the minor QTL for cap color in our study. The minor QTL for cap color, however, might represent important modifier genes those influence the color intensity. Since a very white or dark brown cap color is an important trait it is useful to map these modifiers for future breeding programs. In a parallel study with all these three sets of heterokaryons, cap color was measured with CIELAB system revealing indeed more QTLs for cap color (to be published).

A shorter crop cycle is relevant for the production costs. CHR6 is a major QTL for earliness of Set 1 explaining 15% of the phenotypic variation. This QTL might indicate a correlation between earliness and compost colonization, since it also explains 17% of the phenotypic variation for compost colonization.

The high value alleles of this QTL for earliness (high=late) and compost colonization (high=early) are from different parents, i.e., Mes09143 and H97 respectively, which explained the negative correlation between earliness scores and compost colonization scores (r=-0.43). However, since later individuals have the higher scores for earliness, production earliness was actually positively correlated with the speed of compost colonization, i.e., the faster the compost colonization, the earlier the mushroom production. In addition, a QTL for earliness was found on CHR 8 explaining 14% of the phenotypic variation in Set 2 and 7% in Set 3; Five QTLs for earliness were detected in an intervarietal segregating population on Chromosome 1, 2, 5, 10, and 11 (Foulongne-Oriol et al., 2012a). Four of these were also detected in this study, but not the one on CHR11. The QTL on CHR10 was a major QTL for earliness in Set 3 explaining 15% of the phenotypic variation, and the high value (late) allele was from H39. Overall, apart from CHR6 the high value alleles of major QTLs (CHR8 & CHR10) for earliness were mostly from parents generated from the commercial line Horst U1 (H97 or H39). Since high value for earliness represented later mushroom production, the beneficial alleles for earlier mushroom production were in both cases contributed by the wild parent (Mes09143 or Z8). If these QTLs can be assigned to smaller chromosome regions, which may facilitate marker assisted breeding for an earlier producing cultivar.

Firmness and maturation are two crucial traits for good quality. Firm and closed mushrooms are more appreciated and have a longer shelf life than soft and open mushrooms. As stated previously, the two traits are very likely interdependent to a large extent since the developmental stage (maturation) explains the firmness to a large extent. It is thus not surprising that both traits have major QTLs on CHR10 explaining 31% variance for firmness and 35% variance for maturation in flush 2 of Set 1. The beneficial alleles are from the same parent (H97). CHR10 was also detected as a major QTL for firmness in Set 3 with the beneficial allele from H39. Both H97 and H39 are derived from the commercial variety Horst U1, known for its firm and late maturing mushrooms.

Smooth caps are an important quality trait because the scales on the cap discolor easily post-harvest and reduce thus the quality. A major QTL was detected on CHR 10 explaining 32% of the phenotypic variation for scaling in Set 1. Although the two original heterokaryons of Set 1 were not significantly different in scaling, the high value allele beneficial for smoothness in this case was from the wild parent Mes09143, which might be useful for breeding smoother mushroom caps. This QTL was also detected as a significant QTL in Set 3, but the "smooth" alleles were from different parents in the two flushes (the wild parent Z8 for flush 1 and the commercial H39 for flush 2). This correlates with the performance of this trait in the wild heterokaryotic parent WW7 and Horst U1. WW7 was significantly smoother than the commercial parent Horst U1 in flush 1, but they were not significant different in flush 2. A large difference in scaling between flushes is not often seen in strains examined so far but it is common knowledge that Horst U1 is scaling in flush 1 and hardly in flush 2. That is an interesting observation since we know the pedigree of Horst U1. One of its constituent homokaryons, i.e. H39, is derived from a traditional white variety that is smooth whereas H97 is derived from a scaling off-white variety. It is thus possible that different alleles are expressed in different flushes. If so, this might also have some relevance to differences found in QTL between flushes in this study.

The length of the stipe is relevant for the type of market for which mushrooms are produced. Longer stipes are required when mushrooms are harvested mechanically for the canning industry compared to mushrooms picked by hand for the fresh market. Variation in stipe length can be manipulated by changing environmental factors, *e.g.* the concentration of CO_2 or the density of mushrooms on the growing beds. Elongation of stipe length can be achieved through increasing the concentration of CO_2 in the growing room, which indicates that the variation in stipe shape certainly has an environmental component. However, a high concentration of CO_2 leads to the early maturation and loss of yield, therefore it is important to know the genetic determinants for stipe shape. Consistent and major QTLs for stipe shape were found in this study, and the high value alleles here were mostly from the wild parents Mes09143 and Z8.

Mushrooms of various sizes are required for different cuisines. The size of button mushrooms is influenced by many related traits, e.g. the earliness, density of mushrooms (Foulongne-Oriol et al., 2012a) and maturation (this study). Major QTLs for size were detected in this study, *i.e.* CHR10 for Set 1 & 3 and CHR6 for Set 2. Since there are strong correlations between size and the other traits, QTL effects on size might be indirect effect of other traits, e.g. mushrooms matured late are often big in size.

Six traits (distribution, firmness, maturation, scales, stipe shape, and size) were scored, in total six times in the three heterokaryon sets (two flushes per set). COCO and ER were scored three times (no flush difference). QTLs detected consistently at least twice (with two different data sets) were summarized (Table 9). The overview clearly shows the pleiotropic regions on CHR10 and CHR6, with effects on most traits. CHR10 was also a major QTL for resistance of *Lecanicillium fungicola* (dry-bubble disease) in button mushroom (Foulongne-Oriol et al., 2012b). This study showed that resistance might genetically correlate to earliness, here also mapped to chromosome 10. Consistent QTLs were found for all the tested traits. As shown in the Results, some QTLs for the same traits on the same chromosomes had different high value alleles in Set 1 and Set 2, whereas there is more overlap between Set 1 and 3. Since Set 1 and 2 represent the same set of homokaryotic offspring one would expect a better overlap in QTL between these populations. This seemingly inconsistency can be explained well by examining the genetic relationship between these two sets of heterokaryons. Heterokaryon set 1 contains alleles in one nucleus from either H97 or Mes09143 and in the other nucleus only alleles from the tester H39. Set 3 contains in one nucleus alleles from H39 or Z8 and in the other nucleus alleles from the tester H97. Both sets of heterokaryons share thus, on average, 75% of the alleles derived from Horst U1. In both sets of heterokaryons alleles from the original Horst U1 (H39 x H97) are thus combined and indeed most good traits present in Horst U1 map to the relevant parent in these populations.

Table 9. QTLs consistently detected with at least two data sets

Traits	CHR1	CHR2	CHR3	CHR4	CHR5	CHR6	CHR7	CHR8	CHR10	CHR11	CHR13
CC								* Mes			
COCO						Mes/97					
DS		Mes/97					97/39		* 97/Z8	97	* Z8
ER		* 97/39	97/39	97/39		* Mes/39	Mes/39	* 97/Z8	* 97/39		
FM	39				* 97	* Mes/97/39		Z8	* 97/39		
MT		Mes/39	97/39	* 97/39	Mes/97	Mes/39			* 97/39		
SC			Mes/39			Mes/39	97/39		* Mes/39/Z8	Mes	
SS	Mes		Mes/Z8	* Mes/Z8		97/Z8	97/39	Mes/39	* Mes/Z8		
SZ				39		* Mes/39			* 97/39		

Only significant and consistent QTLs are presented; QTLs explaining larger phenotypic variation (10% ≤ R^2 ≤ 30%, >90% for CC) are indicated with an asterisk. Mes09143 (Mes) and H97 (97) are contributing the high value alleles for traits of Set 1 and Set 2, and H39 (39) and Z8 are contributing high values alleles for traits of Set 3.

Here we made a start with understanding the genetic base for a number of agronomic and quality traits in button mushrooms. This was done using for the first time in mushroom breeding a multi-trait QTL analysis. Although the experimental set up was limited and data sets not complete, this approach shows that QTL can be found in this way and that it also elucidate relationships between traits. Due to the low recombination frequency, mapping for precise QTL locations was not possible. Previous research has indicated that recombination frequency in the tetra-sporic variety *burnetti* (Foulongne-Oriol et al. 2010) is much higher. Identifying genes involved in this important trait would allow the introduction of higher recombination in the bisporic variety. Since the two varieties are fully compatible and the trait is dominant, this seems to be feasible. That would allow a more precise mapping and reduction of linkage drag.

Additional file 1

QTLs of all traits detected by single trait QTL analysis

Heterokaryon set	Locus no.	Locus name	Linkage group	Position	-LOG10(P)	%Expl. Var.	Add. eff.	High value allele	s.e.
COCO/Set 1	2	ChrI_M1	1	1.6	4.895	7.128	0.268	H97	0.06
	45	MHchrV_01	5	8.69	2.858	3.598	0.19	Mes09143	0.059
	59	MHchrVI_02	6	22.9	10.483	16.747	0.41	H97	0.058
	125	ChrIX_T1	9	1.17	5.999	8.556	0.293	Mes09143	0.058
COCO/Set 2	66	MHchrVII_01	7	0	3.86	6.795	0.261	Mes09143	0.067
	126	MHchrIX_03	9	2.23	4.763	8.332	0.289	H97	0.066
	153	C13P2	13	1.98	5.034	9.358	0.307	H97	0.067
ER/Set 1	59	MHchrVI_02	6	22.9	8.922	15.933	0.4	Mes09143	0.062
	125	ChrIX_T1	9	1.17	4.405	7.145	0.268	H97	0.064
	131	ChrX_B1	10	0.62	5.159	8.906	0.299	H97	0.065
ER/Set 2	11	C2P2	2	2.15	5.693	10.082	0.318	H97	0.065
	18	MHchrIII_05	3	1.08	5.522	9.238	0.305	H97	0.063
	34	ChrV_T1	5	0	4.157	6.68	0.259	Mes09143	0.064
	95	MHchrVIII_03	8	29.5	7.744	14.233	0.378	H97	0.064
ER/Set 3	16	HZchrIII_05	3	0	3.95	5.797	0.241	H39	0.061
	24	HZchrIV_06	4	0	4.981	7.952	0.283	H39	0.062
	48	HZchrVI_02	6	0	3.152	4.711	0.218	H39	0.063
	59	C8P2	8	1.59	5.021	8.08	0.285	Z8	0.062
	90	CHR10A1	10	22.74	9.763	17.992	0.425	H39	0.063
	115	C13P6	*13	5.72	3.847	6.964	0.265	H39	0.068
CC1/Set 1	95	MHchrVIII_03	8	29.5	81.699	87.576	0.938	Mes09143	0.028
CC2/Set 1	95	MHchrVIII_03	8	29.5	79.128	87.659	0.939	Mes09143	0.028
CC1/Set 2	95	MHchrVIII_03	8	29.5	76.561	86.431	0.932	Mes09143	0.029
CC2/Set 2	95	MHchrVIII_03	8	29.5	92.912	91.576	0.96	Mes09143	0.024
DS1/Set 1	57	C6P21	6	20.7	2.884	5.397	0.233	Mes09143	0.071
	77	MHchrVII_03	*7	11.37	3.379	6.374	0.253	H97	0.07
DS2/Set 1	131	ChrX_B1	10	0.62	3.824	7.857	0.281	H97	0.073
DS1/Set 2	125	ChrIX_T1	9	1.17	2.976	5.767	0.241	H97	0.072
DS1/Set 3	113	C13P3	*13	2.86	3.643	8.642	0.295	Z8	0.078
DS2/Set 3	52	ChrVII_T3	7	2.32	3.453	6.667	0.082	H39	0.022
	87	C10P18	10	18.19	5.919	15.066	0.123	Z8	0.024
FM1/Set 1	13	MHchrII_04	2	4.65	4.182	5.695	0.239	Mes09143	0.059
	38	C5P7	5	6.51	6.587	10.436	0.324	H97	0.061
	61	MHchrVI_01	6	23.96	4.161	5.671	0.239	H97	0.059
	131	ChrX_B1	10	0.62	9.362	15.135	0.39	H97	0.059
	152	MHchrXIII_05	*13	0	3.716	5.079	0.226	Mes09143	0.059
FM2/Set 1	34	ChrV_T1	5	0	3.877	5.622	0.238	H97	0.061
	131	ChrX_B1	10	0.62	14.077	27.537	0.526	H97	0.062
FM1/Set 2	18	MHchrIII_05	3	1.08	3.231	5.599	0.237	H97	0.068
	62	C6P26	6	25.5	6.077	12.839	0.359	Mes09143	0.07
FM2/Set 2	61	MHchrVI_01	6	23.96	3.75	7.652	0.277	Mes09143	0.072
FM1/Set 3	48	HZchrVI_02	6	0	5.019	10.426	0.324	H39	0.071
	90	CHR10A1	10	22.74	6.801	13.984	0.375	H39	0.069
FM2/Set 3	58	ChrVIII_B1	8	0	3.406	6.612	0.258	Z8	0.071
	94	CHR10B1	10	23.32	6.121	13.092	0.363	H39	0.07

MT1/Set 1	15	ChrII_B1	2	5.88	5.41	8.629	0.295	Mes09143	0.062
	55	ChrVI_B1	6	17.35	4.054	6.22	0.25	Mes09143	0.062
	131	ChrX_B1	10	0.62	6.923	12.208	0.35	H97	0.064
MT2/Set 1	30	MHchrIV_02	4	0.4	5.451	7.245	0.27	H97	0.056
	130	MHchrX_01	10	0	20.397	37.44	0.614	H97	0.057
MT1/Set 2	17	ChrIII_B1	3	0	3.829	7.074	0.267	H97	0.069
	30	MHchrIV_02	4	0.4	3.797	7.263	0.27	H97	0.07
	61	MHchrVI_01	6	23.96	3.917	7.341	0.272	Mes09143	0.069
MT2/Set 2	61	MHchrVI_01	6	23.96	4.26	8.871	0.299	Mes09143	0.072
MT1/Set 3	30	ChrIV_T3	4	8.79	4.369	9.069	0.302	H39	0.072
MT2/Set 3	66	CHR9B1	9	0	2.789	6.267	0.251	Z8	0.078
SC1/Set 1	32	ChrIV_B1	4	1.05	5.024	7.663	0.278	H97	0.061
	131	ChrX_B1	10	0.62	13.833	26.629	0.517	Mes09143	0.062
SC2/Set 1	131	ChrX_B1	10	0.62	2.969	5.885	0.243	Mes09143	0.073
SC1/Set 2	94	C8P28	8	27.79	3.249	6.69	0.259	H97	0.074
SC2/Set 2	20	ChrIII_M1	3	4.36	3.602	7.122	0.268	Mes09143	0.072
SC1/Set 3	87	C10P18	10	18.19	5.15	12.845	0.359	Z8	0.078
SC2/Set 3	19	ChrIII_T2	3	2.92	3.207	6.793	0.261	H39	0.075
	94	CHR10B1	10	23.32	4.576	9.969	0.317	H39	0.073
SS1/Set 1	1	ChrI_B1	1	0	2.824	4.603	0.215	Mes09143	0.067
	32	ChrIV_B1	4	1.05	5.163	9.182	0.304	Mes09143	0.066
	61	MHchrVI_01	6	23.96	5.4	9.713	0.312	H97	0.066
SS2/Set 1	32	ChrIV_B1	4	1.05	10.295	20.234	0.451	Mes09143	0.065
	126	MHchrIX_03	9	2.23	2.897	4.396	0.21	Mes09143	0.064
SS1/Set 2	39	ChrV_B2	5	8.14	2.44	3.748	0.194	H97	0.066
	61	MHchrVI_01	6	23.96	3.901	6.778	0.261	H97	0.067
	70	C7P7	7	6.85	4.668	9.437	0.308	H97	0.071
	93	ChrVIII_B1	8	26.07	3.926	6.633	0.258	Mes09143	0.066
SS2/Set 2	31	MHchrIV_03	4	0.53	7.661	16.375	0.406	Mes09143	0.069
SS1/Set 3	35	CHR4B1	4	9.97	3.372	6.48	0.255	Z8	0.071
	90	CHR10A1	10	22.74	5.763	12.362	0.353	Z8	0.071
SS2/Set 3	90	CHR10A1	10	22.74	3.189	6.92	0.264	Z8	0.076
SZ1/Set 1	14	MHchrII_01	2	5.86	4.15	8.079	0.285	Mes09143	0.07
SZ2/Set 1	26	ChrIV_T2	4	0	3.323	5.686	0.239	H97	0.067
	131	ChrX_B1	10	0.62	6.868	13.839	0.373	H97	0.068
SZ1/Set 2	62	C6P26	6	25.5	6.253	14.019	0.375	Mes09143	0.072
SZ2/Set 2	61	MHchrVI_01	6	23.96	7.118	15.227	0.391	Mes09143	0.07
SZ1/Set 3	90	CHR10A1	10	22.74	3.972	8.299	0.289	H39	0.073

"*" indicates QTLs detected only in single trait analysis but not in multi-trait analysis.

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Chapter 7

General Discussion

Mechanical harvesting is widely used in the Netherlands, which is an effective technique to reduce the high labour costs during mushroom harvesting. However, button mushrooms are sensitive to mechanical bruising and discolor soon after harvesting, making mechanical harvesting not suitable to harvest mushrooms for the fresh market so far. Consequently, a demand of the Dutch mushroom industry is that varieties should be developed which produce mushrooms that can be sorted and packed mechanically after harvest without bruising. In order to assist breeding for cultivars that are less sensitive to bruising, unravelling the genetic determinants that cause bruising sensitivity and/or resistance is the main objective of this study. Since advanced cultivars combine several good characters, QTLs involved in agronomic and quality traits were analysed. Because of the atypical life cycle of *A. bisporus*,quantitative trait loci for these traits cannot be precisely located on small genome regions. Additionally, the meiotic recombination in the life cycle of *A. bisporus* var. *bisporus* was therefore, investigated in-depth.

Breeding mushrooms versus breeding plants

Breeding is about manipulating the attributes, structure, and composition of various organisms to meet the needs of human society. Plants are the primary food producers in the Earth's ecosystem, and plant breeding is considered to be critical to the survival of modern society. Plant breeding is necessary to address the needs of food, feed and industrial usage all around the world. Basically there is a need to enhance the value of food crops by improving the yield and the nutritional quality of products for feeding a growing world population in a healthy way. Next to this, plant breeding adapts plants to environmental stresses and specific production systems, and it also meets the aesthetic demands of humans by means of improved ornamental horticultural varieties. Selection of useful genotypes – one of the key components of plant breeding - was started already many millennia ago for the most important crops all around the world. Archaeological records indicate that selection and domestication started 3 to 5000 years BC and that plant breeding started at 700 BC, and the first reported sexual reproduction in plants is in 1694 in Germany (Acquaah, 2012). The development of modern plant breeding is indispensable with a series of milestones in the history of plant breeding, e.g., the (re)discoveries of the laws of Mendelian inheritance in pea breeding, the development of the Hardy–Weinberg principle in population genetics, inbreeding and outbreeding of maize hybrids, the description of transposable elements in McClintock's maize breeding experiments, etc. The use of genetics in plant breeding has yielded spectacular successes, which brought yield increase, the enhancement of compositional traits and crop adaptation. Commercial plant breeding is undertaken in both private and public sectors for about 150 years now. Profit of plant breeding is primarily gained by private companies. The large number of breeding companies or seed companies indicates the profitability of the plant breeding industry. Since the cosst of the development of new cultivars are usually very high, and it takes several years to complete a breeding program, products from private seed companies are proprietary, which are usually protected by obtaining plant variety protection (PVP) or in specific parts of the world like USA a plant patent. This protects the breeder's rights and enhances the chance of returns from the breeding investment.

Other than plants, mushrooms are not considered as a primary food product. Mushroom breeding has been for long an applied science lagging behind plant breeding. Mushrooms have been cultivated for a long time but only since the last century they are cultivated commercially on a large scale. Of the ca. 5000 species of edible fungi, only 20 have been commercially cultivated (Chang, 1993). As for plants, new mushroom varieties can be protected by plant variety protection (PVP). The first protection was granted for *Agaricus bisporus* (button mushrooms) in 1981. Based on the PVP data of UPOV (International Union for the Protection of New Varieties of Plants) a total 645 varieties of 17 mushroom species were applied and granted for protection (Table 1). This number is small and not comparable with that of plant varieties. For instance, the PVP number of *Solanum lycopersicum* (tomato) is 19,716, and the first PVP for tomato was already granted in 1899. It is clear from this that the effort put into mushroom breeding is much less than that of plant breeding.

Table 1. Cultivated mushroom species and the number of PVP

NO.	Species	Variety	Number of PVP	Year of the first granted variety	Year of the latest granted variety
1	*Agaricus sp.*	*Agaricus bisporus*	39	1976	2013
		Agaricus subrufescens	1		2008
		Agaricus blazei Murrill	1		2007
2	*Pleurotus sp.*	*Pleurotus spp.*	42	1987	2012
		Pleurotus cornucopiae	1		2007
		Pleurotus cystidiosus	3		?
		Pleurotus eryngii	38	2002	2012
		Pleurotus florida	2		1983
		Pleurotus ostreatus	29	1988	2013
		Pleurotus pulmonarius	6	2000	2012
3	*Flammulina sp.*	*Flammulina velutipes*	53	1988	2012
4	*Coprinus sp.*	*Coprinus comatus*	1		1988
5	*Lentinus sp.*	*Lentinus edodes*	213	1981	2011
6	*Pholiota sp.*	*Pholiota adiposa*	27	1987	2010
7	*Stropharia sp.*	*Stropharia rugosoannulata*	2		1986
8	*Auricularia sp.*	*Auricularia polytricha*	3	1990	1996
9	*Hericium sp.*	*Hericium erinaceum*	4	2006	2008
10	*Agrocybe sp.*	*Agrocybe aegerita*	4		2011
		Agrocybe cylindracea	6	1991	2001
11	*Lepista sp.*	*Lepista nuda*	1		?
12	*Cordyceps sp.*	*Cordyceps militaris*	3		2008
		Cordyceps basiana	1		2010
13	*Ganoderma sp.*	*Ganoderma lucidum*	2		?
14	*Hypsizygus sp.*	*Hypsizygus marmoreus*	75	1988	2013
15	*Grifola sp.*	*Grifola frondosa*	44	1986	2010
16	*Sparassis sp.*	*Sparassis crispa*	11	2008	2011
17	*Lyophyllum*	*Lyophyllum decastes*	29	1998	2009
		Lyophyllum shimeji	4	2009	2010
Total			645		

Source: UPOV: Plant Variety Database

Taking button mushroom as an example, the development of button mushroom industry has been mainly attributed to the improvement of cultivation techniques in the last 30 years. The breeding efforts were very limited and worldwide we only find a low number of spawn/breeding companies. The reasons for the slow development of mushroom breeding are diverse. First of all, despite the legal and theoretical protection by PVP, mushroom varieties are difficult to protect in a practical sense. They can be copied easily by making tissue cultures of a mushroom and use this to prepare spawn that is sold under a different name. This applies to most mushrooms species. Second, since the majority of the single spore isolates of button mushroom are heterokaryotic it is difficult to obtain homokaryons as breeding materials. This hampers outcrossing, and limits breeding success (Kerrigan, 1994, Moquet et al., 1998). Third, the low recombination frequency of homologous chromosomes is an obstacle for marker- assisted breeding and breaking linkage during introgression breeding programs (Sonnenberg et al., 2011). Most plant species do not have these obstacles. The result of these obstacles is ample demonstrated by the presence of cultivars that are either direct copies of an existing cultivar or essentially derived varieties (EDV) of existing cultivars (Sonnenberg et al., 2011). EDVs in button mushrooms are often represented by fertile offspring (single spore cultures having received two non-sister nuclei) of a current cultivar. For instance, the first hybrid strain Horst U1 was released in 1980 (Fritsche, 1981), and most new strains which appeared subsequently on the market were either identical or very similar to this hybrid, i.e., EDVs of Horst U1. The whole selection procedure of an EDV takes up to several months. However, a real breeding program is time-consuming, and usually takes years. The convenience of generating EDVs lead to a consequence that no one is willing to invest in a real large breeding program to obtain real new cultivars, but rather pursue “new” cultivars by means of selecting fertile offspring of a currently used and

successful variety. On the spawn market of button mushrooms, there is no real new cultivar, made by breeding, released during the last 30 years that is substantially different and better than the first released hybrid Horst U1. Although the mushroom breeding industry is not as big and as successful as the plant breeding industry, the future of mushroom breeding companies can still be prosperous since the world market of mushrooms is increasing considerably year by year. The investments needed, however, are only done if new varieties can be protected well. Copies can be challenged using existing laws. To consider fertile single spore cultures as essentially derived varieties (EDVs) needs the support and consensus within the breeding and mushroom industry. Attempts to arrange such a consensus have started recently (Sonnenberg, 2013). As in plant breeding, in order to promote the development of mushroom breeding advanced techniques and theories have to be applied to accelerate mushroom breeding programs in terms of evaluating potential parental lines and breeding stocks, high throughput genotyping & phenotyping, and strain protection, etc. Besides this, the healthy function of mushrooms will have to be advertised more and better to consumers to broaden the market.

Mechanisms of mushroom bruising and discoloration

Biochemical cause of mushroom discoloration

The biochemical cause of mushroom discoloration is an oxidization process of phenols catalyzed by the enzyme tyrosinase. Melanin is the final product after a series of chemical reactions, i.e., a consequence of spontaneous polymerization of the reactive quinones (Goldfeder et al., 2013), which is visible as the brown discoloration (Jolivet *et al.*, 1998). This implies that the level of brown discoloration should be correlated with the content level or the color of melanin. Melanin is a brown to black pigment and exists widely in all kinds of organisms, where its function is thought to be protecting organisms from harsh environmental stresses (Eisenman & Casadevall, 2012). Melanin is a component of the cell wall of fungi, which can be found in the inner and outer layers of the cell wall. It is particularly important for strengthening the cell walls under harsh conditions (Free, 2013). The structure of melanin is difficult to study due to its insolubility. Previous studies have been focused on its phenolic precursors. DOPA (L-3, 4-dihyroxyphenylalanine) -melanin and GHB (γ-L-glutaminyl-4-hydroxybenzene)-melanin mainly exist in *A. bisporus*, and GHB-melanin pathway is mainly involved in bruising-induced discoloration (Jolivet, 1995). Melanin is not only attributed to the brown pigment of the native cap color, gill & spore color, but also to the postharvest discoloration, and bruise-induced discoloration of button mushroom. Since the brown color of the four situations mentioned above are apparently not on the same level, different types of melanin may be present in different tissues and developmental phases indicating different mechanisms of melanin synthesis.

Bruise-induced discoloration and postharvest discoloration

Bruise-induced discoloration only happens to the bruised area on the cap skin, and postharvest discoloration happens to the whole mushroom; so both skin and flesh. The process of bruised-induced discoloration is faster and the level is more severe than post-harvest discoloration. This suggests that these two types of discoloration have different mechanisms (Burton & Noble, 1993). The tyrosinase and phenolic compounds are present separately in cells, and intracellular membrane disintegration was considered to be the cause for both of these two types of discoloration but in different time points. However, exudate released from the mycelium cell was observed in a damaged cap surface but not in an aging cap surface (Rama et al., 2000). Since the postharvest discoloration proceeds gradually, the exudate might be too little to be observed. In this thesis, the sensitivity of the bruise-induced discoloration (bruising sensitivity) is the trait of interest.

Bruising sensitivity (BS) is a highly inheritable trait, i.e., the difference in bruising sensitivity of different strains is mainly determined by genetic factors, although it is also slightly influenced by environmental

factors (Chapters 2 & 3). Three environmental factors (relative humidity in the growing room, casing depth and water content of casing) were tested on four strains in Chapter 2. The main purpose of this chapter was to find an environmental condition generating the largest phenotypic variation between different strains. It showed that the combination of environmental conditions now used for commercial cultivation is the one generating the largest phenotypic variation among the four tested genotypes. Although four genotypes might be insufficient to represent the entire genotypic variation, the results showed that strains of high bruising sensitivity were more prone to changes in climate conditions with respect to bruising sensitivity. Since different genotypes might have divergent production times and the climate conditions vary during the cropping period, it might be good to test different genotypes in separate cultivation tests. Next to climate control, watering is an essential procedure during the mushroom cropping period, which has to be included as an experimental factor. Different amounts of water given to the treatment might influence the effect of the other factors, especially the water content of the casing. It would be good to determine the amount of water given to every experimental treatment and make a stringent watering schedule for the whole cropping period in future studies.

Candidate genes involved in the melanin pathway

With the availability of the whole genome sequence of *A. bisporus,* a total of 42 genes which might be involved in the melanin synthesis pathway were identified through homologous alignment. For 26 of those genes, gene expression was determined (Weijn et al., 2012). However, the correlation between gene expression level and level of discoloration has not been established. Since melanin is the pigment for both native skin color and synthetic browning (because of aging or bruise-induced discoloration), the suppression of melanin synthesis might result in both the lightening of skin color and the lower level of discoloration. Mutations in some genes are known to cause an absence of melanin synthesis as seen in the dilution of mouse coat colors and the human oculocutaneous albinism OCA1-4. The genes responsible for these phenotypes are TYR, OCA2, the tyrosinase-related protein 1 gene (TYRP1) and SLC45A2, which are also reported to be associated with normal variations in skin, hair or eye color traits (Sturm, 2009). The major locus for cap color has been mapped on chromosome 8 (Callac *et al.*, 1998, Foulongne-Oriol *et al.*, 2012), and several genes involved in the melanin pathway have been identified on this chromosome, e.g., PPO1, PPO6, and 4CL_9 (Weijn et al., 2012). Knockouts of genes involved in the melanin pathway can be tried to see if the melanin synthesis in the cap skin could be stopped or suppressed. Although there is a long list of candidate genes, it is still unknown which one is the key gene that determines the bruising sensitivity. This problem can be studied through genetic analysis of bruising sensitivity in segregating populations.

QTL analysis of bruising sensitivity

Two segregating populations were generated and used for QTL analysis in Chapter 4. The parental homokaryons of the two populations were selected by evaluating the breeding value of candidate parental lines in a diallel scheme. The four parental lines showed the highest breeding values of either direction, i.e., bruising resistant or bruising sensitive (Chapter 3). Lines with the lowest general combining ability (GCA) might be good donors for bruising resistance, and lines with highest general combining ability might be good donors for bruising resistance. Parental homokaryons with low GCA can be used for introgression breeding to stack positive alleles for bruising resistance, whereas parental homokaryons with high GCA can be used as parental lines of segregating populations to map the genetic determinants of bruising resistance and sensitivity. Mainly because of the incompatibility, the diallel scheme generated in Chapter 3 is incomplete. It would be wise to make a broad selection for all compatible lines in the scheme to get a better estimation of the breeding value.

Because of the low recombination frequency of var. *bisporus,* the resolution of the genetic linkage maps used in this study was too low to precisely locate the beneficial or detrimental alleles on the genome. As

mentioned in Chapter 4 and 5, *A. bisporus* var. *burnetti* has a normal recombination frequency although it is poor for most agronomic and quality traits. A screening for a collection of the tetrasporic strains could be made to find ones with better performance, which can be used as further breeding materials in order to remove the linkage drag during introgression breeding. QTL analysis could be performed in an intervarietal population for a more precise localization as was done in previous studies (Foulongne-Oriol et al., 2012).

In order to have enough crossover recombinants, a pre-selection for crossover recombinants was done while generating the population. This might have led to the distortion of SNP genotypes detected in Chapter 4. The distortion of markers might also be caused by the segregation distortion loci, which are subject to gametic or zygotic selection (Xu, 2008). The segregation distortion could decrease the power of QTL mapping and cause QTL inconsistencies (Foulongne-Oriol et al., 2012). However, it was also found that regions of the genome with severe segregation distortion are equally if not more likely to contain QTL. If these markers are deleted from the map in QTL analysis, more QTLs will be missed (Wang *et al.*, 2005). Xu (2008) found that segregation distortion loci (SDL) are not always harmful to QTL mapping, QTL mapping can potentially but not necessarily benefit from them. However, an extra mathematical model should be used considering both SDL mapping and QTL mapping (Xu, 2008). According to Xu, the presence of only a few SDL can cause the entire chromosome to distort form Mendelian segregation. We did find several entire chromosomes distorting from Mendelian segregation in Chapter 4. Nevertheless, whether they contain SDL and how they influence QTL detection needs to be further investigated.

The two segregating populations are homokaryotic, and were crossed with three homokaryotic tester lines to produce mushrooms for the evaluation of bruising sensitivity and cap color in Chapter 5. Due to the significant interaction effect of genotype by tester lines, the phenotype was significantly influenced by different tester lines. This is a reason for the difference in QTL locations detected in the same segregating populations crossed with two different tester lines. To avoid the interference of tester lines, heterokaryotic offspring could be used alternatively for QTL mapping, which is equivalent to a F2 population in plant breeding.

Cap color (CC) and bruising sensitivity (BS) are two relevant traits, and it is not surprising to see the co-localization of QTLs of CC-QTL and BS-QTL. With the interference of the brown cap color, a major BS-QTL was co-localized with the major CC-QTL on chromosome 8 in population 1 (the offspring of a brown resistant and a white resistant line). However, it is hard to tell if they are the same QTL or two closely linked QTLs due to the low resolution of the genetic linkage map. With cap color as a group factor QTL mapping was also performed in the non-white and white groups for bruising sensitivity. Consistent QTLs with relatively large explained variance were detected for non-white and/or white groups. Thus, the major QTL on chromosome 8 might be an artifact due to the interference of cap color. In addition, QTL mapping for CC and BS was also conducted in a second population (the offspring of a white resistant line and a white sensitive line). Although the co-localization was observed also in population 2, the BS-QTL localized on the same chromosome with CC-QTL was not a major QTL. To avoid the interference of brown cap color and to prove the existence of the BS-QTL on chromosome 8, a pure brown population (the offspring of a brown resistant line and a brown sensitive line) could be used alternatively.

Although a long list of relevant genes of the melanin biosynthesis pathway were reported, and the expression was tested in the PhD thesis of Weijn (2013), the key genes involved in the mechanism of bruising sensitivity remain unknown. The gene expression profile has not been studied in association with the phenotype, i.e., eQTL. The expression of 19 relevant genes was analyzed in a number of extreme resistant offspring and extreme sensitive offspring (population 1 with tester line H39), but no significant difference in the level of expression in resistant offspring and sensitive offspring was found, i.e., the expression level was not correlated with the level of bruising sensitivity (Weijn, 2013). Further gene expression analyses are needed for individuals of population 2. Alternatively, since the genome sequence

of *A. bisporus* is available, gene expression profiling using microarray technique might be feasible to find candidate genes (Yang et al., 2013). In combination with the results of QTL mapping, thousands of genes located on chromosomes where QTLs are located can be simultaneously monitored for the expression.

As the cap color is controlled by a major QTL on chromosome 8, it might be a coincidence that most of the color-related traits in plants seemed to be controlled by major genetic factors. Flower color (white, pink, brick red) of pot azalea (*Rhododendron simsii*) was measured as well with an image system, and a major QTL was identified with explained variation from 57.5% to 67.2%. This major QTL determined whether the flower color would be white or non-white. Some minor QTLs seemed to be related to pink coloration (De Keyser et al., 2012). In rose and lily flower color was mapped as a single qualitative marker (Debener & Mattiesch, 1999, Abe et al., 2002). Two putative flower color QTLs were mapped in Alstroemeria (Han et al., 2002), and carnation (Yagi et al., 2008). For Brassica carinata, petal and anther tip color are two Mendelian-inherited traits, and one major QTL was identified for seed coat color (Guo et al., 2012). Major alleles have been found at a variety of other loci in a number of organisms, with alleles having a large effect on one trait often showing a wide range of pleiotropic effects on the other traits (Lynch & Walsh, 1998). Thus, this major allele determining the cap color of button mushroom might have pleiotropic effects on other related traits, which can be found in QTL analysis for multiple traits.

Breeding for multiple agronomic and quality traits

Good cultivars combine optimal characters of multiple traits, and traits are interrelated. It is wise to evaluate multiple traits rather than a single trait in a breeding scheme. Multi-trait QTL analyses were conducted to study the genetic architecture of 8 agronomic and quality traits (Chapter 6). It was observed that some of these traits were indeed significantly correlated. For traits having general heritability (not lower than 0.4), genetic correlations are comparable with phenotypic correlations usually having the same sign and the same magnitude (Cheverud & Routman, 1995, Roff, 1995). Three types of mechanisms might result in genetic correlations (Lynch & Walsh, 1998). One is pleiotropy, where a single gene is influencing two or more traits, and such genes are often involved in complex biochemical, developmental, and regulatory pathways. Linked QTLs may be another mechanism, i.e., multiple genes controlling different traits are linked in the same chromosome regions. A third possible mechanism of genetic correlation is linkage disequilibrium (LD) between linked or unlinked genes affecting different traits, i.e., some combinations of alleles occur in a population more often or less often than the expected 1:1 ratio. LD between linked loci is high in this study since the recombination frequency is so low, but LD between unlinked loci can be ruled out since there was no significant correlation between markers on different chromosomes (Chapter 4). Pleiotropy or linked QTLs controlling multiple correlated traits were detected in Chapter 6. However, in the case of suppressed recombination frequency as is observed in *A. bisporus var. bisporus*, it is difficult to distinguish pleiotropy from (closely) linked QTLs, and also difficult to reliably distinguish different QTLs on the same chromosome. The availability of the tetrasporic variety with a normal level of recombination and the compatibility with the bisporic variety will be helpful for a more precise QTL mapping for multiple traits. It also has been suggested that linkage disequilibrium (LD) mapping, TILLING (Targeting induced local lesions in genome), and gene replacement or knockdown allow not only dissecting genetic correlations between traits but also discriminating between pleiotropy and linkage of QTL causing trait correlations (Wagner & Zhang, 2011). These approaches can be applied in further studies to distinguish pleiotropy from linked QTLs involved in multiple agronomic and quality traits of button mushroom with the availability of a large number of divergent accessions.

Meiosis in *Agaricus bisporus var. bisporus*

According to the high-level of phylogenetic classification, *Agaricus bisporus* belongs to the Phylum of *Basidiomycota*, the sub-Phylum of *Agaricomycotina*, the Class of *Agaricomycetes*, the sub-Class of *Agaricomycetidae*, the Order of *Agaricales*, the Family of *Agaricaceae*, and the Genus of *Agaricus* (Hibbett

et al., 2007). It differs from most basidiomycetes in the sexuality system. *Agaricus bisporus* has two sub-varieties (sub-species), i.e., *A. bisporus* var. *bisporus* and *A. bisporus* var. *burnetti*. Like all the other basidiomycetes, it reproduces sexually in basidia and generates meiospores (basidiospores) on basidia. Ca. 90% of the basidia of *A. bisporus* var. *bisporus* produce 2 spores, each receiving 2 non-sister nuclei. As a consequence, these spores have different mating types and can produce mushrooms. For a long time the life cycle of this variety has thus been considered as homothallic. Ca. 10% of the basidia, however, produce 3 or 4 spores and most of these spores receive one nucleus. They germinate into homokaryons and must be crossed with compatible homokaryons to produce mushrooms. Upon the discovery of the rare homokaryotic offspring, the sexuality of the var. *bisporus* has been designated as secondary homothallic. The subvariety *A. bisporus* var. *burnettii* produces predominantly 4-spored basidia (90% of the basidia, with a minor number producing 3-spores). The majority of the single spores germinate thus into homokaryons and this variety has been considered as heterothallic. Since both varieties produce 2- and 4-spored basidia they should be considered as amphithallic.

Although these two varieties are both edible, only var. *bisporus* is commercially cultivated because of the poor quality of var. *burnetti* (Callac, 1993). Almost all the breeding efforts are put into the breeding of advanced cultivars of var. *bisporus*. However, due to the atypical life cycle of var. *bisporus*, i.e., most of the offspring are heterokaryotic and fertile which cannot be used as breeding materials, outcrossing is hampered. It is laborious to isolate and identify homokaryons out of large amounts of heterokaryons. Besides, several previous studies (Kerrigan et al., 1993, Foulongne-Oriol et al., 2010) and this study (Chapter 4) showed that the recombination frequency between homologous chromosomes is very low. It means that the generation of crossover recombinants of var. *bisporus* is limited, and the genetic variation in the offspring is mainly due to a re-assorting of parental chromosomes. The crossover suppression poses a barrier to introgressive hybridization during breeding of button mushroom, which leads to difficulties of reducing linkage drag.

The low recombination frequency hampers precise marker-assisted breeding. Offspring of the intervarietal cross of var. *bisporus* and var. *burnetti* showed a normal recombination frequency (Foulongne-Oriol et al., 2010). This suggested that the recombination frequency is suppressed in bisporic var. *bisporus* but normal in the tetrasporic var*. burnetti.* The elevated number of spores per basidium (BSN locus) was mapped to chromosome I on some distance of the mating type locus (Imbernon *et al.*, 1996). A cross was made between a homokaryotic single spore culture having the mating type and the BSN allele from the tetrasporic variety and a homokaryon of a bisporic variety. Offspring of this cross showed a reduced recombination indicating that the differences in recombination frequency are controlled by more than one gene (Foulongne-Oriol et al., 2011). The compatibility between var. *bisporus* and var. *burnetti* provides a convenience for elucidating the genetic factors controlling the meiotic recombination frequency. Since tetrasporic varieties and bisporic varieties have normal and suppressed crossover frequency respectively, it can be expected that the locus mainly controlling a high crossover frequency of *A. bisporus* is linked to the loci of basidiospore number (BSN) on chromosome 1 (Imbernon et al., 1996).

The pairing of non-sister nuclei into one spore combined with the low recombination frequency leads to the preservation of heterozygosity in most of the individuals in the offspring of the *bisporic* variety. As mentioned previously, altered and sometimes improved varieties can be generated by screening fertile single spore cultures as most mushroom breeding companies do. It suggested that the segregation of whole chromosomes or reshuffling of homologous chromosomes over the two constituent nuclei during meiosis could be used to fine tune traits in some way. This leads to the other direction of applying meiotic recombination, i.e., reverse breeding as in plants, a technique based on the generation of gametes with non-recombinant chromosomes by suppressing crossovers in the preceding meiosis (Dirks et al., 2009). In reverse breeding, crossovers are suppressed and each chromosome segregates as a complete and single unite. Chromosome substitution lines (CSLs) can be constructed during reverse breeding for

different species and used for breeding per chromosome, as has been shown for Arabidopsis (Wijnker et al., 2012) and wheat (Efremova et al., 2013). The crossover recombination in plant reverse breeding has to be suppressed on purpose, while that of *A. bisporus* is suppressed naturally. This offers opportunities for mushroom breeders to do breeding per chromosome (reverse breeding). Nonetheless, the applicability of reverse breeding also depends on the chromosome number and the degree of crossover suppression. *A. bisporus* has 13 chromosomes, and the chance of having a CSL of a particular chromosome is really low ($1/2^{13}$). That means screening for CSLs is a very laborious work. Because of the low recombination frequency of *A. bisporus* var. *biporus*, the QTL mapping in Chapter 5 and 6 only assigned QTLs on chromosomes but not on accurate chromosome regions, although large segregating populations were used. Main effect QTL have to be fine mapped by constructing additional segregating populations for chromosomes of interest. Nevertheless, a library of chromosome substitution lines will accelerate the mapping process. Provided that the chromosome substitution lines are available, an initial screen could be conducted on 26 single chromosome substitution lines for bruising sensitivity and other traits. Chromosomes having main effects on a trait of interest could therefore be selected more efficiently. In addition, based on the findings of Chapter 4 of this thesis the crossover recombination is not completely absent but locates in the very distal regions of chromosomes. So far, it is still unknown whether alleles at distal chromosome ends are important to various traits of interest. Unraveling the genes involved in crossover formation in *A. bisporus* and controlling the crossover frequency is a prerequisite to manipulate meiosis in *Agaricus bisporus*.

Genome-wide SNP markers were used for genotyping to understand the meiotic behavior in the offspring of *A. bisporus.* Crossovers in the distal chromosome regions were detected by resequencing the two lines Horst U1 and Sylvan A15. The recombination at chromosome ends were ignored when using regular SNP genotyping (Chapter 4) since only one (occasionally 2) SNP markers had not the parental allele. They were thus considered as unreliable. Whole-genome resequencing is a novel genotyping strategy for more effective genomic mapping and genome analysis. It has been used for genotyping a RIL population of rice (Huang *et al.*, 2009). In that study a sequencing-based genetic map was generated for rice with an average density of 1 SNP every 40 kb. With this map, a QTL explaining up to 30% of the variance for plant height was located in a 100 kb chromosome region. This genotyping by re-sequencing method was proven to be faster in data collection and more precise in recombination breakpoints determination compared to PCR-based marker genotyping. With this method the genome coverage of markers was not a limitation for genotyping any more. Using techniques to score individual SNPs (such as the Kaspar technology) a single allele switch on a chromosome is often considered as an error (Huang et al., 2009). When the whole genome is sequenced, an allele switch will be confirmed by flanking SNPs and each SNP is thus scored with a high reliability. In this way we could unambiguously confirm that recombination in the bisporic variety is confined to approximately the last 50 kb on each chromosome arm. Genotyping by resequencing can be used in further studies to screen exact meiotic behaviours and to map QTLs on precise locations.

Meiosis manipulation and the control of meiotic recombination to support breeding

Meiotic recombination is a fundamental event to generate evolution and genetic diversity. Various new alleles or allele combinations are generated through meiotic recombination and this provides breeders with a countless source of variation. In order to manipulate meiotic recombination and take advantage of the variation for breeding purposes, the mechanism of meiotic recombination has to be understood. The core mechanisms of meiosis are widely conserved in various organisms (Villeneuve & Hillers, 2001), which consists of a single round of DNA replication and two consecutive rounds of chromosome segregation, thereby generating haploid gametes from diploid cells (Martinez-Perez & Colaiacovo, 2009). Briefly, double strand breaks (DSBs) are produced along the chromosomes after duplication. Homologous chromosomes join together and form synaptonemal complexes (SC). DSBs are repaired through

homologous recombination generating reciprocal exchanges. These so-called crossovers are known to play an essential role in most eukaryotic meiosis process, which facilitate the accurate chromosome segregation. At least one crossover must occur per pair of homologues, which is so-called obligate crossover. A crossover converts the inter-sister cohesion into an inter-homologue link. If the crossover is absent, there is no link between the homologous chromosomes. Consequently, chromosomes segregate randomly generating a high level of aneuploid gametes (Crismani et al., 2013). The fact that most cases of human aneuploidy display altered crossover numbers and/or distribution confirmed the essential role of crossovers in accurate meiosis (Lamb et al., 2005). Homologues segregate at anaphase I, and a second division follows. Sister chromatids disjoin like in mitosis. Tetrad gametes are produced at the end of meiosis. In the whole meiosis process, chromosome number is normally fixed, but recombination frequency varies between bivalents, chromatids, cells, sexes, individuals and species (Baudat *et al.*, 2010, Lynn *et al.*, 2005, Webster & Hurst, 2012). In many cases of meiosis no more than 1 or 2 crossovers per chromosome are observed due to crossover interference (Wijnker, 2013, Henderson, 2012).

Meiotic recombination has been applied for plant breeding in terms of two directions. One of the directions is suppressing crossover to preserve allele combinations. In this way doubled haploids can be generated that can be used to reconstruct favorable hybrids (reverse breeding). Crossover formation was abolished by the deletion of DMC1, which is a gene required for crossover formation in most eukaryotes (Wijnker et al., 2012). Homozygous parental lines from heterozygous plants can be generated with this strategy. The other direction of using meiosis is to increase recombination frequency (RF) in order to obtain enough genetic variations. The FANCM mutant of Arabidopsis has a threefold-increased CO frequency as compared to the wild type, where FANCM is a suppressor of crossover formation (Crismani et al., 2012). AtMSH2 has also roles in suppression of recombination in Arabidopsis, which has a broad range of anti-recombination effects, i.e., it suppresses recombination between divergent direct repeats in somatic cells or between homologues from different ecotypes during meiosis (Emmanuel et al., 2006). DNA methylation mutant Atmet1 of Arabidopsis shows boosted centromere proximal and distal crossovers (Yelina et al., 2012). Besides, changes in karyotype composition can result in an increase of RF, i.e., aneuploidy or polyploidy (Leflon et al., 2010). Due to the essential role of the obligate crossover per pair of homologous chromosomes and the fact that RF of var. *bisporus* is really low (<0.2 per linkage group) (Kerrigan et al., 1993), one would expect unbalanced chromosome segregation in var. *bisporus.* Analysis of recovered constituent nuclei of heterokaryotic offspring (Chapter 4) has shown that var. *bisporus* shows a perfectly balanced chromosome segregation. Plenty of SNP markers distributed over the genome were used for genotyping three segregating populations in Chapter 4, and the RF was proven to be not higher than that found by Kerrigan (1993). However, with the resequencing data of Sylvan A15 and Horst U1, we could conclude that the obligate crossovers exist in *A. bisporus* var. *bisporus*, but exclusively located at distal chromosome regions. The genes detected in plants controlling meiotic recombination might be conserved in different species. With these as references, genes involved in the meiotic recombination will have to be investigated in order to manipulate meiosis in *A. bisporus*.

Apart from genetic factors, meiotic recombination can also be influenced by environmental factors. It has been reported that RF can be modified by a number of environmental factors and treatments. Successive studies were conducted by Lu *et al.,* (1969-1974) in *Coprinus lagopus* with a morphological marker (den) and an auxotrophic marker (me-1). The two markers were located on linkage group III, which were 3.5 map units away from each other. There is evidence that recombination rates can be changed by temperature treatment. For example, both high (35°C) and low (5°C) temperature treatments can increase RF in *C. lagopus*, and cold treatment appeared to be more effective than heat treatment. The time that recombination can be modified is determined at the stage of pachytene with light microscopy (Lu, 1969). The higher increase in RF by low temperature can be attributed to the increased duration of pachytene and therefore RF is a function of time (Lu, 1974a). Moreover, accurate control of the initiation of meiosis was achieved by using a temperature and light system in *C. lagopus* (Lu, 1974b). Some clues about the influence of temperature were also observed in *A. bisporus*. For instance, temperature could

have a large influence on basidial spore numbers in a tetrasporic species of *Agaricus*, and the diurnal periodicity for spore number traits is probably driven by temperature in nature (Kerrigan & Ross, 1987). Besides, RF in *C. lagopus* can be increased by non-lethal doses of gamma-irradiation when treatments were given at the beginning of karyogamy (Raju & Lu, 1973). A 2 or 4 h treatment with hydroxyurea during karyogamy of *C. lagopus* caused a decrease in spore viability and a dramatic increase in RF (Raudaskoski & Lu, 1980). Chemical treatments are also known to modify RF in plants. For instance, tritium applied as 3H-orotic acid to the anthers of tomato during interphase-early prophase of meiosis increased recombination between two marker loci by about 50% (Singh, 1974). Although spores were collected under different temperatures in Chapter 4, no analysis was conducted to screen the difference in recombination frequency of offspring from different spore prints. The influence of temperature on recombination frequency in *A. bisporus* needs to be further studied.

A. bisporus is not only an economically important fungus but could serve as a good model system to study meiosis. Its short life cycle, small genome and availability of whole genome sequences makes this fungus an ideal model to study genes involved in meiotic recombination, next to genes responsible for basidial spore number (BSN). Understanding the genetics behind these different life styles might generate tools to manipulate meiosis towards reducing linkage drag (high recombination frequency) or preserve favorable genome region (low recombination frequency) and even substitute whole chromosomes. This will be a supportive and essential foundation for breeding for advanced mushroom cultivars.

Comparison of genetics and breeding of button mushrooms and plants (specifically tomatoes)

Mushroom breeding is similar to plant breeding but differs in a number of aspects (Chapter 3). Taking tomato as an example, a comparison was made between tomato breeding and mushroom breeding (Table 2). Similar to tomatoes (Viquez-Zamora et al., 2013) button mushrooms also have plenty of wild germplasm resources. However, the cultivars of button mushroom are almost genetically identical to the first released hybrid (Chapter 4). The nuclei of fertile diploid mycelium (heterokaryon) stay apart (n + n) and do not fuse as in diploid tomato (2n). The constituent nuclei can thus be recovered from the heterokaryons as haploid homokaryons, and the intact and original genome combination can be maintained forever. This is a practical advantage of mushroom breeding over plant breeding. Homokaryotic offspring are usually used as segregating populations and are in fact gametes that can be propagated vegetatively as mycelia. They can be considered as an equivalent to doubled haploids or recombinant inbred lines used nowadays in breeding of tomato and many other plant species. Multiple population types are used in tomato breeding and selection (Zhang et al., 2013, Salinas et al., 2013). Tomatoes can only be propagated via seeds (Bal & Abak, 2007), but button mushrooms can be propagated via basidiospores, vegetative mycelium or tissue culture. This is an important reason why mushroom varieties are more difficult to protect than tomato varieties. Tomatoes and button mushrooms can both reproduce through selfing and outcrossing. The mating system of button mushroom, however, limits the possibilities for selfing. The compatibility is controlled by one mating type locus and only homokaryons with different mating type are compatible. That means that ca. 50% of the pairings of siblings in an offspring will lead to heterokaryons. In addition, the button mushroom has been shown to suffer from inbreeding depression that hampers a clear expression of traits. Tomatoes can be planted both in the field as well as in greenhouses, but mushrooms are generally cultivated in climate-controlled growing facilities. Mushrooms have a relatively short life cycle, and the whole commercial cropping period is about 35 days. Tomatoes on the other hand need 6 to 10 months in the greenhouse. Although a number of differences in terms of genetics and breeding exist between tomato and button mushrooms, the basic principles and techniques can be applied to both tomato breeding and mushroom breeding. Since mushroom breeding lags behind plant breeding as mentioned in a previous section, many techniques and methods applied in plant breeding are used as references in mushroom breeding.

Table 2. Comparison between tomato breeding and button mushroom breeding

Popular name Scientific name	Tomato *Solanum lycopersicum*	Button mushroom *Agaricus bisporus*
Genetic resources	wild species and modern cultivars	wild strains and very limited number of modern cultivars
Ploidy level	diploid (2n)	heterokaryotic (n+n)
Propagation mode	seeds	spores, vegetative mycelium, or tissue culture
Sexuality	selfing (allogamy) outcrossing	Restricted selfing (controlled by mating type loci) outcrossing (controlled by mating type loci)
Segregating population	F2, F3, F4, RILs, etc.	homokaryotic offspring (equivalent to DH), heterokaryotic offspring (equivalent to F2)
Marker assisted breeding (MAS)	yes	yes
Genetic modified plants (GMO)	possible, but not available	possible, but not allowed to use in Europe.
Cropping condition	field, greenhouse	climate-controlled growing facilities
Cropping period	6 to 10 months	35 days

Conclusions

The mushroom industry worldwide is in need for new varieties adapted to new cultivation techniques or new market demands. One of the demands of the Dutch mushroom industry is that varieties should be suited for mechanical harvest for the fresh market and mushrooms that can be sorted and packed mechanically after harvest without bruising. At present, mechanical harvesting is an effective technique to reduce the high labour costs during mushroom harvesting. Button mushrooms are sensitive to mechanical bruising, thus mechanical harvesting cannot be applied to harvest mushrooms for the fresh market. The work presented in this thesis showed that bruising sensitivity of button mushroom is a highly heritable trait and allows thus breeding for new varieties that are less sensitive to bruising and can be mechanically harvested for the fresh mushroom market. Since a new variety is supposed to combine multiple good traits, also several relevant agronomic and quality traits were investigated in this thesis. These traits are mostly quantitative and complex traits. Before applying marker-assisted breeding, precise locations of traits of interests and genetic markers closely linked to traits have to be determined. Due to the low recombination rate of *A. bisporus* var. *bisporus,* QTL were only assigned to chromosomes without precise positions. This causes a major problem in breeding due to the high linkage drag and can only be solved by introducing the normal meiotic recombination frequency of var. *burnetii* into breeding stock. This thesis also showed that traits in button mushrooms are considerably affected by genetic backgrounds and by certain environmental factors. This study contributes to the understanding of meiosis behaviour and the genetic basis of bruised-induced discoloration of button mushroom and several other traits, and it will enable marker-assisted breeding for button mushrooms of good quality in the future. In combination with further studies on gene expression, phenolic compounds and metabolites, candidate genes and mechanisms determining bruising sensitivity and other traits of interest can be identified.

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Summary

Labor cost is one of the main cost factors of the Dutch mushroom industry, which can be reduced substantially if mushrooms can be harvested mechanically for the fresh market. The present-day hybrids are, however, too sensitive to mechanical damage. Mushrooms are bruised after mechanical damage and post-harvest handling, and discoloration is the first sign of a bad quality. Consequently, machines can only be used to harvest mushrooms for the processed products but not for the fresh market. Mushrooms for the fresh market are still handpicked. Although a number of studies have been done previously on the discoloration of mushroom caps none of these has used the phenotypic variation within the culture collections to study the genetic determinants explaining the differences in bruising sensitivity and thus generating knowledge for breeding superior strains. The main objective of this thesis is to fill this knowledge gap at least to some extent and generate breeding stocks that can be used to construct commercial varieties suitable for mechanical harvest for the fresh market. In addition, the genetic basis of other quality-related traits was investigated. For this, segregating populations were generated and used to study in more detail the life cycle of the bisporic variety. Since the whole genome sequence for the button mushroom is available, the use of SNP markers distributed evenly over the whole genome allows a more detailed study of recombination frequencies generating knowledge invaluable for further mushroom breeding.

Phenotypes are determined by both genetic and environmental factors. Bruising sensitivity of button mushroom can be influenced by a number of environmental factors, especially relative humidity in the growing room, water content of the casing, and casing depth. In Chapter 2, the influence on bruising sensitivity was studied of these three environmental factors and the genotype (G) of strains in a factorial design. Two levels were used for each of the three environmental factors, and four different strains (two resistant and two sensitive to mechanical bruising) were grown. The main purpose of this setup was to test factors known to influence discoloration in order to find environmental conditions that maximise the difference in bruising sensitivity between resistant strains and sensitive strains. The outcome was that the conditions used currently for the commercial mushroom productions cultivation gave the largest differences in discoloration between the resistant strains and sensitive strains.

In order to select suitable parental lines for the construction of segregating populations, the genetic variation in bruising sensitivity (BS) of Agaricus bisporus was studied through an incomplete set of diallel crosses (Chapter 3). This allows estimating the heritability of BS, the estimation of combining ability and the breeding value of candidate parental lines. To this end nineteen homokaryotic parental lines recovered from wild strains and cultivars were inter-crossed in a diallel scheme. Fifty-one successful hybrids were grown under controlled conditions, and BS of these hybrids was assessed. BS was shown to be a trait with a very high heritability. In the population that also segregated for cap color, bruising insensitivity was linked to the brown offspring. The diallel scheme allowed to estimate the general combining ability (GCA) for each homokaryotic parental line and to estimate the specific combining ability (SCA) of each hybrid. One line had a low GCA (low for BS) and is seen as an attractive donor for improving resistance to bruising. The study of SCA indicates that heterosis might play a role in resistance to bruising. This study demonstrated for the first time how to estimate breeding value of parental homokaryons in button mushroom breeding. This can be used to further study the genetic factors underlying bruising sensitivity and other quality-related traits, and to select potential parental lines for further heterosis breeding.

Since the atypical life cycle of Agaricus bisporus var. bisporus leads to difficulties of obtaining homokaryons as breeding materials, it hampers outcrossing, and thus breeding. In addition, the low recombination frequency results in considerable linkage drag and difficulties to generating a dense genetic linkage map. New details were generated by next generation sequencing technologies on meiotic behaviour in A. bisporus var. bisporus described in Chapter 4. Using SNP markers on a segregating

homokaryotic population derived from the bisporic variety Horst U1 it was demonstrated that meiotic recombination in the var. bisporus is infrequent and restricted to the ends of chromosomes. By recovering constituent nuclei of heterokaryotic offspring of Horst U1, it was shown that non-sister nuclei are paired and that this phenomenon combined with the low recombination frequency leads to the preservation of the heterozygous state. The same analysis on present-day commercial varieties reveals that it is very likely that these are directly derived from the first commercial hybrid released in 1980 through the isolation of fertile single spore cultures. This chapter deepens our understanding of the life cycle of A. bisporus var. bisporus and is useful for button mushroom breeding.

New cultivars that do not discolor after mechanical damage allow a full automated harvesting of mushrooms for the fresh market and can in this way reduce the production costs considerably. Chapter 5 described the identification of quantitative trait loci (QTL) as a start to unravel the genetic basis for mushroom bruising sensitivity. In order to unravel its genetic basis and to generate lines for breeding programs, quantitative trait loci (QTL) were identified in two segregating populations: population 1 consists of homokaryotic single spore cultures (homokaryons) of a cross between a white resistant line (CH2B) and a brown resistant line (Mes09143); population 2 consists of homokaryons of a cross between a resistant line (CH2A) and a sensitive line (Z8). Population 1 was crossed with two different tester lines creating two sets of heterokaryons that allowed the phenotyping of mushrooms in different genetic backgrounds. A third tester line was used to generate heterokaryons of population 2. The resulting three sets of heterokaryons were used for QTL detection. Due to the low recombination frequencies, QTL were in most cases assigned to whole chromosomes. Since cap color and discoloration after bruising display the same phenotype, QTL mapping was done for bruising sensitivity (BS) and cap color (CC). CC and BS of the resulting three sets of heterokaryons were measured and analyzed at 60 min and 24 h after bruising (flush 1 & 2), and the segregation of BS and CC was observed. The major QTL for BS and CC were consistently found in all three sets of heterokaryons. QTL identified in this chapter will provide a basis for the breeding of advanced mushroom cultivars that are less sensitive to mechanical bruising. Since QTL analyses for edible fungi are scarce, this chapter contributes considerably to our knowledge on understanding complex traits in edible fungi.

One of the main problems in introducing new traits using wild lines is the restoration of quality. Agronomic and quality traits are often correlated and should be evaluated in combination with each other, rather than as single traits. Modern breeding practices tend to breed for multiple traits. Chapter 6 describes the analysis of multiple traits of A. bisporus and the genetic correlations between traits. Significant phenotypic correlations were observed among different traits. Genetic correlations were the main cause for phenotypic correlations, which were confirmed by pleiotropic QTL shared by different traits. The two major pleiotropic QTL on chromosome 10 and chromosome 6 were controlling almost all the tested traits. This chapter presents the first multi-trait QTL analysis of a mushroom species involving multiple agronomic and quality traits. This will contribute to our understanding of the genetic architecture and correlation between complex traits and as such generates a basis for marker-assisted mushroom breeding for multiple agronomic and quality traits.

This thesis describes the first fundamental approach in breeding for edible mushrooms comparable to what is common used in plant breeding. This will contribute to move mushroom breeding from an applied science to a more advanced science. This study especially contributes to the understanding of the genetic basis of the bruised-induced discoloration and, in addition, meiotic behavior of button mushrooms, and facilitates marker-assisted breeding for button mushrooms of good quality in the future.

Arbeid is een van de belangrijkste kostenfactoren voor de Nederlandse champignonsector. Het mechanisch oogsten van champignons voor de versmarkt kan deze kosten aanzienlijk reduceren. De huidige rassen zijn echter te gevoelig voor beschadigingen. Een mechanische oogst kan beschadiging van de hoed veroorzaken en deze kneuzing leidt tot verkleuring en een lagere kwaliteit. Mechanische oogst wordt daarom nu alleen gebruikt voor champignons die direct verwerkt worden en voor de versmarkt worden champignons nog steeds met de hand geplukt. Eerder zijn al diverse studies gedaan aan het verkleuren van paddenstoelen tijdens en na de oogst. Echter in geen van deze projecten is de variatie in kneusgevoeligheid van stammen in collecties gebruikt om studies te doen naar de genetische componenten die deze variatie kunnen verklaren en dus gebruikt kunnen worden om rassen te verbeteren.

Het belangrijkste doel van het onderzoek beschreven in dit proefschrift is om een aanzet te geven om deze kennisleemte in te vullen en daarnaast ook uitgansmateriaal te genereren dat gebruikt kan worden om kneusongevoeliger rassen te maken. In het onderzoek is ook gekeken naar de genetische basis van andere kwaliteit gerelateerde eigenschappen. De segregerende populaties die zijn gegenereerd zijn ook gebruikt om meer inzicht te krijgen in de levenscyclus van de champignon en met name in de ondersoort die alle commerciële rassen en de meeste wilde lijnen vertegenwoordigt. Omdat het hele champignongenoom nu in kaart is gebracht zijn SNP merkers gebruikt waarmee de recombinatie in de meiose goed bestudeerd kon worden waardoor nuttige kennis voor de veredeling is gegenereerd.

Het fenotype van organismen wordt bepaald door zowel genetische als omgevingsfactoren. De kneusgevoeligheid van witte champignons wordt dan ook door een aantal omgevingsfactoren beïnvloed, zoals relatieve vochtigheid in de lucht, watergehalte van de dekaarde en de dikte van de dekaarde. In hoofdstuk 2 is de invloed van deze drie omgevingsfactoren op de kneusgevoeligheid bestudeerd in samenhang met het genotype van de stammen in een factoriele experimentele opzet. Voor elke factor zijn twee niveaus getest en vier verschillende genotypen gebruikt (twee ongevoelige en twee gevoelige stammen). De belangrijkste reden voor deze opzet was om factoren te testen waarvan bekend is dat ze verkleuring beïnvloeden om daarmee condities te kunnen kiezen die de verschillen tussen ongevoelige en gevoelige stammen maximaliseren. Het resultaat was dat de huidige teeltcondities in de commerciële teelt de grootste verschillen in verkleuring geven tussen de ongevoelige en gevoelige stammen.

Om geschikte ouders te kiezen voor het genereren van segregerende populaties is de kneusgevoeligheid (KG) bepaald in een set incomplete diallel kruisingen (hoofdstuk 3). Hiermee kon de overerfbaarheid (H2) van KG, de *combining ability* (expressie van de eigenschap in verschillende kruisingen) en de *breeding value* van de gebruikte lijnen ingeschat worden. Hiervoor zijn negentien homokaryotische lijnen geïsoleerd uit wilde en commerciële heterokaryons op zoveel mogelijke manieren met elkaar gekruist (diallel matrix). Eenenvijftig geslaagde kruisingen zijn vervolgens geteeld en getest op KG. Hieruit is gebleken dat KG een zeer hoge overerfbaarheid heeft en de genetische variatie bepaalt dus voornamelijk de variatie in KG. In de populaties waarin ook de hoedkleur segregeerde (witte versus niet-witte champignons) bleek ongevoeligheid voor kneuzing gekoppeld te zijn aan niet-witte hoeden. Uit de dialle matrix kon de *general combining ability* (GCA) geschat worden voor elke homokaryons en de specific combining ability(SCA) voor elke kruising (hybride). Een van de lijnen heeft een lage GCA (lage KG) en kan beschouwd worden als een geschikte donor om rassen kneusongevoelig te maken. De analyse van SCA wijst erop dat heterosis een rol kan spelen bij kneusgevoeligheid. Deze analyse laat voor het eerst zien hoe *breeding value* geschat kan worden van homokaryons in de champignonveredeling. Dit kan gebruikt worden om de genetische factoren te bestuderen die kneusgevoeligheid en andere kwaliteitskenmerken bepalen en voor de selectie van ouderlijnen voor heterosis veredeling.

Omdat de atypische levenscyclus van *Agaricus bisporus* var. *bisporus* het isoleren van homokaryons bemoeilijkt, is uitkruisen lastig en wordt veredeling daardoor belemmerd. Daarnaast veroorzaakt de lage recombinatiefrequentie veel *linkage drag* (insleep van ongewenst wild genoom) en kan geen gedetailleerde genetische koppelingskaart gemaakt worden. Er zijn nieuwe inzichten gegenereerd met behulp van *next generation sequencing* technologie in de meiose van *A. bisporus* var. *bisporus* en dat is beschreven in hoofdstuk 4. Door segregatie van SNP merkers te bestuderen in homokaryotische nakomelingen van het ras Horst U1 is aangetoond dat de meiotische recombinatiefrequentie erg laag is en beperk tot de uiterste uiteinden van de chromosomen. Door de beide kerntypen te isoleren van

heterokaryotische nakomelingen van Horst U1 kon worden aangetoond dat voornamelijk niet-zuster kernen worden gepaard in elke spore. Gecombineerd met een laag recombinatiefrequentie leidt dat tot het behoud van heterozygositeit in de nakomelingen. Dezelfde analyse is uitgevoerd op de huidige commerciële rassen en de uitkomst maakt aannemelijk dat deze allemaal zijn afgeleid van de eerste hybride die in 1980 op de markt is gebracht via de isolatie van heterokaryotische eensporeculturen. Dit hoofdstuk verdiept de kennis van de levenscyclus van *A. bisporus* var. *bisporus* hetgeen erg nuttig is voor de veredeling van champignons.

Nieuwe rassen die minder snel verkleuren na mechanische beschadiging maken een volautomatische oogst voor de versmarkt mogelijk en kunnen productiekosten op deze manier aanzienlijk reduceren. Het identificeren van *quantitative trait loci* (QTL) voor KG beschreven in hoofdstuk 5 is een begin voor het ontrafelen van de genetische basis voor deze eigenschap in champignons. Hiervoor zijn 2 segregerende populaties gebruikt: populatie 1 wordt gevormd door homokaryotische eensporeculturen afkomstig van een kruising tussen wit ongevoelig ras (CH2B) en een bruin ongevoelig ras (Mes09143); populatie 2 wordt gevormd door homokaryotische eensporeculturen afkomstig van een kruising tussen een resistente wit ras (CH2A) en een gevoelige lijn (Z8). Populatie 1 is gekruist met twee verschillende testerlijnen om daarmee twee sets heterokaryons te genereren. Hiermee kon het fenotype van deze populatie in twee verschillende genetische achtergronden bestudeerd worden. Een derde testerlijn is gebruikt om van populatie 2 heterokaryons te genereren. De drie sets heterokaryons zijn gebruikt voor QTL analyse. Door de lage recombinatiefrequentie konden QTL meestal alleen aan chromosomen worden toegewezen. Omdat de hoedkleur en verkleuring na kneuzing hetzelfde fenotype laten zien is een QTL analyse gedaan voor zowel hoedkleur variatie als variatie in verkleuring na kneuzing. Variatie voor beide fenotypen zijn in alle drie sets heterokaryons bepaald op 60 minuten en 24 uur na het toebrengen van de kneuzing voor vlucht 1 en vlucht 2 paddenstoelen. De belangrijkste QTL bleken in alle drie populaties op vrijwel dezelfde chromosomen te liggen. Deze kennis vormt hiermee een basis om rassen te veredelen die minder kneusgevoelig zijn. Het identificeren van QTL in paddenstoelen is nog maar rg weinig gebeurt en dit hoofdstuk brengt onze kennis omtrent complexe eigenschappen in paddenstoelen en stap verder.

Een van de grootste obstakels in het introduceren van nieuwe eigenschappen in rassen vanuit wilde lijnen is het herstellen van de kwaliteit. Deze complexe agronomische en kwaliteit eigenschappen zijn vaak aan elkaar gecorreleerd en het is beter om deze eigenschappen niet afzonderlijk maar samen te evalueren. Hedendaagse veredelingsprogramma's richten zich meestal op meerdere eigenschappen. Hoofdstuk 6 beschrijft de analyse van meerdere eigenschappen tegelijk en de mogelijke genetische correlatie tussen deze eigenschappen. Er zijn significante correlaties gevonden tussen verschillende eigenschappen en een genetische correlatie lijkt hiervoor de belangrijkste reden te zijn. Dit wordt onderbouwd door pleiotropische QTL die door verschillende eigenschappen gedeeld worden. De twee belangrijkste QTL op chromosoom 10 en 6 hebben betrekking op bijna alle geteste eigenschappen. Dit hoofdstuk beschrijft voor het eerst een *multi-trait* QTL analyse voor paddenstoelen en dat draagt bij aan de kennis over de genetische basis voor complexe eigenschappen en correlaties tussen complexe eigenschappen. Als zodanig is dit nuttig voor veredeling voor complexe eigenschappen.

Dit proefschrift beschrijft voor het eerst een fundamentele benadering voor de veredeling van paddenstoelen op een manier die gebruikelijk is in de plantenveredeling. Dat zal de paddenstoelveredeling verschuiven van een toegepaste naar een meer fundamentele wetenschap. Deze studie draagt ook vooral bij aan het begrijpen van de genetische basis van complexe eigenschappen zoals kneusgevoeligheid en brengt veredeling met gebruik van genetische merkers voor champignon met een goede kwaliteit een stap dichterbij.

It takes me a while to believe the fact that I am going to defend my thesis. I looked back on this PhD journey and felt the time passed over the last four years. I have to say it is a great journey to do my PhD with a group of great colleagues and friends. I would like to convey my sincere gratitude to all of you who gave me lots of help on my work and even my life.

First of all, I would like to thank my supervisor and co-promotor Dr Anton S.M. Sonnenberg whom I first knew your face on a picture in China. At that moment, I could never imagine that you would be my PhD supervisor. It is a great honor to study and work under your guidance. I was unconsciously influenced by your enthusiasm and precise attitude for mushroom research. You inspired me to challenge myself for scientific thinking and discussed with me to find solutions and new ideas for many subjects. You encouraged me to go to conferences to present my work. Meanwhile, you put great efforts to help me on improving my presenting and writing ability. My thesis would not be complete without your support. Large pieces of red text and comment frames in my manuscripts are the endless efforts you put on my improvements. My writing ability was substantially improved depending on all these straightforward remarks. You are the one who raise me up to more than I can be. Thank you very much for everything.

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Wageningen gathers people from different cultures over the world. It is a great opportunity to get involved in an atmosphere of mixed cultures and to enjoy the cuisine of different countries with friends. Gradually, I got used to European life style, especially Dutch life style. I enjoyed cycling to the neighborhood and the tranquil & serene in this small but lovely city. Marian and Bart, thanks for the good time you brought to me. The dinners we had together all have unforgettable and special taste. Marian, although you were not directly involved in my project, but you really gave me lots of support at work. Arwa, Mirjana, Ram, Yusef, Gert, Peter, Marcela and Rafael, I would like to give my gratitude to you for all your kindness and all the good time together with you. Johan, Yuri, Mina, Madhuri, Danny, Koen, Wendy, Doret, Christel, Fien, Hanneke and many other colleagues, I am glad to know you and have the opportunity to let you know me at Wageningen.

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My project was mainly founded by TTI Green genetics (TTI06201), WUR-Plant Breeding, Bromyc, Sylvan, Product Board for HorticuIture, Greenery, Banken, Lutèce, WeBe Engineering, and Institute of Agricultural Resources and Regional Planning, CAAS. Thus, my utmost gratitude also goes to members of the user's committee of this project and my external superviser Prof. Jinxia Zhang. This project could not run smoothly without your support and the critical discussions we had.

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Offices for us PhD students are the second homes. I was so lucky to spend my PhD journey in an office that is full of nice officemates. Narges, Marie, Freddy and Nasim, thanks for your company to listen to my ups and downs. The time we spent together was incredible memories for my whole life. I hope you all can have your dreams come true.

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Wei

About the author

Wei Gao was born on 4th of Jun 1982 in Liaoning, China. She started her academic studies for her BSc degree in the field of Biology in Shenyang Normal University from 2000 to 2004. After obtaining her Master's degree in Microbiology at Fujian Agriculture and Forestry University in 2007, she started her work on mushroom genetic and breeding in Institute of Agricultural Resources and Regional Planning of Chinese Academy of Agricultural Sciences (CAAS) in Beijing. She then started her PhD in the Plant Breeding Department, Mushroom Research group at Wageningen University in September of 2009. This thesis presents the outcome of her four years research on project of "Breeding for Bruising Insensitive Mushrooms for Cost Efficient Mechanical Harvest".

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: **Wei Gao**
Date: **21 January 2014**
Group: **Plant Breeding, Wageningen University & Research Centre**

1) Start-up phase	*date*
► **First presentation of your project**	
Genetic linkage map construction of Agaricus bisporus with SNPs	Oct 10, 2010
► **Writing or rewriting a project proposal**	
Breeding for brusing insensitive mushrooms for cost efficient mechanical harvesting	Oct 2009
► **Writing a review or book chapter**	
► **MSc courses**	
Genetic analysis tools and concepts(GATC)	Sep 05-Oct 05, 2011
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*10.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
2nd Joint Retreat of PhD Students in Experimental Plant Science, Max Panck, Germany	Apr 15-17, 2010
EPS PhD student day, Utrecht University	Jun 01, 2010
ExPectationS 2010, EPS Career Day Event	Nov 19, 2010
EPS PhD student day, Wageningen University	May 20, 2011
3nd Joint Retreat of PhD Students in Experimental Plant Science, Orsay, France	Jul 05-08, 2011
► **EPS theme symposia**	
EPS theme 4 'Genome Biology', Wageningen University	Dec 10, 2010
EPS theme 4 'Genome Biology', Wageningen University	Dec 09, 2011
EPS theme 4 'Genome Biology', Radboud University	Dec 07, 2012
► **NWO Lunteren days and other National Platforms**	
ALW meeting "Experimental plant Science" Lunteren 2011	Apr 04-05, 2011
ALW meeting "Experimental plant Science" Lunteren 2012	Apr 02-03, 2012
ALW meeting "Experimental plant Science" Lunteren 2013	Apr 22-23, 2013
► **Seminars (series), workshops and symposia**	
Networking Event of TTI Green Genetics 2010	Sep 22, 2010
Networking Event of TTI Green Genetics 2011	Sep 21, 2011
Networking Event of TTI Green Genetics 2012	Sep 19, 2012
Plant Research Day 2010	Feb 8, 2010
Plant Research Day 2011	Mar 8, 2011
Plant Research Day 2012	Feb 28, 2012
The annual NVvM meeting 2010	Nov 26, 2010
The annual NVvM meeting 2011	Nov 25, 2011
The annual NVvM meeting 2012	Nov 30, 2012

	Skills to speak, present and debate	July 24, 2012
	How to Write a Convincing Research Proposal	May 21, 2013
►	**Seminar plus**	
►	**International symposia and congresses**	
	10th European Conference on Fungal Genetics, ECFG 10	Mar 29-Apr 01, 2010
	7th International Conference on Mushroom Biology and Mushroom Products, ICMBMP 7	Oct 04-07, 2011
	18th Internation Society of Mushroom Sciences Conference, ISMS 18	Aug 26-30, 2012
	Conference Next Generation Plant Breeding, 100 years Plant Breeding	Nov 11-14, 2012
►	**Presentations**	
	Meiotic recombination in homokaryotic offspring of Agaricus bisporus, Lunteren (poster)	Apr 04-05, 2011
	Agaricus bisporus as a model to study effects of chromosomes on complex traits, ECFG 10 (poster)	Mar 29-Apr 01, 2010
	Bruising sensitivity trait expression in hybrids of Agaricus bisporus, TTI Networking Event (poster)	Sep 21, 2011
	QTL mapping for bruising sensitivity of Agaricus bisporus, Next Generation Plant Breeding (poster)	Nov 11-14, 2012
	Inheritance of bruising sensitivity in Agaricus bisporus (button mushroom), ICMBMP 7 (oral)	Oct 06, 2011
	Meiotic recombination in the button mushroom Agaricus bisporus, EPS symposium (oral)	Dec 09, 2011
	QTL mapping for bruising sensitivity of Agaricus bisporus var. bisporus, ISMS 18 (oral)	Aug 28, 2012
	QTL mapping for bruising sensitivity and cap color of Agaricus bisporus, Lunteren (oral)	Apr 23, 2013
►	**IAB interview**	Nov 15, 2012
►	**Excursions**	
	Visiting mushroom farms	Oct 08-09, 2009
	Visiting mushroom farms, CNC compost	2010
	Visiting Sylvan spawn company, Hooijmans Compost and Farm Peter van den Berg	Dec 11, 2012
	Enza zaden student experience	May 15, 2013
	Subtotal Scientific Exposure	*22.4 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	Bioinformatics: A Users Approach	Aug 29-Sep 02, 2011
	Mixed model based QTL mapping in Genestat	May 14-16, 2012
	Basic statistics	Jun 19, 20, 25-27, 2012
	Identity by Desecent (IBD) Approaches to Genomic Analyses of Genetic Traits	Jul 03-06, 2012
►	**Journal club**	
	Literature discussion at Plant Breeding	2011
►	**Individual research training**	
	Subtotal In-Depth Studies	*6.6 credits**

4) Personal development		*date*
►	**Skill training courses**	
	Scientific Publishing	Nov 19, 2009
	PhD Competence Assessment	Nov 2009
	Project- and Time Management	Nov-Dec 2009
	Information Literacy, including Introduction Endnote	Dec 07-08, 2009

	Searching for Science on the Web	Nov 11, 2010
	Acdemic Writing I	Mar 03-Jun 30, 2010
	Techniques for Writing and Presenting a Scientific Paper	Oct 19-22, 2010
	Acdemic Writing II	Sep 23-Feb 03, 2011
►	**Organisation of PhD students day, course or conference**	
	Member of Seminar organising Team of Plant breeding	Oct 2010-Sep 2011
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*6.9 credits**

TOTAL NUMBER OF CREDIT POINTS*	**46.4**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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