Unravelling the genetic base of the meiotic recombination landscapes in two varieties of the button mushroom, *Agaricus bisporus*

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Content

Chapter 1

General introduction

Breeding and genetic variation

The aim of breeding is to identify and combine optimal alleles involved in important agricultural phenotypes. For this it is crucial to understand and control the mechanisms that generate genetic variation in organisms, i.e. meiosis. Genetic variation in meiosis is generated in two ways: crossover recombination (CO) between homologous chromosomes and independent assortment of chromosomes, so-called inter-chromosomal recombination, both of which are described in more detail later on in this chapter. While the latter affects the combination of parental alleles existing in different chromosomes, CO recombination affects parental allele combinations in one chromosome. Since breeders aim to introduce only those alleles relevant to the trait of interest and knowledge in mechanisms that affect CO recombination are crucial. A lack of CO in genomic regions of interest is one of the main problems in breeding, causing the introduction of unwanted alleles also known as linkage drag. On the other hand, retaining optimal allele combinations is also an important issue especially in breeding seed varieties in which the desired allele combinations of elite genotypes are not existent in the following generation because seeds are produced after meiosis and thus *per se* genetically changed (Wijnker and de Jong, 2008). It is important for breeders to have a better understanding of how CO recombination is generated during meiosis, and, in particular, what regulates the positions and frequencies of CO which could lead to the ultimate tool for breeders: management and control of meiosis.

COs are needed for creating new allele combinations as well as to generate physical contact between homologous chromosomes. The latter is needed to ensure a balanced segregation of homologous chromosomes in meiosis I (Hillers, 2004). In reverse breeding, CO re-combinations are eliminated to preserve allele combinations in elite genotypes by knocking out essential genes such as *dmc1*, mediating the formation of CO recombination. Double haploids are then generated and these can be used to reconstruct elite genotypes. Eliminating the CO recombination leads to gametes having an unbalanced number of chromosomes, and if they are viable, they will produce individuals low in fitness and vigour. Only gametes with low frequencies have a balanced number of chromosomes and are thus viable. The chance of obtaining gametes with a balanced number of chromosomes decreases in plants with a higher number of chromosomes (Dirks et al., 2009; Wijnker et al., 2012). Simply switching CO on and off is not a solution either to generate new alleles or preserve existing allele combinations. Controlling the CO position and frequency is not yet possible, but studying the recombination behaviour of organisms having different CO patterns would bring about new options in breeding strategies. Therefore, I will pose some questions as to which factors can regulate CO patterns/distributions in chromosomes and which organism has a typical CO pattern that can help improve breeding programmes by managing CO recombination.

Meiosis and CO recombination

As stated above, meiosis is key to genetic variation in sexually reproductive organisms. In diploid organisms, cells contain two sets of chromosomes, one of maternal and one of paternal origin. Two consecutive divisions, meiosis I and II, which follow a single round of DNA replication, mark the transition from the diploid to the haploid phase (gametes). During DNA

replication, so-called pre-meiotic S-phase, each chromosome is doubled resulting in homologous chromosomes (homologues) each consisting of two sister chromatids attached to each other at the centromere. The sister chromatids remain joined together until the metaphase of meiosis II (Griffiths, 2005). In prophase, the first stage of meiosis I, double strand breaks (DSBs) are generated, a process that initiates homology search, co-alignment of homologues and meiotic recombination. Concurrently, a proteinaceous structure known as the synaptonemal complex (SC) begins to nucleate at sites of recombination interactions. The axial elements (AE) from each pair of sister chromatids are linked together closely to assemble two lateral elements (LEs) of the SC in each of the homologues, first likely to appear at the chromosome ends (Zickler, 2006), which link the axes of the homologues paired lengthwise. The areas between two LEs are connected by central elements of the SC, consisting of transverse filaments which carry structures called recombination nodules (Figure 1). The latter contains proteins involved in the recombination process and indicates the position of the CO. The process of installing the SC, which links two homologous chromosomes together lengthwise, is known as synapsis (Zickler and Espagne, 2016).
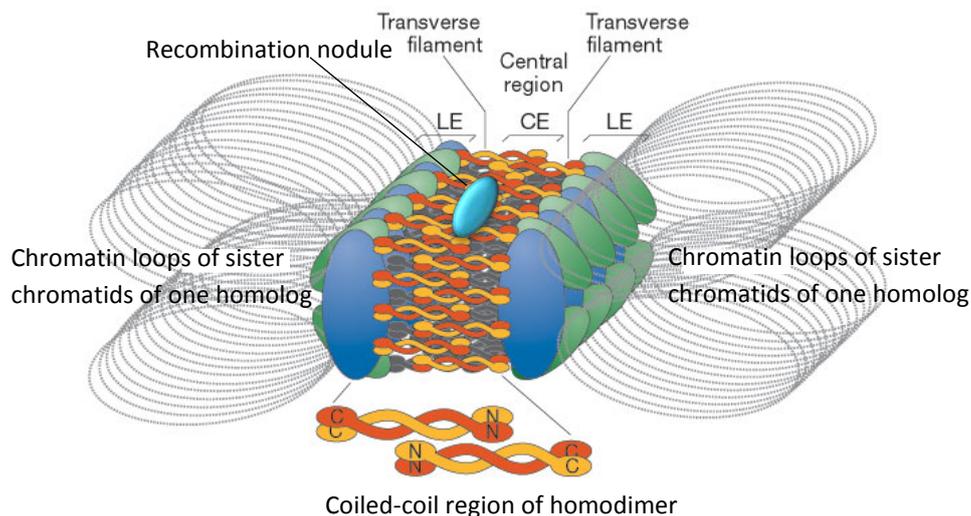

Figure 1. Diagram showing major elements of the synaptonemal complex (SC).
SC is a proteinaceous structure formed by a lateral element (LE), central element (CE) and transverse filaments. (Adapted from Page & Hawley (2004) Annual Review of Cell and Developmental Biology 20 ©2004 by Annual Reviews www.annualreviews.org).

As synapsis proceeds, DSBs are processed and repaired along DNA repair pathways by the DSBs ends resection and invasion of one end of the double-stranded homologue, creating a displacement loop (D-loop) structure. Stable heteroduplex DNA intermediates created in this way are known as single-end invasion intermediates (SEIs). The single strand is extended by DNA polymerase using a non-sister chromatid as a template. The D-loop structure can subsequently be processed by a number of mechanisms having different outcomes (Figure 2). The decision to repair the DSBs seems to be made early on in the recombination process possibly at the time of the SEIs formation (Allers and Lichten, 2001; Hunter and Kleckner, 2001). In the first mechanism which is synthesis-dependent strand annealing (SDSA), the extended single strand is disrupted by helicases, then the break is repaired by the separation of

single strands from non-sister chromatids and its re-association with the other end of the break, resulting in non-crossovers (NCO) or so-called gene conversions (GC) (Allers and Lichten, 2001). In fact, GC is a non-reciprocal transfer of the genetic material in the region where DSBs occur.
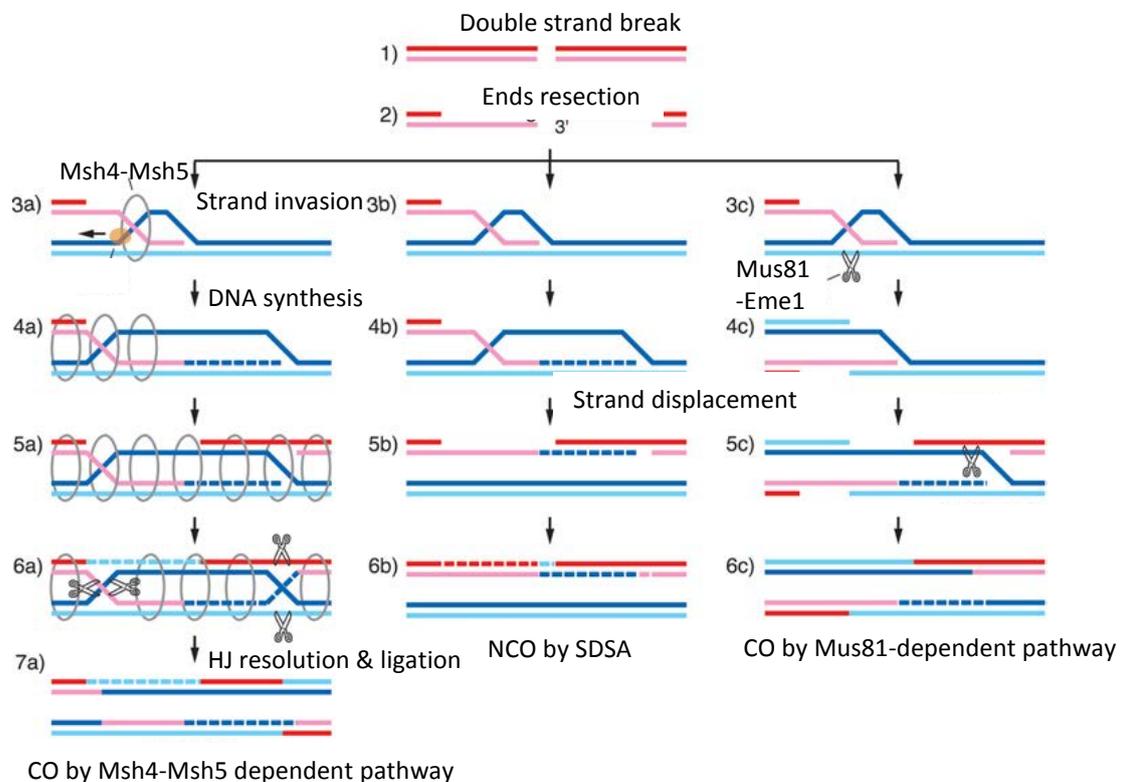

Figure 2. Model for meiotic crossover (CO) and non-crossover (NCO) formation.
Adapted from (Whitby, 2005).

Unlike the SDSA mechanism, in the second mechanism the extended single strand is not separated from the non-sister chromatid, resulting in the formation of double holiday junctions (dHjs). It is currently known that dHj can be resolved by a subset of enzyme/resolvases, the so-called Pro-CO via two pathways. Two Pro-COs known as Msh4-Msh5 and Mus81-Mms4 determine along which pathway dHjs can be resolved. The first pathway is also designated as the ZMM pathway according to the group of proteins involved (Zip1-4, Mer3, and Msh4-5). If Msh4-Msh5 is the Pro-CO, dHjs are resolved in such a way that COs are generated. This pathway exhibits interference, which means that the occurrence of another CO close to the existing one is unlikely. If Mus81-Mms4 is the Pro-CO, dHjs are also resolved by creating COs, but this pathway does not manifest interference. Non-interfering COs, however, are not the sole product of Mus81-Mms4 dependent pathway, as NCOs/GC are also generated along this pathway (Whitby, 2005; Youds and Boulton, 2011; Kohl and Sekelsky, 2013). Thus, three different products result from repairing DSBs: COs associated with interference (along Msh4-Msh5 dependent pathway), COs without interference (along Mus81-Mms4 dependent pathway) and NCO/GC (along SDSA and Mus81-Mms4 dependent pathway).

The presence of at least one CO per chromosome is essential for an equally (balanced) segregation of chromosomes on the metaphase I (Hillers, 2004). In addition to CO recombination, interchromosomal recombination contributes to the total genetic blending of parental chromosomes. That means each homologue can end up in one gamete or the other. After meiosis I, the sister chromatids are disjoined, similar to mitosis, to generate haploid gametes each of which contain half the number of parental chromosomes. The genetic content of each haploid gamete is a mosaic of the parental genomes due to CO recombination and this depends on the frequency and position of the CO and its interchromosomal recombination. In most organisms large numbers of CO per chromosome are uncommon and each chromosome arm often has only 1-2 COs which mainly occur in genomic regions called hot spots (Lichten and Goldman, 1995; Pryce and McFarlane, 2009). Contrary to hot spot regions, some are defined as cold spots such as centromere-proximal regions, telomeres and DNA repeats where a considerable repression of DSBs and CO has been observed (Baudat and Nicolas, 1997; Gerton et al., 2000).

Making the choice of *Agaricus bisporus* to study meiotic recombination

The mushroom *A. bisporus* belongs to the fungal phylum Basidiomycota. As in many other genera of this phylum, meiosis occurs in special cells termed basidia, positioned on gills in fruiting bodies. Fruiting bodies are formed from heterokaryotic mycelia in which each cell contains two types of nuclei and the quantity of each nucleus type can vary (Raper and Kaye, 1978). The nuclei remain side by side during most of the life cycle and fusion (karyogamy) which takes place in basidia prior to meiosis. After meiosis, four post-meiotic nuclei are packed into spores called basidiospores (meiospores).

A. bisporus is represented mainly by two interfertile varieties i.e., *bisporus* and *burnettii* which differ in their life cycle and meiotic recombination behaviour. Recent researches have indicated that the two varieties have a very contrasting meiotic recombination landscape (MRL) (Foulongne-Oriol et al., 2010; Foulongne-Oriol et al., 2011; Sonnenberg et al., 2016). Unravelling mechanisms that control these landscapes might offer opportunities to control meiosis and thus be very useful for breeding programmes of the button mushroom. If genes that play a general role in meiosis are also involved, this knowledge could also be applied to breeding programmes for other crops.

The life cycle and MRL of the bisporus variety

The button mushroom, *A. bisporus* (Lange) Imbach var. *bisporus*, is one of the most widely cultivated mushrooms in the world. In 1981, the first hybrids of the *bisporus* variety, Horst U1 and U3, were released onto the market and subsequent new hybrids which also came on the market were either genetically identical or highly similar to the first ones (Sonnenberg et al., 2011). Despite the economic value of the button mushroom, there is hardly any investment in breeding new varieties (Gao et al., 2013). This low investment can result from the typical life cycle of this variety which makes it difficult to introduce new traits without a considerable linkage drag and in addition allows the generation of essentially derived varieties (look-a-likes) in very little time without large investments (Sonnenberg et al., 2017).

The *bisporus* variety is an example of amphithallic sexuality (Figure 3). About 80%-90% of the basidia produce two fertile spores, each of which preferentially receive two non-sister nuclei (ploidy level of n+n) with opposite mating types which will, upon germination, result in heterokaryotic mycelium. The mating type loci are the determinants of compatibility between individuals. Around 10%-20% of the basidia produce three or four spores, of which most will have one nucleus and germinates into homokaryotic mycelia (Evans, 1959; Saksena et al., 1976; Callac et al., 1993; Kerrigan et al., 1993). As only 10% of randomly selected single spores in the *bisporus* variety germinate into homokaryons which can only be used for outcrossing, breeding is hampered by this typical life cycle (Kerrigan et al., 1992).

a: Karyogamy and meiosis

b: Basidiospores formation; two types of basidia, i.e., 2-spored and 4-spored basidia

c: Germination of basidiospores with one nucleus (homokaryons)

d: Fusion between compatible homokaryons, resulting in formation of heterokaryotic mycelia

e: Germination of basidiospores with two nuclei (heterokaryons) which can directly form heterokaryotic mycelia

f: Heterokaryotic mycelia formed either from fusion of two compatible homokaryons or heterokaryons produce fruiting bodies.
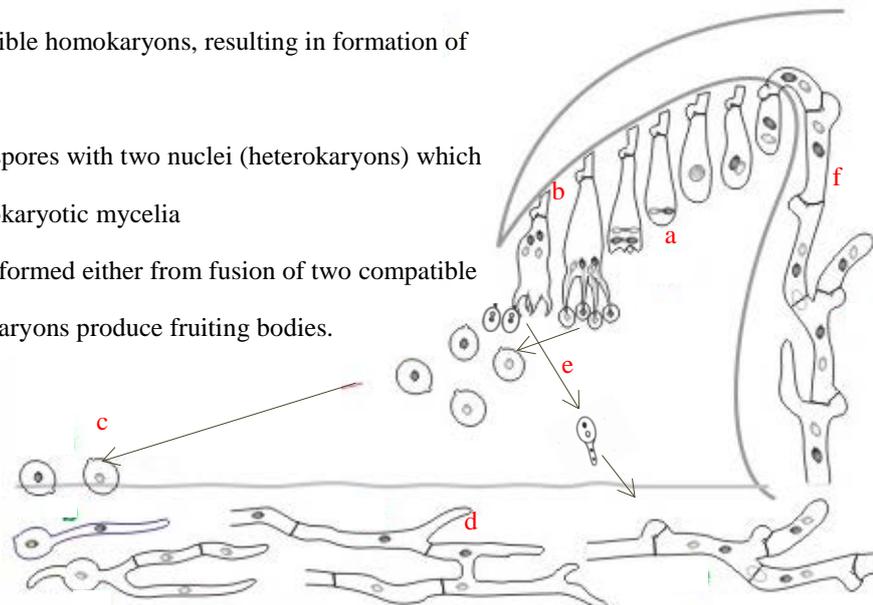

Figure 3. **life cycle and sexuality of *Agaricus bisporus*.** All stages from karyogamy to spore formation occur in one basidium but to better illustrate the life cycle, each stage is shown in a separate basidium.

Recently, a thorough study of MRL of the *bisporus* variety using whole genome sequencing (WGS) revealed that 92% of all mapped COs occurs in the first/last 85 kb of each chromosome. There is no indication of aneuploidy, suggesting that each chromosome experiences one obligate CO per meiosis, at least, in most organisms as a vital means for proper segregation of chromosomes (Sonnenberg et al., 2016).

Having CO restricted at the extreme ends of chromosomes, the offspring of the *bisporus* variety receives nearly intact parental chromosomes after meiosis. This meiotic behaviour together with non-sister pairing of nuclei preserves heterozygosity. The majority of the heterokaryotic offspring differ from their parental genotypes only by reshuffling homologous chromosomes over the two nuclei. The rare occurrence of homokaryotic spores and the absence of CO in major parts of chromosomes makes breeding button mushrooms a difficult and laborious task.

Elevated levels of recombination in euchromatic regions near chromosome ends have been reported in yeast (Barton et al., 2008) and other non-fungal organisms (Barlow and Hulten, 1998; Jensen-Seaman et al., 2004) but an almost complete absence of CO in the rest of the chromosomes has not been seen in yeast.

Life cycle and MRL of the burnettii variety

A. bisporus var. *burnettii* was identified by Mr. Jerry Burnette in 1978, which shows a more common life cycle. It produces a differing ratio of heterokaryons and homokaryons; only ~10% of its spores are heterokaryotic and thus naturally fertile, while the majority of the spores are homokaryons (Callac et al., 1993; Kerrigan et al., 1993). The life cycle of the *burnettii* variety is also regarded as amphithallic but with heterothallism dominating.

Where the life cycle of the *bisporus* variety hampers outcrossing, the life cycle of the *burnettii* variety allows more outcrossing, and thus is more suitable for breeding programmes. Up to now, no genetic linkage map of the *burnettii* variety has been made. There is only indirect evidence showing that *burnettii* and *bisporus* varieties have different MRL (Foulongne-Oriol et al., 2010).

Life cycle and MRL of the inter-varietal hybrid

As stated earlier, *bisporus* and *burnettii* varieties are interfertile. The number of spores on basidia is controlled by a locus known as basidial spore number (BSN) which was mapped on chromosome I and linked with the mating type locus (Imbernon et al., 1996). The predominant expression of the BSN locus from the *burnettii* variety was observed in an inter-varietal hybrid (var. *bisporus* × var. *burnettii*), as the elevated percentage of basidia having four spores and the proportion of basidia having two spores reduced to 1.2%-3.6 % on average (Kerrigan et al., 1994).

A number of genetic studies using segregation analysis in the inter-varietal hybrids (var. *bisporus* × var. *burnettii*) revealed increased recombination frequencies in hybrids (0.86 CO per chromosome; Callac et al., 1997; Foulongne-Oriol et al., 2010; Foulongne-Oriol et al., 2011). Using an inter-varietal hybrid in the study performed by Foulongne-Oriol et al. (2010), a map length of 1156 cM was constructed with an almost equal distribution of CO recombination across the genome with some clustering. Interestingly enough, the distal region of the chromosomes revealed a somewhat greater CO frequency. In addition, in a second generation hybrid (between *bisporus* variety and progeny from a *bisporus* variety × *burnettii* variety) the recombination frequency was 0.67-fold lower than in the first generation hybrid (Foulongne-Oriol et al., 2011). These results indicate that the recombination frequency in the *burnettii* variety is higher than in the *bisporus* variety and as such, likely to be a quantitative trait.

Molecular breeding in two varieties of *A. bisporus*

A. bisporus var. *bisporus* is a variety with a common heterokaryotic phase, a rare homokaryotic phase and a short diploid phase in the basidia during karyogamy with 13 chromosomes. In plants, a nuclear fusion of parental haplotypes occurs after fertilisation. Genetic analysis of parental haplotypes, which are usually constructed indirectly is, therefore, difficult in plants. The life cycle of basidiomycetes, including *A. bisporus*, enables the recovery of parental

haplotypes in addition, to the propagation of single meiotic products (spores). This produces an accurate analysis of genomic changes due to meiosis. From Horst U1, two parental haplotypes i.e., H39 and H97 are available and they were sequenced *de novo*. The genome size of H39 and H97 is approximately 31 Mb which is currently assembled into 16 scaffolds for H39 and 13 scaffolds for H97. The WGS of these two haplotypes is known to have one of the best complete genome sequences i.e., telomere to telomere, which is hardly seen in fungi whose genome has already been sequenced *de novo*. As all information of an individual is in the genome, the availability of high quality WGS will help on gaining insight into the order of the genes between or within species through comparative genome studies. A previous study has shown a very high collinearity between two genomes of the *bisporus* variety (Sonnenberg et al., 2016).

The majority of spores in *A. bisporus* var. *burnettii* are homokaryons. Little is known about MRL of the *burnettii* variety. Meiotic recombination are thought to be spread more equally over the chromosomes in the *burnettii* variety, although this has never been directly made known (Foulongne-Oriol et al., 2010). Since both these varieties are interfertile and are likely to have contrasting MRL, they represent an applicable model for unravelling mechanism(s) which control MRL. As far as the *burnettii* variety is concerned the high quality genome sequence was not available, therefore we used PacBio RSII technology to do the *de novo* sequencing of one of the haplotypes of the *burnettii* variety.

The method most predominantly used to study CO recombination is via the construction of genetic linkage maps. The first genetic linkage map was created in 1913 (Sturtevant, 1913). The basic principle of this technique is to find polymorphic molecular markers for the parental lines used in order to generate a segregating population from these parents. The segregation analysis of markers in the offspring (mapping population) allows the positioning of markers on the genome. As each of the parental chromosomes has an equal chance of ending up in nucleus one or two after meiosis I, a 50% segregation of parental markers indicates a location on the same chromosome. Segregation of parental markers lower than 50% indicates localisation on the same chromosome and recombination frequencies between pairs of markers enables the sorting of markers on each chromosome (the closer the markers, the lower the recombination frequency (Sturtevant, 1913). Thanks to WGS technologies, more and more molecular markers such as single nucleotide polymorphism (SNP) markers can be generated, allowing us to study meiotic recombination much faster in detail.

In order to apply molecular breeding in button mushroom, the construction of linkage maps has been one of the major bottlenecks for a long time. The first genetic linkage map of the *bisporus* variety contained 11 linkage groups with a total map length of 543.8 cM (Kerrigan et al., 1993). It was apparent at that time that *A. bisporus* had a low recombination frequency of less than 0.2 CO per linkage group per meiosis, nevertheless chromosomes were segregating properly during meiosis. In addition, recombination frequencies in the distal regions of the chromosomes were found to be relatively higher. The earliest cytological study of the *bisporus* variety by Evans (1959), showed 12 chromosomes in haploids. Furthermore the CHEF (clamped homogeneous electric field) analysis revealed that this variety had 13 chromosomes with a genome size of 31 Mb (Royer et al., 1992; Lodder et al., 1993; Sonnenberg et al., 1996). Observing large groups of non-recombining markers in the linkage studies led to the conclusion that either the CO

hardly occurred in large genome regions or that such recombinants cannot germinate successfully, because the germination rate of the spores is low (Kerrigan et al., 1993).

A recent linkage map of a population derived from the *bisporus* variety Horst U1 was constructed using 629 SNP markers. Most SNP markers in this study co-segregated and grouped together due to the low recombination frequency leading to a short linkage map. The total map length of 321 cM with 13 linkage groups was made with an average CO ratio of 0.89 at the ends of the chromosomes, i.e., the number of CO in the first/last 100 kb of all chromosomes over all CO (Sonnenberg et al, 2016). As with other previous linkage studies in this variety (Kerrigan et al., 1993; Xu et al., 1993), COs were found mainly at the chromosome ends. In another recent study two other segregating populations were also made using one of the parental homokaryons of Horst U1 and two homokaryons obtained from heterokayons which were distantly related to a *bisporus* variety. The constructed linkage maps were somewhat short having a total map length of 166 cM and 86 cM with COs mainly at the chromosome ends (Gao et al., 2015). During the application of molecular breeding, a very short linkage map will possibly result when assigning quantitative trait loci (QTL) controlling a trait of interest to almost complete chromosomes (Gao et al., 2015).

Contrary to what has been assumed so far in terms of CO recombinations using segregation analyses in the *bisporus* variety, a thorough study of its MRL was carried out using WGS which revealed that COs are predominantly restricted at the ends of chromosomes, as stated earlier, therefore, low recombination frequencies observed in other studies are likely to occur due to difficulties in generating markers for chromosome ends or missing sequences of chromosome ends (Sonnenberg et al, 2016).

In our project we used genotyping by sequencing (GBS) to genotype a segregating population derived from the *burnettii* variety and constructed the first genetic map of this variety. This infomation was used in this thesis to assemble the scaffolds generated by *de novo* sequencing into a high quality WGS, in order to understand the MRL of the *burnettii* variety, and perform a comparative genome sequence study to detect the degree of collinearity between the *bisporus* and *burnettii* varieties.

QTL study of MRL

Insight into the mechanisms for determining the localisation of CO, whether at chromosome ends or distributed throughout chromosomes, can be very useful for breeding all kinds of mushroom and even plant species. The quantitative inheritance of recombination in *A. bisporus* was defined by Foulongne-Oriol et al (2011). In order to perform QTL study and search for MRL, we made a mapping population (F1 inter-varietal hybrid) initially from which a genetic map was constructed. An assessment of the MRL can only be made in the offspring of the second generation hybrids which are generated by crossing the individuals of the F1 hybrid with a compatible homokaryon. We generated three different types of segregating populations on a small scale to verify which type is most feasible for phenotyping i.e., assessment of MRL. After selecting the type of segregating populations and subsequently isolating the homokaryotic offspring, the homokaryotic offspring were genotyped. We attempted using the frequencies and

fractions of recombination per chromosomal region to determine the phenotypic value for QTL analysis.

Aim and layout of the thesis

Two interfertile varieties of *A. bisporus* with contrasting MRL make it possible to elucidate genetic regions underlying MRL which is the main objective of this thesis. Generated knowledge is important and extremely relevant for breeding programmes relating to this type of mushroom and other plant species because linkage drag is the main obstacle for introducing new traits. Controlling meiosis during the propagation of elite hybrids in e.g. plant seeds is almost impossible due to the necessary steps of meiotic recombination which leads to a change in phenotype. This can be circumvented by reverse breeding. However, reverse breeding is much less efficient due to a significant increase in unviable gametes.

At the outset of this Ph.D. project, the MRL of the *bisporus* variety was not studied precisely at the molecular level, therefore the initial aim focussed on mapping the presence or absence of CO recombinations. As mentioned earlier, due to a predominant restriction of CO recombination at chromosome ends in *bisporus* variety, during my research I attempted to map the "recombination landscape" as a phenotype i.e., CO at the ends of the chromosomes and CO distributed throughout the chromosomes. In my research, two strains were used, i.e., Horst U1 as the *bisporus* variety and Bisp119/9 as the *burnettii* variety. From a parallel project high quality genome sequences of the parental haplotypes of Horst U1 are available (H97 v3.1 & H39 v 3.1), which gave us the opportunity to generate molecular markers and perform a detailed study of meiotic recombination which is also necessary for the *burnettii* variety.

In chapter two I report on the *de novo* sequencing of a parental haplotype of the Bisp119/9, i.e., Bisp119/9-P4 using the PacBio RSII. The second parental haplotype, Bisp119/9-P1, was re-sequenced using Illumina HiSeq 2000. The assembly procedures as well as the constructed genetic linkage map of the Bisp119/9 using genotyping by sequencing (GBS) were used to anchor contigs to scaffolds. The reconstruction of the *de novo* assemblies of Bisp119/9-P4 resulted in 16 scaffolds, containing telomeric sequences on almost all of the chromosome ends (Bisp119/9-P4 lacks 3 telomeric sequences). The genetic linkage map of the Bisp119/9 is the first to have been produced for the *burnettii* variety. The GBS technique was performed for the first time in *A. bisporus* which enabled us to generate a set of genetic markers to construct a linkage map. A set of homokaryons of Bisp119/9 were isolated and genotyped by GBS. The *burnettii* variety showed a recombination pattern spread evenly over the genome, in contrast to the *bisporus* variety where COs are restricted to chromosome ends. Using the alignment of the *burnettii* genome versus *bisporus* genome, a comparative analysis of genome collinearity and rearrangement was performed, as the changes in genomic architecture of the genotypes for instance inversions and other chromosomal rearrangements might affect the recombination behavior of the inter-varietal hybrid. In order to detect significant rearrangements, the reconstructed genome sequence of Bisp119/9-P4 was compared to the H39 and H97 sequences, showing only one large inverted region (~800 kb) between the haplotype Bisp119/9-P4 relative to those of the *bisporus* variety. The results revealed that the Bisp 119/9-P4 genome is highly collinear with the genomes of H97 and H39.

Having examined the MRL of the *burnettii* variety and the degree of collinearity between the *bisporus* and *burnettii* varieties, in chapter three an F1 inter-varietal hybrid was generated by crossing the *bisporus* and *burnettii* varieties. A homokaryotic offspring was isolated from an F1 hybrid and genotyped by SNP markers to construct a linkage map. This enabled us to perform the QTL analysis. There are now three genetic linkage maps of the *A. bisporus*, i.e., *bisporus* variety (Sonnenberg et al., 2016), *burnettii* variety (This thesis, Chapter 2) and the inter-varietal hybrid of the *bisporus* and *burnettii* varieties gives a comparison of the MRL between these three genomes. Using markers for all three maps made it possible to compare the type of MRL in these three different populations. The MRL trait cannot be assessed immediately on the F1 mapping population because this phenotype is expressed as a CO in the offspring of each individual in this population. Therefore, chapter four describes the analysis of a pilot experiment to clarify what type of phenotypic segregation (MRL) is visible in the following generation (F2 population) and what type of F2 population e.g. outcrossed or intercrossed population will give a clear variation of the phenotype. This experiment was carried out using a limited set of offspring from three different F2 populations, i.e., 1) intercrossed population, 2) outcrossed population with the *burnettii* type parent, and 3) outcrossed population with the *bisporus* type parent. The results revealed that a comprehensive study of the phenotype (MRL) is virtually impossible to carry out on the first two populations due to large amounts of homozygosity in each of the homokaryotic pairs of intercrossed population and poor quality of fruiting bodies which have a low spore germination rate of outcrossed population with the *burnettii* type parent. Thus, the third population, i.e. outcrossed population with the *bisporus* type parent seemed to be the most feasible in which the MRL phenotype could be assessed. Studying the phenotype in the entire genome was possible because of the high SNP density between the three genomes. The results of this chapter provide us with the knowledge to study the MRL further and select potential F2 populations to identify QTL.

In chapter five, identifying QTL is described to unravel the genetic basis explaining the difference of MRL in *A. bisporus*. The selected outcrossed population in chapter four was generated and used to quantify MRL. This chapter describes different methods to quantify CO using a limited set of markers. The identified QTL indicates that there is a genetic base for the different MRLs observed in the two *A. bisporus* varieties. The resolution of QTL is, however, low and suggestions are made on how to fine map QTL.

Chapter six covers the various topics addressed in this thesis, and integrate the outcome of the experimental chapters with reference to our current understanding of MRL. I discuss the implications of what we explored in *A. bisporus* for other species such as crop plants and the prospects of management and control of meiosis towards a more efficient breeding programme in relation to our findings.

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Chapter 2

Genome analysis of the button mushroom *Agaricus bisporus* var. *burnettii*

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Abstract

The button mushroom *Agaricus bisporus* is represented mainly by two varieties i.e., the *bisporus* and the *burnettii* variety. While the former variety has a secondary homothallic life cycle producing mainly two-spored basidia, the latter is mainly heterothallic producing predominantly four-spore basidia. Previous research has revealed that in the var. *bisporus* meiotic crossovers (CO) are restricted to chromosome ends. Until now, there has only been an indirect indication of the CO landscape of the *burnettii* variety. To study meiosis in this variety in more detail, we have *de novo* sequenced one of the constituent nuclei of a heterokaryotic strain of the *burnettii* variety and re-sequenced the other constituent nucleus. Using Genotyping by Sequencing (GBS) on the homokaryotic offspring of this heterokaryon, a genetic linkage map was constructed and used to map the entire scaffold of the *de novo* sequenced homokaryon. This resulted in a telomere to telomere genome sequence of almost all thirteen chromosomes. A comparison of this genome to the previously sequenced genomes of two var. *bisporus* homokaryons revealed an almost perfect collinearity between all three genomes. An exception was discovered in chromosome ten, where an inversion of ca 800 kb in the *burnettii* variety was found compared to the var. *bisporus* genomes. To the best of our knowledge, the availability of three high quality genome sequences within one species, representing the two main varieties has not been reported so far. The linkage map generated with GBS demonstrated clearly that, in contrast to the var. *bisporus*, CO events are distributed more or less evenly over chromosomes. This research will contribute to unravelling the genetic base for the differences in life cycles and especially the CO landscape of both *bisporus* varieties.

Introduction

The haploid genomes of fungi are the most compact eukaryotic genomes known in general (Corradi et al., 2010). They are relatively small in size compared to those in animals and plants, varying from 2.3 Mb to 177.57 Mb with an estimated total of around 10,000 genes (Hamer et al., 2001). The small genome size of fungi and few repetitive sequences facilitate the generation of high quality genome sequences at a relatively low cost. Fungi have the greatest number of sequenced genomes (Haridas et al., 2011) which makes them more attractive for fundamental research (http://www.ncbi.nlm.nih.gov/genome/browse/). Recently, a project was initiated by the Joint Genome Institute (JGI) to sequence 1000 fungal genomes from different species in order to provide a better insight into fungal biology and evolution (1000 fungal genome project, [http://1000.fungalgenomes.org/home/]). The Ascomycetes fungus, *Mycosphaerella graminicola*, is known to have one of the most complete genome sequences (Goodwin et al., 2011). However, not all fungal genome sequences have a high quality *de novo* sequence. This means that there are still many holes in the assembled genomes with the number of scaffolds exceeding the number of predicted chromosomes. A complete genome assembly, i.e., telomere to telomere, is barely visible in fungi whose genome has already been *de novo* sequenced.

As all the information pertaining to an individual is in the genome, the availability of high quality whole genome sequence (WGS) gives scientists a better view in various aspects, one of which is to detect different genomic elements e.g. the gene, repetitive families and their distribution within the genome and also compare different genomes to detect chromosomal rearrangements. To gain insight into the order of genes between or within certain species, comparative genome studies are recommended. High quality WGS enables the precise comparison of genomes to detect changes in the genomic architecture of the compared genotypes for instance inversions and other chromosomal rearrangements. The latter are known to influence the meiotic recombination of an organism (Emanuel and Shaikh, 2001; Rieseberg, 2001). Meiotic recombination occurs during the first meiotic division and has two outcomes, i.e., crossovers (COs) and gene conversion/non-crossovers (GC/NCOs) which create genetic variations.

The basidiomycete *Agaricus bisporus* is mainly represented by two interfertile varieties, i.e., *A. bisporus* var. *bisporus* and *A. bisporus* var. *burnettii* (Callac et al., 2003), which differ in their ecology, life cycle, and morphological characteristics. However, little is known about the variation between their genome structures. *A. bisporus* var. *burnettii* found in the Sonoran desert of California has a predominantly heterothallic life cycle. The spores of the *burnettii* variety have two ploidy levels, i.e., heterokaryons (n+n) and homokaryons (n). Homokaryons and heterokaryons are the sexual spores which are formed in basidia where meiosis takes place. The majority of the basidia in this variety produce four spores of which most will have one nucleus (ploidy level of n) and germinate into homokaryotic mycelia. Homokaryons need to be mated with a compatible partner to form fruiting bodies. Only ~10% of its spores are heterokaryotic with some indications of non-sister nuclei pairing and thus self-fertile (Callac et al., 1993; Kerrigan et al., 1993; Kerrigan et al., 1994). *A. bisporus* var. *bisporus*, also known as button mushroom*,* is a widely cultivated mushroom which has a considerable economic value (Kameda et al., 2006; Adams et al., 2008; Savoie et al., 2008). Its edible fruiting bodies have

been part of the human diet over the last centuries. Apart from its consumption value and other features which distinguish it as a model fungus (Fermor and Wood, 1981; Burton et al., 1997), it can also be used as a model fungus for studying meiotic recombination. The *bisporus* variety has mainly homothallic sexuality and produces both homokaryons and heterokaryons in different ratios. Most of the basidia in this variety produce two spores, each receiving preferentially two non-sister nuclei (ploidy level of n+n) with opposite mating types which will, upon germination, result in heterokaryotic mycelium. Around 10%-15% of the basidia produce three or four spores, of which most will have one nucleus which germinates into homokaryotic mycelia (Evans, 1959; Saksena et al., 1976; Callac et al., 1993; Kerrigan et al., 1993).

In contrast to the *burnettii* variety, genetic studies on the meiotic behaviour of the *bisporus* variety are more advanced. However, in the past, studies mainly focussed on random isolation of homokaryons and genotyping using a limited number of molecular markers to construct genetic linkage maps. All studies resulted in a low recombination frequency with COs positioned mainly at chromosome ends (Summerbell et al., 1989; Kerrigan et al., 1993; Gao et al., 2015). Recently, the high quality genome sequence of both haplotypes of the *bisporus* variety became known publically (H97 v3.1 & H39 v3.1). The precise mapping of CO events has also been carried out recently using WGS on a limited number of homo- and heterokaryotic single spore isolates of a *bisporus* variety. We were able to see that 92% of all mapped COs occurs in the first/last 85 kb of each chromosome. In fact, the low recombination frequency of the *bisporus* variety observed in other studies was probably due to the fact that many COs are unlikely to be detected during the genotyping of homokaryons using a limited number of molecular markers. It is not always possible to cover the chromosome ends with molecular markers due to the existence of the repetitive elements at chromosome ends (Sonnenberg et al., 2016). The telomeric and centromeric regions of *A. bisporus* mainly harbour clusters of repetitive elements (Morin et al., 2012). The *bisporus* variety, therefore, has a typical recombination landscape with an almost exclusive CO position at the chromosome ends. The availability of high quality genome sequence is very important to depict a complete picture of the recombination landscape of an organism. It allows the choice of markers covering chromosomes from telomere to telomere and will detect inversions or other significant differences between genomes which could affect COs. In a previous study there was evidence of a very high collinearity between two genomes of the *bisporus* variety (Sonnenberg et al., 2016).

The meiotic behaviour of the *burnettii* variety has not been studied so far. A study on the meiotic behaviour of the inter-varietal hybrid made from a cross between *bisporus* and *burnettii* strains revealed greater recombination rates in the *burnettii* variety (Callac et al., 1997; Foulongne-Oriol et al., 2010). This evidence is inconclusive to back up the hypothesis that both *burnettii* and *bisporus* varieties do indeed have different recombination landscapes because until now no genetic linkage map has been made of the *burnettii* variety.

A typical recombination landscape combined with a large number of heterokaryons generated with the non-sister pairing of nuclei make the breeding of the *bisporus* variety challenging and also laborious. Meiotic recombination has an important role to play in introducing desirable traits or discarding undesirable ones. If the *burnettii* variety does have a meiotic recombination

spread all over the genome, this would be much more suitable for breeding programmes. Outcrossing is simpler in this variety as the majority of its spores are homokaryons. However, other features of the *burnettii* variety, such as rapid maturation make it unsuitable for commercial production. Having a better knowledge of the meiotic recombination landscape in the *burnettii* variety necessitates a high-quality genome sequence which has not been established yet.

We used the PacBio RSII technology to do the *de novo* sequencing of one haplotype of the *burnettii* variety with two aims: (1) to perform a comparative genome sequence study between the *bisporus* and *burrnettii* varieties to detect the degree of collinearity as a factor affecting recombination landscape of the inter-varietal hybrid. (2) to generate molecular markers required for our long-term objectives to study the meiotic recombination landscape. As far as we know, this is the only example of a fungal genome in which three high quality genome sequences occur in one species. The knowledge gained gives hope for improving *A. bisporus* value as a model organism for studying the meiotic recombination landscape. We have also constructed the first *A. bisporus* var. *burnettii* linkage map. For that, we used the Genotyping-by-Sequencing (GBS) technique (Elshire et al., 2011) to obtain a set of genetic markers. This linkage map proved to be an ideal tool for assembling the scaffolds generated by *de novo* sequencing into a high quality WGS. The present linkage map of the *burnettii* variety also contributes to a better understanding of meiotic recombination landscape as an important feature in breeding programmes of the *A. bisporus* species.

Materials and methods

Strains

Two heterokaryons used in this study were obtained from the fungal collection of Plant Breeding at Wageningen University & Research; Horst U1 a white commercial *bisporus* strain and Bisp119/9 and a wild type *burnettii* strain originating from the ARP collection (Kerrigan, 1996). The constituent nuclei as homokaryons (H97 and H39) from Horst U1 heterokaryon were available from another study (Sonnenberg et al., 2016). The constituent homokaryons of Bisp119/9 (Bisp119/9-P4 and Bisp119/9-P1) were recovered by protoplasting in accordance with the method previously described (Sonnenberg et al., 1988).

DNA preparation for sequencing

Obtaining high amounts of molecular genomic DNA having both a high yield and good quality is essential for sequencing. After two weeks of growth on agar plates covered with cellophane, the mycelia of Bisp119/9-P4 and Bisp119/9-P1 were harvested, freeze-dried and ground to a fine powder in an Eppendorf tube (2ml). Genomic DNA was prepared by the method previously described (Sonnenberg et al., 2016).

De novo sequencing and assembly

Large amounts of molecular DNA of one of the constituent nuclei from Bisp119/9 (homokaryon Bisp119/9-P4) were *de novo* sequenced using PacBio RSII technology applying P5-C3 and P6-C4 chemistry (Pacific Biosciences, Menlo Park, California, USA). The sequence data (373,492

reads have an N50 length of 8106 nt, totalling 2,238,359,064 nt representing a coverage of 70x) were assembled into scaffolds using HGAP2 and polished using an SMRTportal 2.3.0 with a Quiver algorithm and resulted in 50 scaffolds having a total sequence length of 31,634,749 bp. Further strategies were applied to improve the *de novo* assembled genome sequence of the homokaryon Bisp119/9-P4. First of all the SMRT® View BridgeMapper was used to detect misassemblies, and connect potential links of contigs to one another (SMRT® portal, Pacific Biosciences, Menlo Park, California, USA). The BridgeMapper was subsequently used to re-align unaligned examined ends to the rest of the assembly. The genetic linkage map (see "Genotyping by Sequencing" session) generated from a homokaryotic offspring of Bisp119/9 was used to assemble scaffolds into a complete genome. Finally, a BLAST search (CLC Genomics Workbench version 7.5.1, Qiagen, Aarhus, Denmark) was done on the whole genome sequence of the scaffold ends that did not show overlap on one side to see if the few gaps remaining could be removed. From Bisp119/9-P4 and the other constituent homokaryon, Bisp119/9-P1, re-sequencing data using Illumina HiSeq was also available before *de novo* sequencing was carried out.

Genotyping By Sequencing

The heterokaryotic strain, Bisp119/9, was cultivated in the growing facility of Unifarm, Wageningen University & Research as previously described (Weijn et al., 2012). Basidial spores of Bisp119/9 were collected at room temperature and used to isolate 170 homokaryotic single spore isolates (SSIs). The entire genomic DNA was extracted from homokaryotic SSIs as well as two constituent parental homokaryons of Bisp119/9 as previously described (Sonnenberg et al., 2016). DNA quantification, A260/A280 ratio and A260/A230 ratio were determined using a NanoDropTM spectrophotometer and Qubit® 2.0 Fluorometer (Thermo Fisher Scientific Corporation, Waltham, MA).

All 170 homokaryotic SSIs of Bisp119/9 and the constituent nuclei of Bisp119/9 (Bisp119/9-P4 and Bisp119/9-P1) were genotyped using the GBS technique (Keygene N.V. owns patents and patent applications protecting its Sequence Based 257 Genotyping technologies) as previously described (Elshire et al., 2011). In GBS technology, an appropriate restriction enzyme (RE) is used to reduce the complexity of the genome. In our lab, barcodes and adapters were available for three REs, i.e., ApeKl, EcoT22l, and Pstl. In order to test which RE is suitable for our experiment, we performed an *in silico* restriction site analysis on the *de novo* sequencing data of Bisp119/9-P4 using a CLC Genomic Workbench (version 7.5.1, Qiagen, Aarhus, Denmark). Using an RE that is methylation sensitive is much more efficient as the repetitive regions of the genome are not targeted and will thus be avoided in the following sequencing analysis. Adapters and barcodes were used according to Elshire et al.(2011). All samples were sequenced using Illumina© HiSeq.

Raw GBS sequence data containing all forward and reverse HiSeq reads (pair end data) were mapped against the *de novo* reference sequence version 2.1 of *A. bisporus* 119/9-P4 using the Burrows-Wheeler Alignment tool (BWA) backtrack algorithm of the BWA version 0.7.7-r441(Li and Durbin, 2009). This data was used as an input to generate variance calls using FreeBayes version v0.9.18-g4233a23 with default settings (Garrison and Marth, 2012). The

VCF file output was initially filtered for read depth > 5 and the positions of type "SNP." This resulted in 605,199 SNP positions which were filtered once more using a filtering pipeline to obtain enough markers and individuals to construct a genetic linkage map. The filtering pipeline was based on the filtering pipeline previously published as a blueprint (Schilling et al., 2014). The steps in the filtering pipeline were as follows: As we genotyped haploid genomes (homokaryons), no heterozygote scores were anticipated. Therefore, all positions that were heterozygous for > 10% of all individuals were removed. Positions having either an unknown for at least one of the two parents or an identical genotype for both parents were also removed. The segregation ratio of 0.5 was expected as our dataset had biparental segregation. That means that both parental alleles are found equally in the offspring. However, deviation of the segregation ratio has been observed in many experimental systems. In our dataset, all positions with deviant segregation ratio (segregation ratio < 0.20 or > 0.80) were removed. In the last step individuals and positions with too many missing data points were also removed.

Linkage mapping

Due to the large amount of GBS data, the DOS version of RECORD was used prior to JoinMap 4.1 and constructs a genetic linkage map (Van Os et al., 2005). All SNP positions were arranged based on the RECORD position, enabling us to remove all identical loci from the dataset. This dataset containing unique loci was used as an input for JoinMap 4.1 software and construct the genetic linkage map of Bisp119/9 based on the independence LOD score using a haploid model (HAP1) with default parameter settings (independence LOD score; significance level from 2.0 to 10.0 LOD; Van Ooijen, 2011). Chi-square analysis ($P < 0.05$) was used for checking the accuracy of fitting to the expected Mendelian ratios and linkage groups (LGs) were set up at the level of LOD $\geq$ 5. Centimorgan (cM) distances were expressed by the Kosambi function (Kosambi, 1943) and crossover frequency was calculated as the crossover number per individual per chromosome. We plotted the number of map units of SNP markers per linkage group calculated with JoinMap 4.1 against their respective physical distance (bp) to detect the relationship between genetic and physical distance.

Genome comparison

The NUCmer (NUCleotide MUMmer) version 3.1 was used to compare the genome of homokaryons Bisp119/9-P4 version 2.1 and H39 version 3.1 (Kurtz et al., 2004). NUCmer setting was adjusted to mincluster = 1000, minmatch = 100 for the whole genome and mincluster= 500 and nosimplify for comparing individual scaffold. Individual chromosomes were blasted against the RepBase database for a visual presentation of the repetitive elements of both Bisp119/9-P4 version 2.1, and H39 version 3.1 (Tempel et al., 2008). The setting was as follows: fungi for "sequence source" together with "forced translated search". The size of each visual presentation of the repetitive elements was adjusted to each chromosome and displayed graphically along the chromosomes in the MUMmer plots.

Telomeric repeated sequences were analyzed in all three genomes, i.e., H97 version 3.1, H39 version 3.1 and Bisp119/9-P4 version 2.1 *de novo* sequences using CLC Genomics Workbench version 7.5.1. The number of telomeric motifs in all three genomes was calculated.

The SNP density between the Bisp119/9-P4 version 2.1 and H39 version 3.1 was calculated by mapping the HiqSeq reads of Bisp119/9-P4 against H39 version 3.1 as a reference, using the Burrows-Wheeler Alignment tool (BWA) backtrack algorithm of BWA version 0.7.7-r441(Li and Durbin, 2009), followed by arranging the aligned reads using the SAMtools version 0.1.19-44428 cd (Li et al., 2009). Variant calls were generated by using the Freebayes version v0.9.18-1-g4233a23 (Garrison and Marth, 2012). The VCF file output was filtered to retain only positions with type SNP having a read depth of DP > 5.

Results

Sequence data and assembly

From *bisporus* variety Horst U1, two constituent parental homokaryons (H97 and H39) were previously recovered and *de novo* sequenced. H97 has been sequenced by JGI (http://genome. jgi-psf.org/) and was described previously (Morin et al., 2012). Recently, some misassemblies of this genome were repaired using the linkage map of Horst U1 and this updated genome is now available at NCBI (H97 v3.1). The other constituent nucleus (homokaryon H39) was also recently *de novo* sequenced and submitted to NCBI (Sonnenberg et al., 2016).

The two constituent parental homokaryons of the *burnettii* variety Bisp119/9 were available from previous projects. The homokaryon Bisp119/9-P4 was selected for *de novo* sequencing using PacBio RSII technology with coverage of 70×. We assembled the sequence of 373,492 reads into 50 scaffolds with a total sequence length of more than 31 Mb. The use of pipelines described in M&M and the genetic linkage map reduced the number of scaffolds to 16 representing a genome size of 30.7 Mb, which is very similar to those of the *bisporus* homokaryons H39 and H97 (Sonnenberg et al., 2016). Chromosomes 1, 3 and 5 each consist of 2 non-overlapping scaffolds. All other chromosomes were complete or nearly complete and for chromosomes 3, 9, and 12 the telomere sequence on one side was missing (Table 1).

Table 1. Overview of the length and completeness of the homokaryon Bisp119/9-P4 v2.1 chromosomes

Scaffold	Size (bp)	5′ Telomere	3′ Telomere
1*	3,639,502	Yes	Yes
2	3,265,402	Yes	Yes
3*	3,132,325	Yes	No
4	3,158,235	Yes	Yes
5*	2,380,213	Yes	Yes
6	2,426,497	Yes	Yes
7	2,254,680	Yes	Yes
8	1,867,134	Yes	Yes
9	1,867,033	Yes	No
10	1,897,446	Yes	Yes
11	1,931,181	Yes	Yes
12	1,559,381	Yes	No
13	1,323,473	Yes	Yes
Total size:	30,702,502		

* Chromosome consists of 2 non-overlapping scaffolds.

A linkage map of the heterokaryon Bisp119/9

Because of our interest in constructing the first genetic linkage map of the *A. bisporus* var. *burnettii* to study its meiotic recombination landscape and to further improve the *de novo* sequencing of Bisp119/9-P4, we needed to identify polymorphic markers for its constituent nuclei that are evenly distributed throughout the genome. For this we used a spore print of Bisp119/9 to generate a population of homokaryotic offspring and used GBS data to obtain a set of genetic markers. GBS reduces the complexity of the genome sequencing data by selecting genome fragments between 300 and 700 bp using an appropriate restriction enzyme. An *in silico* restriction site analysis on *de novo* sequencing of Bisp119/9-P4 revealed that EcoT22I and PstI will not generate sufficient fragments shorter than 1kb (Supplementary materials Figure S1), whereas ApeKI will. Theoretically, a total of 16,095 fragments ranging between 300 and 700 bp were found, representing 7,655,497 bp (=24.9%) of the genome (Figure 1). Genome fragments generated by ApeKI were also distributed evenly over the genome. Almost every 10 kb region of the Bisp119/9-p4 genome contains at least 1 restriction fragment (Supplementary materials Figure S2). However, restriction enzyme ApeKI is partly methylation sensitive. This means that ApeKI does not digest the entire DNA in the methylated regions, resulting in larger fragments that will be excluded during size selection prior to sequencing. Methylation in *A. bisporus* has mainly been found in repetitive elements which comprise less than 15% of its genome (Morin et al., 2012; Foulongne-Oriol et al., 2013). Therefore, ApeKI may not, or hardly, be cut in the major repetitive sequences of *A. bisporus*. Based on the outcome of the *in silico* digest test, it was expected that the number of genomic fragments targeted by ApeKI would be more than adequate to generate SNP markers spread evenly over the whole genome.
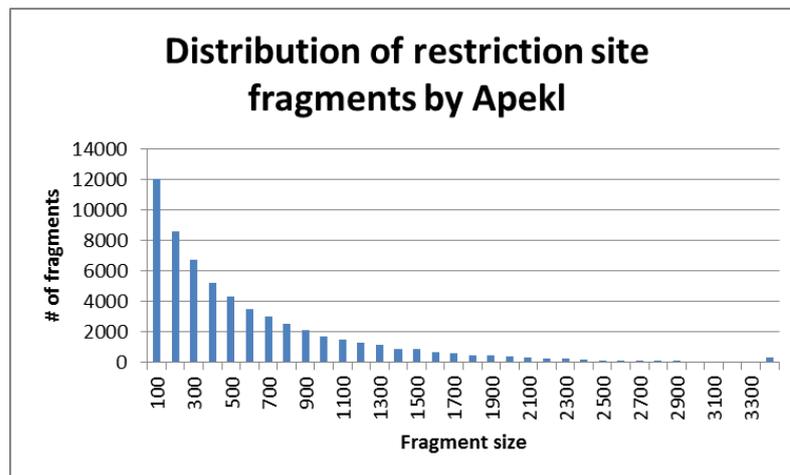

Figure 1. Distribution of fragments with different size digested in silico by ApeKI using *de novo* sequence of Bisp119/9-P4.

Overall genome sequence comparison

The most recent WGS of Bisp119/9-P4 (Bisp119/9-P4 v2.1) was compared to H39 v.3.1 and H97 v.3.1, the constituent nuclei of Horst U1. The WGS of H39 and H97 were previously

carried out using PacBio RSII technology and Sanger sequencing, respectively. The WGS of both H39 (Sonnenberg et al., 2016) and Bisp119/9-P4 (Table 1) consist of 16 scaffolds with 3 chromosomes each containing 2 non-overlapping scaffolds. The assembled genome of H97 consists of 13 scaffolds which correspond to the number of chromosomes (Morin et al., 2012; Sonnenberg et al., 2016).

The alignment of all three genomes reveals that they are mainly collinear (Figure 2; Supplementary materials Figures S3 & S4 for individual chromosome comparisons). One large inversion of ~ 800 Kb was found on chromosome 10 of Bisp119/9-P4 compared to the same chromosome of the *bisporus* variety (Figure 3), indicating a structural difference between the *bisporus* homokaryons and this *burnettii* homokaryon. A smaller inversion of ~ 250 Kb was found on chromosome 4 of Bisp119/9-P4 compared to the H97 genome (Supplementary materials Figure S4). This inversion was not seen relative to the H39 genome (Supplementary materials Figure S3) and might thus be a misassembly in the H97 genome (Sonnenberg et al., 2016). The appearance of the gaps or shifts in the Mummer plots viewed for all chromosomes coincides with clusters of repeats, as has been described previously (Sonnenberg et al. 2016).
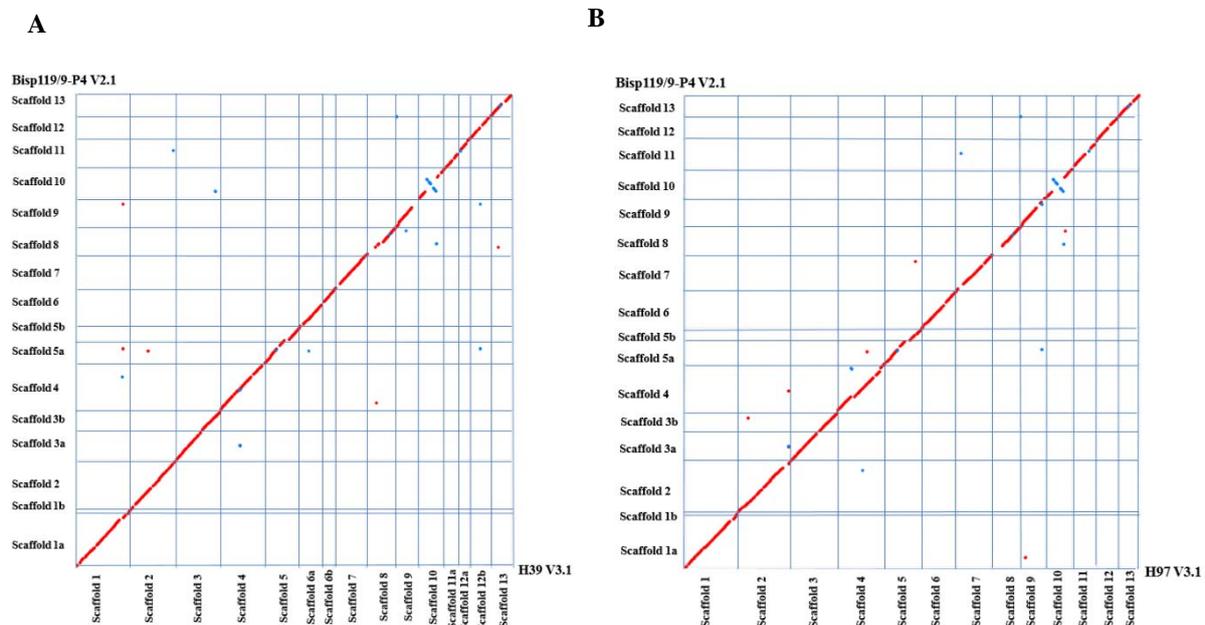

Figure 2. Collinearity of the genome of Bisp119/9-P4 v2.1 with two genomes of H39 v3.1 and H97 v3.1 using MUMmer plots. A) Comparison of all chromosomes of Bisp119/9-P4 and H39 genomes. Chromosomes 6, 11 and 12 of H39 and chromosomes 1, 3 and 5 of Bisp119/9-P4 each consist of 2 non-overlapping scaffolds. Red colour corresponds to collinearity of the sequences; Blue colour corresponds to inverted sequences in H39 compared to Bisp119/9-P4. The red and blue dots correspond to repetitive sequences scattered throughout the genome. B) Comparison of all chromosomes of Bisp119/9-P4 and H97 genome. The genome of H97 consists of 13 scaffolds corresponding to the estimated chromosome number of *A. bisporus*.

The other homokaryon Bisp119/9-P1 was re-sequenced using Illumina HiSeq. The reads were mapped against the genome of Bisp119/9-P4 v2.1 to detect SNPs. In total, there are 148,788 SNPs between Bisp119/9-P4 v2.1 and Bisp119/9-P1 which is equal to an SNP density of 4.8 SNPs per kb. This number is almost three times less than the SNP density between Bisp119/9-P4 and H39 version 3.1 (449,084 SNPs equal to 14.6 SNPs per kb).
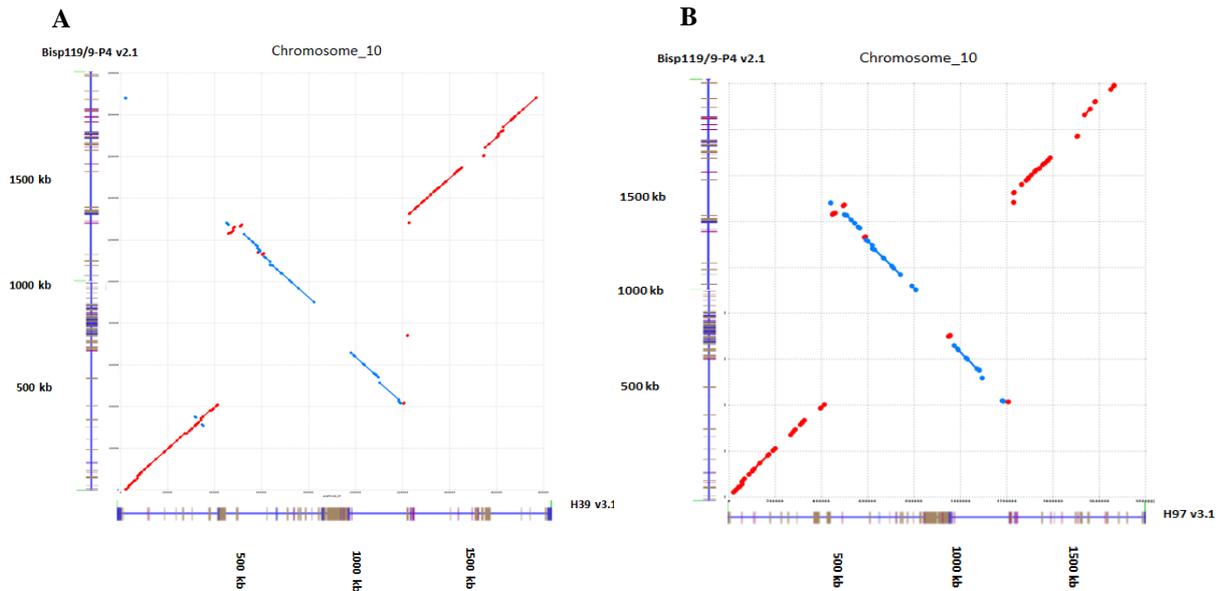
Figure 3. Comparison of chromosome 10 of Bisp119/9-P4 v2.1 with H39 v3.1 and H97 v3.1. A) MUMmer plot of the chromosomes 10 of Bisp119/9-P4 and H39 homokaryon. The plot shows an inversion (blue) in chromosome 10 of Bisp119/9-P4 v2.1 relative to the respective region in chromosome 10 of H39 v3.1. B) MUMmer plot of the chromosomes 10 of Bisp119/9-P4 and H97 homokaryon. The plot shows the same inversion (blue) in chromosome 10 of Bisp119/9-P4 v2.1 relative to the respective region in chromosome 10 of H97 v3.1.

Mapping the sequence reads from GBS data against the *de novo* sequence of Bisp119/9-P4 v2.1 and the subsequent filtering steps resulted in retaining SNPs that met all the criteria. Out of 170 homokaryons used in this study, 16 homokaryons were discarded for all downstream data processing due to either heterozygote scores or missing scores. The coverage of the retained SNPs per scaffold in the *de novo* sequence of Bisp119/9-P4 v2.1 was checked by calculating the number of SNPs per 10,000 bp for each scaffold. Many regions were not covered by SNPs (Figure 4).

While an SNP density of 4.8 SNPs per kb in the Bisp119/9 genome should, in principle, be more than adequate to generate a linkage map, SNPs should be distributed evenly over the genome. This was clearly not the case. Many areas had none, or very low numbers of SNPs. Chromosomes 2, 5, 7, 9 and 12 are examples of those with hardly any SNP and had regions without SNPs at all (Figure 4; Supplementary martial Figure S5). As a result, a linkage map could only be made for scaffolds 1(1a and 1b), 3 (3a and 3b), 10 and 11. Out of 4,229 SNP markers positioned on the four chromosomes indicated, 2,504 SNP markers were retained by removing identical markers (DOS version of RECORD) which were subsequently used as the input for JoinMap 4.1. The co-segregating markers were discarded from the final map construction. A total of 353 SNP markers were ultimately mapped on four chromosomes of *A. bisporus* var. *burnettii* genome (Figure 5), and together these markers cover ~ 416 map units (cM).

Figure 4. An overview of the SNP coverage per scaffold in *de novo* sequence of Bisp119/9-P4 version 2.1.
SNP coverage after all filtering steps. Red; regions without SNP coverage. Green; regions with SNP coverage.

The analysis of segregation ratios showed that 150 markers (42%) deviate from the expected Mendelian ratio (1:1 ratio, chi-square test, P < 0.05). The length of the haploid *A. bisporus* var. *burnettii* (Bisp119/9-P4) chromosomes 1, 3, 10 and 11 is estimated to be approximately 10,600 kb meaning that, on average, 1 cM equals approximately 25 kb (Table 2). To better visualize the recombination along the four indicated chromosomes, the map units (cM) positions of each marker per chromosome were plotted against its respective physical position (bp). These plots reveal that crossover events were more widely spread over the entire chromosomes compared to the *bisporus* variety (Sonnenberg et al., 2016), and chromosomes 3 and 10 showed somewhat more recombinations at the ends (Figure 6).
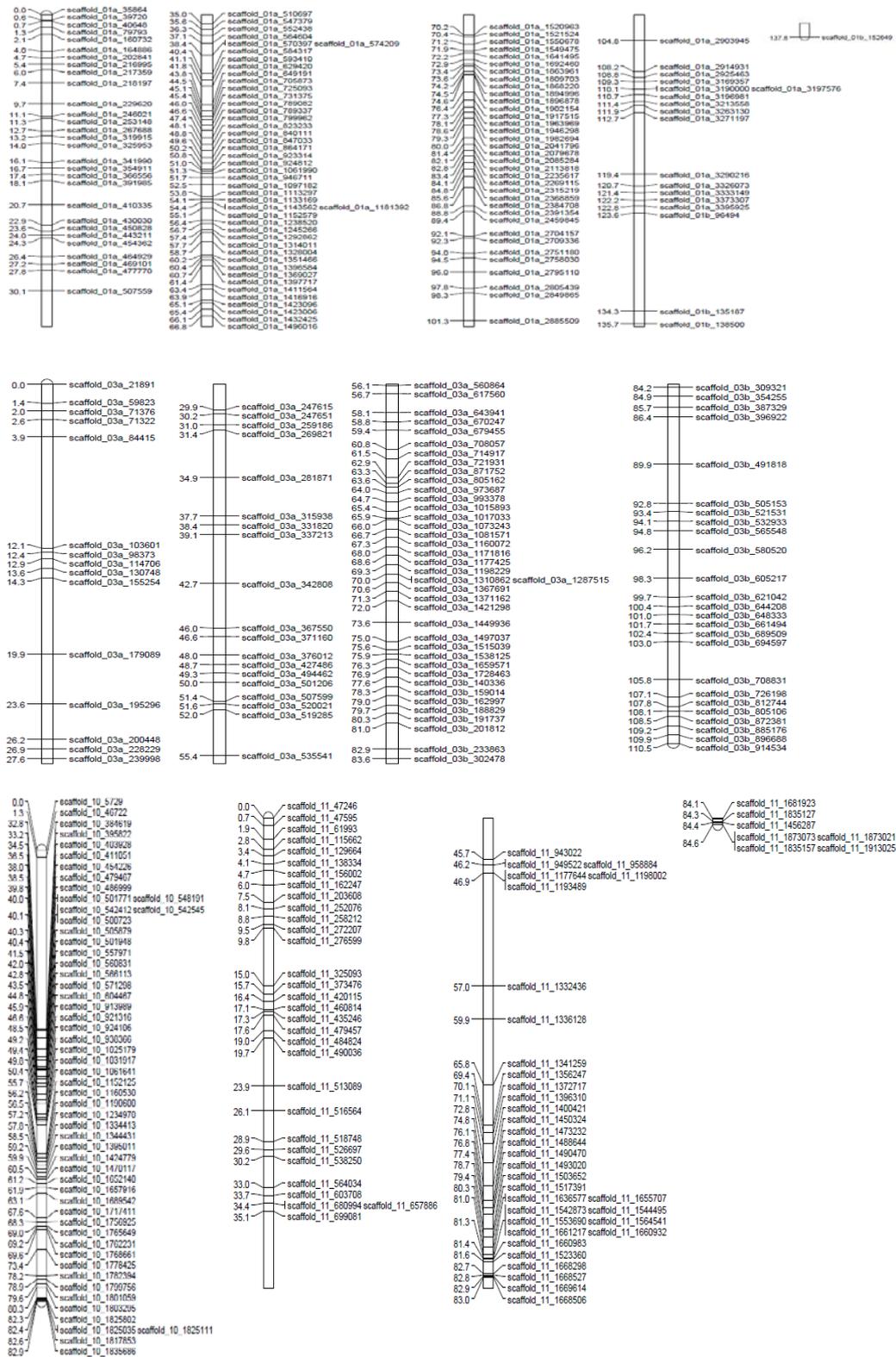
Figure 5. Genetic linkage map of Bisp119/9 population. Four linkage groups i.e., 1 (consists of two scaffolds), 3 (consists of two scaffolds), 10 and 11 are only presented. The SNP markers were labelled as scaffold number followed by their physical positions on that particular chromosome on the right side of each chromosome. The Centimorgan (cM) distance is given per marker pairs on the left side of each chromosome. All of the marker information can be seen in supplementary Table 1.

Table 2. Map statistics of a segregating population of 154 homokaryons of Bisp119/9.

LG	Chromosome length		Marker range[a]		No. Markers	No. skewed markers	No. of crossovers/chromosome						CO frequency per chromosome	Parental types (%)
	Physical length(bp)	Genetic length(cM)	Top	Bottom			0 Bisp119/9-P4[b]	0 Bisp119/9-P1[c]	1	2	3	4		
I	3639502	137.83	35864	3616694	128	78	7	22*	56	52	17	0	1.37	0.19
III	3132325	110.54	21891	2806024	98	69	9	22*	84	35	2	2	1.09	0.20
X	1897446	82.912	5729	1835686	55	3	27	30	71	25	1	0	0.81	0.37
XI	1931181	84.614	47246	1913025	72	0	27	27	73	25	1	1	0.84	0.35
Totals	10600454	415.896			353	150	70	101	284	137	21	3	1.03	0.28

[a] Marker range relative to the Bisp119/9-P4 v 2.1 genome.

[b] Number of individuals with intact linkage group of Bisp119/9-P4.

[c] Number of individuals with intact linkage group of Bisp119/9-P1.

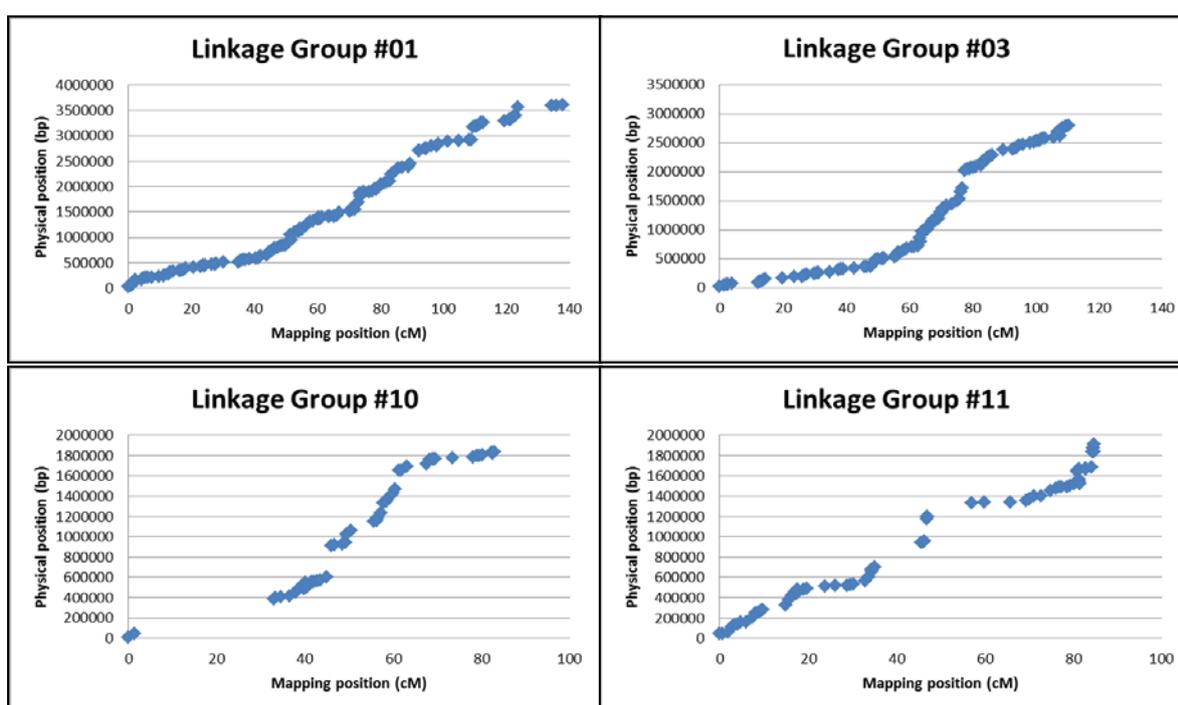* Significant deviation from the expected 1:1 ratio (chi-square test, P < 0.05).

Figure 6. Centimorgan (cM) distribution of SNP markers per chromosome along their physical position. The genetic position in cM of each marker per chromosome was calculated by JoinMap software and plotted against the physical positions.

All SNP markers retrieved from the GBS data regardless of their uneven distribution across the genome were used to construct the linkage map of the Bisp119/9 population with the aim of further improving the assembly of the *de novo* sequencing in Bisp119/9-P4 and validate the order of and orientation of its 16 scaffolds (Table 1).

Analysis of the motifs in telomeric regions

The ends of most eukaryotic chromosomes, so called telomere, contain repeated sequences that protect the chromosome from degradation or from fusion with other chromosomes. The assembled sequences of H97 v3.1, and H39 v3.1 contain telomeric sequences in most chromosomes except the telomere on the left side of chromosome 7 and the telomeres on the

right side of chromosomes 9 and 13 in H97 v3.1 and the telomere on the right side of chromosome 9 in H39 v3.1 (Sonnenberg et al., 2016). In the assembled sequence of Bisp119/9-P4 v2.1, the telomeres on the right side of chromosomes 3, 9, and 12 are missing.

The analysis of the assembled sequences of all three genomes revealed that T_2AC_5 is the consensus sequence of the telomeres in all three genomes with some variation in the number of C's. This was previously reported in the genome of H97 v3.0 and JB137-S8. The latter is a strain of *A. bisporus* var. *burnettii* (Foulongne-Oriol et al., 2013). The JB137-S8 strain has previously been sequenced using 454 pyrosequencing and Illumina HiSeq, and is available on the JGI website (http://genome.jgi-psf.org/) (Morin et al., 2012).

Discussion

Today, many researchers are able to study genomes in more detail due to the availability of DNA sequencing technologies at an affordable cost. They are also able to compare the genome architectures of different organisms. *A. bisporus* represents an organism with two completely different recombination landscapes and provides the opportunity to unravel the mechanisms involved (Sonnenberg et al., 2016). From one of the main varieties of this species, i.e., var. *bisporus*, there are two high quality genome sequences (H97 & H39) available (Morin et al., 2012; Sonnenberg et al., 2016). From another main variety of this species, i.e., var. *burnettii*, a strain named JB137-S8 has already been sequenced, but the quality is not high (>2000 scaffolds; (Morin et al., 2012). In our study, we obtained a high quality WGS in one of the parental haplotypes of the *burnettii* variety Bisp119/9 and only three telomeric sequences are missing.

As far as we know, the existence of three high quality genome sequences (H97, H39, and Bisp119/9-P4) within one species, representing the two main varieties has not been reported so far. Although the use of WGS as a research tool is rapidly on the increase, complete genomes of such a quality are still rare even for small genomes as in fungi. Most WGS of fungi lack the sequence of telomere regions. Although for many types of fungi the exact number of chromosomes is not known, the number of scaffolds generated by WGS seems to be much higher than that of chromosomes (Table 3 is an example of some WGS from the 1000 fungal genome sequencing project). Here is evidence that *de novo* sequencing and a linkage map can generate a whole genome sequence telomere to telomere.

In order to detect all COs in a segregating population, including chromosome ends, a complete whole genome sequence is required. However, covering the ends of the chromosomes with molecular markers in this variety seems to be challenging due to the presence of *Penelope* sequences in subtelomeric regions of the genome (Sonnenberg et al., 2016). Analysis of the telomeric sequences of *A. bisporus* revealed that the most frequent telomere motif is T_2AC_5 as previously reported (Foulongne-Oriol et al., 2013). Additionally, the three genome sequences (Bisp119/9-P4, H97 and H39) all have in common the fact that a telomere is missing at the right end of chromosome 9 in the region where the ribosomal gene cluster is located.

Table 3. The number of scaffolds in whole genome sequencing of 11 species of basidiomycete used in 1000 fungal genome sequencing project.

Fungal species	# No. of scaffolds
Coprinopsis cinerea	68
Armillaria solidipes	198
Trametes cingulata	279
Pleurotus eryngii	487
Lepista nuda	603
Coprinellus micaceus	703
Coprinopsis marcescibilis	710
Hydnum rufescens	817
Xerocomus badius	1092
Suillus brevipes	1132
Paxillus adelphus	1335
Pluteus cervinus	1392

This observation prompted us to ask whether the absence of telomere in that particular chromosome is related to the neighbouring ribosomal gene cluster. Telomeres are important for genome stability and are thought to have a specialized mechanism enabling them not to get lost during replication (Palm and de Lange, 2008). During DNA replication, chromosome ends always end in single-stranded overhangs that are normally seen as damage and removed by nucleases. The last telomere repeat can generate a loop and thus masks free overhangs that prevent degradation. Nevertheless, DNA polymerase can only elongate from the 3′ end of a DNA chain. Therefore, DNA synthesis on one strand (lagging strand) occurs in the wrong direction (5′ to 3′) and thus cannot go on continuously. This leads to shortening of the telomeres in each cycle of replication. Telomerase can counteract this by using an RNA template (Griffiths, 2005). It might be that this function at the right end of chromosome 9 is carried out by a mechanism that maintains the ribosomal DNA cluster, explaining why there would be no need for telomeres in this area. However, it is possible that this telomere has not been captured by our sequencing process. A more detailed genetic analysis is necessary to determine the reason for the absence of telomere in chromosome 9 of these three genomes.

High quality genome sequences also enable us to compare genome structures between or within species to explore chromosomal rearrangements as a factor influencing meiotic recombination. Here, we compared the genome sequence of Bisp119/9-P4 to those of H39 and H97, revealing a high degree of collinearity. The only large-scale inversion that appears to be real is the one on chromosome 10 of Bisp119/9-P4 relative to those of H39 and H97. Previously, the genome sequence comparison between H97 and H39 also revealed a high degree of collinearity (Sonnenberg et al., 2016). We also provided visible evidence that the inversion found by Sonnenberg et al. (2016) in chromosome 4 of H97 relative to that of H39 might be a misassembly in the H97 genome. It has long been known that inversion and other chromosomal rearrangements influence recombination behaviour as well as the fitness of an organism (Navarro et al., 1997; Rieseberg, 2001). COs are reduced within the inverted regions in the heterozygote phase of an organism and increased along collinear regions of two homologues (inter-chromosomal effect) (Schultz and Redfield, 1951). No major chromosomal rearrangements have been found in the *bisporus* variety (Sonnenberg et al., 2016) or in the current study. It is therefore unlikely that the recombination landscape of either *bisporus* variety or inter-varietal cross is due to the differences in the genomic architectures of these genomes.

In our study, genotyping the homokaryotic offspring of Bisp119/9 heterokaryon using the GBS technique revealed the first genetic linkage map of the *burnettii* variety as a tool to study meiotic recombination. GBS is an ideal technique to develop markers required for genetic map construction especially for the unknown genome. However, because the GBS library in our study concurred with the *de novo* sequencing of the *burnettii* variety, we used the *de novo* sequencing data to analyse the GBS dataset. In GBS, there is no prior need to identify markers e.g. to select SNPs for a linkage map and many molecular markers can be obtained if there is sufficient polymorphism in the genome. To our surprise, large parts of the genomes of the constituent homokaryons of Bisp119/9 (Bisp119/9-P4 & Bisp119/9-P1) were very similar in sequence resulting in a very low SNP density (Supplementary material Figure S5). Bisp119/9 was not isolated in our lab and obtained from the ARP collection (Kerrigan 1996). Most strains of the *burnettii* variety were distributed as pieces of lamellae and recovered as tissue cultures. Bisp119/9 could well originate from a cross between two homokaryons originating from spores on these lamellae and such crosses can indeed lead to homozygous regions. Another strain of the *burnettii* variety known as Bisp015 which was also obtained from the ARP collection (Kerrigan 1996), was re-sequenced on a previous occasion (data not shown). The constituent homokaryons of Bisp015 have an even more similar sequence than the Bisp119/9 with a lower SNP density and display areas without any SNPs (Supplementary material Figure S6). The SNP distribution between the constituent nuclei of the cultivar Horst U1 (H39 and H97) also display areas with a low SNP density (Sonnenberg et al. 2016). Although very similar, sufficient SNPs can be found in these areas to generate linkage maps. These areas might represent genomic parts that share an anchestor (Supplementary material Figure S7).

In our study, only four chromosomes (1, 3, 10 and 11) are completely covered with markers. The linkage map displaying these chromosomes shows a normal recombination frequency with a tendency of more recombinations towards chromosome ends e.g. in chromosome 10. The first ~385 kb of chromosome 10 is covered with three markers that have ~33 cM distance on the genetic map. Because there are not more markers on this side of the chromosome, we cannot position the area in that particular region of chromosome 10 where more recombination takes place. Nevertheless, the great cM distance shows some tendency towards more recombination possibilities in this area.

The recombination behaviour of the *burnettii* variety, which has been observed, contrasts considerably with the *bisporus* variety where a linkage map construction has been challenging for a long time. All linkage maps of this variety already constructed are quite short, and only indicate a low recombination frequency in the *bisporus* variety. The most recent linkage map of the *bisporus* variety was also very short (321 cM). Sonnenberg et al. (2016) used 139 homokaryons of Horst U1 genotyped by 629 SNP markers for constructing the linkage map of this variety. Outcrossing of the constituent nuclei of Horst U1 (H97 and H39) with the non-related *bisporus* variety homokaryons generated a similar CO pattern indicating that this is common for the variety *bisporus* (Gao et al., 2015). Only by resequencing Horst U1 offspring could all recombinations be detected and it was revealed that >90 of CO occur within the last 100 kb of each genome. Therefore, CO frequency was not low in the *bisporus* variety but mainly restricted to the chromosome ends where they are difficult to detect unless WGS is carried out

on the offspring (Sonnenberg et al. 2016). This emphasizes the need for having a complete genome sequence, i.e. telomere to telomere in each chromosome.

The direct evidence emenating from the recombination landscape of the *burnettii* variety corresponds with indirect evidence found during previous work where a normal recombination frequency was found in the offspring of a cross between var. *bisporus* × var. *burnettii* (Foulongne-Oriol et al., 2010). Recombination in the inter-varietal cross took place throughout the whole genome with a somewhat higher frequency at the chromosome ends. After considering the recombination landscape of both *bisporus* variety, i.e., it was mainly restricted to the chromosome ends and the *burnettii* variety, i.e., a more even distribution of recombination, the recombination landscape of the inter-varietal cross seems to be at the intermediate to that of both varieties. However, comparing the recombination landscape of both varieties can only be done properly if all COs have been captured.

Despite the fact that *A. bisporus* is one of the most cultivated mushrooms worldwide, the effort for breeding this species lags behind that of other organisms. Since 1980 when the first hybrid of the *bisporus* variety, Horst U1, was marketed, almost all cultivars had either identical or highly similar genomes to that variety. The typical life cycle of this variety is known to be the main reason for such low breeding efforts. The majority of the spores in this variety are self-fertile, and the low frequency of homokaryotic single spore isolates (SSIs) hampers outbreeding. Having recombination exclusively at the ends of chromosomes will lead to passing on the almost intact parental chromosomes to the next generation. This unique recombination landscape combined with the non-sister pairing of nuclei into spores is a big challenge to motivate breeders to introduce new traits into the new cultivars as this leads to a high linkage drag in breeding programmes of the button mushroom. The *burnettii* variety with its greater frequency of homokaryotic SSIs and more distributed recombination throughout the genome looks more promising for breeding. The high-quality genome sequences of both *bisporus* and *burnettii* varieties and the knowledge of the recombination behaviour of the *burnettii* variety will contribute to identifying the factors involved in the different recombination landscapes in *A. bisporus*. These findings offer tremendous possibilities to button mushroom breeders when introducing a desired trait into a new genetic background. In addition, these data also provide a resource for future genetic studies of this species.

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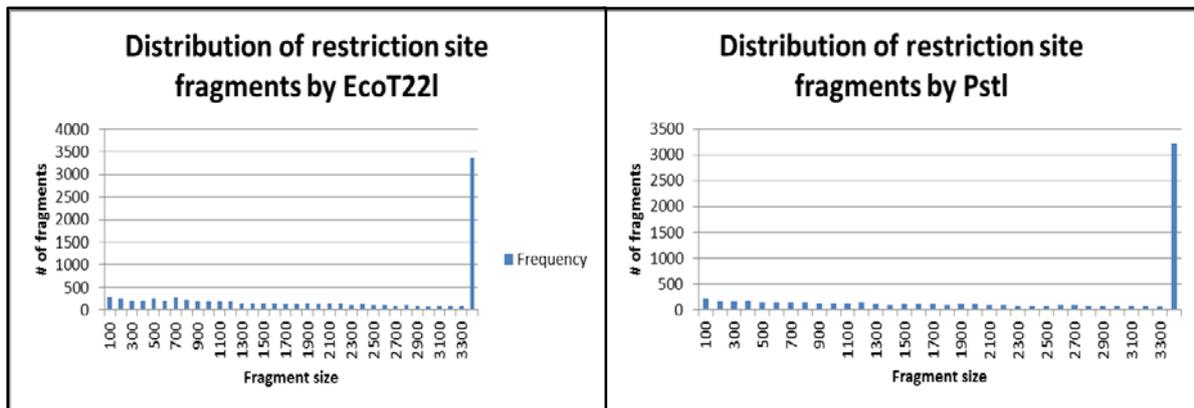
Supplementary materials

Figure S1. Frequency distribution of the fragments digested in silico by EcoT22l and Pstl enzymes using *de novo* sequence of Bisp119/9-P4.
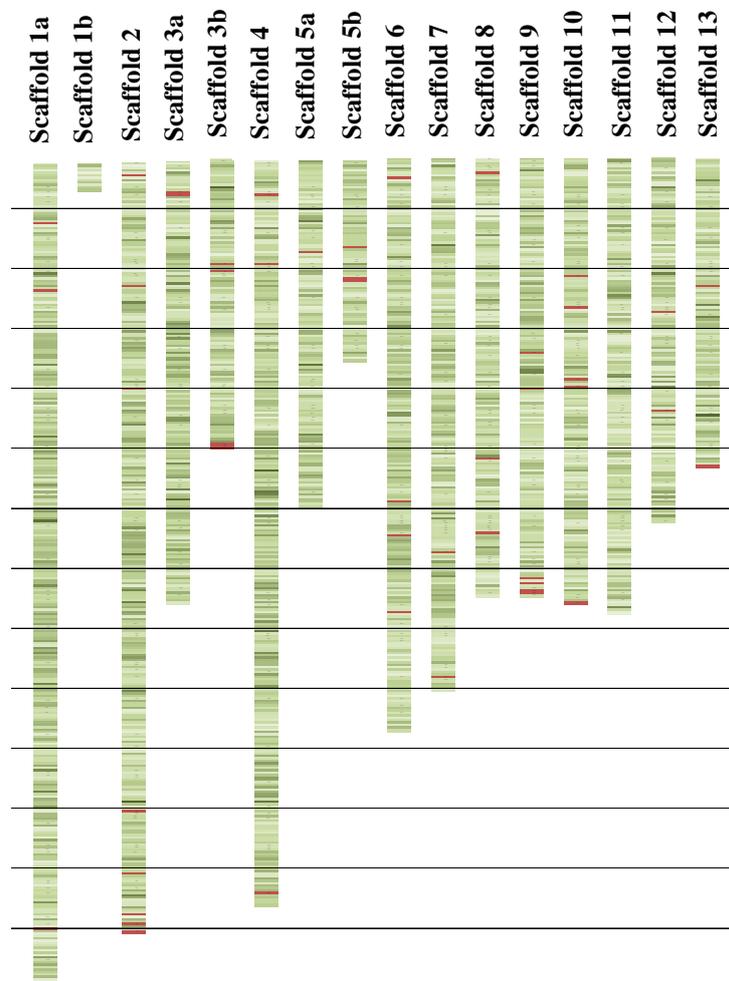

Figure S2. An overview of the distribution of the genome fragments between 300 and 700 bp in size digested by ApeKI. All graphic scaffolds in Bisp119/9-P4 are divided into regions of 10 kb and the number of restricted fragments within each region has been calculated. The shade of green indicates the number of restricted fragments within the region (darker green = more fragments). The red regions do not contain restricted fragments of the size indicated. Each horizontal black line represents an area of 250 kb.
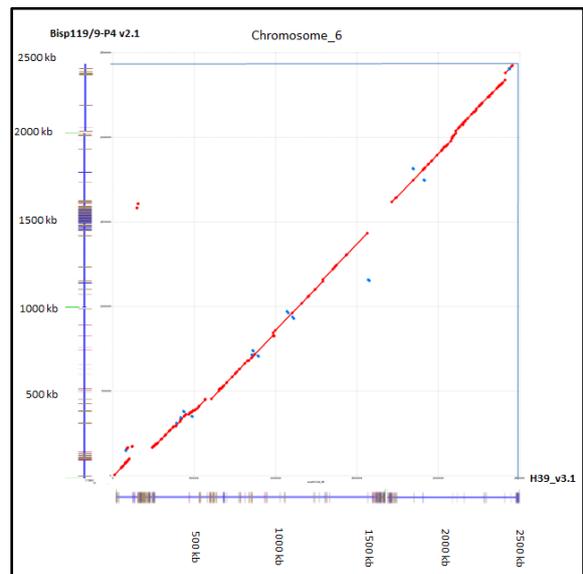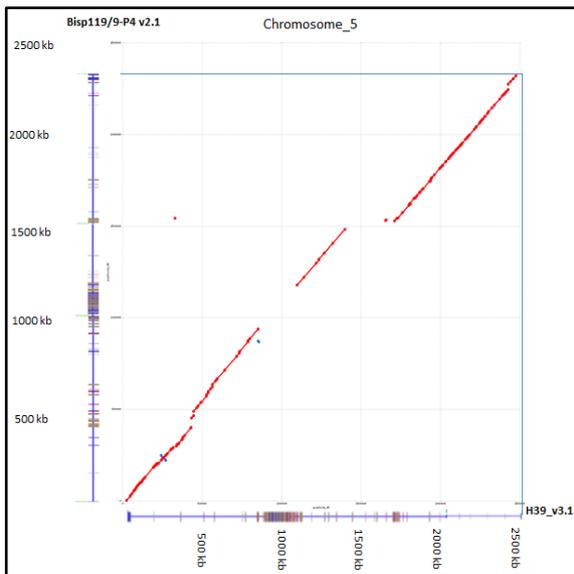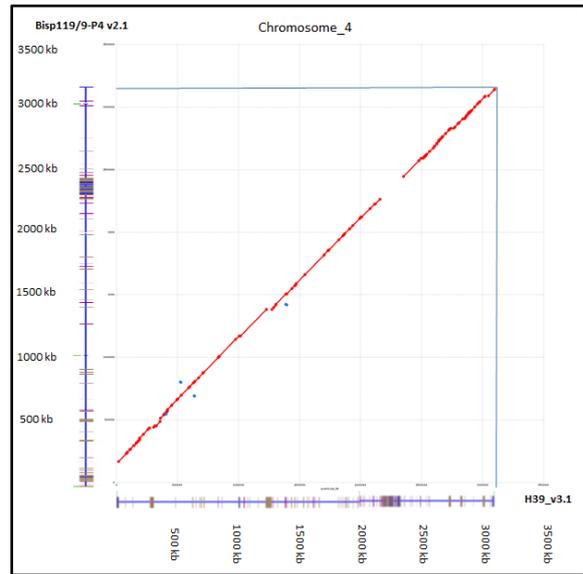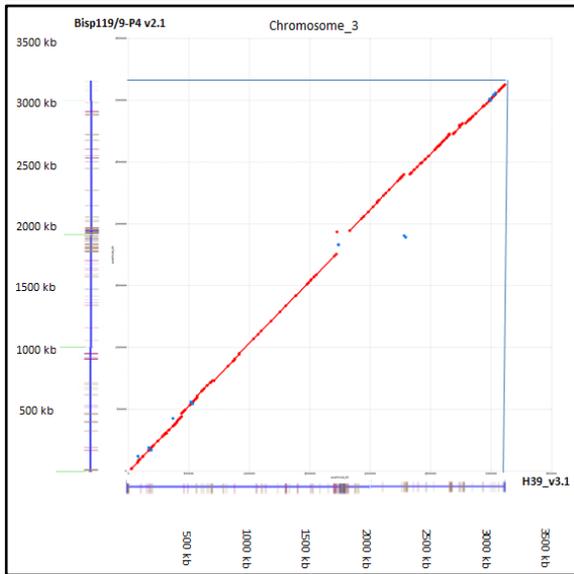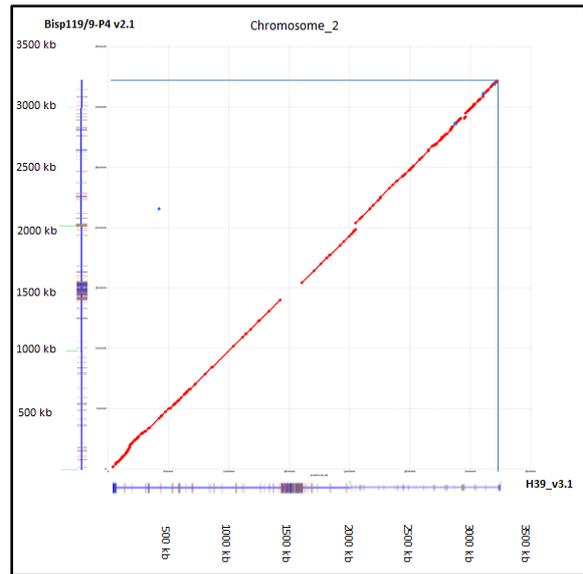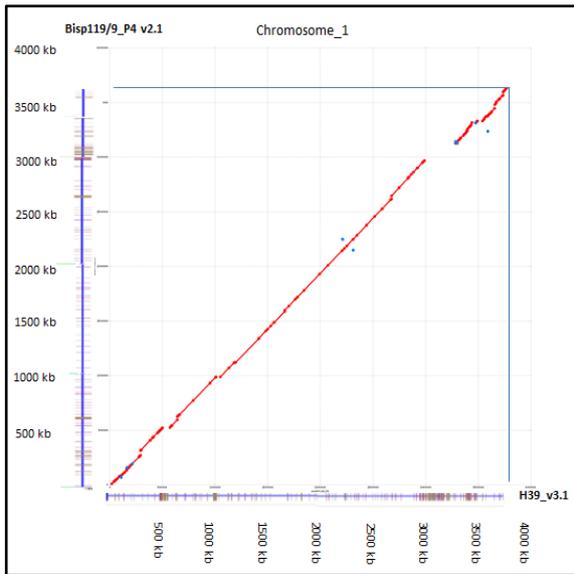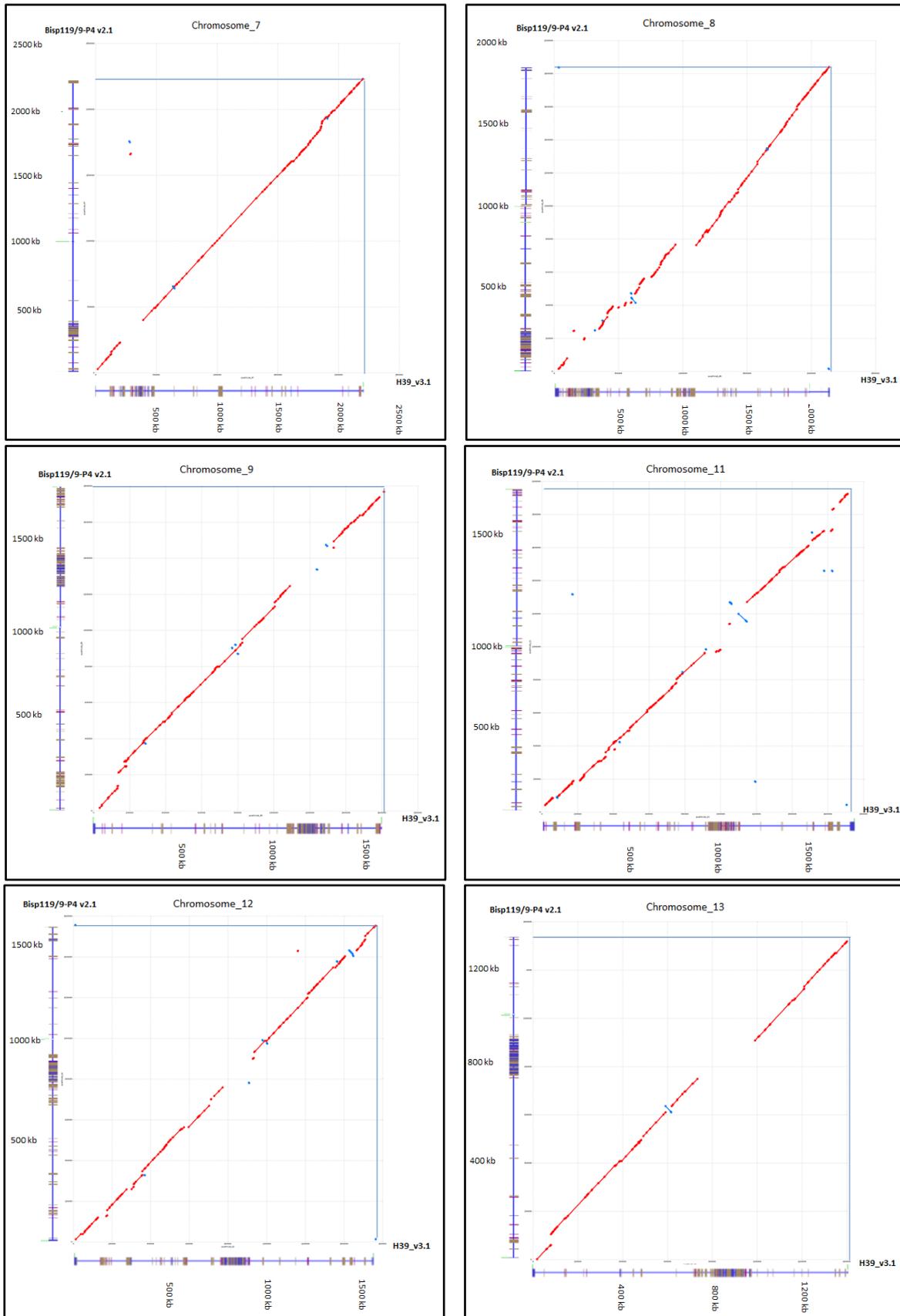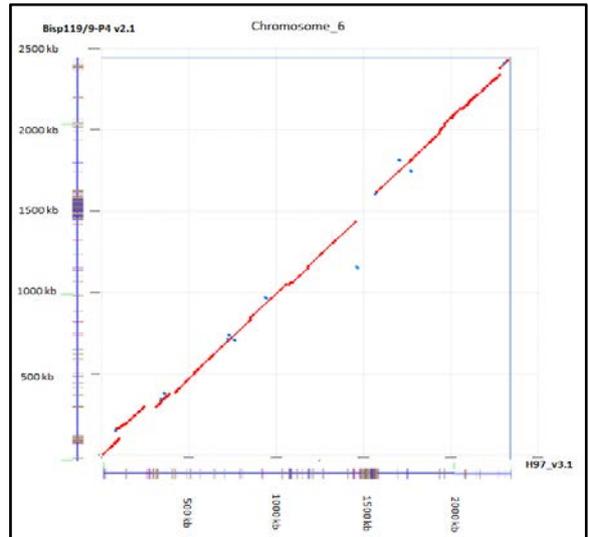
Figure S3. MUMmer plot of each individual chromosome comparison of Bisp119/9-P4 v2.1 and H39 v3.1. Along both X and Y axis the position of repetitive elements plotted along the chromosome length is seen. Repetitive elements of these two genomes differ in length and sequence content resulting in the gaps seen in the alignment.
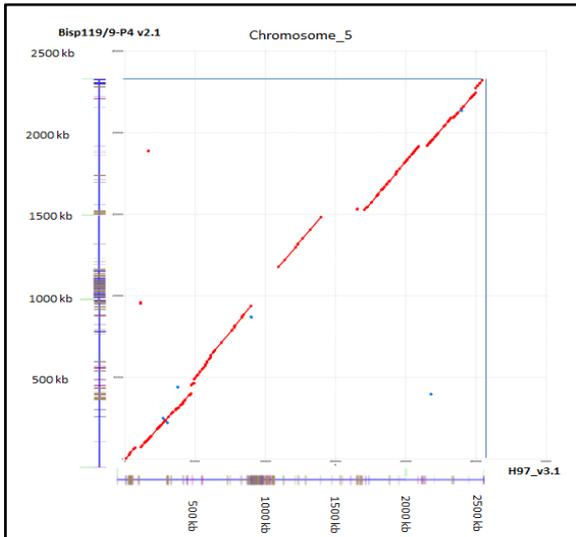
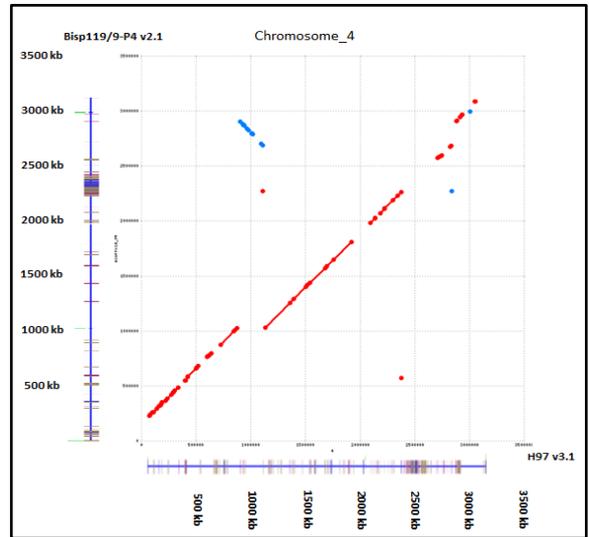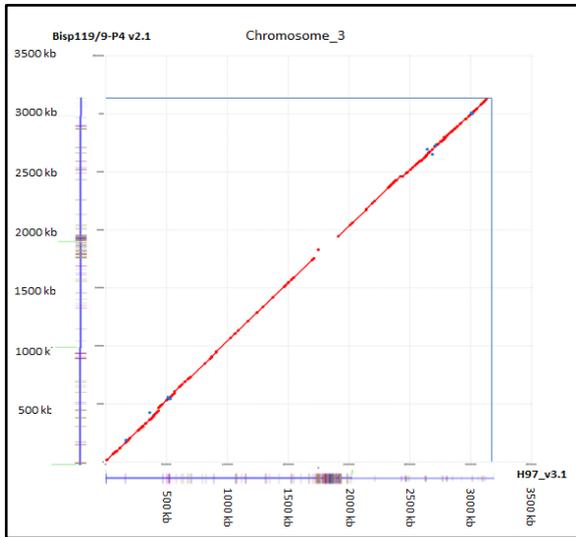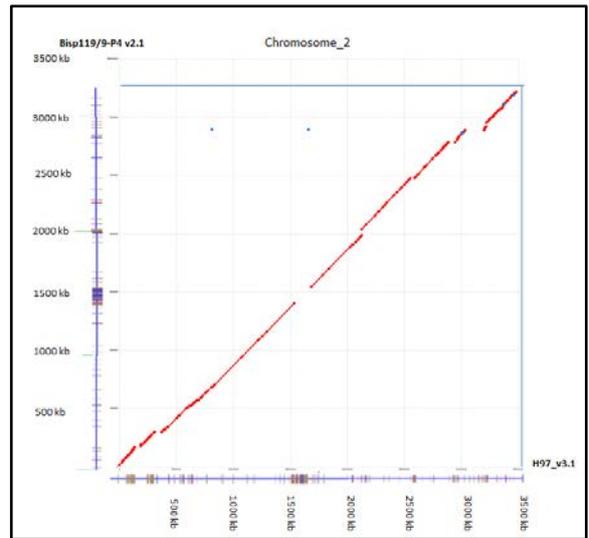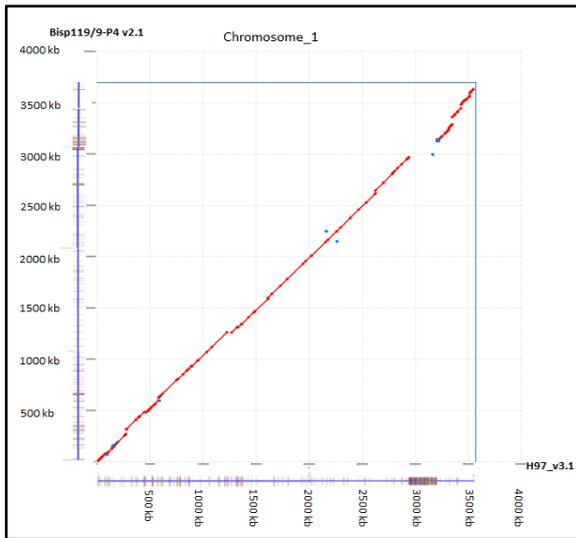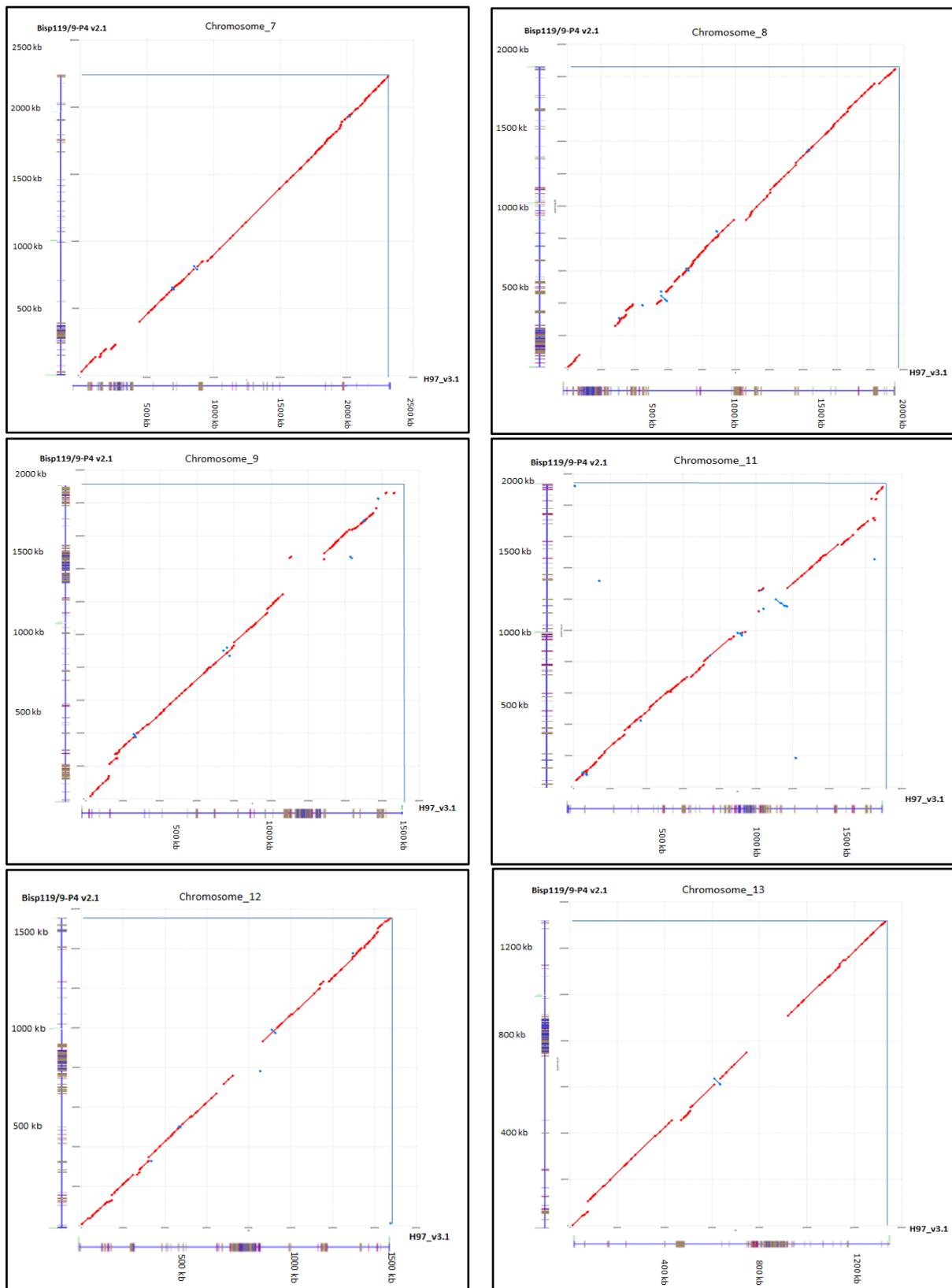**Figure S4.** MUMmer plot of each individual chromosome comparison of Bisp119/9-P4 v2.1 and H97 v3.1. Along both X and Y axes the position of repetitive elements plotted along the chromosome length can be seen. Repetitive elements of these two genomes differ in length and sequence content resulting in the gaps seen in the alignment.
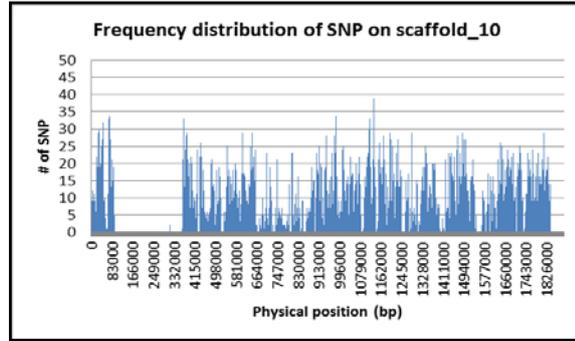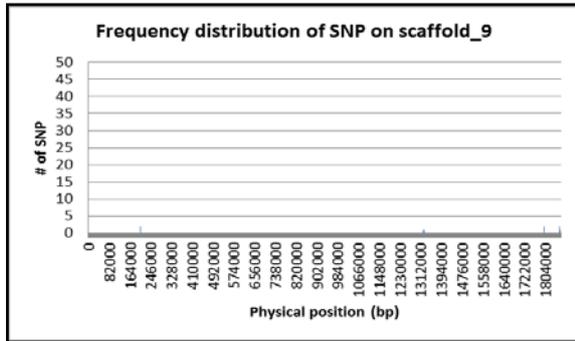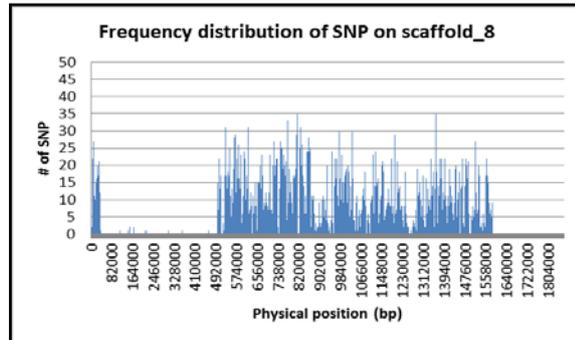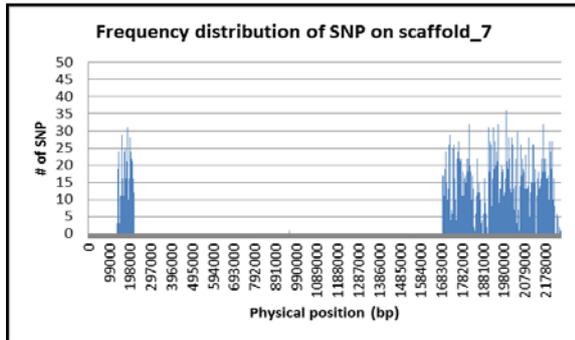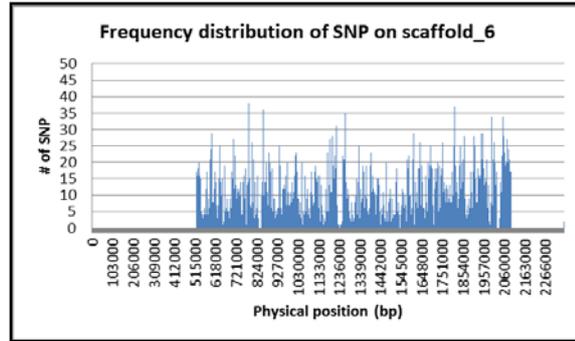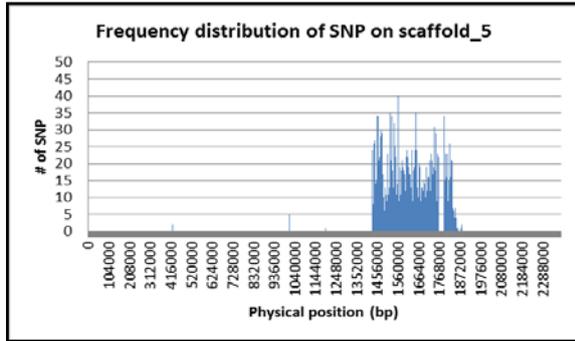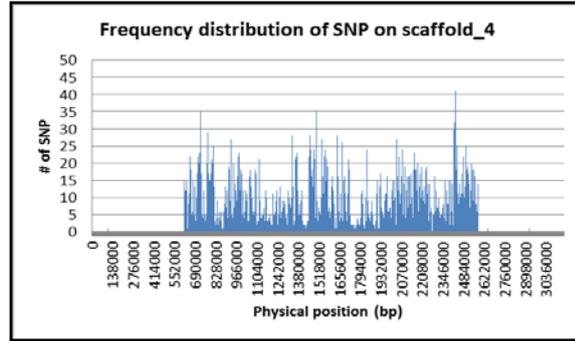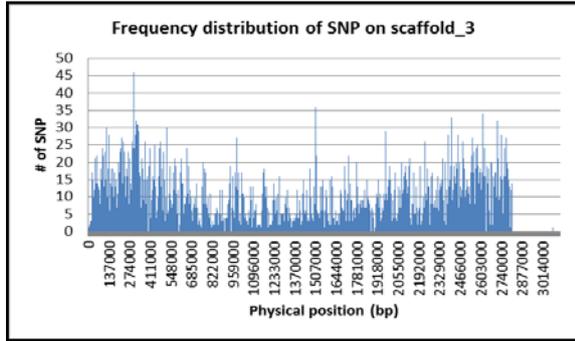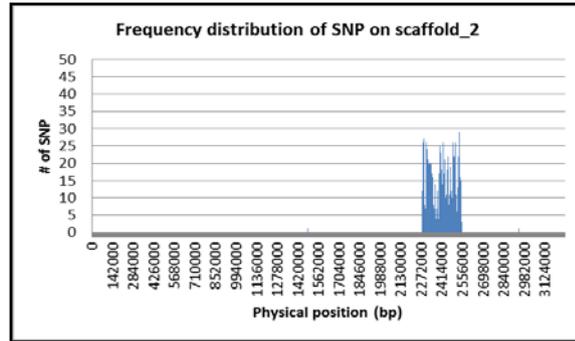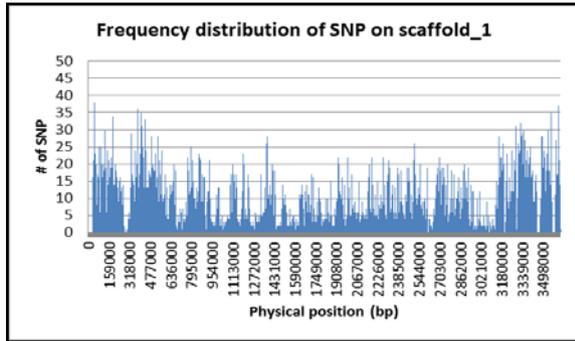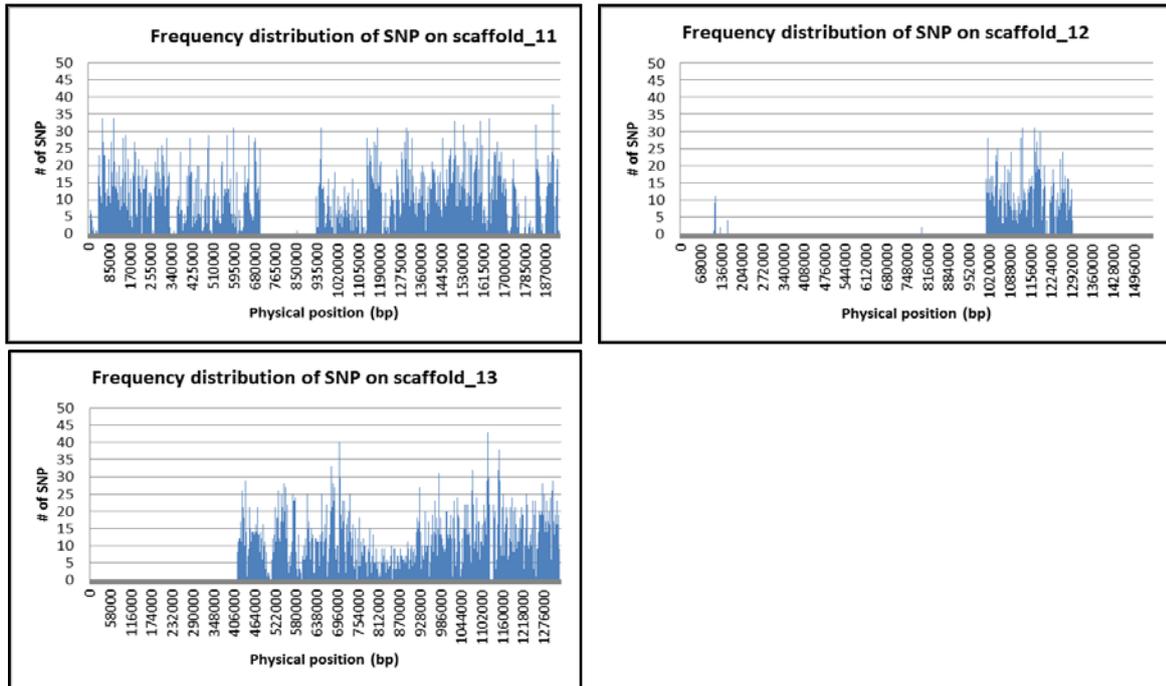
Figure S5. Distribution of SNP across the genome of the *burnettii* variety Bisp119/9 using the genome of Bisp119/9-P4 and Bisp119/9-P1. The position of SNP is expressed in bp throughout the genome in each scaffold.

Frequency distribution of SNP on scaffold_1

Frequency distribution of SNP on scaffold_2

Frequency distribution of SNP on scaffold_3

Frequency distribution of SNP on scaffold_4

Frequency distribution of SNP on scaffold_5

Frequency distribution of SNP on scaffold_6

Frequency distribution of SNP on scaffold_8

Frequency distribution of SNP on scaffold_7

Frequency distribution of SNP on scaffold_9

Frequency distribution of SNP on scaffold_10

Figure S6. Distribution of SNP across the genome of the *burnettii* variety Bisp 015. The position of SNP is expressed in bp throughout the genome in each scaffold.
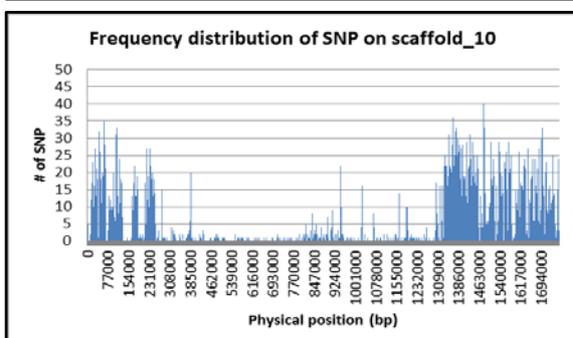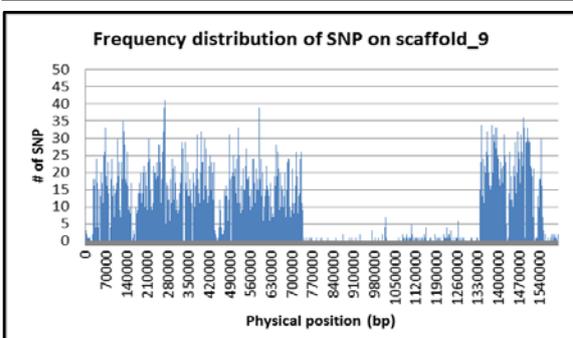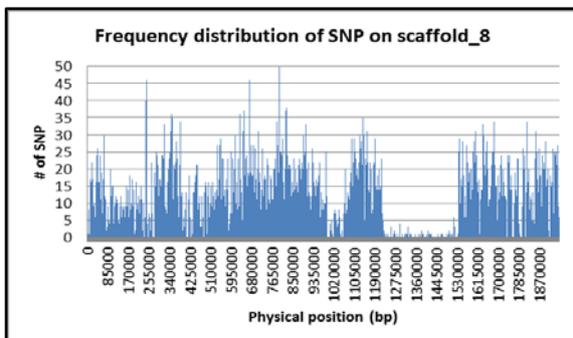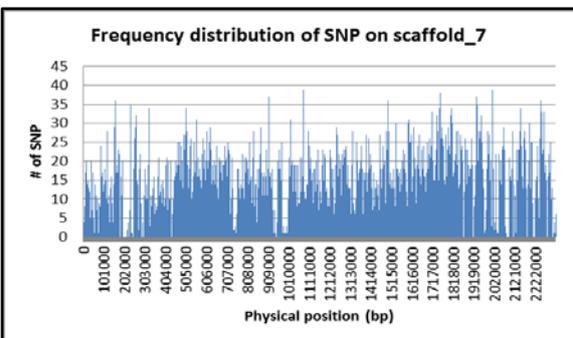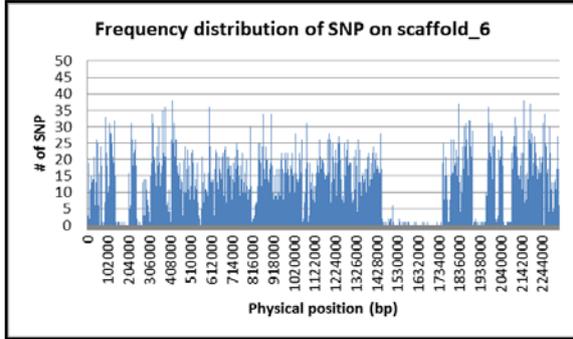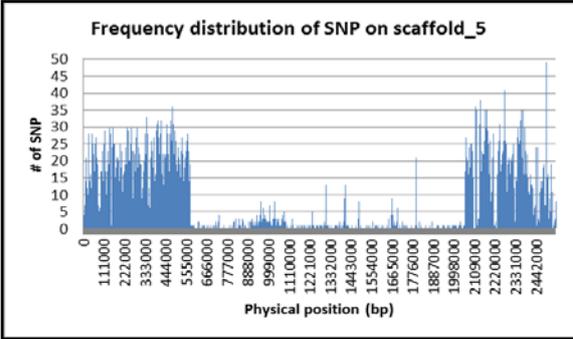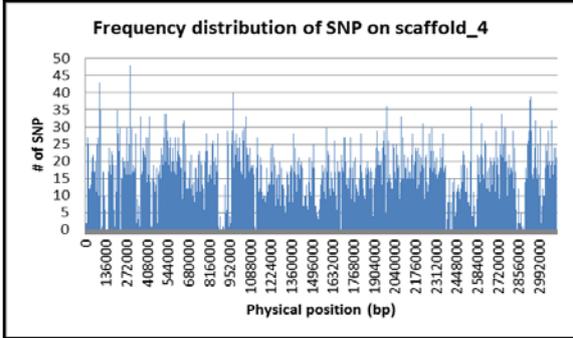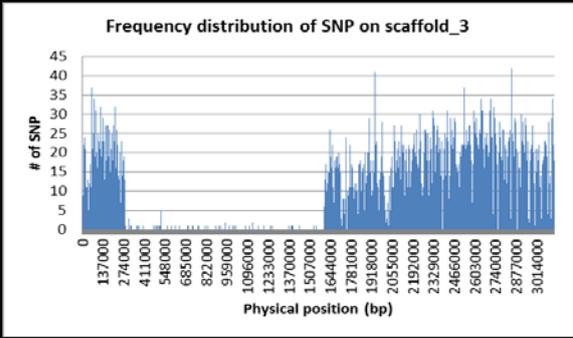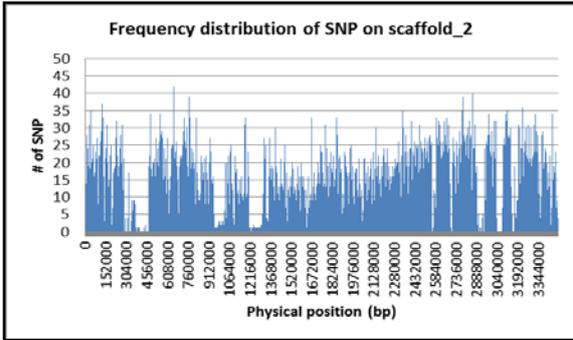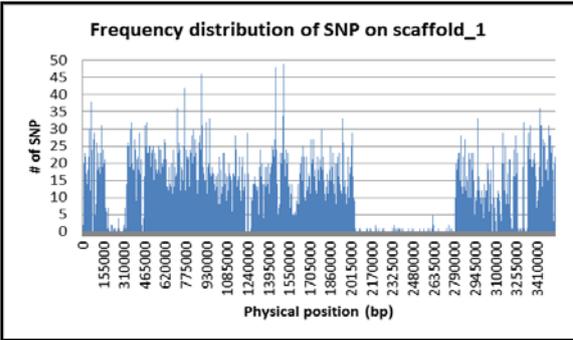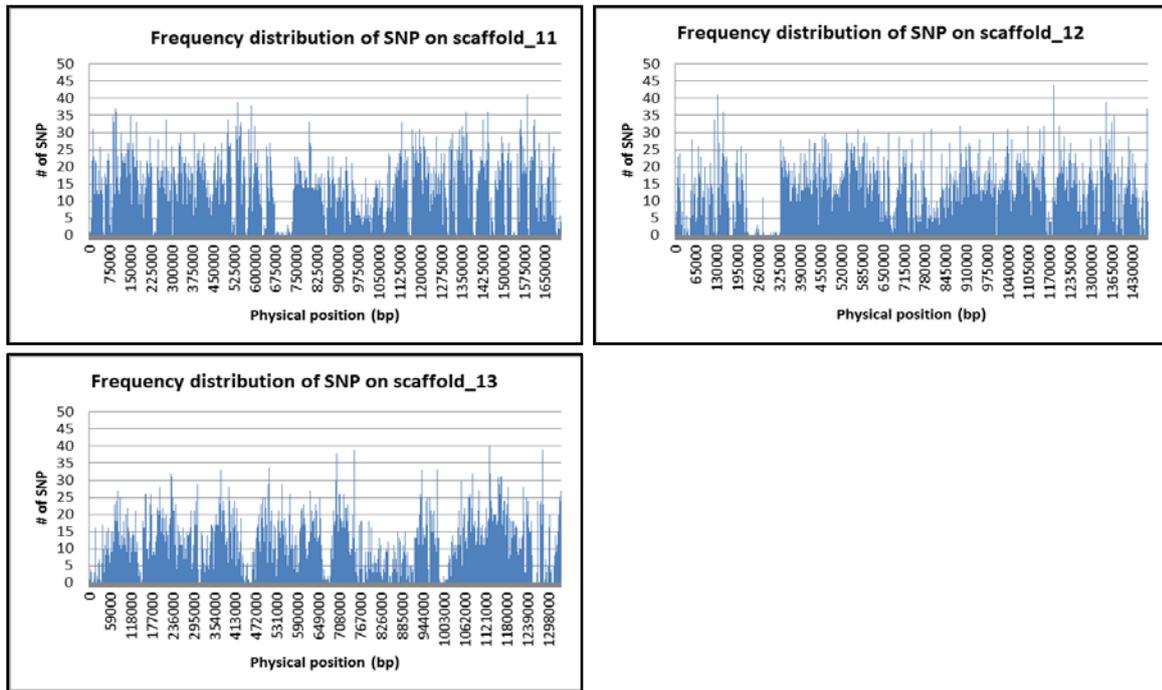
Figure S7. Distribution of SNP across the genome of the *bisporus* variety Horst U1 using the genome of H97 and H39. The position of SNP is expressed in bp throughout the genome in each scaffold.

Chapter 3

Patterns of crossover events in *Agaricus bisporus* using comparative linkage mapping

Authors:

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To be submitted

Abstract

Agaricus bisporus is represented by two varieties, i.e. the var. *bisporus* and the var. *burnettii*. The former is typified by a meiotic recombination landscape (MRL) where crossovers (CO) are restricted to chromosome ends, whereas the latter shows CO distributed over the entire chromosome. A genetic linkage map was constructed by genotyping 178 homokaryotic offspring of an inter-varietal hybrid (var. *bisporus* × var. *burnettii*) using 210 SNP markers. The map covers ~1196 cM with 13 linkage groups corresponding to the 13 chromosomes of *A. bisporus*. CO were found genome-wide in the inter-varietal hybrid. In order to have a proper comparison of the MRL of the parental varieties and the inter-varietal hybrid, markers were selected at comparable positions in all three maps. This showed a clear contrasting MRL of the parental types: CO restricted to chromosome ends for the var. *bisporus* and CO distributed over the entire chromosome for the var. *burnettii*. The inter-varietal hybrid had an intermediate phenotype, with CO distributed all over the chromosome at an increased CO frequency at the chromosome ends. The present linkage map provides sources for exploring the genetic control of different MRL of *A.bisporus*.

Introduction

The button mushroom *Agaricus bisporus* appears to be an ideal model organism for studying mechanisms that affect the position of meiotic crossovers (COs). Two varieties, i.e., *A. bisporus* var. *bisporus* and *A. bisporus* var. *burnettii* differ extremely in their recombination landscape. The former is one of the most widely produced mushrooms worldwide with a typical recombination landscape, i.e., COs are mainly restricted to chromosome ends (Sonnenberg et al., 2016), and produces mainly basidia with two spores (heterokaryons). The heterokaryons carry preferentially non-sister nuclei and are thus fertile (Summerbell et al., 1989; Callac et al., 1993; Kamzolkina et al., 2006). *A. bisporus* var. *burnettii* is a wild variety found in the Sonoran Desert in California (Callac et al., 1993) and has been shown to have a normal recombination landscape, i.e.; COs are more evenly distributed across the entire genome (This thesis, Chapter 2). It mainly produces basidia carrying four homokaryotic spores (Kerrigan et al., 1994). Homokaryons are the source for outcrossing which can be isolated easier from the *burnettii* variety (Summerbell et al., 1989; Callac et al., 1993; Kamzolkina et al., 2006).

The typical characteristics in the life cycle of the *bisporus* variety hamper breeding of this variety. Especially the restriction of CO to chromosome ends results mainly in the inheritance of parental type chromosomes which cause a considerable linkage drag, i.e., transferring the undesirable segment of the genome linked to the gene of interest. In a breeding programme, COs recombination plays a major role in introducing new traits into the recipient genome and also in breaking the linkage drag. The first hybrid of the *bisporus* variety, Horst U1, was generated in 1980 (Fritsche, 1982). Since then many hybrids coming onto the market were genetically identical or very similar to Horst U1. Generating a large number of fertile heterokaryons combined with a typical recombination landscape is known to be the main reason for generating essentially derived varieties (EDV) in the *bisporus* variety (Sonnenberg et al., 2017). This makes the breeding of the *bisporus* variety challenging, as well as laborious yet unattractive from an economic point of view.

The *bisporus* and *burnettii* varieties are interfertile and thus enable us to create segregating populations to unravel the genetic control of CO positioning. Many agronomical traits related to quality, yield or resistance to disease are complex and thus explained by QuantitativeTrait Loci (QTL). A prerequisite for identifying QTL is a genetic linkage map. Linkage mapping is an approach that determines the order of the genetic markers and their relative position to one another on the chromosome based on recombination frequency. The phenotypic data of the trait of interest will then be correlated with the linkage map to locate genes controlling the trait. Alongside linking phenotypes to genomic regions, genetic linkage maps are also used to perform comparative studies of recombination frequency within a species or between different species. In genome sequence assembly, linkage mapping can also help to anchor scaffolds and find misassemblies in the sequence. The most recent linkage map of the offspring derived from *burnettii* variety Bisp119/9 has proven to be an important component for such purpose. It also gave a better understanding of the recombination landscape of the *burnettii* variety (This thesis, Chapter 2).

Previously, a linkage map of an inter-varietal hybrid derived from homokaryons of Horst U1 and a *burnettii* variety JB3 was constructed using AFLP, CAPS and SSR markers. An entire linkage map of 1156 cM derived from the inter-varietal hybrid revealed an elevated recombination frequency compared to the *bisporus* variety (Foulongne-Oriol et al., 2010). Later, an offspring from this inter-varietal hybrid was crossed with an offspring of the *bisporus* variety, resulting in a shorter linkage map (851cM; Foulongne-Oriol et al., 2011). It was suggested that the map reduction was due to the cross with *bisporus* variety. Studies on recombination frequency of this hybrid also indicated that the recombination landscape is likely under genetic control. All constructed linkage maps of the *bisporus* variety are very short (Summerbell et al., 1989; Kerrigan et al., 1993; Gao et al., 2015) considering the estimated chromosome number (n=13) of *A. bisporus* (Sonnenberg et al., 1996). As COs are mainly restricted at the chromosome ends in the *bisporus* variety, in the offspring of a cross between the individuals of an inter-varietal hybrid and homokaryons of the *bisporus* variety, COs might shift towards the ends of the chromosome. Thus, many COs might not be detected in linkage map studies due to the difficulty in covering the ends of the chromosome with molecular markers (Sonnenberg et al., 2016).

The uniform coverage of the genome with molecular markers is needed in order to study genome-wide recombination frequency. The recent release of whole genome sequences (WGS) of three haplotypes in *A. bisporus* (Morin et al., 2012; Sonnenberg et al., 2016) provide a source to develop molecular markers e.g. Single Nucleotide Polymorphism (SNP) markers for linkage map construction (Gao et al., 2015; Gao et al., 2016). The primary aim of this study was to build a linkage map of the inter-varietal hybrid, named HBT03, that can be used as a basis to unravel the genetic control of the recombination landscape. For that, we used one of the constituent homokaryons of Horst U1 and Bisp119/9 for which WGS are available. A proper comparison of the recombination landscape of the *bisporus* variety, *burnettii* variety and inter-varietal hybrid has not been made so far. A comparison can be made by doing comparative mapping studies which are rare in fungi. The prerequisite for a comparative mapping study is to have a set of markers that have more or less the same position on all maps.

The data available in the Horst U1, Bisp119/9 and HBT03 linkage maps makes a comparison of recombination landscape possible and is the second objective of the present study. A linkage map of 139 offspring derived from Horst U1 using 629 SNP markers has recently been constructed. The total map length was very short (321 cM; Sonnenberg et al., 2016). Previously, a linkage map was constructed of Bisp119/9. Only for four chromosomes evenly distributed SNPs were available as for most of the other chromosomes the constituent nuclei of Bisp119/9 were remarkably identical. The map length of these four chromosomes was ~ 416 cM, which can be assumed from the map size of four chromosomes (This thesis, Chapter 2). From the inter-varietal hybrid HBT03, a linkage map of 178 offspring genotyped with 210 SNP has been constructed and is presented here. To make a proper comparison between the three maps, markers with similar positions for each one were selected. If necessary, additional SNP markers were designed. The comparative mapping presented here depicts a clearer picture of the recombination landscape between the two varieties and the expression in a cross between these

varieties. The usefulness of the present inter-varietal hybrid HBT03 linkage map in identifying the QTL involved in meiotic recombination landscape is explained.

Materials and Methods

Segregating population

Two heterokaryons used in this study were obtained from the fungal collection of Plant Breeding Wageningen University & Research; Horst U1 a white commercial *bisporus* strain and Bisp119/9 a wild type *burnettii* strain originating from the ARP collection (Kerrigan et al. 1996). The constituent nuclei as homokaryons from Horst U1 (H97 and H39) and Bisp119/9 (Bisp119/9-P4 and Bisp119/9-P1) were available from another study (Sonnenberg et al., 2016). One of the constituent parental nuclei of Horst U1 and Bisp119/9 were selected to make the mapping population in this study. The inter-varietal hybrid, named HBT03, was obtained by a confrontation between Bisp119/9-P4 and H97 which were subsequently cultivated at the mushroom farm of Unifarm in Wageningen in a controlled climate. The HBT03 hybrid was grown on commercial compost (CNC Substrates), spawned in a flower box filled with 2 kg of compost. The fruiting body was collected to make spore-prints. Quantifying the number of spores per basidia was conducted in three fruiting bodies of HBT03 as a replication and confirmed that HBT03 was predominantly tetrasporic (data not given). The spores of the HBT03 hybrid were plated as described previously (Sonnenberg et al., 1988). Petri dishes with two compartments were used as follows: one compartment was filled with commercial spawn (Sylvan A15) as a germination stimulus and the second with malt-mycological peptone (MMP) agar medium to plate spore suspension. Petri dishes were placed in an incubation room (25^OC). The single spore isolation (SSI) began with the germination of the first spore.

Marker selection and genotyping

The constituent nuclei of Horst U1 (H97 and H39) and one of the constituent homokaryon of Bisp119/9 (Bisp119/9-P4) were previously *de novo* sequenced (Morin et al., 2012; Sonnenberg et al., 2016). Bisp119/9-P4 was also re-sequenced using Illumina HiSeq (This thesis, Chapter 2). For the mapping strategy, the level of polymorphism between two parental lines is important (Young, 2001). To check the level of polymorphism between Bisp119/9-P4 and H97, the High-seq sequence reads of Bisp119/9-P4 were mapped against the H97 v3.1 genome as a reference using Burrows-Wheeler Alignment tool (BWA) backtrack algorithm of BWA version 0.7.7-r441 (Li and Durbin, 2009), followed by arranging the aligned reads using SAMtools version 0.1.19-44428 cd (Li et al., 2009). The variant calls were generated using Freebayes version v0.9.18-1-g4233a23 (Garrison and Marth, 2012). The VCF file output was filtered to retain only the positions with type SNP having a read depth of DP > 5.

A selection of SNP markers was made to genotype homokaryotic SSIs. Three important criteria were considered in our selection. First, the flanking regions up to 60bp of the selected SNPs were checked for the presence of additional SNPs. Second, the flanking region of SNPs was blasted against H97 v3.1 sequence to obtain unique SNPs in the genome. Third, markers were selected in such a way to ensure that the whole genome is evenly covered with SNPs. High quality DNA was extracted from freeze-dried mycelium as described previously (Sonnenberg

et al., 2016). DNA concentration was adjusted to 30 ng/µl and samples were stored at -20C. Genotyping of the individuals was performed in the Dr. van Haeringgen Laboratorium (VHL) using the KASPar SNP Genotyping System.

Linkage mapping

The homokaryotic SSIs isolated from the HBT03 hybrid were genotyped by SNP markers to construct a linkage map, which was generated using JoinMap 4.1 (Van Ooijen, 2011) based on the independent LOD score using a haploid model (HAP1) with default parameter settings (independence LOD score; significance levels from 2.0 to 10.0 LOD). Chi-square analysis (P < 0.05) was used to check if it aligned with Mendelian ratios and linkage groups (LGs) which were established at the level of LOD $\geq$ 5. Centimorgan (cM) distances were expressed by the Kosambi function (Kosambi, 1943) and the crossover frequency was calculated as the crossover number per individual per chromosome.

Comparative mapping

A comparison was made of the genetic mapping statistics of the *bisporus* variety Horst U1, *burnettii* variety Bisp119/9 and the inter-varietal hybrid HBT03. In order to make a fair comparison of the maps, markers had to be placed in more or less the same position on all three maps. For this purpose, markers were selected in such way so that the position of each marker was similar in each map (less than 50 kb apart). Additional markers were added to the existing dataset of the Bisp119/9 linkage map for the positions without markers which had been placed in the other two maps. The new genetic maps were re-constructed with selected markers using JoinMap 4.1 (Van Ooijen, 2011) and parameter settings as described above. In order to visualize the *bisporus* variety, *burnettii* variety and inter-varietal hybrid recombination landscapes in a better way, we plotted the number of mapping units of the SNP markers per linkage group against their respective physical distance (bp).

Results

Genetic linkage map

The inter-varietal hybrid HBT03 was obtained by crossing the constituent homokaryons of Horst U1 and Bisp119/9, i.e., H97 and Bisp119/9-P4, respectively. Horst U1 and Bisp119/9 were selected because of their contrasting phenotype, i.e., recombination landscape. The positive mating interaction was visually determined by the formation of fluffy mycelia in the contact zone. Previous research has shown that the germination rate of the spores varies within varieties of *A. bisporus* which can cause bias in the offspring analysed (Elliott, 1972; Callac et al., 1993; Kerrigan et al., 1993). Therefore, the homokaryotic SSIs of HBT03 were selected randomly at different stages, i.e., early, mid and late germination of spores. The first spore began germinating on the 11th day after spreading and the isolation period lasted for 39 days at a germination rate of 42%. This indicates that spores of the HBT03 hybrid germinate non-synchronously over a long time of period. However, the germination rate could possibly be underestimated due to an excessive growth rate of the earlier SSIs. As the majority of basidia were 3-spore and 4-spore (data not given), we assumed that many SSIs are putative

homekaryons and thus did not check the ploidy of the SSIs with molecular markers linked to mating types.

A comparison of the re-sequenced genome of Bisp119/9-P4 with the *de novo* sequencing of H97 v3.1 revealed 508,912 SNPs which is equal to an SNP density of 16.5 SNPs per kb. This should, in principle, be more than adequate for selecting markers that are evenly distributed across the genome. However, this was not the case for the right end of chromosome 9 which harbours the ribosomal DNA cluster (Sonnenberg et al., 2016). The right telomere of chromosome 9 was missing in both H97 and Bisp119/9-P4 *de novo* sequences. A total of 178 homokaryotic SSIs of the HBT03 hybrid were genotyped with 210 SNP markers. The total genetic map length is ~1196 cM, and consists of 13 linkage groups (LGs; Figure 1) corresponding to the estimated chromosome number (n=13) of this species (Kerrigan et al., 1993; Sonnenberg et al., 1996).

The analysis of segregation ratios revealed that 82 markers (39%) deviate from the expected Mendelian ratio (1:1 ratio, chi-square test, P < 0.05) especially markers on LGll and LGlll. There were more H97 than Bisp119/9-P4 alleles on both LGII and LGIII. The means of recombination frequency was calculated per chromosome per individual and varied from 0.30 (LGlX) to 1.04 (LGV) with an average of 0.88. The characteristics of the linkage groups derived from HBT03 population are summarized in Table 1. The average number of COs per individual was ~8 in the HBT03 population (Figure 2). The number of individuals having non-recombined chromosomes for each LG was determined. Individuals with no recombination events were found more for LGIX (Table 1).

Comparative mapping

Here we have compared the genetic linkage maps of the *bisporus* variety Horst U1 (Sonnenberg et al., 2016), *burnettii* variety Bisp119/9 (This thesis, Chapter 2) and inter-varietal hybrid HBT03 (this study). A complete comparison of the linkage maps could only be made for the *bisporus* variety and the inter-varietal hybrid due to a very high similarity between the genomes of the constituent homokaryons of Bisp119/9 (Bisp119/9-P4 & Bisp119/9-P1), resulting in a very low SNP density.

A total of 154 SNPs markers altogether were selected from each of the linkage maps of Horst U1 and HBT03 at more or less the same positions, i.e. less than 50 kb apart. The entire map length of the *bisporus* variety is ~303 cM and consists of 13 linkage groups (Table 2). Analysing the segregation ratios reveals that 57 markers (37%) deviate from the anticipated Mendelian ratio (1:1 ratio, chi-square test, P < 0.05). To visualize the recombination along chromosomes in a better way, the map units (cM) positions of each marker per chromosome were plotted against its respective physical position (bp) (Supplementary materials Figure S1). These plots reveal that crossover events are clustered at the ends of the chromosomes as already manifested by Sonnenberg et al. (2016).
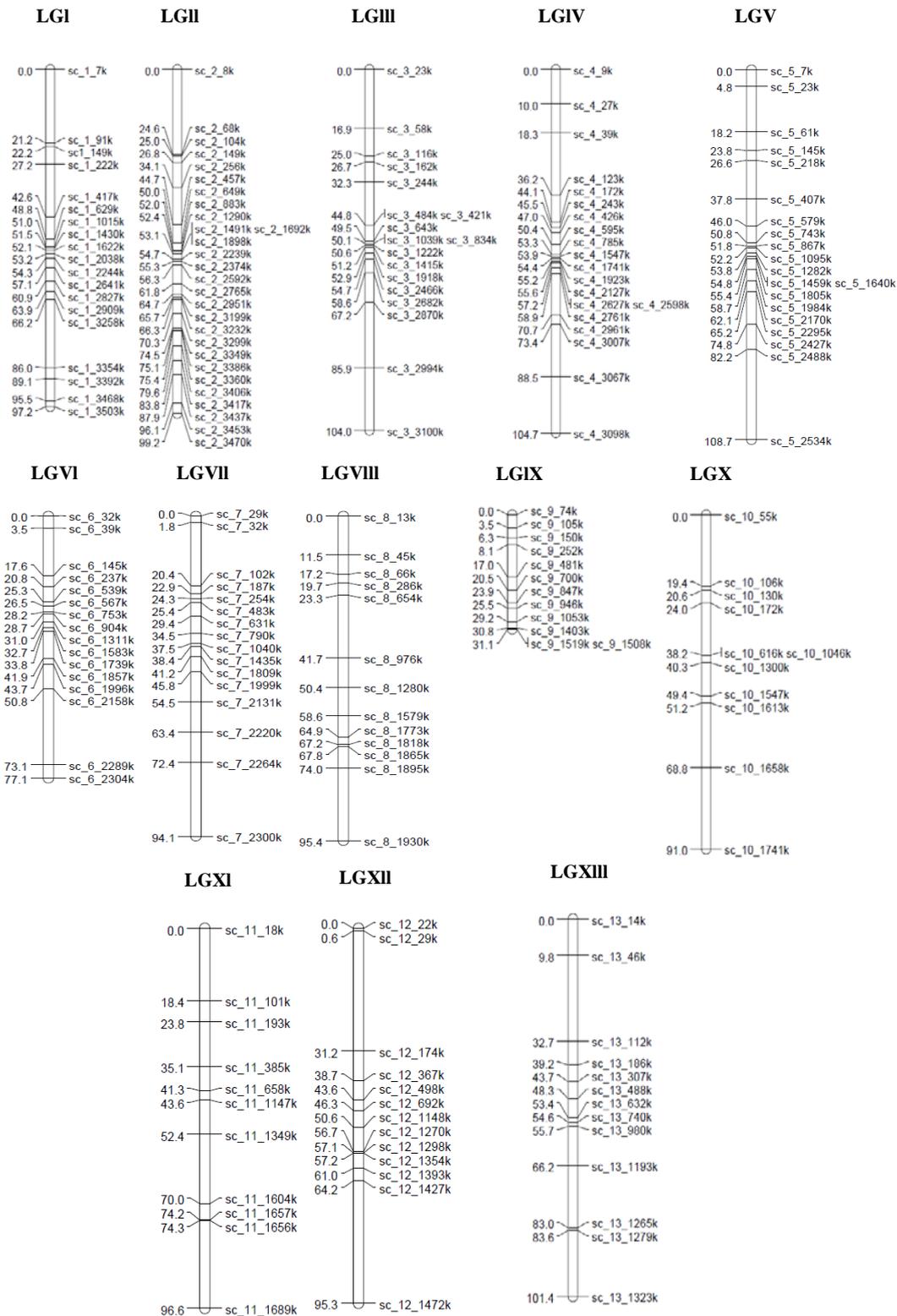
Figure 1. **Genetic linkage map of inter-varietal hybrid HBT03 based on 210 SNP markers.** The positions of the markers per chromosome are indicated on the right side of each linkage group (e.g. sc_1) followed by the position on that particular chromosome with their positions in Centimorgan to the left. Thirteen linkage groups (LG1-LG13) are revealed corresponding to the estimated number of chromosomes in *A. bisporus*.

Table 1. Map statistics of the genetic linkage map of the inter-varietal hybrid HBT03

LG	Chromosome length			Marker range[a] (kb to Mb)	No. markers	No. Distorted markers	No. of crossovers/ chromosome					CO frequency per Chr.	Parental types (%)
	H97vs3.1 (bp)	Bisp119/9-P4 vs2.1 (bp)	Length (cM)				0 H97[b]	0 Bisp119/9-P4[c]	1	2	3		
I	3550205	3639502	97.2	7 to 3.50	19	2	25	29	85	35	4	0.94	30%
II	3489786	3265402	99.2	8 to 3.47	28	26	34*	18	84	39	3	0.96	29%
III	3131856	3132325	104.0	23 to 3.10	18	16	44*	0	91	42	1	1.00	25%
IV	3112789	3158235	104.7	9 to 3.09	20	2	20*	34	79	39	6	0.98	30%
V	2550681	2380213	108.7	7 to 2.53	20	9	28*	18	81	48	3	1.04	26%
VI	2329815	2426497	77.1	32 to 2.30	16	5	30	40	88	19	1	0.72	39%
VII	2323169	2254680	94.1	29 to 2.30	16	10	36*	18	90	32	2	0.90	30%
VIII	1953186	1867134	95.4	13 to 1.93	13	2	39*	19	80	39	1	0.90	33%
IX	1688379	1867033	31.1	74 to 1.52	12	0	69	56	52	1	0	0.30	70%
X	1762792	1897446	91.0	55 to 1.74	11	3	40*	20	84	33	1	0.86	34%
XI	1708529	1931181	96.6	18 to 1.69	11	0	28	25	87	38	0	0.92	30%
XII	1482581	1559381	95.3	22 to 1.47	13	0	23	25	97	33	0	0.92	27%
XIII	1334073	1323473	101.4	14 to 1.32	13	7	27	24	83	44	0	0.96	29%
Totals			1195.8		210	82	443	326	1081	442	22		33%

[a] Marker range relative to the H97 vs3.1 genome.
[b] Number of individuals with intact linkage group of H97
[c] Number of individuals with intact linkage group of Bisp119/9-P4
* Significant deviation of the expected ratio 1:1 ($p < 0.05$)
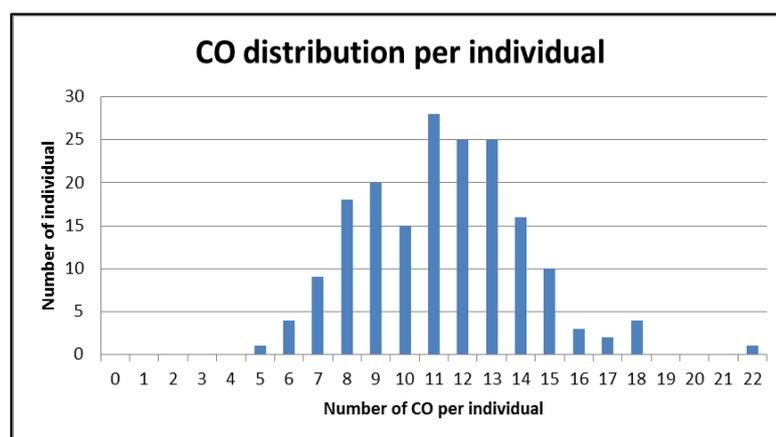
Figure 2. **Distribution of crossover recombination per individual in 178 homokaryons of HBT03 population.**

The total map length of the inter-varietal hybrid is ~ 1004 cM and consists of 13 LGs (Table 2). Here, the total map length is shorter than the actual map of this hybrid (Table 1) and this was most likely caused by reducing the number of markers used to make both maps comparable. Five markers which co-segregated were discarded from further analysis. Analysing the segregation ratios reveals that 55 markers (36%) deviate from the expected Mendelian ratio (1:1 ratio, chi-square test, $P < 0.05$). Plotting map unit (cM) positions for each marker per chromosome against its respective physical position (bp) reveals that CO events occur somewhat evenly across the genome, yet have a tendency happen at the ends of the chromosomes (Supplementary materials Figure S2).

In the recent linkage map of Bisp119/9, only a few LGs (I, III, X and XI) were evenly covered by polymorphic markers (This thesis, Chapter 2). Of these four LGs only the position of the

markers on LGI and LGXI enabled us to compare the respective LGs on the two other maps. In addition, LGIII and LGX were compared with the respective LGs of the two other maps even though the number of the markers and their positions were not same. A total of 105 homokaryons genotyped with 47 markers were used to remap LGI, LGIII, LGX and LGXI of Bisp119/9. Three markers were co-segregated. The map length of the four LGs mentioned above is ~ 419 cM which can be expected from mapping the size of four chromosomes (Table 2). Plotting map unit (cM) positions of each marker on these four LGs against its respective physical position (bp) show a clear contrasting recombination pattern to those of the *bisporus* variety (restricting the recombination at chromosome ends) and inter-varietal hybrid (more or less intermediate to that of the *bisporus* and *burnettii* varieties; see Figure 3).

Table 2. Map statistics of the genetic linkage map of three varieties, i.e., var. *bisporus*, var. *burnettii* and intervarietal hybrid

LG	Genetic length (cM)			Post meiotic Chromosome (%)												CO frequency per Chromosome	
	Intervarietal hybrid	*bisporus* variety	*burnettii* variety	Parental type			1 CO			2 CO			3 CO			HBT03	Horst U1
	HBT03	Horst U1	Bisp119/9	HBT03	U1	Bisp119/9	HBT03	U1	Bisp119/9	HBT03	U1	Bisp119/9	HBT03	U1	Bisp119/9		
I	95.8	22.5	132.2	31	85	19	48	15	41	19	0	34	2	0	6	0.92	0.15
II	50.4	2.8	-	56	96	-	38	2	-	7	1.4	-	0	0	-	0.51	0.05
III	104.4	20.9	115	25	82	19	51	16.5	53	24	1.4	26	0.5	0	1.9	1.00	0.19
IV	52.3	6.8	-	57	94	-	38	6.5	-	6	0	-	0	0	-	0.49	0.06
V	91.0	16.7	-	34	85	-	46	14	-	18	1.4	-	1.7	0	-	0.87	0.17
VI	71.3	13.7	-	42	74	-	48	9	-	9	14	-	0.5	2.1	-	0.68	0.45
VII	92.7	36.8	-	31	71	-	51	27	-	16	2	-	1.7	0.72	-	0.89	0.33
VIII	67.5	33.9	-	44	76	-	48	24.5	-	8	0	-	0	0	-	0.65	0.24
IX	31.3	5.4	-	70	94	-	29	6.5	-	0.5	0	-	0	0	-	0.30	0.06
X	91.7	58.8	85.5	33	57	35	48	37	49	19	6.5	15	0.5	0	0.95	0.87	0.50
XI	89.0	14.3	86.4	36	87	37	54	12	47	10	0.7	15	0	0	0.95	0.74	0.14
XII	64.8	26.2	-	41	81	-	51	19	-	8	0	-	0	0	-	0.67	0.19
XIII	102.6	44.7	-	29	63	-	47	35	-	24	1.4	-	0	0	-	0.96	0.38
Totals	1004.6	303.5	419.1	41	80		46	17		13	2.2		0.5	0.22		0.73	0.22

However, comparing the recombination landscapes of these varieties can only be done properly if all COs are captured. In this way, positions and frequencies of recombination events can be characterized in the entire genome. As COs mainly take place in the first/last 100 kb of the chromosomes in the *bisporus* variety, a meaningful comparison between these three varieties is only possible if the ends of the chromosome are well covered with markers. The chromosome ends of *A. bisporus* contain non-LTR Penelope sequence and it is not always possible, therefore, to cover the ends with molecular markers. Consequently, many COs may be overlooked in all three maps especially the *bisporus* variety. For example, both ends of LGII, one end of LGIV, LGIX, LGXI, and LGXII are not completely covered with SNP markers (Table 3), resulting in shorter linkage maps for these LGs.
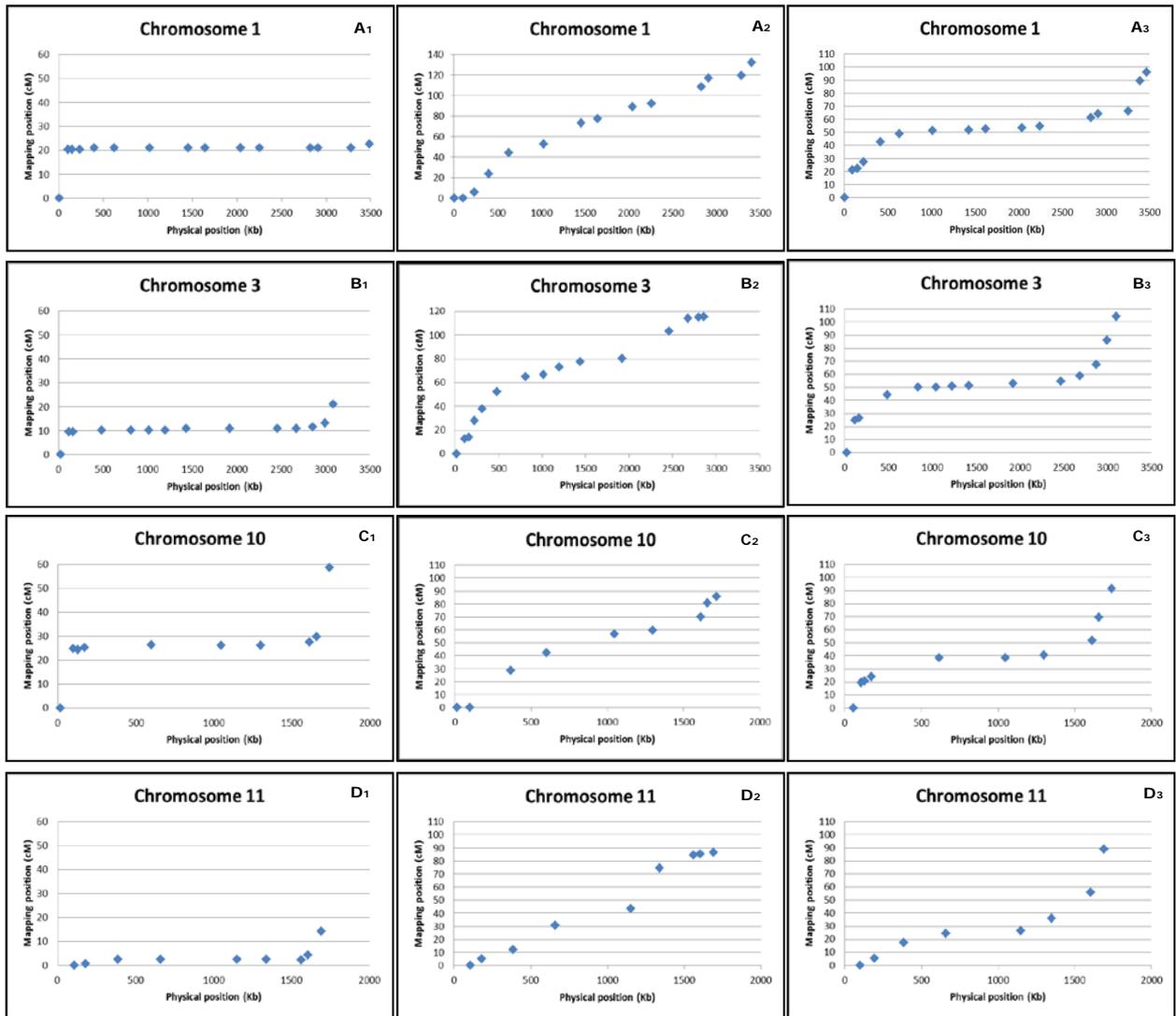
Figure 3. Comparison of the *bisporus* variety recombination landscape, *burnettii* variety and intervarietal hybrid of *A. bisporus* using the linkage map strategy. A-D; Genetic distance of scatter plots (cM) of markers against their respective physical position. A_1-D_1; Plots of the *bisporic* variety. A_2-D_2; Plots of the *burnettii* variety. A_3-D_3; The plot of the intervarietal hybrid.

Table 3. Characteristics of the markers used in comparative mapping of *bisporus* variety Horst u1, *burnettii* variety Bisp119/9 and inter-varietal HBT03 hybrid

LG	No. of markers			Distance to the left end			Distance to the right end			No. distorted markers		
	HBT03	Horst U1	Bisp119/9	HBT03	Horst U1	Bisp119/9	HBT03	Horst U1	Bisp119/9	HBT03	Horst U1	Bisp119/9
I	17	17	14	7	6	6	82	67	103	2	0	10
II	14	14	-	68	104	-	104	101	-	14	0	-
III	14	14	15	23	23	23	32	42	271	12	13	7
IV	15	15	-	123	123	-	46	57	-	1	16	-
V	12	12	-	61	59	-	17	41	-	4	12	-
VI	10	12	-	32	10	-	41	38	-	3	0	-
VII	14	14	-	32	50	-	23	23	-	9	12	-
VIII	9	9	-	13	13	-	88	88	-	1	0	-
IX	9	9	-	74	60	-	180	189	-	0	0	-
X	10	10	9	55	17	79	22	20	57	3	1	1
XI	7	9	9	101	108	108	20	20	17	0	0	0
XII	7	7	-	174	180	-	11	11	-	0	1	-
XIII	11	12	-	14	14	-	11	11	-	6	2	-
Totals	149	154	47							55	57	18

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Discussion

The current linkage map of an inter-varietal hybrid of *A. bisporus* is composed of 210 SNP markers on 13 linkage groups covering a total genetic length of 1196 cM. The homokaryons that constitute the inter-varietal hybrid have been derived from two main varieties of *A. bisporus* i.e., *bisporus* and *burnetii* variety and differ in meiotic recombination landscape. The current segregating population of the inter-varietal hybrid has been a basis for studying the genetic control of the recombination landscape as well as making comparative mapping studies. The level of polymorphism between two parents is essential for a QTL study in addition to the contrasting difference in the trait of interest. In our study, the coverage of the genome with SNP markers was adequate enough for building a relatively saturated linkage map.

A linkage map of an inter-varietal hybrid was previously constructed using other molecular markers (SSR, CAPS and AFLP; Foulongne-Oriol et al., 2010). The availability of WGS of *A. bisporus* made it possible to use SNP markers for genotyping. The use of SNPs in genotyping appears to be less problematic than other techniques. For instance, scoring of alleles is done far simpler in SNP genotyping because SNP is a bi-allelic marker which leads to a YES/NO allele call. However, if one of the two alleles is not detected in SNP genotyping, the heterozygote individual might be considered to be a homozygote or vice versa. In such a case, it is better to avoid any assumption for known alleles in individuals in order to minimise this error. SNPs are more often likely to be found in areas that might be responsible for variation in QTL analysis (Vignal et al., 2002). The total map length; the number of linkage groups equating to the estimated number of chromosomes and almost uniform genome coverage indicate that the genetic map of this study is relatively saturated. Here, the best possible selection of SNP markers is made especially for those at chromosome ends. Covering the ends of the chromosome with SNP markers is challenging due to repetitive elements clustered at chromosome ends (Sonnenberg et al., 2016).

Distorted segregation of loci is commonly reported in linkage map analysis which correlates with the deviation from the anticipated Mendelian ratio. The level of distorted loci observed in the current study (39%) is much higher than that observed in a previous inter-varietal hybrid linkage map (8.95%) (Foulongne-Oriol et al., 2010). A quite similar level of distorted loci was found in the recent linkage map of the *bisporus* variety (32%; Sonnenberg et al., 2016). Previous study on the linkage map of inter-varietal hybrid suggested that mechanisms underlying distorted segregation are different in the inter-varietal hybrid and *bisporus* variety (Foulongne-Oriol et al., 2010; Foulongne-Oriol et al., 2011). Although the two populations of *bisporus* variety and inter-varietal hybrid in this study share one parent (H97), there is hardly any consistency in distortion. For example, LG III is strongly biased in the linkage map of both populations, but towards different parental genotypes.

Distorted segregation can be caused by a number of factors. The biased selection of spores during sampling was considered to have contributed to the phenomenon of distorted segregation. Although the spores used for mapping in our study were randomly selected at different points in time as well as growth rate, the germination rate of (40%) was in the range (3%-44%.) as previously reported for inter-varietal hybrids (Callac et al., 1993), more than half

the spores could not be isolated. Moreover, of those which were isolated, only 178 homokaryons were used for mapping. Therefore, the sampling process in our study might cause partial distorted segregation. The biased selection on mating types can also induce skewed segregation in loci linked to mating types. The epistatic effects between certain combinations of alleles might influence spores' viability and thus distorted segregation is induced differently in various genetic backgrounds.

The loci revealing segregation distortion can affect QTL power and QTL additive effects (Xie et al., 2013). The areas that are strongly biased in the genome can have a QTL linked to the trait of interest. However, distorted segregation does not always influence a QTL especially for the traits that are not dominant. There are models by which segregation distortion loci (SDL) can be mapped and used jointly with QTL mapping. Combining SDL mapping and QTL mapping has been proposed to improve the statistical power of QTL detection in some circumstances which are contrary to what is commonly believed (Xu, 2008). In a breeding programme, distorted segregation leads to an under/over allele representation and has an effect on the efficiency of the breeding strategy. For example, for LGlll the allele from H97 is the preferred allele in the current study. Only the markers at the ends of LGlll are not associated with segregation distortion. Selection against undesirable H97 alleles in that particular chromosome would be laborious. Which are the genetic factors that control segregation distortion in the genome? If a gene that causes segregation distortion is segregating in a population, other genes that are closely linked to it tend to be distorted as well (Zamir and Tadmor, 1986). As markers on different LGs show segregation distortion in the genetic maps of *A. bisporus*, the genetic control of such a phenomenon would seem complex. In the genetic map of *A. bisporus*, the whole chromosome on which these distorted segregating markers are located is often distorted. Further studies need to be carried out to clarify such a mechanism.

The average number of recombinations per chromosome (0.88) found in this study is quite similar to that of inter-varietal hybrid (0.86) observed by Foulongne-Oriol, et al. (2010). The lowest recombination frequency was observed in linkage group IX which is possibly due to the fact that the far right end of chromosome IX was not well covered with SNP markers. The last SNP marker was placed far from the right end at ~170kb. The telomere on the right side of chromosome IX is missing in both WGS of H97 and Bisp119/9-P4. Therefore, the actual end of this chromosome has not been identified yet. The right side of chromosome IX also harbours a cluster of ribosomal DNA and thus SNP markers were not easily detectable in this area (Sonnenberg et al., 2016).

Recently, a WGS comparison between H97 and Bisp119/9 genomes revealed the high degree of collinearity. The only rearrangement that appeared to be realistic was an inversion on chromosome 10 of Bisp119/9-P4 relative to that of H97 (this thesis, Chapter 2). A ~800 kb inverted region (starting from ~400 kb to ~1200 kb) of chromosome 10 is covered with three SNP markers in the current study only covering a distance of ~2cM. This manifests a lower rate of recombination in this area in relation to the rest of chromosome 10. In the inverted region, recombination is reduced during the heterozygote phase of an organism and increased along collinear regions of two homologues (inter-chromosomal effect) (Schultz and Redfield, 1951). The genotyping of more individual genomes having more markers in this region would help us

to understand whether the number of COs has been reduced or not. Recombination events are found genome-wide in an inter-varietal hybrid. The map expansion at the end of the chromosomes shows a tendency of more recombination in distal regions. Recombination events were almost equally distributed across the genome of the *burnettii* variety Bisp119/9 (this thesis, Chapter 2) in contrast to *bisporus* variety Horst U1 where CO recombination was mainly restricted to the end of the chromosomes (Sonnenberg et al., 2016). Therefore, the recombination landscape of the offspring derived from inter-varietal hybrid is intermediate in that of both varieties. Considering the current linkage map and the level of collinearity, it is unlikely that the recombination landscape of the inter-varietal hybrid is due to the differences in the genomic architectures of H97 and Bisp119/9-P4. However, a clear comparison can only be made if markers are placed at the same position in linkage maps of these varieties and all COs are captured.

Genetic maps were re-constructed using the raw dataset of the linkage maps of Horst U1, Bisp119/9 and HBT03 hybrid. Comparative mapping was accomplished by selecting the markers placed at more or less the same position in all three datasets. The type and the size of the population are of two differing parameters, which affect the accuracy of the genetic mapping. The larger population confidently improved the accuracy of mapping by obtaining the loci in an accurate order. Inverting the marker order and establishing more linkage groups was observed in populations having a smaller size (Ferreira et al., 2006). Populations were not equal in size in our study. To reconstruct the genetic maps, Bisp119/9 had fewer individuals in the population compared to Horst U1 and HBT03. The segregating offspring of HBT03 and Bisp119/9 share a similar parental type (Bisp119/9-P4). Therefore, the population size might affect the evaluation of recombination frequency and marker order. This was not the case in our study because the order of markers was well maintained in these maps. However, only two chromosomes of Bisp119/9 were reconstructed in our study for comparative mapping due to the low level of polymorphism in the genome of Bisp119/9. Chromosomal rearrangements on LG1 were previously hypothesized due to the difficulties in putting the markers in order (Kerrigan et al., 1993; Xu et al., 1993). This hypothesis is ruled out by the current study as well as the WGS comparison of *A. bisporus*.

Infrequent recombination has long been reported in the *bisporus* variety. When comparing the genetic map length of the *bisporus* variety, *burnettii* variety, and inter-varietal hybrid, that of *bisporus* variety is much shorter. A much greater number of non-recombined chromosomes (parental chromosomes) was found in *bisporus* variety. Given the fact that COs are mainly placed at the end of the chromosomes in the *bisporus* variety, genotyping offspring with a limited set of molecular markers would not make it possible to detect all recombination events. Using WGS, Sonnenberg, et al. (2016) found a much lower percentage of parental chromosomes in the *bisporus* variety (45%) which was quite similar to that of the inter-varietal hybrid (48%) as observed by Foulongne-Oriel, et, al. (2010). The percentage of parental chromosomes found in the current study (33%) was lower than that of the inter-varietal hybrid studied by Foulongne-Oriel et, al. (2010). It is noteworthy that the positions of the markers are not the same in these two studies. Recombination normally occurs across the genome of the inter-varietal hybrid with a tendency to recombine at the ends of the chromosome. Therefore,

some recombination sites might not be detected at the ends of chromosomes if these are not covered with markers.

Here, we have proven by doing comparative mapping that the significant differences in recombination landscape of the *bisporus* variety, *burnettii* variety, and inter-varietal hybrid are mainly the position of recombination sites. In many organisms, CO recombinations are not randomly distributed across the genome, and many hot and cold spots have been reported (Gerton et al., 2000; Pryce and McFarlane, 2009). Each chromosome must have at least one CO to ensure proper chromosome segregation (Hillers, 2004). An elevated number of recombination COs were found on the smaller chromosome in budding yeast suggesting that recombination is affected by the size of the chromosome (Kaback et al., 1992). Here we have no indication of a relation between the number of COs and chromsosome lengths. The number of COs on all LGs (except LGIX) are comparable.

This chapter clearly identifies the differences in CO landscape along the parental lines of the *bisporus* and *burnetii* varieties and the expression of this trait in the inter-varietal hybrid line. The linkage map of the hybrid can now be used to study the segregation of the CO landscape and map QTL involved. In addition to meiotic recombination landscape, the current map is useful for the QTL analysis of other traits of economic importance such as resistance to pathogens. The extended linkage map enables a more precise location of QTL. The very short linkage maps of *A. bisporus* resulted previously in the assignment of QTLs controlling cap colour and bruising sensitivity covering almost complete chromosomes (Gao et al., 2015). In addition, we have proven a distinct recombination landscape between three strains of *A. bisporus* using a comparative mapping approach. For localising recombination sites in the *burnettii* variety as well as the inter-varietal hybrid more precisely, a WGS of their offspring is important as it has been done in the *bisporus* variety.

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Supplementary materials
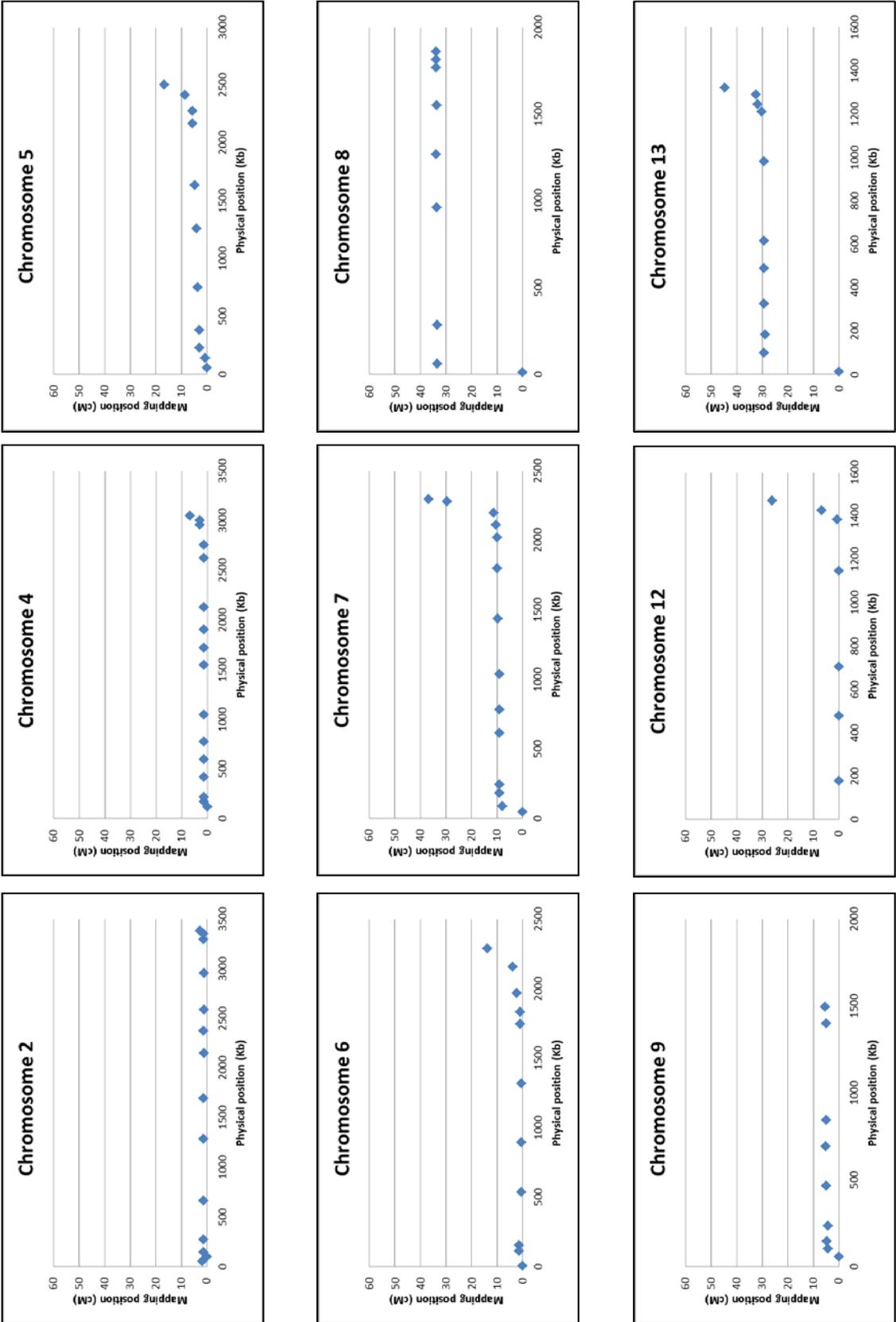
Figure S1. Scatter plots of centimorgan (cM) distribution of SNP markers per chromosome along their physical position in the linkage map of Horst U1.
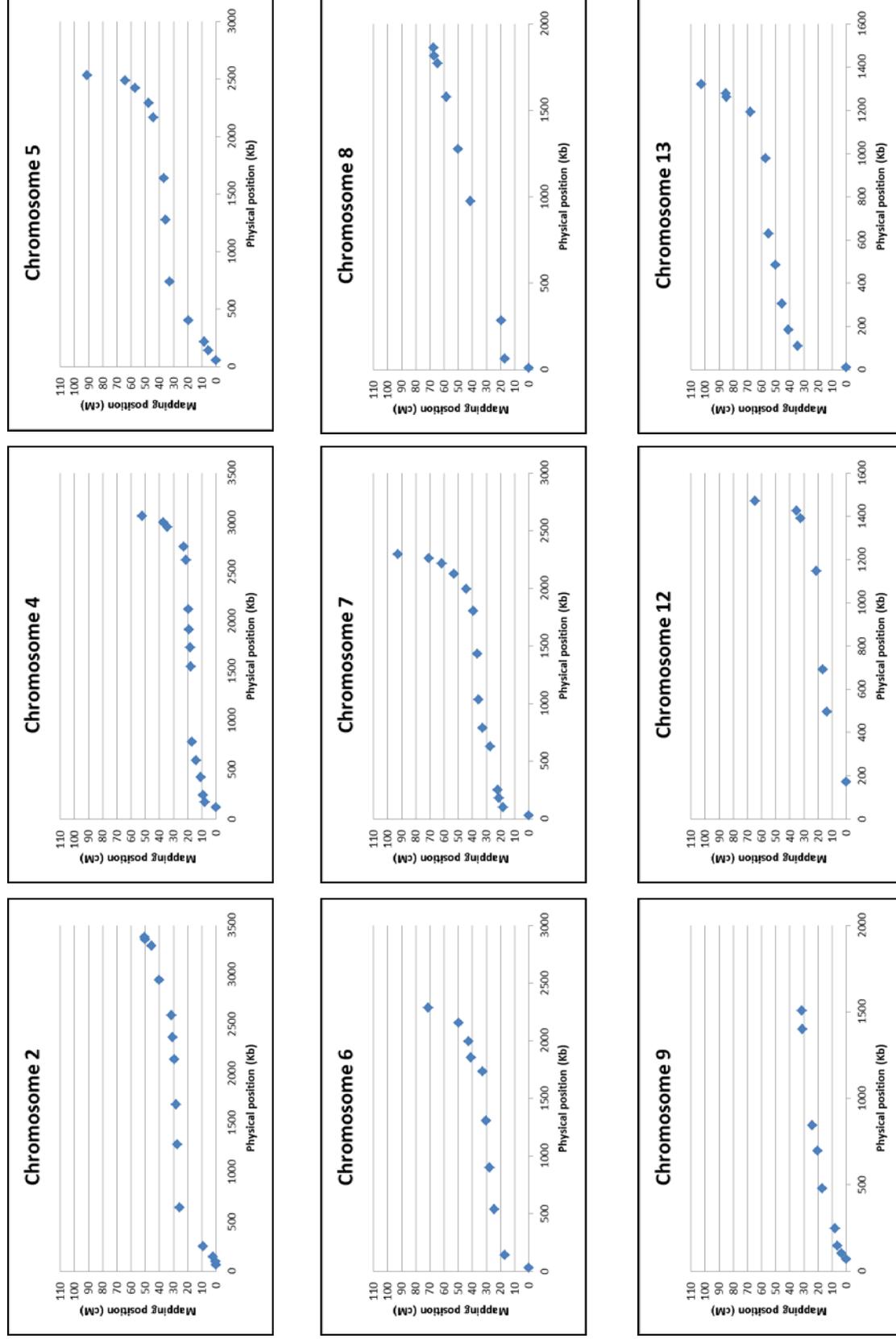
Figure S2. Scatter plots of centimorgan (cM) distribution of SNP markers per chromosome along their physical position in the linkage map of inter-varietal hybrid HBT03.

Chapter 4

Influence of a genetic background on the meiotic recombination landscape of *Agaricus bisporus*

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Abstract

The main varieties of the button mushroom, *Agaricus bisporus* var. *bisporus* and *A. bisporus* var. *burnettii*, both have a contrasting meiotic recombination landscape (MRL). Whereas the var. *burnettii* displays crossovers (COs) distributed over the entire chromosomes, in the var. *bisporus* COs are restricted to the extreme ends of chromosomes. Previously, a genetic linkage map was generated by genotyping homokaryotic offspring of an inter-varietal hybrid (var. *bisporus* × var. *burnettii*). This map displays an intermediate MRL: COs distributed over the entire chromosome with increased COs at chromosome ends. To map this quantitative trait, offspring of the mapping populations must be generated and assessed for MRL. In this study, the individuals of the mapping population were intercrossed and outcrossed to generate three types of segregating populations. Two compatible tester homokaryons derived from both *bisporus* and *burnettii* varieties were used for outcrossing. The homokaryotic offspring isolated from each type of segregating populations were genotyped with SNP markers covering the entire length of the chromosomes. Recombination frequencies were determined at distal and elsewhere on the chromosomes and used to compare recombination landscapes between chromosomes across populations as well as between segregating populations across chromosomes. The MRL in the segregating populations was clearly affected by the type of crosses made. The current study indicates that segregating populations generated by outcrossing the mapping population with the *bisporus* tester homokaryon are most suitable for QTL analysis of the MRL phenotype.

Introduction

A. bisporus var. *burnettii* reveals crossovers (COs) distributed more or less evenly over the entire genome. This more common recombination landscape contrasts to the *A. bisporus* var. *bisporus* recombination landscape which is restricted at chromosome ends. As COs at the extreme ends of chromosomes are difficult to detect due to the presence of repetitive sequences, the CO frequency of the *bisporus* variety is often underestimated (Sonnenberg et al. 2016). Foulongne-Oriol et al. (2011) observed that there was a reduction in the recombination rate in the second generation hybrids generated from a cross between homokaryons of the *bisporus* variety and a homokaryotic progeny from an inter-varietal hybrid (var. *bisporus* × var. *burnettii*). This reduction indicates a shift of COs towards chromosome ends due to the cross with the *bisporus* homokaryon. This quantitative change suggests that meiotic recombination is likely to be influenced by more than one gene which can be explained by quantitative trait loci (QTL). Quantitative inheritance of recombination also appeared in plants (Pfeiffer and Vogt, 1990; Tulsieram et al., 1992; Fatmi et al., 1993). In order to study the quantitative inheritance of meiotic recombination, populations are required that segregate for this trait, i.e., variation in meiotic recombination has to be visible, and recombination has to be assessed over the entire length of chromosomes.

Segregating populations can be generated in different genetic backgrounds which may affect the recombination rate (Pfeiffer and Vogt, 1990; Williams et al., 1995; Sebastian et al., 2000). Hence, it is important to understand meiotic recombination in the different genetic backgrounds to make an important decision about the experimental design. However, meiotic recombination might be a qualitative rather than a quantitative trait. Two examples of qualitative recombination modifiers, i.e., *Rm1* and *Ph1* which affect the genome-wide recombination were identified in plants (Cornu et al., 1989; Zickler and Kleckner, 1999).

Alongside the recombination landscape, the two varieties also differ according to the average number of spores that are formed on basidia. The *bisporus* variety has predominantly two spores per basidium and the *burnettii* variety mainly four spores per basidium. The basidial spore number is determined by the *BSN* locus on chromosome I (Imbernon et al., 1996). The *Bsn-t* (tetrasporic) locus of the *burnettii* variety appears to be dominant over the *Bsn-b* (bisporic) locus of the *bisporus* variety. Previous research suggests that the recombination rate observed in chromosome I of the first generation hybrids depends on the genotype of the *BSN* locus (Callac et al., 1998). This conclusion was drawn from studying recombinations between a few markers on chromosome I in the offspring between homokaryons which were derived from *bisporus* and *burnettii* strains. However, the recombination landscape is a trait that is not expressed in these segregating populations but in its offspring. Each individual in these first generation segregating populations must therefore be crossed with compatible homokaryons (second generation hybrids) to generate offspring in which recombination can be studied.

Second generation hybrids can be generated by outcrossing using a compatible tester homokaryon or by intercrossing (sib-mating) the homokaryotic offspring of the first generation hybrid (mapping population). Here, we define an intercross as mating between single fruiting body offspring. The number of possible crosses will be restricted to those pairs, which differ in

mating type to each individual originating generally from different meiotic events. To assess meiotic recombination landscape (MRL) and subsequent QTL analysis, the homokaryotic offspring of each second generation hybrid must be isolated and tested for CO frequencies and positions. Intercrossing is far more common in plant breeding than in mushroom breeding. The maximum variation of the trait is evident in intercrossed populations due to the fact that the genome of homokaryons derived from a mapping population is involved in segregating the trait. However, intercrossing is subject to three main limitations, i.e., non-segregation for the mating type and its surronding areas, the high level of homozygosity, and inbreeding depression. In outcrossing, the homokaryotic single spore isolates (SSIs) of the mapping population are crossed with a compatibe tester homokaryon. The genetic constitution of the tester homokaryon may affect the trait segregation. However, the entire genome can be assessed for COs due to its high level of heterozygosity.

In this study, we generated three different second generation hybrids and studied a limited number of segregating populations from each type of hybrid to determine which type of cross generates the most informative segregating population for QTL analysis of the MRL. Each segregating population has its own advantages. By using segregating populations, along with whole genome sequences (WGS) and the subsequent detection of SNP markers we were able to assess the MRL. Objectives were: (i) to discover which type of segregating population reveals the clearest MRL segregation, (ii) to determine how to quantify MRL in QTL analysis and (iii) to specify how many markers are needed to assess the MRL. Furthermore, the data generated could be used to determine if the *BSN* locus is linked to MRL.

Materials and Methods

Fungal strains and populations

All strains used in this study were obtained from the Plant Breeding Wageningen University & Research fungal collection. Two original heterokaryotic lines were used, i.e., Horst U1 a white commercial *bisporus* strain and Bisp119/9 a wild type *burnettii* strain, originating from the ARP collection (Kerrigan et al. 1996). The constituent nuclei of Horst U1, i.e.H97 and H39, were available from another study (Sonnenberg et al., 2016). The constituent homokaryons of Bisp119/9 (Bisp119/9-P4 and Bisp119/9-P1) were recovered by protoplasting according to the method described previously (Sonnenberg et al., 1988). Bisp119/9-P4 and H97 were crossed to make the F1 hybrid, known as HBT03, from which the homokaryotic SSIs were collected to generate a mapping population (this thesis, chapter 3). These homokaryotic SSIs were used to generate three types of second generation hybrids, i.e., intercrossed hybrids and two types of outcrossed hybrids. The pedigree of these second generation hybrids is depicted in Figure 1.

To generate intercrossed hybrids, 42 homokaryotic SSIs of the HBT03 population were selected to generate 441 breeding pairs. Three criteria were used for this selection; each homokaryon in a pair had to be different in the mating type in order to be compatible. The homokaryons selected needed have at least 13 COs (1 per chromosome) as CO is important for precise QTL assignment. Both parental genomes of HBT03 hybrid (H97 and Bisp119/9-P4) should on

average be equally represented in the selection. From 441 breeding pairs, 190 heterokaryons were selected to generate fruiting bodies.
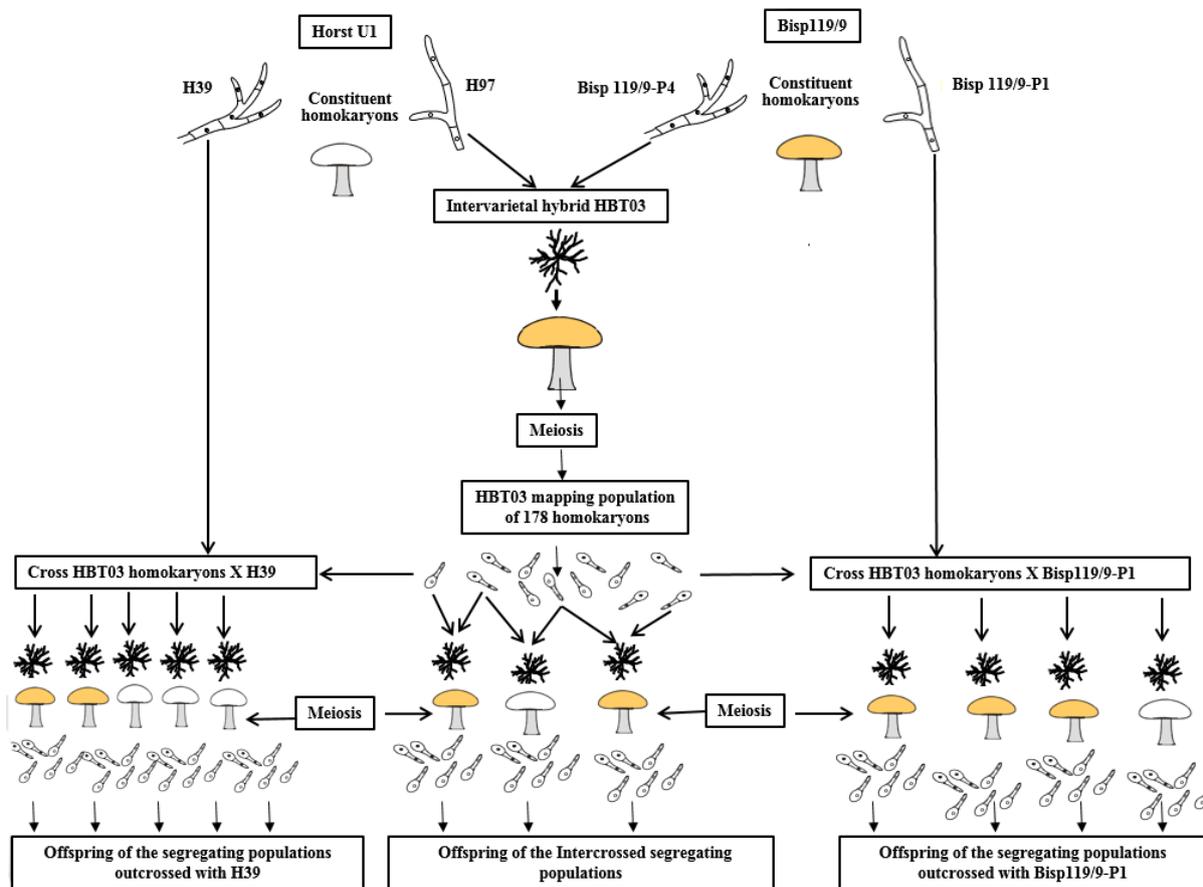
Figure 1. The pedigree presentation of segregating populations used in this study.

In order to generate outcrossed hybrids, the homokaryotic SSIs of the HBT03 population were crossed with two tester lines, i.e., H39 and Bisp119/9-P1. H39 and Bisp119/9-P1 are the constituent homokaryons in the *bisporus* variety Horst U1, and the *burnettii* variety Bisp119/9, respectively. The heterokaryons of each of the three types of second generation hybrids were grown on commercial compost (CNC Substrates), spawned in flower trays filled with 2 kg of compost in separate cultivation rooms. The fruiting bodies were collected to make spore-prints. Single spore isolates were isolated as described previously (Sonnenberg et al., 1988). Petri dishes filled with commercial spawn (Sylvan A15) were used as a germination stimulus, and placed along with spore plates in a box in an incubation room (25^OC). The single spore isolation began immediately following germination of the first spores. Homokaryotic and heterokaryotic SSIs of each spore-print and the mating type of homokaryons were determined by PCR primers close to mating type.

Marker selection and genotyping

Three sets of SNP markers were designed, for the purpose of identifying segregation between the two parental types in the three types of hybrids generated, the intercrossed and the two outcrossed hybrids. The first set was designed by selecting a subset of SNP markers which had already been used in the mapping population of the inter-varietal hybrid HBT03 (this thesis, chapter 3). It was used to genotype homokaryotic offspring of the intercrossed hybrids. The second set was designed to differentiate between the genotypes of homokaryons in the HBT03 mapping population (genotypes of H97 and Bisp119/9-P4) and the tester homokaryon H39. The third set was designed to differentiate between the genotypes of homokaryons in the HBT03 mapping population and the tester line Bisp119/9-P1.

Ten SNP markers were selected per chromosome in all three sets in such a way that each chromosome end (as far at the end as possible) was covered with two SNPs. The area ~100 kb away from each chromosome end was set as a border and covered with one SNP. The position of the border was defined based on the precise MRL assessment in the *bisporus* variety indicating that COs occur mainly in the first/last 100 kb of the chromosomes (Sonnenberg et al., 2016). The other four SNPs were evenly distributed over each the remaining part of each chromosome. It this way, we were able to detect COs at the ends of the chromosome (first/last 100 kb) as well as COs occurring in the rest (middle) of the chromosome. Genotyping the individuals was performed in the Dr. van Haeringen Laboratorium (VHL) in Wageningen, using KASPar SNP Genotyping System.

Quantification of MRL

To quantify MRL in all second generation hybrids involved in this study, the number of COs at both ends and also in the middle of the chromosomes, was calculated per chromosome and per individual. Using this dataset for each population, CO frequencies at different positions, i.e., chromosome ends and middle part were calculated in the following way:

Ends COs fraction is the sum of COs at both ends divided by the total number of COs per chromosome per segregating population. *Middle COs fraction* is the sum of COs in the middle divided by the total number of COs per chromosome per segregating population. Finally, the middle CO frequency was calculated by the number of COs in the middle of the chromosome divided by the number of individuals per chromosome per population.

Results

In order to study the linkage of the individuals' genotype in the mapping population HBT03 with the MRL, three different second generation hybrids were created to generate offspring. This was done to determine the types of hybrids which are most useful to study CO frequencies and positions in its offspring. This will depend on the accuracy with which we can assess the phenotype, hence the recombination between markers. Homokaryotic SSIs of the mapping population were thus intercrossed or outcrossed with the *bisporus* H39 or *burnettii* Bisp119/9-P1 homokaryon. Homokaryotic SSIs were isolated from each hybrid and genotyped using SNP markers.

Intercrossed population

A selection of homokaryons in the HBT03 mapping population was made as already described in the "Materials and Methods" section to generate 190 hybrids that were cultivated to collect spore-prints. Eighteen heterokaryons did not fruit and the remaining that did fruit was mainly unable to shed spores during the spore-print collection. For those that did not shed spores on paper, the spores were rinsed off the gills. As is the case for intercrosses, many genomic areas are homozygous for parents used in each breeding pair and thus do not segregate.

The initial aim of this study was to design SNP markers for the desired positions described in the "Materials and Methods" section for all 13 chromosomes of *A. bisporus*. As anticipated with the intercrosses, homozygosity between homokaryons in pairs (intercrossed hybrids), restricted the study for CO positions to only three chromosomes. Only chromosome I, IV, and XIII showed adequate heterozygosity. Three intercrossed hybrids were selected, namely HBTI043, HBTI081, and HBTI084 to generate homokaryotic segregating populations. These three hybrids were selected for two reasons. First of all, these hybrids were able to shed spores on paper. Secondly, the HBT03 homokaryons used in generating these three hybrids had also been successful crossing with H39 and Bisp119/9-P1 (two tester homokaryons used in this study). The genotype file of these hybrids is presented in supplementary materials Figure S1.

The homokaryotic SSIs derived from HBTI043, HBTI081, and HBTI084 hybrids were genotyped for chromosome I, IV, and XIII using 30 SNP markers. Four SNPs were excluded from further analysis due to homozygosity of the area, i.e., one SNP at the right end of chromosome I, another at the left end of chromosome XIII and two at the ends of chromosome IV. The characteristics of the SNP markers used for genotyping are listed in supplementary materials Table S1.

As described in the Materials and Methods section, our initial attempt to assess the MRL of each chromosome per segregating population was to calculate ends CO fraction, middle CO fraction and the middle CO frequency. For that, we set SNP markers as close as possible to the ends of the chromosomes, approximately 100 kb away from the chromosome ends and distributed over the rest of the chromosome. For the intercrossed population, however, we had no specific data point for the left end of chromosome XIII in the hybrid HBTI043 due to homozygosity of the area. Only three chromosomes could be compared and because the end of one chromosome was missing, it was difficult to assess the MRL landscape using the CO number at chromosome ends. Even though *A. bisporus* depicts CO interference, CO at chromosome ends and outside this area is more or less complementary. An assessment of CO frequency in the middle of chromosomes should thus reflect the MRL. The number of COs was divided by the number of individuals for which data was available to correct the varying number of individuals in each population. The middle CO frequencies were lower in chromosome IV than those in chromosomes I and XIII (Figure 2).

Outcrossed with H39

To gain an impression of the type of data generated by outcrossing with a *bisporus* tester homokaryon, we selected ten homokaryotic SSIs from the HBT03 population and crossed these

with H39 (a constituent homokaryon of the Horst U1 variety). Most of the HBT03 homokaryons used in intercrosses were also used for the two types of outcrosses. This enabled us to see how the MRL of each HBT03 homokaryon is expressed in the different genetic backgrounds.
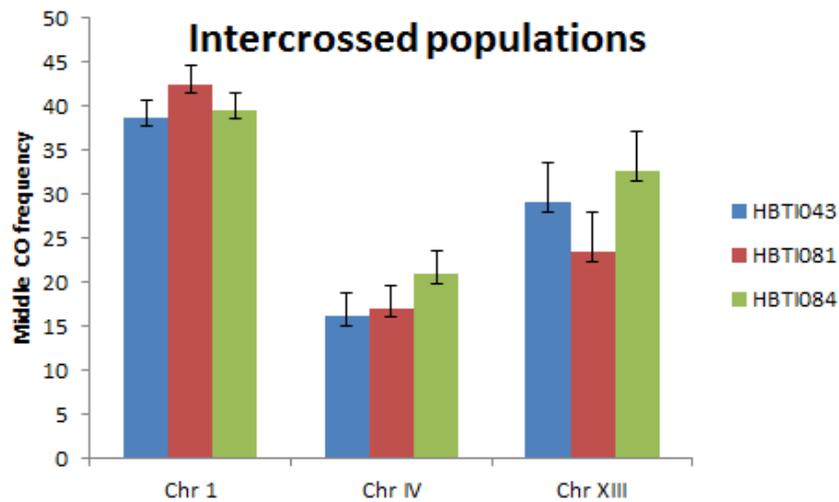
Figure 2. Comparison of the middle CO frequencies between intercrossed populations.
Error bars indicate standard error.

Five of the homokaryons used for outcrossing with H39 carried the *Bsn-b* allele, and the other the *Bsn-t* allele. The hybrids therefore represent a group with a *Bsn-b/b* genotype and the *bisporus* phenotype, another group with a *Bsn-b/t* genotype and the *burnettii* phenotype, because *Bsn-t* more or less dominates over *Bsn-b*. This was done to see if the *BSN* locus is linked to the CO position. The genotype file of 10 homokaryons of the HBT03 population used for outcrossing with H39 is presented in supplementary material Fig. S2. Ten segregating populations were generated by isolating homokaryotic progenies from each outcrossed hybrid. In contrast to the intercrossed populations, we were not restricted to a few chromosomes in populations outcrossed with H39 in order to detect polymorphism between the genotypes of homokaryotic SSIs derived from the HBT03 population and H39 tester homokaryons and then discover the COs. However, the aim of this study was to assess and compare the MRL in three types of hybrids generated in this study, therefore only chromosomes I, IV, and XIII were genotyped in the H39 outcrossed population as they were in the intercrossed populations.

Of the 30 SNPs used to genotype homokaryotic progenies, only one on chromosome XIII did not reveal a reliable marker score and was thus excluded. The characteristics of the SNP markers used for genotyping are listed in supplementary materials Table S2.

As already mentioned, we have fewer drop outs for the markers in the middle of the chromosome which makes it possible to calculate the middle CO frequency per chromosome per population. A considerable variation of the middle CO frequency has been observed between populations as expected and also between chromosomes (Figure 3). Despite some inconsistency, we generally found the middle CO frequencies to be statistically different across populations and chromosomes (Generalized linear model, logit link function; $p < 0.01$).
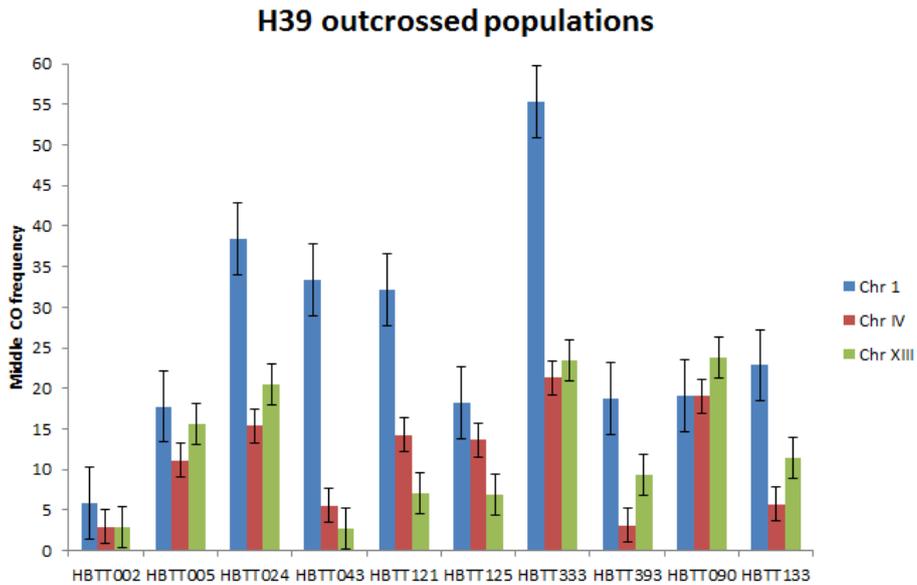
Figure 3. Comparison of the middle CO frequencies between H39 outcrossed populations.
Error bars indicate standard error.

Having accumulated data on ten different populations in the outcross with H39, we decided it would make sense to compare the middle CO frequencies of the three chromosomes. If the number of COs are compared pairwise, a positive correlation results (Figure 4), indicating that the mechanisms, regulating COs might act similarly on these chromosomes.
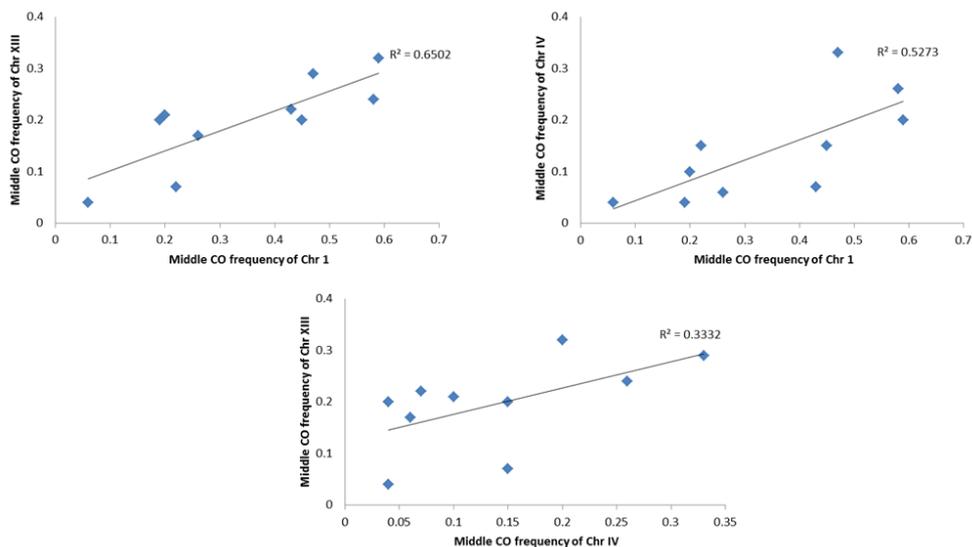
Figure 4. Correlation between middle CO frequency of chromosomes I, IV and XIII in H39 outcrossed populations. Each dot represents one segregating population (n=10), the straight line represents the best-fit line obtained by linear regression analysis.

If the BSN locus were linked to the type of recombination landscape, one would expect a difference in average between the number of COs in the middle of chromosomes in offspring of the *Bsn-b/b* and *Bsn-b/t* genotypes. The variation in CO frequencies between these two *Bsn-*

type populations were, however, very similar indicating that there is no linkage between *Bsn* and the MRL (Figure 5).
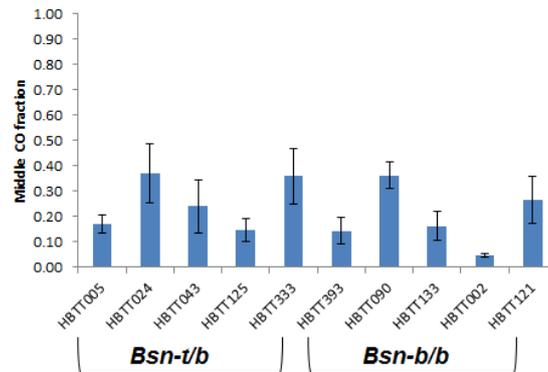
Figure 5. The average of middle CO fraction of 10 segregating populations outcrossed with H39 ($p < 0.01$). Error bars indicate standard error.

In contrast to SNP markers developed at the ends of the chromosomes for intercrossed populations, SNP markers at the ends of the chromosomes developed for H39 outcrossed populations manifested reliable marker scores. Therefore, the middle CO fraction could also be calculated in H39 outcrossed populations (# CO in the middle/(# CO at the end + # CO in the middle)). The variation in the middle CO fraction of chromosome I is larger than those of chromosomes IV and XIII (Figure 6). The lowest middle CO fraction was found in the HBTT002 population, meaning that most COs occurred at the ends of the genotyped chromosomes in this population.
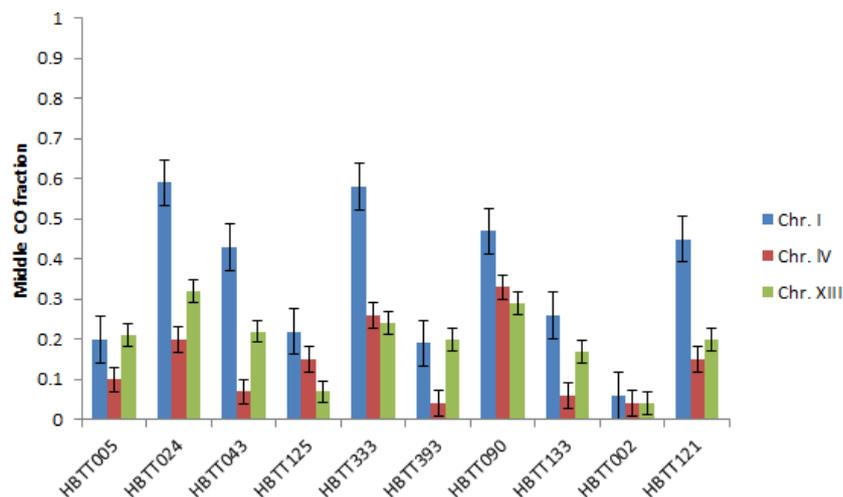
Figure 6. Middle CO fraction of 10 segregating populations outcrossed with H39 ($p < 0.01$). Error bars indicate standard error.

Outcrossed with Bisp119/9-P1

To get an impression of the type of data generated by outcrossing with a *burnettii* tester homokaryon, we selected a subset of homokaryons in the HBT03 population and crossed these

with Bisp119/9-P1 (a constituent homokaryon of Bisp119/9). The homokaryons of HBT03 used in outcrosses with Bisp119/9-P1 were also used for the outcrosses with H39 and intercrosses. Most of these crosses, however, did not fruit well during cultivation. Of those few fruiting, four hybrids were used to isolate spores. The germination rate of the spores was very low (data not shown). Due to a high similarity between two constituent homokaryons of Bisp119/9, i.e., Bisp119/9-P4 and Bisp119/9-P1, we were unable to find SNPs on all chromosomes at similar positions as were used in the other two types of population. Only useful markers were found for chromosome I and also for chromosome XI. Two markers on the left at the end of chromosome I and one on the left at the end of chromosome XI unfortunately fell away during genotyping. Therefore, we only calculated the middle CO frequency in the population outcrossed with Bisp119/9-P1. The characteristics of the SNP markers used for genotyping are listed in supplementary materials Table S3.

The higher middle CO frequency calculated in Bisp119/9-P1 outcrossed populations indicated that many COs occurred in the middle of the chromosomes in these populations (Figure 7). This is an indication of the effect of Bisp119/9-P1 genome could have on the CO position. However, chromosome XI was not genotyped in the other two types of segregating populations.
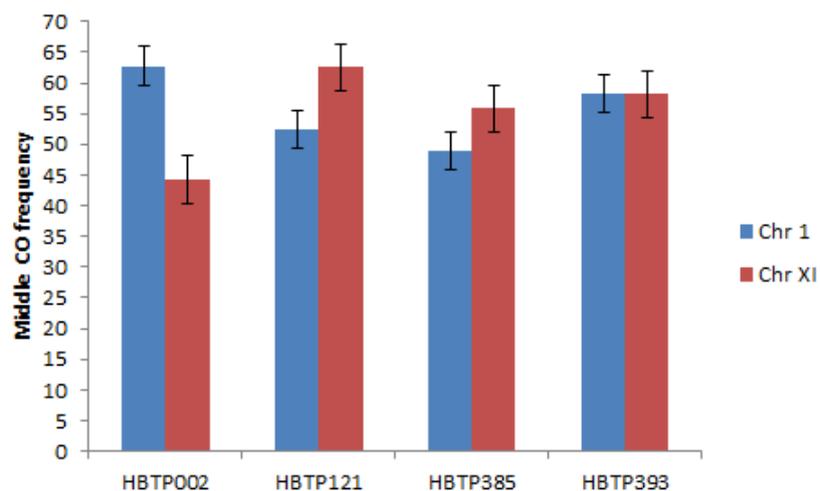
Figure 7. Comparison of the middle CO frequencies between Bisp119/9-P1 outcrossed populations. Error bars indicate standard error.

Overall comparison between populations

Because the main aim of the current study was to gain a better understanding as to which of the segregating populations would be the most feasible to perform a QTL study for MRL, we now compare three types of crosses generated in this study. A proper comparison could only be made between middle CO frequencies of chromosome I. The middle CO frequency could be calculated in all three types of crosses regardless of COs taking place at the ends of the chromosomes and thus overcome problems with SNP markers at the ends of the chromosome. To depict MRL, however, all CO events need to be taken into account. Thus, middle CO fraction (# CO in the middle/(# CO at the end + # CO in the middle)) of chromosome I could be compared between intercrossed and H39 outcrossed populations. The middle CO frequency

and fraction of chromosome I in H39 outcrossed populations which share parental homokaryons derived from HBT03 mapping population with intercrossed and Bisp119/9-P1 outcrossed populations are listed in Table 1.

Table 1. Comparison of the middle CO fraction and frequency of chromosome I genotyped in three types of segregating populations

Population		Chromosome I Middle COs		Parental homokaryon [b]
		Frequency (%)	Fraction [a]	
X H39	HBTT005	17.78	0.20	HBT03005
	HBTT125	18.18	0.22	HBT03125
	HBTT393	18.75	0.19	HBT03393
	HBTT002	5.88	0.06	HBT03002
	HBTT121	32.14	0.45	HBT03121
X Bisp119/9-P1	HBTP002	62.79	-	HBT03002
	HBTP121	52.5	-	HBT03121
	HBTP385	48.84	-	HBT03385
	HBTP393	58.14	-	HBT03393
Intercrossed	HBTI043	38.71	0.81	HBT03005 & HBT03385
	HBTI081	42.55	0.71	HBT03385 & HBT03125
	HBTI084	39.53	0.56	HBT03125 & HBT03121

[a] missing value due to the absence of SNPs at the end of the chromosome.
[b] homokaryotic SSIs derived from mapping population HBT03.

This comparison will enable us to determine the effect that genetic backgrounds of two tester homokaryons have on MRL. Although the position of the SNP markers used in the intercrossed and two types of outcrossed populations were not identical, it seems that COs had shifted towards the ends of the chromosomes in H39 outcrossed populations due to the genetic background of the tester homokaryon H39. The higher frequency of the middle CO of chromosome I in populations outcrossed with Bisp119/9-P1 compared to those of intercrossed populations and H39 outcrossed populations can be seen as an indicator of the effect of the tester homokaryon Bisp119/9-P1 has on segregation in MRL. For example, HBTP121 is a population outcrossed with Bisp119/9-P1 which shares one parental homokaryon with HBTT121 (H39 outcrossed population) and HBTI084 (intercrossed population). The middle CO frequency of chromosome I is higher in the HBTP121 population than that of HBTT121, and HBTI084. This is the case for HBTP393 and HBTP002 (Bisp119/9-P1 outcrossed populations) in which each shares one parental homokaryon with HBTT393 and HBTT002 (both H39 outcrossed populations), respectively. The middle CO frequency of chromosome I

is almost three times higher in HBTP393 than in HBTT393 and 10 times higher in HBTP002 than in HBTT002.

The middle CO fraction could only be calculated for chromosome I in the intercrossed populations and in the populations outcrossed with H39. The middle CO fractions of chromosome I in the intercrossed populations are greater than those of outcrossed populations. For example, the intercrossed HBTI084 population shares one parental homokaryon with H39 outcrossed HBTT125 population and one parental homokaryon with H39 outcrossed HBTT121 population. The middle CO frequency and fraction of chromosome I of HBTI084 are greater than those of HBTT125 and HBTT121. The frequencies of CO in the middle of chromosome I clearly show the effect of the tester line. Outcrossing with H39 reduces the CO frequencies in the middle considerably and thus indicates a shift of CO towards the chromosome ends.

Discussion

To make an accurate assessment of MRL first of all necessitates a sufficiently large sample of segregating populations as well as the markers covering the entire length of chromosomes. In mushroom breeding, homokaryotic SSIs (the haploid meiotic products) are used to construct linkage maps. As homokaryotic SSIs are haploid, mating with a compatible homokaryon is necessary to generate second generation hybrids in which the trait of interest is assessed. Phenotyping of traits such as colour or bruising sensitivity (Gao et al., 2015; Gao et al., 2016), can be done directly on mushroom hybrids (fruiting bodies). To assess the MRL trait, however, progenies must be generated from the mapping population and these need to be crossed with a compatible homokaryon to generate mushrooms. From each of these next generation hybrids, a number of homokaryotic SSIs have to be isolated and genotyped to assess the MRL. Hybrids generated for the intercrosses and outcrosses with H39 have the *Bsn-b/b* locus as a substantial part. Therefore, many basidia will produce two spores that are almost entirely heterokaryotic. Mapping MRL thus is laborious, costly and time-consuming. This process requires that SSIs are pre-screened for slow growth rates as homokaryons generally grow slower than heterokaryons. Subsequently, the ploidy level of pre-screened SSIs is confirmed by molecular markers linked to the mating type region.

To obtain information on segregating populations generated on different genetic backgrounds, the homokaryotic SSIs derived from the mapping population HBT03 were intercrossed and outcrossed using tester homokaryon H39 (*bisporus* type) and Bisp119/9-P1(*burnettii* type). By generating and genotyping the homokaryotic SSIs derived from each segregating population, we found that the segregating populations outcrossed with H39 are currently the best populations to be chosen for QTL analysis of MRL.

In both types of outcrossed populations, each hybrid shares the tester homokaryon genome, and the segregation of MRL is due to genetic differences of the homokaryotic SSIs derived from the HBT03 hybrid. The tester homokaryons' genome can affect the expression of MRL either by shifting COs towards the chromosome ends, when using H39 or more evenly spread over chromosomes, when using Bisp119/9-P1. Reduced recombination was observed in second generation hybrids outcrossed with *bisporus* homokaryon (Foulongne-Oriol et al., 2011). This

reduction can be explained by the fact that recombinations are mainly restricted to chromosome ends in the *bisporus* variety, hence the outcrossing with homokaryons of the *bisporus* variety might shift recombination towards the ends of the chromosome where it is difficult to generate molecular markers (Sonnenberg et al., 2016).

An outcross with a *burnettii* homokaryon will reduce the number of COs at chromosome ends and might solve this problem. When we performed these outcrosses, resequencing data from Bisp119/9-P1 was not available at that time. It appeared that the two constituent homokaryons of Bisp119/9 (Bisp119/9-P1 and Bisp119/9-P4) were homozygous in the largest part of the genome (this thesis, Chapter 2). The cultivated Bisp119/9 heterokaryon does fruit and only produces mushrooms of a low quality (data not given). The outcross of the mapping population (H97 x Bisp119/9-P4) with Bisp119/9-P1 again resulted in hybrids having large homozygous genomic parts. That might have caused difficulties in fructification as well as a low germination spore rate. It remains unclear why the inbreeding symptoms seem to be more severe in the Bisp119/9-P1 outcrossed populations than in the original Bisp119/9 strain.

Intercrossing has some restrictions in mushroom breeding. *A. bisporus* has a unifactorial mating system in which the compatibility between homokaryons is controlled by one locus (Miller, 1971). Consequently, half of the homokaryotic SSIs derived from one mushroom can be crossed with the other half meaning that mating type region remains heterozygous and will not show segregation. In addition to that, intercrossing often causes inbreeding depression (Xu, 1995) due to large regions of homozygosity. During fructification of the intercrossed hybrids generated in the current study, we ran into a problem. The intercrossed hybrids that did fruit often showed proteolysis or were not able to shed spores (data not given) which could indicate inbreeding depression. The level of homozygosity also made it difficult to generate sufficient markers to study segregation. Informative markers could be generated for a limited number of chromosomes in only a few of the hybrids. The genotype of homokaryons has to be carefully selected in order to reach as much heterozygosity as possible in each pair. Heterozygosity between homologous chromosomes is not only necessary for detecting COs, it could also affect the recombination rate. The higher level of sequence similarity between homologous chromosomes was assumed to have an effect on the recombination rate (Salomé et al., 2012). In the *Schizophyllum commune*, COs preferentially occur in areas where there is a higher similarity between parental genomes (Seplyarskiy et al., 2014). In contrast, *Arabidopsis thaliana* has reduced recombination rates in homozygous chromosomes (Barth et al., 2001).

Some of the hybrids outcrossed with the tester homokaryon H39 did not fruit as well (data not given), but we did not find any indication for severe inbreeding depression in this type of hybrid as those of intercrossed and outcrossed with the *burnettii* tester homokaryon. H39 and H97 are the constituent Horst U1 homokaryons; the first mushroom hybrid that came onto the market. The similarity between the genome of these two haplotypes is less than those of Bisp119/9 (this thesis, chapter 2). Therefore, by outcrossing the homokaryotic SSIs of the HBT03 hybrid with H39 inbreeding depression is not expected. It is not evident why some hybrids outcrossed with H30 did not fruit.

The expression of inbreeding depression in the *A. bisporus* var. *bisporus* is hypothesized by its secondary homothallic life cycle where recessive lethal alleles can be masked by pairing post-meiotic non-sister nuclei and typical MRL. Sonnenberg et al., (2016) have re-sequenced homokaryotic offspring of the *bisporus* variety Horst U1 and none of the parental type (non-recombined) chromosomes were missing in the offspring, suggesting that the hypothesis of masking recessive lethal alleles is less likely in the *bisporus* variety. It might be that certain combinations of alleles result in lower fitness levels and that can have an epistatic effect .

The level of similarity between genomes contributing to hybrids is also important for evaluating the MRL. The higher the similarity, the lower the chance of finding SNP markers to cover the entire length of chromosomes with informative markers. This was the case when hybrids were outcrossed with Bisp119/9-P1. The precise evaluation of MRL requires an appropriate coverage of the genome with molecular markers particularly at the ends of the chromosome where it is difficult to be well covered by markers. The more or less similar position of the SNP markers also has to be taken into consideration when comparing MRL in hybrids generated in different genetic backgrounds which was our initial aim. Where possible, we designed SNPs markers. However, some markers at the ends of the chromosome failed during genotyping, resulting in an incomplete evaluation of the MRL in some populations. Only three chromosomes out of the thirteen were sufficiently heterozygous in intercrossed populations and were used to compare the CO frequencies and positions between the intercrossed and outcrossed populations. The MRL of the two parental types derived from two varieties involved in our study are very contrasting. Restriction of recombination to the ends of the chromosome is a pattern of MRL observed across the genome of the *bisporus* variety (Sonnenberg et al. 2016). An almost even recombination distribution has been seen for a limited number of chromosomes in the *burnettii* variety (this thesis, Chapter 2 & 3). Although a limited number of chromosomes were assessed for MRL in the current study, the effects of the genetic background of the tester line in outcrosses was very clear, COs shifted towards chromosome ends in H39 outcrossed populations, and to the middle of the chromosome in Bisp119/9-P1 outcrossed populations.

A variation on the CO position in different populations derived from the second generation hybrids is anticipated because we expect segregation for this trait. We have also seen considerable differences between chromosomes within one population. Correlation analysis in the outcrossed population with H39 reveals that there is a positive correlation in middle CO frequencies between the three chromosomes examined, indicating a mechanism that might act similarly towards all chromosomes. The correlation is not high and, therefore, might have an effect on the accuracy of QTL analyses. Part of the variation might be caused by the different positions of the SNP markers between the populations for the three chromosomes examined. The lower CO frequency in the middle of chromosome IV in intercrossed populations might be due to the fact that the position of the SNP marker on the left side border of this chromosome is more than 100 kb further away from the left side end of the chromosome than the SNP markers for chromosomes I and XIII. This means a smaller region was used to count the middle COs of chromosome IV. CO frequencies might, however, also vary between chromosomes. Foulongne-Oriol and co-workers (2010 & 2011) also found a lower recombination frequency

in chromosome IV compared to that of chromosome I in the offspring of crosses between *bisporus* var. *x bisporus* var. and *bisporus* var. *x* inter-varietal hybrid.

Conclusion

The main purpose of this study was to determine what type of population would be best suited to assess MRL. Our analyses have revealed how different genetic backgrounds impact on the position of COs using three types of second generation hybrids generated by intercrossing and outcrossing. It seems that the second generation hybrids outcrossed with H39 is currently the best option to be further used for segregating populations in order to map MRL. Covering the entire length of chromosomes with molecular markers was found to be important, while assessing MRL. Fractions and frequencies of CO in different areas of the chromosomes e.g. the ends and the middle seem to be a suitable method to quantify MRL. In order to make a proper evaluation of the MRL the entire length of chromosomes, especially the chromosome ends, have to be covered with molecular markers. Intercrossing raises the level of homozygosity which does not enable us to detect COs and phenotype the MRL to perform a QTL analysis. Outcrossing with a tester homokaryon of the *burnettii* variety directs COs towards the middle of the chromosomes where it is easier for them to be covered with molecular markers and thus phenotype MRL, but the high level of inbreeding depression and similarity between the genome of two haplotypes of the *burnettii* variety limited our ability to use Bisp119/9-P1 for further QTL study. The use of the *bisporus* tester homokaryon for outcrossing directs COs towards the ends of the chromosomes where is difficult for them to be covered with molecular markers. Nevertheless, there were sufficient polymorphisms in the genome of this type of crosses compared to the other two types of crosses which will enable us to detect COs genome-wide. Genotyping more chromosomes in the populations outcrossed with the *bisporus* tester homokaryon (H39) will reveal detailed information on CO events in this type of crosses and enable us to phenotype MRL for further QTL study. The power, accuracy and precision of the QTL study of MRL can be influenced by the number of second generation hybrids used to generate segregating populations, the number of homokaryotic SSIs isolated from each hybrid, the type of segregating populations and the precision of the assessment of MRL. In general, the number of populations used in our study was not adequate to make a proper evaluation of MRL in different genetic backgrounds due to the major difficulties encountered and described above.

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Supplementary materials

Figure S1. A genotype plot of the selected intercrossed hybrids. Red indicates the genotype of Bisp119/9-P4; green indicates the genotype of H97.
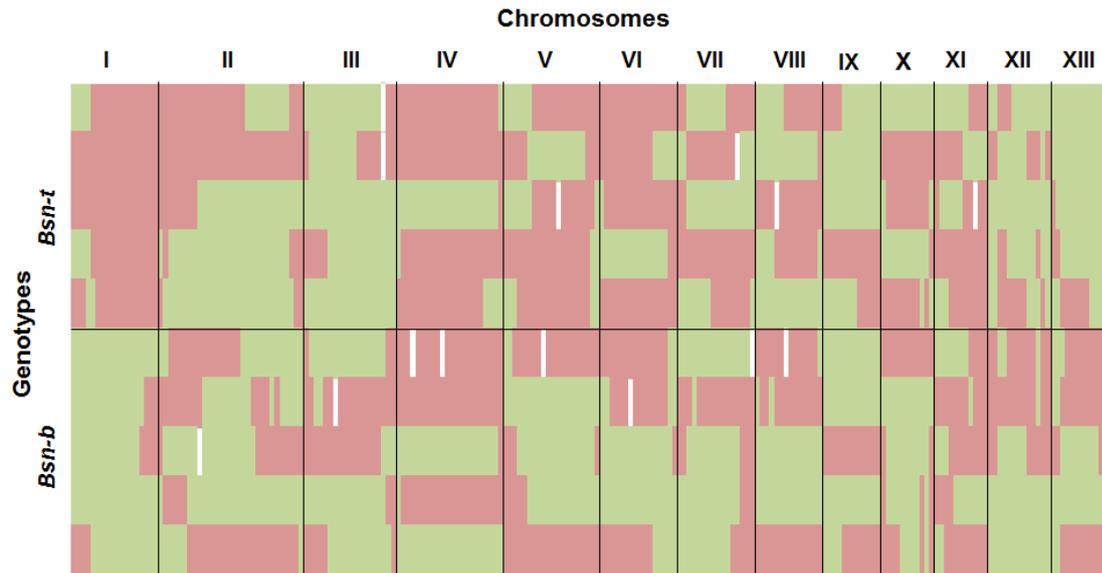
Figure S2. A genotype plot of selected homokaryons of the HBT03 population outcrossed with H39. Red indicates the genotype of Bisp119/9-P4; green indicates the genotype of H97. White indicates missing values.

Table S1. Characteristics of the SNP markers used to genotype intercrossed populations

SNP marker (bp)	Chromosome	Definition
SC1-6563	I	Left end
SC1-91233	I	Left border
SC1-221517	I	Middle
SC1-1015084	I	Middle
SC1-1622071	I	Middle
SC1-2243645	I	Middle
SC1-2908540	I	Middle
SC1-3391608	I	Right border
SC1-3467554	I	Right end
SC4-27395	IV	Left end
SC4-.242972	IV	Left border
SC4-595353	IV	Middle
SC4-1547237	IV	Middle
SC4-1923481	IV	Middle
SC4-2597741	IV	Middle
SC4-2960726	IV	Right border
SC4-3067040	IV	Right end
SC13-112057	XIII	Left border
SC13-306871	XIII	Middle
SC13-488296	XIII	Middle
SC13-632198	XIII	Middle
SC13-740118	XIII	Middle
SC13-979595	XIII	Middle
SC13-1192524	XIII	Right border
SC13-1265238	XIII	Right end
SC13-1279474	XIII	Right end

Table S2. Characteristics of the SNP markers used to genotype populations outcrossed with H39

SNP marker (bp)	Chromosome	Definition
SC1-5616	I	Left end
SC1-6661	I	Left end
SC1-113301	I	Left border
SC1-1004064	I	Middle
SC1-1621994	I	Middle
SC1-2165210	I	Middle
SC1-2929089	I	Middle
SC1-3459715	I	Right border
SC1-3539552	I	Right end
SC1-3541642	I	Right end
SC4-16848	IV	Left end
SC4-21732	IV	Left end
SC4-117209	IV	Left border
SC4-594554	IV	Middle
SC4-1303748	IV	Middle
SC4-1924960	IV	Middle
SC4-2593914	IV	Middle
SC4-2989173	IV	Right border
SC4-3103567	IV	Right end
SC4-3105663	IV	Right end
SC13-15402	XIII	Left end
SC13-104876	XIII	Left border
SC13-306397	XIII	Middle
SC13-488066	XIII	Middle
SC13-740720	XIII	Middle
SC13-979194	XIII	Middle
SC13-1191186	XIII	Right border
SC13-1321587	XIII	Right end
SC13-1321725	XIII	Right end

Table S3. Characteristics of the SNP markers used to genotype populations outcrossed with Bisp119/9-P1

SNP marker (bp)	Chromosome	Definition
SC1-112735	I	Left border
SC1-116099	I	Middle
SC1-1025156	I	Middle
SC1-2195550	I	Middle
SC1-3411633	I	Middle
SC1-3427186	I	Right border
SC1-3538595	I	Right end
SC1-3539952	I	Right end
SC11-16547	XI	Left end
SC11-100527	XI	Left end
SC11-101596	XI	Left border
SC11-583930	XI	Middle
SC11-1116509	XI	Middle
SC11-1593672	XI	Middle
SC11-1600318	XI	Right border
SC11-1690776	XI	Right end
SC11-1692175	XI	Right end

Chapter 5

Identifying QTL underlying the meiotic recombination landscape in *Agaricus bisporus*

Authors:

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Abstract

The two main varieties of *Agaricus bisporus* i.e., var. *bisporus* and var. *burnettii* demonstrate a contrasting meiotic recombination landscape (MRL). Crossovers (CO) are mainly restricted to chromosome ends in the *bisporus* variety while the *burnettii* variety reveals an almost even distribution of CO across the chromosomes. *A. bisporus* is therefore an excellent model in which to study the genes involved in a CO position. A linkage map of an inter-varietal hybrid (var. *bisporus* × var. *burnettii*) has already been constructed by genotyping its homokaryotic offspring. To map genomic regions involved in the differences of the recombination landscape, 71 homokaryotic offspring of the inter-varietal hybrid were outcrossed with an unrelated tester homokaryon of the *bisporus* variety. The homokaryotic offspring isolated from each hybrid were genotyped with SNP markers. Marker pairs were generated for the regions at the ends of chromosomes to assess the CO there or elsewhere on chromosomes for each segregating population. QTLs were identified that signify a substantial part of the variation in the recombination landscape of *A. bisporus*. Because the identified QTLs span large parts of chromosomes, further strategies are being considered to assess the recombination landscape more precisely.

Introduction

The genetic variation has been largely created by changing allele combinations on chromosomes during meiosis. This change mainly relies on the formation of crossovers (CO) between homologous chromosomes. The formation of CO also generates contact between homologous chromosomes and ensures in this manner a proper segregation of each homologue to opposite poles in the first meiotic division (the reductional division) thus halving the chromosome number of the cell. The formation of novel allele combinations on chromosomes is the key to the creation of a new variation in breeding programmes. Understanding mechanisms influencing CO positions and frequencies are thus crucial to optimize breeding programmes (Wijnker and de Jong, 2008; Dirks et al., 2009). Breeding programmes can benefit from a lack of CO when creating an elite genotype in which desired allele combinations need to be preserved through generations. In plant breeding this is achieved by generating inbred families and double haploids (Forster et al., 2007). When introducing new allele combinations into a genetic background, CO are essential to minimize the linkage drag, i.e., transfer of the undesirable genome segment linked to the gene of interest. This means that the CO on either side of the desired locus needs to introduce that locus into a new genetic background. Therefore, by understanding the mechanisms governing the patterns of the CO recombination we will be able to control its position and frequency and manage it for breeding programmes.

The button mushroom *Agaricus bisporus* var. *bisporus* cultivated worldwide (hereafter referred to as the *bisporus* variety) has a typical meiotic recombination landscape (MRL) where COs are predominately restricted to chromosome ends (Sonnenberg et al., 2016). In contrast, the wild type *A. bisporus* var. *burnettii* (hereafter referred to as the *burnettii* variety) reveals that COs are more or less evenly distributed over the entire genome (this thesis, chapter 2). The constantly recurring MRL of the *bisporus* variety therefore causes problems in breeding due to the considerable linkage drag. Studying the traits revealing quantitative inheritance is also considerably cumbersome in the *bisporus* variety due to this restriction of recombination at chromosome ends (Gao et al., 2015; Gao et al., 2016). The linkage maps of the *bisporus* variety are very short considering they only display 13 chromosomes of *A. bisporus* (Summerbell et al., 1989; Kerrigan et al., 1993; Gao et al., 2015; Sonnenberg et al., 2016). The most recent linkage map of 139 homokaryotic single spore isolates (SSIs) of the *bisporus* variety Horst U1 genotyped by 629 SNP markers was only 321 cM in size (Sonnenberg et al., 2016). In contrast, the first linkage map of the *burnettii* variety Bisp119/9 manifested a much greater length of the linkage groups (this thesis, Chapter 2). An inter-varietal hybrid generated by a cross between the *bisporus* and *burnettii* varieties revealed an intermediate recombination pattern inheritance; in which recombination was distributed over the entire length of chromosomes with a tendency of CO at the ends of the chromosomes (Foulongne-Oriol et al., 2010; this thesis, Chapter 3). The reduction of the recombination rate in second generation hybrids generated from a cross between homokaryons of the *bisporus* variety and a homokaryotic offspring from an inter-varietal hybrid (var. *bisporus* × var. *burnettii*) suggested that meiotic recombination is likely to be influenced by more than one gene and can be controlled by quantitative trait loci (QTLs; Foulongne-Oriol et al., 2011).

As *bisporus* and *burnettii* varieties are interfertile and also reveal contrasting MRLs, they were crossed to generate segregating populations to map MRL. Crossing these two varieties was done by recovering the parental haplotypes using protoplasting. The H97 (parental haplotype of the *bisporus* variety) was crossed with Bisp119/9-P4 (parental haplotype of the *burnettii* variety) to make a haploid mapping population from which a genetic linkage map was constructed (this thesis, Chapter 3). Assessing of MRL cannot be done directly on the mapping population because this phenotype is expressed as COs in the offspring of each individual in this population. That infers that the offspring of a mapping population must be crossed with a compatible tester homokaryon (outcrossing) or by intercrossing (sib-mating) the homokaryotic offspring of a mapping population to generate mushroom hybrids (next generation hybrids). This has already been tested. It was revealed that outcrossing the offspring of the mapping population with H39 (parental haplotype of the *bisporus* variety) is currently the most feasible hybrids for generating haploid offspring in which COs are assessed (this thesis, Chapter 4).

The availability of the whole genome sequence of three haplotypes used in the current study (H97, Bisp119/9-P4, and H39) enabled the development of SNP markers which discriminated between the genotype of the mapping population (H97/Bisp119/9-P4) and tester homokaryon H39. The SNP markers developed were used to measure recombination frequencies in the segregating populations at the offspring level. Marker positions were selected in such a way so that we could differentiate between CO at chromosome ends (first/last 200 kb from chromosome ends) or elsewhere on the different chromosomes. QTLs were identified indicating a genetic base for the MRL.

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 University & Research. Two original heterokaryotic lines were involved, i.e., Horst U1 a white commercial *bisporus* strain and Bisp119/9 a wild type *burnettii* strain, originating from the ARP collection (Kerrigan, 1996). Figure 1 describes how segregating populations are generated. The constituent nuclei as homokaryons (H97 and H39) from Horst U1 heterokaryon were available from another study (Sonnenberg et al., 2016). The constituent homokaryons of Bisp119/9 (Bisp119/9-P4 and Bisp119/9-P1) were recovered by protoplasting in accordance with the method previously described (Sonnenberg et al., 1988). Bisp119/9-P4 and H97 were previously crossed to make an F1 hybrid, known as HBT03, from which homokaryotic SSIs were collected to generate a mapping population. The population was mapped with 210 SNP markers at an average distance of 5.7 cM between the markers (this thesis, chapter 3).

The homokaryotic SSIs of the HBT03 hybrid were outcrossed with a compatible tester homokaryon H39 to generate heterokaryons (second generation hybrids). The generation of spore prints from these heterokaryons and the subsequent isolation of homokaryotic SSI were done as previously described (this thesis, chapter 4).
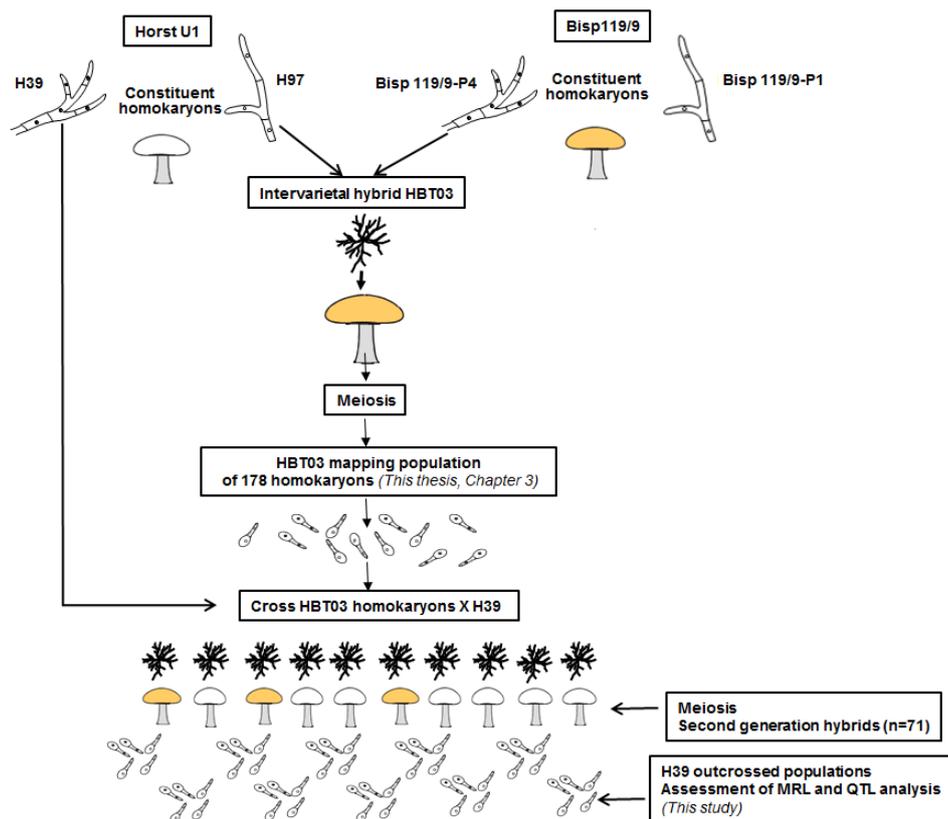
Figure 1. The pedigree presentation of segregating populations used in this study.

The hybrids having the *Bsn-t/b* loci were mainly selected to isolate homokaryotic SSI. The screening of hybrids having *Bsn-t/b* loci was carried out on the genotype file of homokaryotic SSIs of the HBT03 mapping population. There were three SNP markers near to the *BSN* locus on chromosome I in the genotype file (SC1_2244K, SC1_2641K, and SC1_2827K). If the score of these three SNP markers for a homokaryotic SSI of the mapping population was from Bisp119/9-P4 (constituent homokaryon of the *burnettii* variety Bisp119/9; *Bsn-t*), the cross of that SSI with H39 (*Bsn-b*) was selected (Figure 2).

The segregating populations were generated from the selected hybrids (homokaryons derived from HBT03 × H39). To reach ca. 50 SSI in each hybrid, we needed to isolate 200-500 SSIs, depending on the genotype of the hybrid, e.g. *bsn-t/b* or *bsn-b/b*. The process of isolating SSIs was laborious and time-consuming. The time frame in which SSIs germinated and were isolated varied considerably between populations and could take up to 50 days in some cases (data not provided). Furthermore, the ploidy of SSIs derived from each population had to be identified using molecular markers linked to the mating type. This means an extensive number of PCR had to be carried out in order to obtain a number of homokaryotic SSIs from each population.

Marker selection and genotyping

With the availability of the genome sequence and the physical position of the SNP in three haplotypes, i.e., H97, H39, and Bisp119/9-P4 SNP markers were designed. The homokaryotic SSIs isolated from each second generation hybrid represent recombinants between the genome of HBT03 hybrid (H97 and Bisp119/9-P4) and H39.
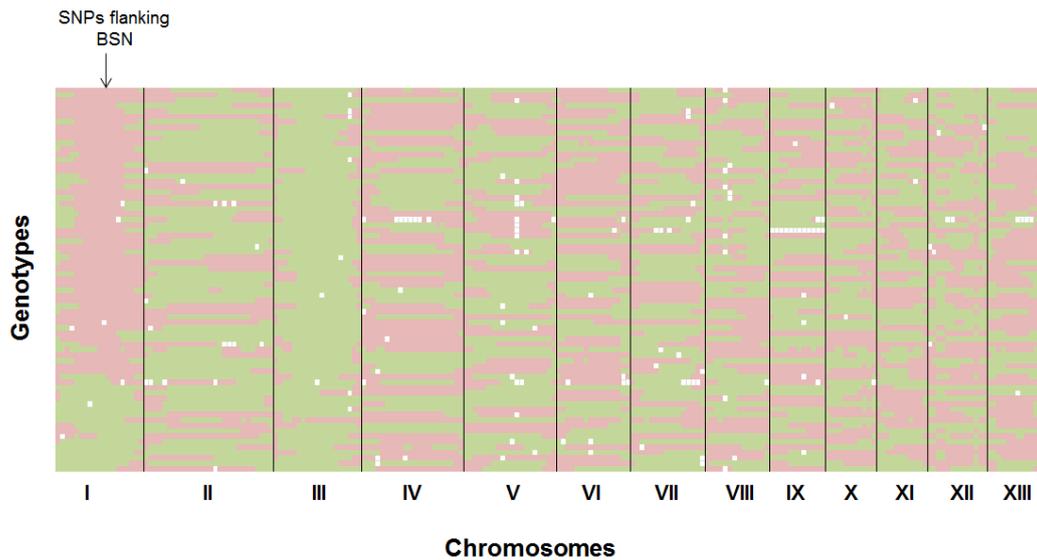
Figure 2. A genotype plot of selected homokaryons of the HBT03 population outcrossed with H39.
Red indicates the genotype of Bisp119/9-P4; green indicates the genotype of H97. White indicates missing values.

Therefore, SNP markers were selected differentiating between H97/Bisp119/9-P4 on the one hand and H39 on the other. Two SNP markers were generated for both chromosome ends, one as far towards the chromosome end as possible and the other in an area 150-200 kb away from chromosome ends, thus four markers per chromosome (Figure 3). In this way, we were able to detect COs at the ends of the chromosome (first/last 150-200 kb) as well as COs taking place in the rest (middle) of the chromosome. This marker setup did not allow for a detection of double COs in the larger middle part of the chromosome. DNA samples of all homokaryotic SSIs were used for genotyping in the Dr. van Haeringen Laboratorium (VHL), Wageningen, using the KASPar SNP Genotyping System.

Quantification of MRL

As not all homokaryotic SSIs in each segregating population genotyped with SNP markers revealed a reliable marker score especially for SNP markers at the far end of the chromosomes, a method was designed to use as much data as possible. Throughout the initial step of MRL quantification, the frequency of individuals having COs for each pair of markers (pairs at chromosome ends as well as pairs flanking the middle) was calculated. This frequency is given by the number of individuals having COs between marker pairs divided by the number of individuals with data for both markers in a pair.

(1)

$$Fraction\ of\ individual = \frac{\#\ individuals\ with\ CO\ between\ marker\ pair}{\#\ individuals\ with\ data\ for\ both\ markers\ of\ the\ marker\ pair}$$

Using this kind of normalisation a correction is made for the varying number of individuals in each population. Three initial frequencies were calculated using the above-mentioned formula,

i.e., freq. 1 (COs at the left end of the chromosome), freq. 2 (COs in the middle) and freq. 3 (COs at the right end of the chromosome) as illustrated in Figure 3.
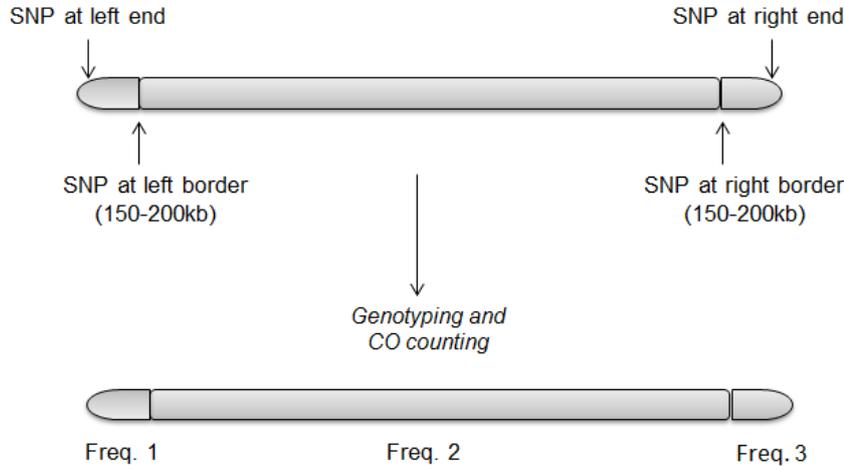
Figure 3. The position of the SNP markers on the chromosome to phenotype MRL.

The calculated frequencies for each region per chromosome (freq. 1, 2, and 3) were subsequently used to calculate the total number of CO frequencies at different positions in various ways as follows.

Let n be the number of frequencies being averaged for each chromosome in each segregating population, xi be the value of each frequency (freq. 1, freq. 2, and freq. 3), and j be the chromosome number. Therefore, COs at the ends of each chromosome is expressed as

(2)

$$\overline{Ch_j\ Ends} = \frac{1}{n} * \sum_{i=1}^{n} xi$$

Using $Ch_j\ Ends$ of each chromosome where j is the chromosome number and n is the number of chromosomes being averaged for $Ch_j\ Ends$, we estimated the ends average in each segregating population as follows:

(3)

$$Endsaverage\ (\overline{Ch_j\ Ends}) = \frac{1}{n} * \sum_{j=1}^{n} \overline{Ch_j\ Ends}$$

Given the number of COs in the middle of the chromosomes (freq. 2), the middle average was estimated in each segregating population as follows:

(4)

$$Middle\ average = \frac{1}{n} * \sum_{i=1}^{n} mi$$

where n is the number of chromosomes so that their freq. 2 is being averaged and mi is freq. 2 for each chromosome in each segregating population.

Using freq. 1, freq. 2, and freq. 3 for each chromosome per population, MRL can also be expressed as a fraction of the CO at the chromosome ends and also in chromosome middle. For each chromosome, the ends fraction and middle fraction can be estimated as follows.

$$End_j\ fraction = \frac{freq.1 + freq.3}{freq.1 + freq.2 + freq.3} \qquad (5)$$

$$Middle_j\ fraction = \frac{freq.2}{freq.1 + freq.2 + freq.3} \qquad (6)$$

Where j is the chromosome number.

Given the ends and middle averages calculated above, the total end fraction and total middle fraction for each segregating population can be calculated as follows.

$$(7)$$
$$Total\ end\ fraction\ = \frac{Average\ (Ch_j\ Ends)}{Average\ (Ch_j\ Ends) + Middle\ average}$$

$$(8)$$
$$Total\ middle\ fraction\ = \frac{Middle\ average}{Middle\ average + Average\ (Ch_j\ Ends)}$$

Ultimately, end CO/middle CO ratio is estimated as to which is the ratio of COs at both ends of the chromosomes by COs in the middle of the chromosomes per segregating population.

QTL detection

The CO frequencies and fractions described above were used as phenotypic traits for QTL detection. QTL detection was carried out with Simple Interval Mapping (SIM), Multiple QTL Mapping (MQM), and stepwise QTL (SW) using the "qtl" package of the R statistical language and environment (R/ qtl) version 1.40.8 (Broman et al., 2003; Arends et al., 2010). The explained variance (EV %) by each QTL indicates that the phenotypic variation and the additive effects of each QTL have been estimated. The genome-wide significant LOD threshold was estimated by doing permutation tests. Altogether we carried out 1000 permutations of all the phenotypic values relative to those of the genotypes.

Results

Phenotyping MRL

In order to map MRL in *A. bisporus* we used 71 second generation hybrids that were generated by outcrossing the homokaryotic SSIs of the HBT03 population with H39 (constituent homokaryon of the *bisporus* variety, Horst U1). This type seemed to be the most feasible hybrids at the time this experiment was carried out (this thesis, Chapter 4). As we were unable to find any significant correlation between *BSN* locus and MRL (this thesis, chapter 4), we generated populations mainly from hybrids having *Bsn-t/b* loci. Following this method, isolating homokaryotic SSIs was less laborious because hybrids having *Bsn-t/b* are expected to produce more homokaryons than heterokaryons.

The homokaryotic offspring of these second generation hybrids have been allocated to either the mating type of the mapping population (H97 or Bisp 119/9-P4) or the mating type of the tester homokaryon (H39). The molecular markers used to determine the ploidy were designed to differentiate between the mating type of Bisp119/9-P4 or H97 on one hand and the mating type of H39 on the other. The offspring in each population were isolated randomly by time period and often showed a bias in mating type loci towards H39 (*bisporus* tester homokaryon) genome (Figure 4).
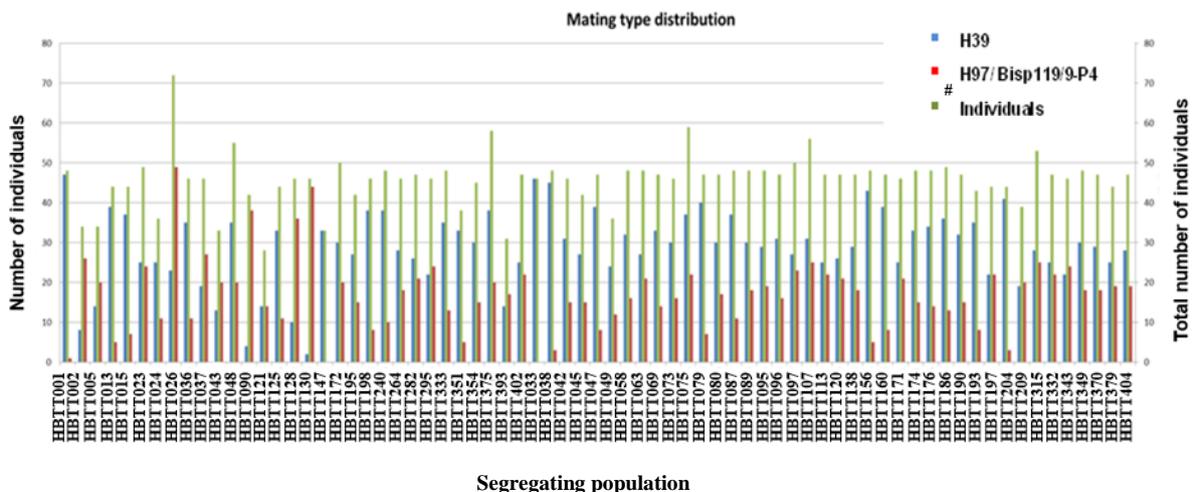
Figure 4. Distribution of mating types in H39 outcrossed populations used in the current study.

The SNP markers designed to genotype homokaryons are listed in Table 1. In order to make a proper comparison of CO positions on different chromosomes it is advisable that SNP markers are generated for comparable areas (this thesis, Chapter 4). We were only able to design SNP markers at desired positions described in the Materials and Methods section for ten out of 13 chromosomes. In this study, chromosomes III, VIII, and IX were not genotyped due to the absence of SNP markers either at extreme ends of chromosome or in the area 150-200 kb away from chromosome ends. It was not always possible to design molecular markers at the extreme ends of the chromosome due to the quality of the genome sequence and presence of the repetitive elements in these areas (Sonnenberg et al., 2016). Therefore, there is some variation

in the position of the most outward markers for the different chromosomes (Table 1), varying from 5.6 to 25 kb from the end of a chromosome. We might therefore miss CO in these uncovered end regions.

Table 1. Characteristics of the SNP markers used to genotype homokaryotic SSIs derived from segregating populations outcrossed with H39

Chromosome	Position of SNP markers (bp) [a]			
	left end	left border	Right end	Right border
I	5,616	193,190	8,563	159,055
II	10,804	176,416	16,785	145,501
IV	16,848	168,191	7,126	168,308
V	10,280	162,104	15,276	142,108
VI	15,265	139,296	19,586	151,671
VII	14,598	154,944	25,164	167,863
X	14,118	176,685	18,752	127,650
XI	16,359	169,013	9,625	184,095
XII	12,208	139,671	10,518	183,114
XIII	15,402	169,133	12,348	176,368

[a] Marker range relative to the H97 v3.1 genome.

A total of 2507 homokaryotic SSIs derived from 71 segregating populations (ca. 35 homokaryons per population) were genotyped with 40 SNP markers covering ten chromosomes. The simulation experiment previously revealed that a minimum of 24 homokaryons per population is required to assess the CO position per population (data not given). As the SSIs were selected for haploidy in the mating type, we expected to have homozygous marker scores. However, unexpected heterozygous scores as well as missing values of some markers, especially those at the ends of the chromosomes, complicated our analysis. Of the ten genotyped chromosomes, sufficient and reliable marker scores were only obtained for chromosomes I, II, IV, V, VI, VII, and X. Hence chromosomes XI, XII and XIII were excluded from further analysis. If we assume that MRL is controlled by the same mechanism over the entire genome, assessing MRL on seven chromosomes might provide valuable information to perform the QTL analysis.

The numbers of homokaryons among 71 segregating populations were corrected for variability by calculating the average frequencies of CO at the end and middle positions on each chromosome. In order to obtain data for each population, the frequencies of CO at the ends and middle regions was calculated over all chromosomes as well as the fractions of these CO and of the total CO. In order to have as much data as possible, the average CO frequency in the middle of the chromosomes (middle average) per segregating population, was calculated by

including or excluding freq. 2 of chromosomes XI, XII and XIII. As explained above, the data for chromosomes XI, XII and XIII was excluded for calculating freq. 1 and freq. 3, due to unreliable marker scores in three SNPs, i.e. one per chromosome, placed at the end of the respected chromosomes. However, CO frequency in the middle of chromosomes XI, XII and XIII could be calculated (freq. 2) and be further used to make an average of recombination in the middle of the chromosomes per segregating population. This value is expressed as "middleaveragec" in our analysis. If freq. 2 of the chromosomes XI, XII and XIII had not been used, the average CO in the middle of the chromosome would have been named "middle average."

QTL mapping of MRL

Fractions and frequencies of CO and the genetic linkage map of the HBT03 population were used to perform QTL analysis using r/qtl software implemented in the statistical package of R. Segregation of the SNP markers in the genetic linkage map of HBT03 revealed quite an extreme distortion for the large part of chromosome III and to a lesser extent in chromosome II towards H97 genome (Figure 5). The models of SIM, MQM and SW mapping were used to identify and characterize QTL for MRL. In the SIM model the existence of a QTL between marker pairs in many positions has been tested (Lander and Botstein, 1989), while in MQM models co-factors (markers) are selected by multiple regressions and a backwards elimination which can help to analyse multiple traits simultaneously and identify QTLs that were previously unknown. The stepwise QTL is an approach in which the likelihood of the QTL model is optimized by using penalties for including more QTL and interactions between QTLs.
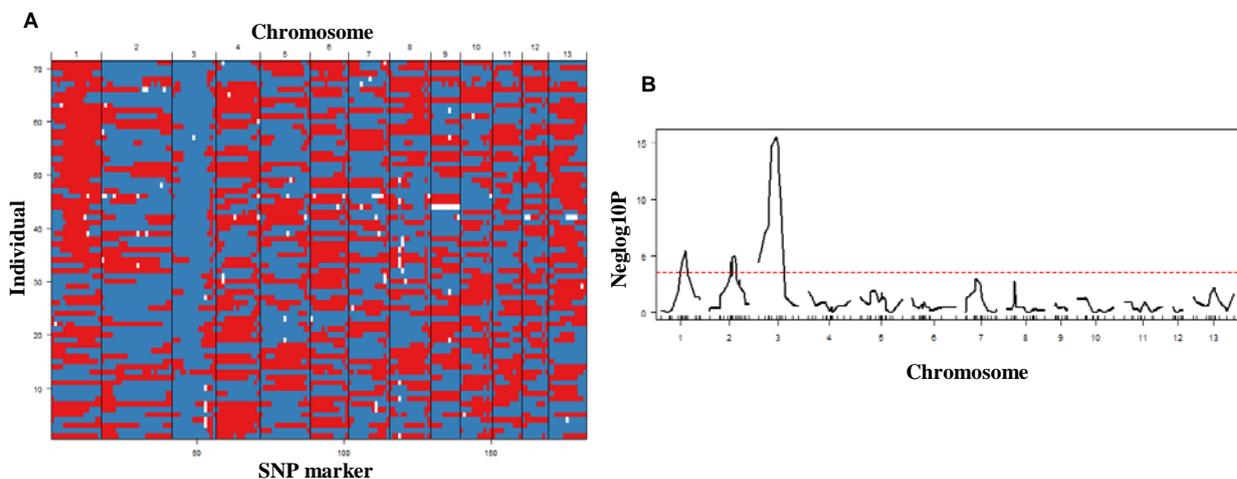
Figure 5. Pattern of segregation distortion in HBT03 mapping population. A) Genotype plot of individuals of the HBT03 mapping population; Red indicates the genotype of Bisp119/9-P4; blue indicates the genotype of H97. White indicates missing values. B) Segregation pattern of the genome of Bisp119/9-P4 and H97 in the offspring of the HBT03 population. Chromosome III is very skewed. A red dash is a significant threshold at a p-value of 5%.

Several steps need to be checked while performing a detailed QTL analysis. After imputation of all missing genotypic data, a plot of recombination fractions of pairwise markers along with LOD scores for the testing r = ½ is generated (Figure 6A). The recombination plot helps to reveal errors in the order of markers. This plot indicates that markers used in the HBT03 population linkage map actually belong to the corresponding chromosomes. The results of

MQM and SW mapping were always compared to the SIM model using Haley-Knott regression (Figure 6B; Haley and Knott, 1992). MQM and SW analysis often confirmed the results of SIM (scan-one). The effect of each QTL was estimated on a phenotype's mean as half the difference between the phenotype's mean for two homozygotes (Figure 6C). The estimated effect plot (additive effect) reveals the difference between the phenotype's mean when the A-allele (Bisp119/9-P4) is present minus the phenotype's mean when the B-allele (H97) is present. This plot helps us to perceive how likely it is that QTL is evident in a specific position, which, in the end, also depends on the allele distribution (segregation of A and B-alleles) in that position.
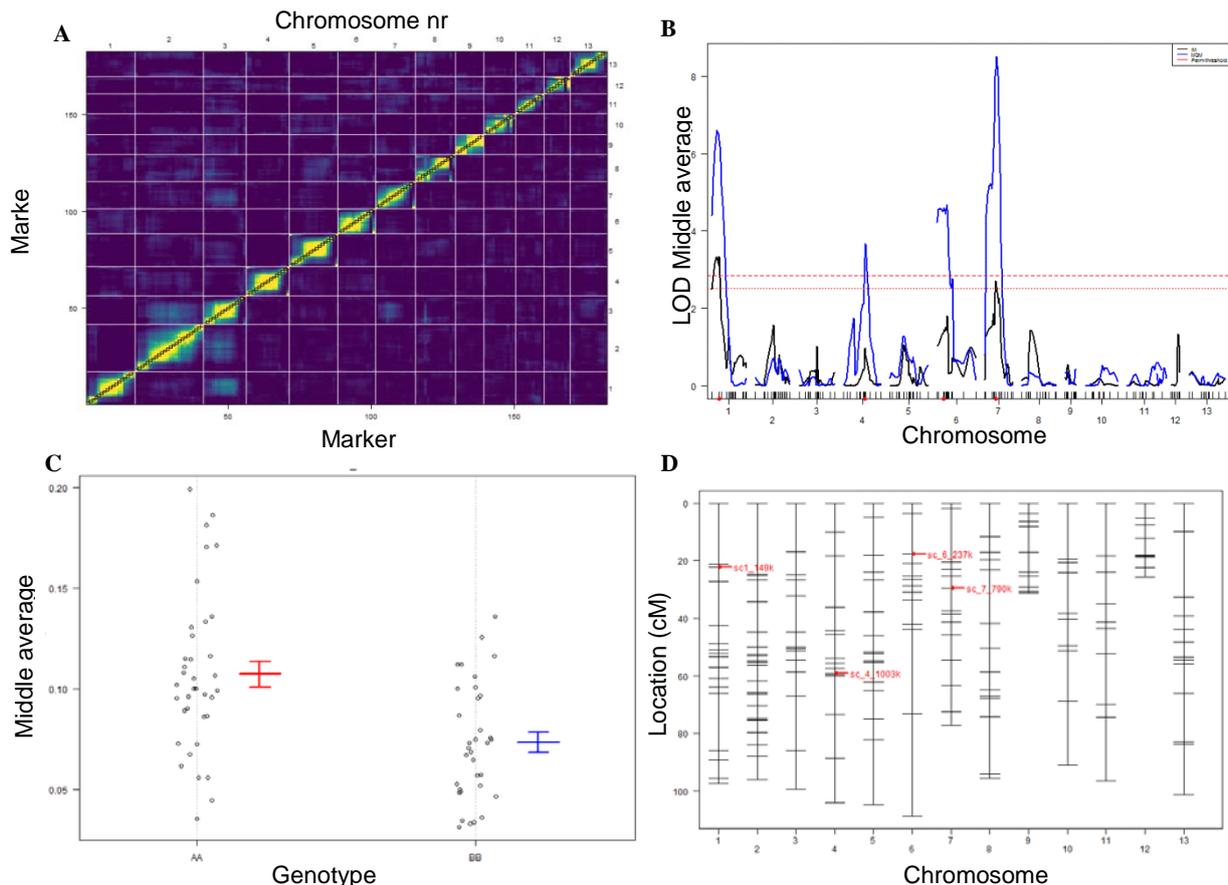
Figure 6. R/qtl output for MRL in *A. bisporus*. A) pairwise recombination fractions. all markers against all markers. Markers are in mapping order in both axes. The upper triangle reveals the estimated recombination fractions and the lower triangle the LOD score. Areas of low recombination frequency (strong linkage/high LOD) are represented by yellow squares; Areas of recombination frequency> 0.5 (no linkage/low LOD) are represented by blue squares. Green squares identify values between those two extremes; B) LOD profile comparison between MQM (blue) and Haley-Knott interval mapping (black) for the average recombination at the ends of the chromosomes. Dashes indicate the LOD treshholds; C) Plot of the average of recombination in the middle of the chromosomes against the genotype group (A corresponds to Bisp119/9-P4 and B corresponds to H97) at marker SC1-149k. D) Genetic map showing the significant QTL markers using average values of recombination in the middle.

In R/qtl MQM a backward elimination of cofactors is applied. Initially we added five cofactors as possible cofactors to the number of QTL that were detected by the SIM method. The QTL having the highest LOD score and at a certain minimum distance from each other were selected. We chose the distance of 15 cM as a default value, meaning that when two cofactors are at 15 cM from one another, the one with the highest LOD is retained and another cofactor elsewhere

in the genome is selected. Then an automated backward elimination of cofactors was performed on the initially selected cofactors which would help to eliminate cofactors that do not significantly contribute to the model fit (how many QTLs are controlling a trait). The remaining cofactors were finally used in the MQM algorithm as covariates during mapping. A genome-wide permutation test provided an LOD threshold of 3 to declare significant QTLs. The final selected QTL model was ultimately used to map MRL, and for each trait value a plot showing significant markers are automatically produced (Figure 6D). We used 36 individual values (CO fractions and frequencies) per segregating population to map MRL (Supplementary material Figure S.1) but to enhance the readability of this chapter we only present those values that resulted in the identification of significant QTL.

A correlation matrix between the frequency of CO at the ends, the fraction of CO at the ends, the frequency of middle CO and the fraction of middle CO for each chromosome was created, all revealing a correlation with R^2 lower than 0.5 (Figure 7A, B). Some fractions and frequencies revealed stronger correlation compared to others. For example, the frequency of CO in the middle of chromosome VI correlated more with the frequency of CO in the middle of chromosomes I and II, indicating that a more or less similar number of CO had been detected for these three chromosomes (Figure 7B).
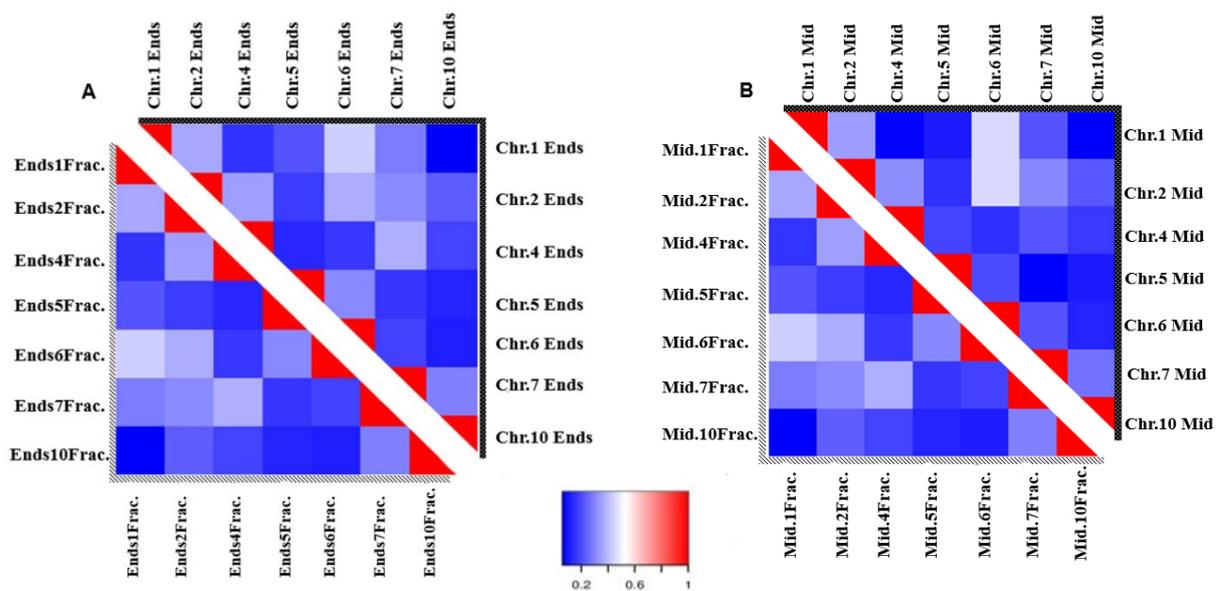
Figure 7. **Correlation matrix between phenotypic traits measured to map MRL.** A) the upper right triangle presents the correlation of ends in CO frequency between chromosomes examined in this study and the lower right triangle presents the correlation of ends in CO fraction between chromosomes; B) the upper right triangle presents the correlation of middle CO frequency between chromosomes and the lower right triangle presents the correlation of middle CO fraction between chromosomes. The light blue corresponds to a stronger correlation and the darker blue corresponds to a weaker correlation.

The phenotypic traits that are quantitatively inherited have a continuous distribution (Liu, 1997). An almost continuous variation was seen in the frequency distribution of traits in all segregating populations. Figure 8 depicts the frequency distribution of traits that revealed significant QTL when using SW mapping. When the homokaryotic SSIs isolated from a cross between Bisp119/9-P4 (*burnettii* type) and H97 (*bisporus* type) were outcrossed with H39

(*bisporus* type) to generate segregating populations, approximately two-thirds of the genome of the individuals carry the alleles of *bisporus* variety. The ends CO fractions and frequencies were, therefore, higher than the middle CO fractions and frequencies.

For all QTL models and most of the phenotypic values used in this study, the most significant QTLs were identified in the middle and on the left side of chromosome I. In order to visualize traits better for which QTLs were detected based on the LOD profiles using SIM, MQM, and SW mapping, heat maps were created (Figure 9). These heat maps enabled a quick overview of all QTLs to interpret further the genetic base of the traits showing significant QTL. When phenotypic values calculated by averaging the CO at chromosome ends were used, a QTL in the middle of chromosome I was detected having major contributions of CO data at the ends of chromosomes IV and X. When phenotypic values calculated by averaging CO in the middle of the chromosome were used a QTL on the left side of chromosome I was detected having major contribution from CO data in the middle of chromosome I. Other significant QTLs were also identified for phenotypic values calculated by CO in the middle of the chromosomes on chromosome IV, VI, and VII using MQM and SW mapping. All estimated QTL positions cover a large part of the relevant chromosomes.

The QTL identified in the middle of chromosome I explained approximately 30%, and the one on the left side of chromosome I about 20% of the phenotypic variation in all three QTL models (SIM, MQM, SW). For both QTLs, a high value allele for the traits was donated by Bisp119/9-P4 (*burnettii* type). Data from the CO fraction at the ends of chromosome I, ratio of the end CO/middle CO and the total fraction of COs at the ends of the chromosome resulted in a high value allele of H97 (*bisporus* type). Significant QTLs were also mapped on chromosomes IV, VI, and VII. The QTL identified on chromosome IV explained ~ 12% using MQM and 14% using SW, the one on chromosome VI, 13% (MQM & SW) and, the one on chromosome VII ~ 23% (MQM & SW) of the phenotypic variation. Here, the high value allele was donated by Bisp119/9-P4. An output file summarized QTL analysis using IM, MQM, and SW mapping, providing an overview of all the significant QTLs, their LOD score, positions, explained variation, confidence interval and high value allele (Supplementary materials, Table S2). Surprisingly enough, the phenotypic values calculated from COs in the middle and at the ends of the chromosomes appeared to be controlled by different QTLs.

The interaction between QTLs (epistasis) detected for a trait can account for a significant part of the variation in QTL analysis (Doerge, 2002; Phillips, 2008) and requires a large sample size. In order to predict the effect of introgression of the QTL into a new genetic background, the epistatic interaction has to be known. Such epistatic effects are usually detected only when population sizes that are studied are substantially larger than ours. Nevertheless, when interactions are significant they might even be detected in smaller populations such as those we used here. We were able to calculate the epistatic interactions between QTLs with the QTLs that were detected by the multiple QTL models from MQM. We could not find a QTL model in which epistatic interactions were significant.
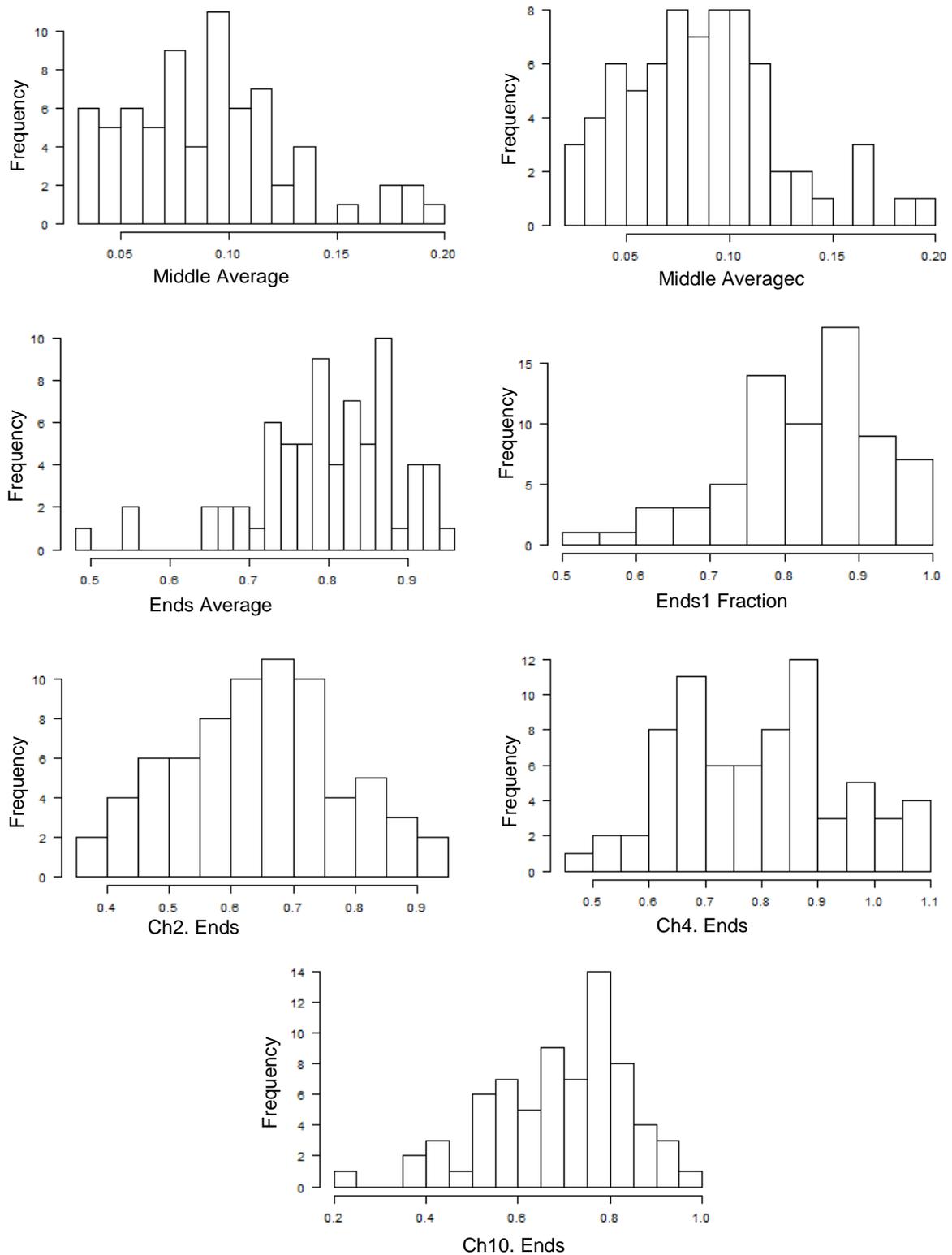
Figure 8. Frequency distribution histograms for traits showing significant QTL in SW QTL mapping of 71 segregating populations. The *horizontal axis* indicates data range traits, and the *vertical axis* indicates the frequency of populations.
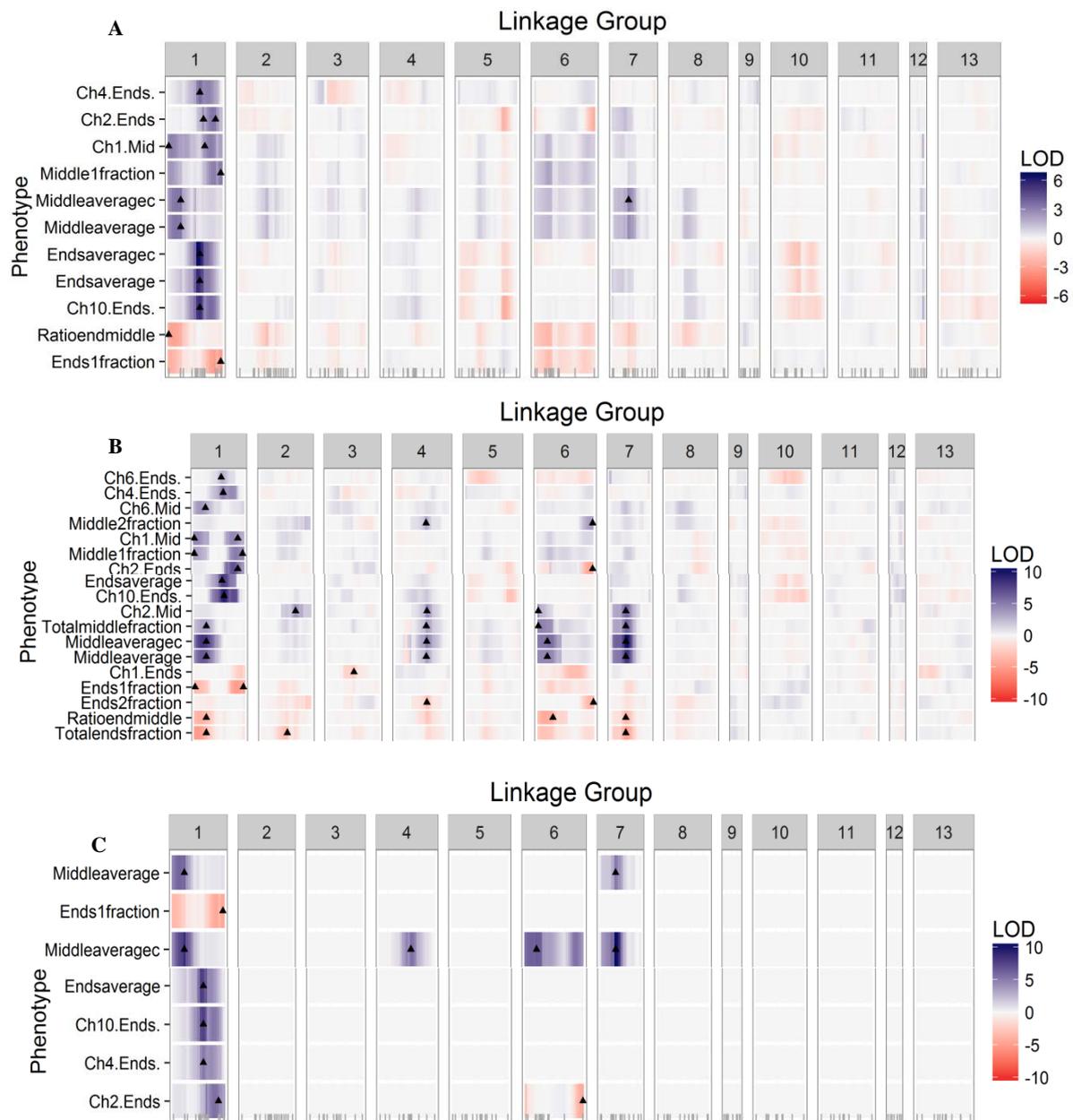
Figure 9. The heats maps showing significant QTLs of the calculated traits. A) a heat map using SIM; B) a heat map using MQM; C) a heat map using SW mapping. Columns indicate linkage groups (chromosomes); rows indicate individual trait LOD profile for which significant QTLs were detected. A false colour scale is used to indicate the QTL significance. Positive value (blue) represents a larger effect of Bisp119/9-P4, negative value (red) of H97.

Discussion

Our analysis presents the first QTL mapping of *A. bisporus* recombination landscape. Using the recombination frequency in two chromosomal regions (at chromosome ends and in the middle of chromosomes) obtained by genotyping the offspring of the second generation hybrids (the offspring of a cross between *bisporus* and *burnettii* variety × *bisporus* tester homokaryon), we

identified genomic regions that are likely to control the recombination landscape. We determined a method for converting genotype to phenotype as values for QTL mapping and found significant QTLs for MRL. However, the broad area covered by QTL also indicates that an improved assessment of CO is needed for the precise mapping of areas involved in MRL in *A. bisporus*.

At the outset of this research we wanted to detect CO for all chromosomes in the offspring of the segregating populations. This appeared to be difficult because markers at the extreme ends of the chromosomes could not always be developed. Data produced by resequencing the haploid offspring of the *bisporus* variety has shown that approximately 30% of the CO occurs in the last 25 kb of the chromosome ends. This data could only be obtained by resequencing whole genomes in haploid SSIs (Sonnenberg et al., 2016). Resequencing a set of offspring from 71 populations was too costly at the time we conducted this research. Surprisingly enough, two significantly distinct QTLs were found on chromosome I using either data from CO at the chromosome ends or CO in the middle of the chromosomes. During meiosis, each chromosome has at least one CO to ensure proper segregation of the chromosome (obligate CO). Because no detailed studies are available on CO interference in *A. bisporus*, we have no indication that interference does not occur (Sonnenberg et al., 2016). Therefore, CO at the ends of the chromosome makes the occurrence of another CO elsewhere on that particular chromosome less likely. This means that the values calculated for CO at the chromosome ends are to some extent complementary to those of the COs in the middle of the chromosomes. If this is the case, one would expect that data from CO at chromosome ends and from CO in the middle would generate QTL in similar positions. It is therefore surprising that QTL generated with CO data at chromosome ends does not always co-localize with QTL generated with data from CO in the middle of chromosomes. Also the total number of CO varies between different populations. Different reasons could account for that.

An incomplete picture of MRL of segregating populations could be the most likely reason due to difficulties that have already been described. The incompleteness might be more dramatic for CO events at chromosome ends. Although the outcross of the mapping population with a *bisporus* tester line might not have an MRL landscape as extreme as the *bisporus* variety, we will certainly have missed CO at the chromosome ends. As we used markers in areas 200 kb further from chromosome ends to estimate CO in the middle of chromosomes, we might also have missed double CO. Nevertheless, significant QTL were detected which could explain a substantial part of the variation in the MRL. Having fewer data points for some populations due to dropouts of some markers could be another reason. In addition, each population's offspring were isolated in specific time periods and they were randomly isolated as well as genotyped. This means that there could be a bias in our selection as observed in the mating type loci.

There could well be QTLs that were not identified in the current study. Resolution of the QTL study can be increased by including more data points. When SW mapping was applied, two more QTLs were found in chromosomes IV and VI by using data from chromosomes XI, XII, and XIII. In addition to the foregoing, there was a significant segregation distortion in chromosome III in the genetic map of the mapping population used in this study. If the genetic map used for the association between the genotype and phenotype indicates segregation

distortion for loci, the power of the QTL and additive QTL effect can also be affected (Xie et al., 2013).

To depict a precise and complete picture of MRL, CO events need to be assessed in more detail. The sequencing based method provides a powerful tool towards a more precise determination of the recombination (Huang et al., 2009). The whole genome resequencing of the haploid SSIs was considered to be capable of capturing all CO events. We can evidently see that the frequency of non-recombinant chromosomes in the haploid SSIs of Horst U1 genotyped by SNP markers is now twice as high as when they are re-sequenced (Sonnenberg et al., 2016). In addition, whole genome resequencing enables the detection of gene conversion which will provide a better insight into a detailed portrait of a recombination landscape.

At the outset meiosis chromosomes are duplicated, generating two sister chromatids. Following duplication, yeast generates 140-170 double strand breaks (DSBs) along the whole length of the chromosomes (Buhler et al., 2007), a process which concurrently initiates homology search, synapsis through the formation of synaptonemal complex and meiotic recombination. While synapsis occur, DSBs are processed and repaired by DNA repair pathways to generate COs and gene conversion if sister chromatids are not used as substrates for recombinational repair (Allers and Lichten, 2001; Puchta, 2005). While CO events are the reciprocal exchange between parental chromosomes, gene conversions are non-reciprocal exchanges between parental chromosomes resulting in 3:1 segregation of the parental nucleotides.

The potential of using a resequencing method to detect gene conversion in the Horst U1 heterokaryotic offspring was recently presented by Sonnenberg et al. (2016) in which CO associated with gene conversion were detected. In *A. bisporus* two non-sister nuclei are preferentially paired in one spore enabling us to dissect half tetrads and detect gene conversion if the chromatids involved in the same recombination event are paired into one spore. It was revealed that gene conversion contributes to a small portion of the new variation in *A. bisporus* and yeast (Mancera et al., 2008; Sonnenberg et al., 2016). However, not all gene conversion is always detected due to systematic errors in sequencing or the distance between SNP markers flanking gene conversion.

Double CO occasionally occurs but not as often as single CO. If two positions on a chromosome are close to each other, double CO occurs less often than between two positions that are farther apart. Consequently, the more double CO there are in the middle of the chromosome might have been missed on the chromosome ends in the current study. A comparison of the frequencies of recombining and non-recombining post-meiotic chromosomes between *bisporus* variety Horst U1 has been made (Sonnenberg et al., 2016), Bisp119/9 (this thesis, Chapter 2), and the inter-varietal HBT03 population (this thesis, Chapter 3) demonstrates how many double CO was observed when using more than four markers per chromosome (Table 2). More double CO was found in Bisp119/9 and HBT03 population than in Horst U1 when using the SNP genotyping method. Using WGS the number of double CO in Horst U1 was found to be almost the same as those in the HBT03 inter-varietal hybrid which indicates that the number of CO observed has clearly been influenced by the genotyping method. Moreover, the difference in CO between the *bisporus* and *burnettii* varieties is considered to be the position rather than

frequency (Sonnenberg et al., 2016). In our comparison we found more double CO in the *burnettii* variety Bisp119/9 than in the *bisporus* variety Horst U1 which indicates that CO in two varieties might also differ in frequency which requires a WSG of the *burnettii* variety offspring in order to reveal a precise assessment of CO frequency in this variety.

Table 2. A comparison of the frequencies of the type of post-meiotic chromosomes observed in offspring of three populations Bisp119/9 (This thesis, Chapter 2), HBT03 (This thesis, Chapter 3) and Horst U1 (Sonnenberg et al., 2016).

| | *bisporus* var. Horst U1 | | *burnettii* var. Bisp119/9 | Intervarietal hybrid HBT03 |
	629 SNP markers (%)	WGS (%)	752 SNP markers (%)	176 SNP markers (%)
Parental types	96	45	32	54
1 CO	2	45	43	34
2 CO	1	9	22	10
3 CO	0	1	2	1.5

As mentioned in the previous chapter, we had three options to generate offspring from the second generation hybrids, i.e. intercrossing and outcrossing with either a *bisporus* or a *burnettii* homokaryon. Outcrossing with a tester homokaryon generates a shift of the phenotype towards either the *bisporus* or *burnettii* MRL, hence reducing the phenotypic range of segregation. An intercross would have generated the best data in principle as no new genome is introduced and for each allele involved in the trait an homozygous state can be generated allowing the maximal effect on the phenotype and therefore optimal for the QTL analysis. This option was rejected due to inbreeding depression and non-segregating genome areas caused by homozygosity. The drawbacks could be overcome when generating two mapping populations parallel to one another, each having parental homokaryons of the *bisporus* and *burnettii* types yet with unrelated genomes. This allows the intercross between individuals of the two mapping populations to generate offspring for which CO can be assessed. This avoids inbreeding and, due to the genetic distance between parents, enough segregating markers are available.

The key to successful QTL mapping is high-quality data. Phenotyping costs are relatively high depending on the phenotype and sample size. Costs for phenotyping MRL may even be higher than for other traits e.g., resistance to a pathogen because in addition to the cost of generating crosses and mushroom hybrid cultivation, the individuals of each hybrid must be isolated which can be time consuming and laborious. Following individual isolation, the genotyping of each individual is an additional cost.

In breeding programmes, the favourable traits of a donor genotype are transferred to the existing genotype by interspecific crossing followed by several series of consecutive backcrossing. Introgression of a desired locus into a recipient genotype requires CO recombination at either side of that locus, while the other loci of two genotypes (donor and recipient) are also combined. Many of these allele combinations are unwanted and are further eliminated by CO recombination in consecutive backcrossing. Thus, the frequency and the position of CO recombination are important in breeding programmes. The different meiotic behaviour of the *bisporus* and *burnettii* varieties allowed us to locate genomic regions that are likely to control

MRL. Introducing the "normal" MRL trait into breeding lines that are mainly *bisporus* types would facilitate breeding button mushrooms considerably. This study can also contribute to the approach of reverse breeding in which the homozygous parents can be re-generated from starting a heterozygote hybrid by eliminating COs and thus creating non-recombining gametes (Dirks et al., 2009). The elimination of CO events has been achieved by using knock-out *dmc1*, which leads eventually to the formation of unviable gametes due to an unbalanced segregation of chromosomes. Restricting CO to the extreme ends of chromosomes ensures a balanced segregation of homologues whereas the greater part of chromosomes remains parental types. Identifying the genes involved in mechanisms directing the two different MRL can therefore be very useful in many breeding programmes

Acknowledgments

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Supplementary materials

Table S1. Cross-object containing all data for the 36 measured traits (in columns) in the 71 segregating populations (in rows) which can be used as an input file for R/qtl.

Table S2. Summary of all QTL results, providing an overview of all detected QTL, their peak LOD score, position, confidence interval and direction for, Microsoft Excel (xls) format.

Chapter 6

General discussion

Managing crossover recombination (CO), in terms of directing CO to the most desirable positions, increasing CO frequency or eliminating CO events along the chromosomes has been of long standing interest for breeding companies (Wijnker and de Jong, 2008). The worldwide cultivated button mushroom *Agaricus bisporus* var. *bisporus* has a typical Meiotic Recombination Landscape (MRL) in which CO events are predominantly restricted to the chromosome ends (Sonnenberg et al., 2016). For mushroom breeders the restriction of CO at chromosome ends has become one of the major limitations for improving or generating new mushroom hybrids due to considerable linkage drag. Previous studies suggested that in another variety of *A. bisporus*, i.e., *burnettii* variety, CO seemed to be spread more evenly across the chromosomes (Foulongne-Oriol et al., 2010; Foulongne-Oriol et al., 2011). These studies were carried out using an inter-varietal hybrid (var. *bisporus* × var. *burnettii*) in which an elevated recombination frequency was observed compared to that of the *bisporus* variety; hence MRL of the *burnettii* variety has never really been studied. We hypothesised that the MRL of two varieties of *A. bisporus* is a quantitatively inherited trait (QTL), and therefore we aimed to identify genomic regions, underlying different MRL of *A. bisporus*, i.e., CO confined at chromosome ends and/or CO spread across the chromosomes. In order to achieve this goal, we evaluated MRL of the *burnettii* variety in more detail using genetic linkage analysis which revealed a contrasting MRL to that of the *bisporus* variety (Chapter 2). Subsequently, *bisporus* and *burnettii* varieties were crossed to generate a mapping population from which a genetic linkage map was constructed (Chapter 3), which was used to analyse the QTL inheritance of MRL (Chapters 4 and 5). In the following part of this chapter, I first of all explain how to assess CO more precisely and which type of segregating population could improve the future QTL study of MRL. Thereafter factors affecting MRL are considered and how these can be studied in more detail. The obstacles in mushroom breeding are addressed and the potential applications of the MRL of *A. bisporus* for mushroom and plant breeding programmes will be taken into consideration in the last section.

Genotyping technologies to assess CO recombination

CO recombination have been non-randomly placed at different positions in various meiotic nuclei along a particular chromosome, which together give rise to a specific MRL. To precisely assess an MRL, all CO events need to be detected. Due to the predominant localization of CO at the ends of the chromosome in the *bisporus* variety, precise assessment of MRL will be difficult using a limited number of molecular markers (Sonnenberg et al., 2016). This is due to the fact that chromosome ends harbour repeats (telomeres and subtelomeric sequences) and the quality of genome sequences at chromosome ends is often low.

The construction of linkage maps have long been in practice for this variety, all resulting in very short linkage maps due to difficulties in generating markers for chromosome ends or missing sequences of chromosome ends (Kerrigan et al., 1993; Gao et al., 2015; Sonnenberg et al., 2016). In Chapter five, we used *bisporus* tester homokaryons to generate second generation hybrids from the mapping population. These were subsequently used to isolate the homokaryotic offspring in which the MRL could be assessed. Due to the use of the *bisporus* type of tester homokaryon, CO events shifted more to the ends of the chromosomes in segregating populations. To assess the phenotype "MRL", we used only four markers per

chromosome in order to limit costs. For each chromosome we tried to generate a marker as far as possible at both chromosome ends and a marker 150-200 kb distal from the end. In this way we were able to assess CO either at chromosome ends or elsewhere, i.e. in the middle. Due to the aforementioned difficulties the data sets were often incomplete when generating reliable markers for chromosome ends. This might have affected the precise assessment of MRL in segregating populations, which is illustrated by the fact that two distinct QTLs on chromosome I were found for traits "recombination at the ends" and "recombination in the middle". Alongside the missing data, we were only able to design SNP markers for seven of the 13 chromosomes. In addition, double COs in the middle of the chromosomes cannot be detected due to this limited set of markers. Because of the high-quality genome sequences of *A. bisporus* innumerable SNP markers can be developed at specific genome positions. However, there are always limitations as to the number of markers that can be used in an experiment due to the cost of PCR-based marker genotyping. Additionally, we are often aware that some SNP markers fail during an SNP assay and thus do not enable the assessment of a genome-wide view on MRL. Therefore, the current genotyping method used to phenotype the MRL of the segregating populations needs to be considered as suboptimal and more attention needs to be paid to using genotyping methods that cover MRL genome-wide.

Contrary to PCR-based marker genotyping, whole genome re-sequencing allows genotyping of the offspring in each segregating population in the greatest detail (Huang et al., 2009; Wijnker et al., 2013; Sonnenberg et al., 2016) and provides a better insight into MRL, especially at chromosome ends that are not easily covered with markers. Whereas in techniques such as the SNP Kaspar assay a single allele switch on a chromosome is often considered to be unreliable (Huang et al., 2009), an allele switch in the whole genome re-sequencing is confirmed by flanking SNP markers. The potential of using whole genome re-sequencing was recently presented by Sonnenberg et al. (2016) in which a limited set of homokaryons and heterokaryons derived from *bisporus* variety were genotyped in this way to detect CO events in the distal chromosome regions. In *A. bisporus*, two non-sister nuclei are preferentially paired in one spore enabling us to dissect half tetrads and detect gene conversion if chromatids involved in the same recombination event are paired into one spore. Detecting both CO and gene conversion provides unparalleled insights into the precise outcome of meiosis.

Genome sequencing techniques improve quickly with reducing costs, but whole genome re-sequencing of offspring for each segregating population will still be too costly because in each segregating population the number of offspring needs to be genotyped for screening the exact meiotic behaviour in each segregating population. Other methods such as genotyping by sequencing (Elshire et al., 2011) and RAD-mapping (Baird et al., 2008) which allow the pooling of 96 samples per Illumina lane are also efficient because specific restriction enzymes followed by specific barcodes are used to reduce the complexity of the genome. The key to using these techniques effectively is to choose an appropriate restriction enzyme to cover the entire genome especially the ends of the chromosome. In chapter two, genotyping by sequencing proved to be a useful technique to detect variations and consequently CO recombination across the genome. Another factor affecting the efficiency of techniques such as genotyping is by sequencing the abundance of polymorphisms between two parents which then can be followed by segregating

populations. Therefore, it is pivotal to ensure the abundance of polymorphism between parental genomes before investing in constructing a mapping population. The parental genomes of the *burnettii* variety used in this study were unexpectedly highly similar which caused two problems. Firstly, the linkage map of the *burnettii* variety had only four chromosomes evenly covered with molecular markers (Chapter 2). Secondly, as mentioned in chapter 4, we observed inbreeding depression in second generation hybrids owing to the choice of the tester homokaryon. The homokaryotic individuals derived from the F1 hybrid (haplotype H97 var. *bisporus* × haplotype Bisp119/9-P4 var. *burnettii*) were crossed with a *burnettii* tester homokaryon (haplotype Bisp119/9-P1) that appeared to have large genome regions identical to Bisp 119/9-P4, and thereby performing SNP genotyping on offspring derived from *burnettii* outcrossed hybrids was challenging.

Types of segregating populations

This thesis presents data, revealing that the MRL of *A. bisporus* is a quantitative trait (Chapter 5). As the MRL trait is the outcome of meiosis, phenotyping cannot be carried out directly on the mapping population. The individuals of mapping populations are haploid and they have to be crossed with a mating partner to generate second generation hybrids which are equivalent to the F2 population in plants. Following mushroom cultivation, the offspring of each hybrid has to be collected for the evaluation of MRL. Prior to the QTL analysis, three types of second generation hybrids were evaluated to see which would generate the most useful population, i.e. in which the "MRL" phenotype can be scored best (Chapter 4).

The segregating populations from which we choose to study the phenotype of a trait are essential for the outcome of the analysis. In selfing, maximal variation in the phenotype is obtained which is optimal for a QTL study. In this thesis, when selfing was performed between individuals of the mapping population (derived from one fruiting body) some drawbacks were observed such as inbreeding depression and non-segregating genome regions caused by homozygosity (Chapter 4). Such drawbacks can be overcome when generating two mapping populations parallel to one another, each having parental homokaryons of the *bisporus* and *burnettii* types, but with unrelated genomes. This enables intercrossing between individuals of the two mapping populations to generate second generation hybrids and subsequent isolation of their offspring for which CO can be assessed. This avoids inbreeding and, due to genetic distance between the parents, enough segregating markers are available.

Callac and co-workers (1998) previously postulated that the basidial spore number (BSN) locus on chromosome I (Imbernon et al., 1996) might confer the increased recombination frequency in the *burnettii* variety. This was based on the observation that hybrids having one or both parents of the *burnettii* type had an elevated number of spores per basidium and its offspring revealed a CO frequency higher than those from pure *bisporus* lines. However, this is an incorrect conclusion owing to the fact that the relevant phenotype (MRL) is not expressed in the mapping population but in its offspring. No clear association between the recombination frequency and the BSN locus was found in our studies (Chapter 4). However, the number of populations used in our studies was a limiting factor to completely rule out the association between the BSN locus and the higher recombination frequency. Foulongne-Oriol et al. (2011)

reported elevated recombination frequencies in the inter-varietal hybrid (var. *bisporus* × var. *burnettii*) compared to that of the *bisporus* variety. Later, Sonnenberg et al. (2016) used a whole genome re-sequencing technique revealing that the *bisporus* variety has an almost similar frequency of non-recombining and recombining chromosomes to that of the inter-varietal hybrid. The difference between MRL of the *bisporus* and *burnettii* varieties are thus mainly due to differences in the position of CO and not their frequency (Sonnenberg et al., 2016). Whether *burnettii* and *bisporus* varieties also display different recombination frequencies remains to be investigated. To enable us to investigate the MRL of the *burnettii* variety, all CO events had to be detected on account of the fact that the CO recombination data is considerably affected by the genotyping methods used in different studies. The number of double COs in the *burnettii* variety was twice that of the *bisporus* variety (Chapter 5). The incidence and distribution of CO recombination depends on the initial step to be taken, i.e., double strand breaks (DSBs) formation and how the DSBs are repaired (Baudat and de Massy, 2007). Detailed knowledge on the formation of DSBs in the two varieties could thus shed some light on the possible mechanisms involved.

Double Strand Breaks (DSBs) and recombination

The variation in CO distribution with regard to the variation in DSBs sites can generally be divided into three parts, i.e., the localization of DSBs sites, the timing of DSBs and the fate of DSBs. The major part of the variation in distribution of CO events across the genome is consistent with the variation in distribution of DSBs sites which mainly depends on the accessibility of chromatin sites to recombination enzyme Spo11 (Wu and Lichten, 1994; Berchowitz et al., 2009). The number of DSBs generated per meiosis usually exceeds the number of COs (Moens et al., 2002), thus not all DSBs are resolved into CO recombination. For example in yeast, 140 -170 DSBs are generated during meiosis (Buhler et al., 2008) of which 90 are roughly repaired as COs and 66 as NCOs events (Mancera et al., 2008).

The formation of DSBs is induced by Spo11 which remains bound to DNA following the DSBs formation. By isolating Immuno-precipitated Spo11-oligonucleotide complexes would help to identify DSBs and use them for sequencing to map the sequenced reads onto a genome (Pan et al., 2011). Elucidating DSBs sites in *bisporus* and *burnettii* varieties could help in understanding the initial step of recombination formation. If these two varieties differ in DSBs incidence or DSBs position, it might explain the differences in CO positions between the two varieties. Examining cytological and mutant studies in *Sordaria macrospora*, an ascomycete fungus, in more detail, might shed some light on how to explain the likely cause of the different MRL in the two varieties of *A. bisporus.*

In *S. macrospora*, a gradual alignment occurs between homologous chromosomes following nuclear fusion (Zickler, 1977). Homologous chromosomes are attached to the nuclear envelope and the telomeric regions have a tendency to co-align initially. DSBs are known to mediate homologous chromosome co-alignment. Homologous chromosomes do not or rarely align in the absence of DSBs (Storlazzi et al., 2003). A complete alignment is observed at the end of leptotene at a distance of ~ 400 nm between homologous chromosomes. A closer alignment (200 nm) and connections between homologous chromosomes are observed when a nascent D-

loop is formed by invading one end of a DSB into a homologous chromosome. The ss-DNA strands might thus play an important role in detecting its homologue and creating an alignment. The distance between co-aligned homologous chromosomes is specified by Msh4, a CO resolvase, suggesting that Msh4 stabilizes a nascent D-loop. The *msh4* series mutants reveal a mixture of completely co-aligned pairs, partial co-alignment in short regions often in telomere regions and the absence of co-alignment. In addition to Msh4, mer3 which is a meiosis-specific helicase also seems to play a role in homologous chromosome co-alignment by ensuring the establishment of long-distance contact between homologous chromosomes in one position rapidly succeeded by axis juxtaposition in that same position (Zickler and Espagne, 2016). Moreover, disruption of condensins, protein complexes involved in chromosome assembly and segregation, also leads to an alteration in the DSBs site and incidence, thereby CO localization and incidence (Mets and Meyer, 2009). After a more narrow alignment (200-400 nm), the homologous chromosomes become closely associated at a distance of 100 nm due to the formation of the synaptonemal complex (SC). Whether the number and positions of DSBs play a role in differences in the MRL observed in the two *A. bisporus* varieties is questionable. We have identified some of the genes (*Rad51, Spo11, DMC1, Rad50, MSH4, MSH5, and MRE11*) possibly involved in the generation of DSBs and resolution into COs in *A. bisporus* (not shown). These genes are coded in both varieties in almost identical proteins and seem therefore to function properly in both varieties. This might suggest that DSBs are formed quite similarly in both varieties and can in principle be resolved during the CO.

The timing of DSBs can have an overall effect on the MRL. It seems that DSBs throughout the genome are formed in an asynchronous way, by favouring the first DSBs to be formed in the sub-telomeric regions of the chromosomes similar to those observed in human and barley meiosis (Higgins et al., 2012; Pratto et al., 2014). These early sub-telomeric DSBs are resolved as CO events and then by means of CO interference the occurrence of another CO close to the existing one is unlikely. In many organisms the number of CO per chromosome varies between 1 and 3. The formation of the first CO in sub-telomeric regions often leads to a preference of CO in these regions.

DSBs seem more likely to occur in certain specific genomic regions (Keeney, 2007). These specific genomic regions across the genome are defined as recombination hot spots where the recombination rates are significantly higher (Lichten and Goldman, 1995; Pryce and McFarlane, 2009). Contrary to hot spot regions, some are defined as cold spots such as centromere-proximal regions, telomeres and DNA repeats where a considerable repression of DSBs and CO events has been observed (Baudat and Nicolas, 1997; Gerton et al., 2000). DNA repeats in the *bisporus* variety have previously been studied genome-wide (Foulongne-Oriol et al., 2013). Sub-telomeric regions of the chromosomes in the *bisporus* variety are mainly associated with non-LTR *Penelope* sequences ranging from a few kb up to 40 kb (Sonnenberg et al., 2016). In chapter two, the position of repetitive elements along the chromosome was illustrated in *bisporus* and *burnettii* varieties. DNA repeats appeared to be widely spread over the whole genome in both varieties with some amounts of clustering, indicating that the differences in MRL for these two varieties are less likely to be defined by differences in genome sequences. In addition, high collinearity was found between the genome sequences of two

haplotypes in the *bisporus* variety (Sonnenberg et al., 2016) and between the *bisporus* and *burnettii* varieties (Chapter 2). It is currently unclear what controls the position of DSBs. There is no particular DNA sequence which corresponds to DSBs sites (de Massy, 2003), although a motif has been recognized that associates with 40% of all human recombination hot spots (Myers et al., 2008). Promoter regions are known to host the majority of DSB sites (Wu and Lichten, 1994; Baudat and Nicolas, 1997; Gerton et al., 2000) which is consistent with CO hot spots in yeast.

The fate of DSBs can also affect the final outcome of meiosis. It might also be possible that DSBs in the *bisporus* variety are repaired mainly via sister chromatids and thus have no genetic consequences while the *burnettii* variety makes use of a non-sister chromatid as a template. The differences in MRL of these two varieties may also be related to steps preceding the CO resolution; e.g. synapsis. In the following paragraphs I will outline some steps for future work on how homologous chromosomes are paired and synapsed in these two varieties.

The process of homologue recognition and correct pairing

Prior to the first meiotic division, homologous chromosomes are brought together to undergo karyogamy or nuclear fusion. To conjugate homologous chromosomes, a series of events are necessary which are only partially understood. First of all they need to find each other in nuclear territory. To bring chromosomes into close proximity with one another, local homologous chromosome pairing occurs in many organisms starting from centromere (Christophorou et al., 2013) or telomeres. The telomeres are randomly bundled in the inner nuclear envelope at the early leptotene stage, forming chromosomal bouquet (Figure 1).
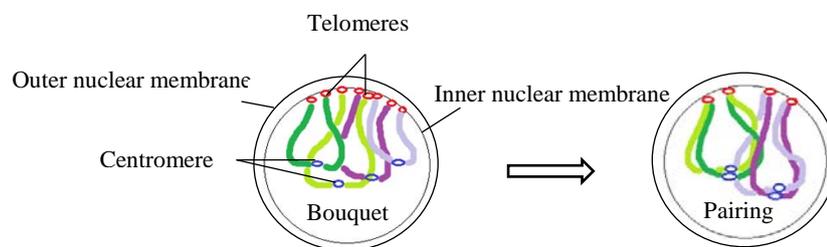
Figure 1. Formation of bouquet and homologous pairing. Two pairs of homologous chromosomes are shown i.e. Bright green and light green and bright purple and light purple. Modified from: http://scienceyoucandigest.blogspot.co.uk/2013/11/the-role-of-centromeres-in-bouquet.html

Initiation of the bouquet is coincidently accompanied by the beginning of homologous pairing and synapsis which is often assumed to promote homologous pairing perhaps by gathering all chromosomes in a limited volume and, thus making the process of homologous search faster (Harper et al., 2004; Tsai and McKee, 2011). However, a complete understanding of bouquet formation and its function as well as its relation to homologous recombination is still unknown. The association of telomeres with nuclear periphery almost concurrently occurs with DSB formation in yeast, implying that DSB formation and homologous chromosome pairing occur non-randomly perhaps to minimize irregular relationships between homologous chromosomes (Zickler and Kleckner, 2015). The SC formation is initiated in the adjacent telomeric region in

flatworm following bouquet formation and the entire length of homologous chromosomes are then synapsed (Xiang et al., 2014). Although bouquet formation facilitates homologous pairing and synapsis, it may not be absolutely necessary for pairing or synapsis, as some degree of pairing and synapsis is achieved before tight bouquet formation or in specific mutants.

In *Sordaria* meiosis, co-alignment of homologues is observed before bouquet formation and only at the late zygotene stage or early pachytene stage when all telomeres are tightly bundled in the nuclear envelope, suggesting that bouquet formation is not necessary for homologues co-alignment and may not have a significant role in pairing (Zickler and Espagne, 2016). In addition, bouquet formation is observed in mutants defective in recombination in yeast and *S. macrospora* which lack *spo11*, suggesting that bouquet formation is a recombination independent event (Trelles-Sticken et al., 1999; Storlazzi et al., 2003). While the entry into bouquet formation is not controlled by the recombination process, studies suggest that this process is probably a mediator to exit from the bouquet stage as bouquet resolution is delayed in the absence of recombination or when observing incomplete recombination processes. The pairing mechanism appears to be more complex because in some organisms it depends on the formation of DSBs hence the initiation of the recombination process (Zickler and Espagne, 2016), whereas in other organisms it is DSB independent (MacQueen et al., 2002; Harper et al., 2004). DSB independent pairing (Recombination independent pairing) has been observed in budding yeast (Cha et al., 2000) and *Caenorhabditis elegans* (Rog and Dernburg, 2013). In *C. elegans*, DSB independent pairing predominantly occurs between pairing centres, which are located near one end of the chromosomes as well as in the interstitial regions along each chromosome.

Cytogenetic studies and the possible role of the synaptonemal complex

The SC is the assembled proteinaceous structure between two homologous chromosomes and has been thought to be important for the meiotic recombination (Petes et al., 1991). According to the state of assembly and disassembly of the SC, meiotic prophase I is subdivided into several stages (Figure 2). In leptotene, two sister chromatids of each chromosome become associated with the axial element which is silver stainable. In zygotene, transverse filaments and central elements become associated with axial elements of homologous chromosomes, a process known as synapsis. At the pachytene stage, the entire length of homologous chromosomes is synapsed. The axial elements, transverse filaments and central elements form the SC and in the mature SC the axial elements are referred to as lateral elements. During the diplotene stage, homologous chromosomes desynapse which is followed by disassembling the axial elements during the diakinesis.

SC has been recognized as having at least two functions. It stabilizes and maintains a pairwise association between homologues (Hayashi et al., 2010; Mlynarczyk-Evans and Villeneuve, 2010). This occurs not only in organisms in which SC formation is promoted at a subset of recombination intermediates, but also in organisms in which SC formation is a recombination independent process. Alongside that, the central elements in SC function as a mediator in the maturation process of CO-related recombination intermediates into mature CO (de Boer and Heyting, 2006).

The association between SC nucleation with recombination sites has been observed in *S. macrospora*. SC is nucleated first at the chromosome ends where DSBs are also formed and later on at different sites where the aligned homologues are located. The formation of short stretches of SC at different points forms a pattern depending on the number of DSBs. Therefore, sufficient initiation points must be generated to form a complete SC (Zickler and Espagne, 2016). The observed recombination nodules between lateral elements of the SC are likely to contain proteins involved in the resolution of DSBs into CO or non-CO events (Zhang et al., 2014).
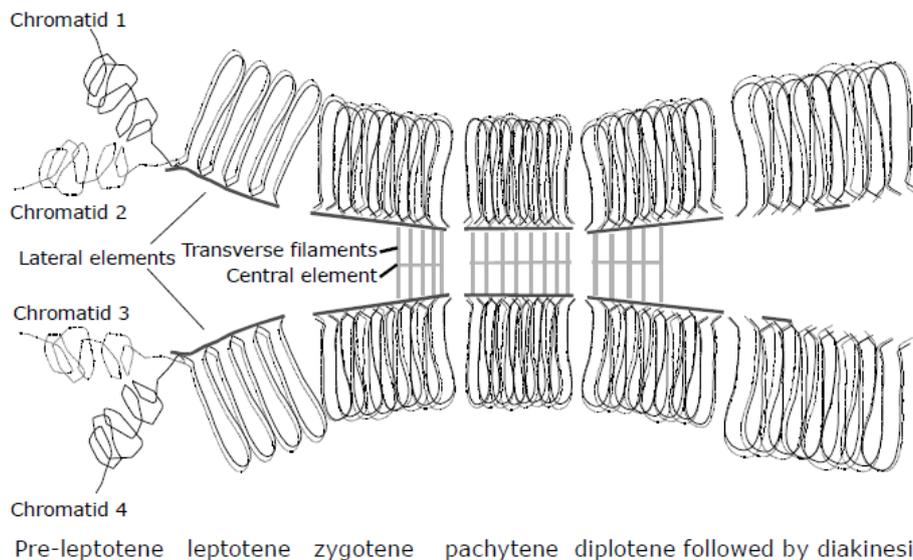
Figure 2. Assembly and disassembly of the SC during meiotic prophase I. Pre-leptotene (each chromosome consists of two sister chromatids replicated during S-phase), leptotene (the beginning of axial element formation along each chromosome), zygotene (connection of the axial elements by transverse filaments), pachytene (full synapsis), diplotene (transverse filaments dissociated), and diakinesis (axial elements disassembled; (Eijpe, 2002).

MSH4 seems to be essential for the correct formation of SC in *Sordaria*. The role of SC in the formation of CO is demonstrated by the fact that the recombination proteins such as Rad51, Mer3 and MSH4 are firstly localised on the lateral elements but shift to the central region of the SC after nucleation. Only then are DSBs repaired into CO events. The conclusion therefore is that DSBs play a role in the initial stages of homologous chromosome co-alignment and the initiation of recombination while the SC plays a crucial role in organizing proteins involved in the resolution of DSBs into CO events.

Beside the linkage studies in *A. bisporus*, a few cytological studies have also been carried out to study chromosome behaviour in meiosis. The earliest two cytological studies had limited optical resolution and could not show all 13 homologous pairs during meiosis of the *bisporus* variety (Evans, 1959; Saksena et al., 1976). Further detailed studies using silver staining of spread protoplasted meiotic prophase I of both *bisporus* and *burnettii* varieties revealed that the *bisporus* variety has very short stretched axial elements and SC and no clear bivalents could be detected. In contrast, in the *burnettii* variety, axial elements and SC in bivalents were observed which led to the conclusion that defects in SC formation in the *bisporus* variety might explain its typical MRL (Mazheika et al., 2006). It is possible that an incomplete synapsis is associated

with CO localization at the ends of the chromosomes in the *bisporus* variety if indeed synapsis starts at chromosome ends and is only completed in the *burnettii* variety. The association between CO localization and a restricted or incomplete synapsis in certain regions has been found in budding yeast, plants and animals (Oakley, 1982; Jones, 1983; Fung et al., 2004; Calvente et al., 2005; Viera et al., 2009). As described above, an alignment between homologous chromosomes in *Sordaria* starts at the telomeres. In addition, some mutations such as *Msh4* lead to a partial alignment usually only in the telomeric region. It is also possible that alignment followed by synapsis in the *bisporus* variety starts in the telomeric regions and that a mutation prevents a full synapsis therefore resolution of DSBs into CO in the chromosomal regions happens further away from the telomeric regions.

In the absence of a normal synapsis, an order structure that highly resembles SC can also be formed outside the chromosomes, i.e., polycomplexes (Öllinger et al., 2005). The observation of large lumps of proteinaceous structures in nuclear spreads of the *bisporus* variety by Mazheika et al. (2006) might indicate that structural components of the SC have been formed but not attached to chromosomes. (Tung and Roeder, 1998) generated mutants of the protein Zip1p forming the transverse filaments connecting the lateral elements in the SC. One of the mutations prevented the localization of Zip1p to chromosomes which led to the assembly of Zip1p into polycomplexes, aggregates not associated with chromosomes. The electron-microscopic pictures of Mazheika et al. (2006) suggest a similar phenomenon. In addition, using Zip1p mutants, Tung and Roeder (1998) demonstrated that there is a correlation between the extent of synapsis and level of CO events. It is not yet understandable how SC can shape the position of COs across the genome. Having established SC first of all at chromosome ends where telomeres are randomly bundled in the inner nuclear envelope to form a bouquet might facilitate CO in this region and then by the means of CO interference the frequency of adjacent COs is also affected.

In order to find leads towards the mechanisms involved, it is also important to develop a good cytological system to study meiosis in *A. bisporus* in more detail. This will not be easy as meiosis does not seem to occur synchronously in *A. bisporus* (personal observations).

The benefits of restricting CO at chromosome ends in the *bisporus* variety

Having CO recombination at the ends of chromosomes ensures that most of the *bisporus* variety offspring acquires parental chromosomes almost intact after meiosis. This meiotic behaviour together with the non-sister pairing of nuclei will promote the preservation of heterozygosity. Heterozygosity can mask lethal and deleterious recessive alleles, and thus increase reproductive fitness (Kerrigan et al., 1994). CO recombination can lead to homozygosity and thus unmask lethal and deleterious recessive alleles. In the smut-fungus *Microbotryum violaceum*, CO events are suppressed in the mating type region, leading to an accumulation of lethal alleles (Fontanillas et al., 2015). Mating type bias observed in this fungus is caused by deleterious recessive alleles linked to mating type. The occurrence of intratetrad mating, i.e., mating between the meiotic products from one meiotic event, provide opportunities for the deleterious recessive alleles to be masked by the diploid stage and hence maintained across the non-recombining regions of the genome (Hood and Antonovics, 2000, 2004). The fusing of meiotic

products from one meiotic event also occurs in the *bisporus* variety to restore the diploid stage (heterokaryotic stage) without genetic contribution from other individuals. If maintenance of heterozygosity occurs to avoid the homozygosity of lethal alleles in *A. bisporus* is questionable. Segregation distortion of a few chromosomes or chromosomal regions was observed in *bisporus* variety, but all parental types were recovered in non-recombining homokaryons. It is possible that certain combinations of parental alleles produce non-viable spores or spores with a very slow growth rate and thus are not used in genetic studies. Additional studies of spores on a large scale are required for the analysis of relationships between segregation distortion and the accumulation of deleterious alleles.

Is the MRL of the *bisporus* variety typical for all strains of this variety? Evidence of the occurrence of a typical MRL of the *bisporus* variety in natural populations can be found in the study of Gao and co-workers (2015). Two segregating populations were made in this study using one of the parental homokaryons of Horst U1 (commercial hybrid) and two homokaryons obtained from wild heterokayons which were distantly related to Horst U1. The linkage maps constructed were also short having a total length map of 166 cM and 86 cM with CO events mainly at chromosome ends (Gao et al., 2015).

Obstacles and troubleshooting in mushroom breeding

Among all edible mushrooms *Agaricus bisporus* var. *bisporus* is the most cultivated mushroom worldwide. The cultivation and consumption of mushrooms have a long history. Mushrooms have not only nutritional and medicinal properties for humankind (Kerrigan, 2005; Firenzuoli et al., 2008; Wisitrassameewong et al., 2012) but can also be used to upgrade agricultural waste (Van Kuijk et al., 2015). In spite of the fact that mushroom breeding has been practised in many areas of interest in the last decade such as generating genetic markers, genetic map analysis, quantitative inheritance of certain traits, etc. there have hardly been any new cross bred varieties released since the first hybrids appeared on the market in 1980, i.e., Horst U1 and Horst U3. While wild isolates of *A. bisporus* reveal a broad genetic variation, a low genetic variation has been observed in the traditional and present-day hybrids (Sonnenberg et al., 2017). Three reasons can account for that:

First of all, making tissue cultures of a variety is making it easier to duplicate the variety and subsequently cultivate them under a different name. Secondly, the homokaryons which are breeding material are formed at a low frequency in the *bisporus* variety, resulting in time-consuming breeding. In contrast, the fertile heterokaryons are formed in larger frequencies which are often used to generate further generations, and are a low investment for generating hybrids by cross-breeding. The next generation hybrids emanated from fertile heterokaryons of a curremt cultivar are considered to be essentially derived varieties (EDVs). Thirdly, due to a typical MRL of the *bisporus* variety, the genetic materials of the parental nuclei are inherited almost intact to the next generation except for chromosome ends where recombination predominantly takes place. This problem, known as linkage drag is one of the major problems in mushroom breeding. Due to the above-mentioned reasons mushroom breeding is lagging behind plant breeding and considerable efforts have been directed mainly towards improving

cultivation techniques such as composting and mechanical harvesting, but rarely to cross-breeding.

The release of the genome sequence of one the parental haplotypes of Horst U1, i.e., H97 in 2012 (Morin et al., 2012), its further improvement and the release of the genome sequence of the other parental haplotype of this hybrid i.e., H39 in 2016 (Sonnenberg et al., 2016) enabled a genome sequence comparison to be made between these two haplotypes, comparisons of the position of markers in the genetic maps to those on the physical map resulting in a precise assessment of the *bisporus* variety MRL. In chapter two, one parental haplotype of the *burnettii* variety was *de novo* sequenced. The existence of three genome sequences in the *A. bisporus* generally provides applicable markers in the commercial mushroom and its wild relative. These three reference sequences open up possibilities for people to refer to physical regions of markers among genotypes. Mapping of reads of re-sequenced offspring can prove successful when using these three genomes as a reference. Sequences from additional lines, especially from *burnettii* variety with high-quality *de novo* assemblies will further facilitate the study of MRL as the parental haplotypes of the *burnettii* variety strain used in our study were highly similar in sequence.

Mushroom breeders just like plant breeders aim at improving the quality and performance of mushroom varieties, e.g., higher yields, disease resistance, increased nutrition, greater beauty, less sensitive to bruising, etc. The traits of interest in one variety or line are introduced into an existing genetic background. For instance, a line having resistance to a certain disease but low-yielding is crossed with the line having a high-yielding capacity although it is sensitive to that disease. The ultimate goal of breeders is to introduce resistance to the high-yielding line without affecting the yield.

In breeding seed crop varieties meiosis and consequently CO recombination proceeds seed formation which leads to an alteration in allele combination of elite genotypes. One way to preserve desired allele combinations is to generate homozygosity. Homozygosity can be achieved through selfing and backcrossing but requires series of generations. The fastest way that has been routinely used in plant breeding to achieve homozygosity is generating double haploids (DH), i.e. a genotype formed by growing the gametes (haploid) into a homozygous diploid genotype doubling the chromosome. In *A. bisporus*, DH cannot be generated because the compatibility between homokaryons is controlled by mating type loci and mating occurs if two homokaryons have different mating types (Kües, 2015). The parental nuclei in *A. bisporus* do not fuse following the mating of two compatible homokaryons. They remain side by side and nuclei fusion occurs just before meiosis. While in plant breeding generating and preserving homozygous parents is challenging, the parental nuclei can in principle be recovered as homokaryons in *A. bisporus*. Haploid homokaryons isolated from mushroom as single spore isolates, are similar to plant gametes which can be propagated vegetatively and genotyped to generate genetic linkage maps. To assess a trait of interest and map quantitative traits/QTLs, the haploid homokaryons from which the genetic map was constructed have to be crossed with a compatible tester homokaryon in order to generate heterokaryons that can be used to produce fruiting bodies.

Heterokaryons from segregating populations can be generated either by selfing/sib-mating (crosses between homokaryons derived from one mushroom/fruiting body) or outcrossing (crosses between homokaryons derived from mapping population and a tester homokaryon). Although the maximum variation of the trait is perceived by selfing, this approach leads to inbreeding depression. More importantly, for the "MRL" trait heterozygosity between homokaryons is needed for detecting COs, while selfing leads to homozygosity (Chapter 3). The use of compatible tester homokaryons for outcrossing will also influence the expression of the trait of interest in QTL studies (Gao et al., 2015; Gao et al., 2016). We screened segregating populations on a small scale to get a better insight into segregating the MRL using selfing and outcrossings (Chapter 3). As the trait that we studied was the outcome of meiosis, assessment of the MRL must be done on the offspring of each segregating population. Studying variation of the MRL trait was thus laborious and time-consuming.

The objective of chapter five was to detect and locate QTLs underlying different MRLs in the *A. bisporus*. Results from the QTL analysis revealed QTLs with explained variations ranging from 20% to 30% with relatively high significance levels. We found two QTLs on chromosome I for two traits which are assumed to be complementary. i.e., COs at the ends and COs in the middle of the chromosomes. Therefore, the question now is which QTLs are reliable and which aren't? To decide which QTLs are reliable some factors have to be taken into account, for instance, the stability of the QTL in cofactor analysis, the influence of extreme phenotypes, phenotyping errors, genotyping errors, the environmental effects and segregation distortion. I personally tend to distrust the QTLs if segregation distortion is seen in the experiment, as the results might have been biased.

Segregation distortion is a common phenomenon, which might occur due to biased selection of individuals during sampling. The SNP genotypes in the linkage map of an inter-varietal hybrid constructed in this thesis manifested extreme segregation distortion on chromosome III and to lesser extent on chromosome II (Chapter 3). The loci showing segregation distortion can affect QTL power and QTL additive effects and cause QTL inconsistencies (Foulongne-Oriol et al., 2012; Xie et al., 2013). The regions that are particularly biased in the genome can have a QTL linked to the trait of interest. However, distorted segregation is not always harmful to QTL mapping and this can potentially but not necessarily benefit from them. There are models by which segregation distortion loci (SDL) can be mapped and jointly used with QTL mapping (Xu, 2008). According to Xu, the entire chromosome can be biased from Mendelian segregation if only a few SDL are present. In Chapter 3, the entire chromosome III except the distal regions was biased from Mendelian segregation. This might have led to an overestimation of QTL effects and an underestimation of QTL numbers calculated in Chapter 5. Nevertheless, how distorted segregation is observed in our study influences QTL detection needs to be further investigated.

From a breeding perspective, segregation distortion of one parental allele or the other can circumscribe the breeding programmes. For example, on chromosome III (linkage group III), the allele from the *bisporus* variety is over-represented in the population of an inter-varietal hybrid (Chapter 3). Selection against unfavourable alleles from *bisporus* variety in this region requires strenuous effort.

Up to now, the resolution of the genetic linkage maps of the *bisporus* variety was too low due to the restriction of recombination at the ends of the chromosomes causing difficulties in detecting CO recombination. This avoids precisely locating QTLs underlying certain traits on the genome. Using the population of an inter-varietal hybrid (var. *bisporus* × var. *burnettii*) leads to a more precise localization of QTLs (Foulongne-Oriol et al., 2012). Although most of the agronomic and quality traits are poor in the *burnettii* variety, the use of this variety in breeding programmes can be a great help in minimizing the linkage drag during introgression breeding. The genome organization in the *burnettii* variety seems to be highly collinear along with the genome of the *bisporus* variety (Chapter 2). Understanding the genome organization is a great help to enable breeders to understand why the expected outcomes are not achieved in some crosses. Fine mapping the *Ty-1* gene in tomato, a virus resistant gene, for instance has failed for a long time due to suppressing the recombination in its region, caused by chromosomal rearrangements between resistant donor (*Solanum chilense*) and recipient cultivated tomato (Verlaan et al., 2011). Comparative analysis of the sequences uncovers not only large but also small chromosomal rearrangements. Hence, the availability of next generation sequence (NGS) technologies enables mushroom breeders not only to use this information for marker assisted breeding (MAS), but also to uncover chromosomal rearrangements in the genomes (Chapter 2). Next generation breeders can benefit from whole genome sequencing and re-sequencing technologies, by predicting whether inter- or intra-varietal hybrids have to be generated in order to integrate traits of interest.

Another issue, yet to be addressed, is the effect of environmental factors on the trait of interest. For example, both high (35 $^\circ$C) and low (5 $^\circ$C) temperatures appeared to increase the recombination frequency in *Coprinus lagopus*.(Lu, 1969; Lu, 1974, 1974) There is some evidence showing that basidial spore numbers of the *burnettii* variety have been influenced by temperature (Kerrigan and Ross, 1987). Although we have collected spore prints from segregating populations practically at the same developmental stage (veil breaking) and temperature, how these factors influence MRL needs to be investigated further.

Reverse breeding approaches

In the *bisporus* variety, the heterozygosity is preserved by preferentially pairing of non-sister nuclei into one spore and restricting recombination at distal ends of the chromosome. Most mushroom breeding companies use these fertile spores to generate varieties that differ from parental genomes mainly due to the reshuffling of homologous chromosomes over the two constituent nuclei. As stated earlier in this chapter, to preserve allele combinations of elite genotypes and be able to fix complex genotypes, plant breeders use methods such as generating DHs and backcrossing to achieve homozygosity. In a breeding technique known as reverse breeding, gametes with non-recombinant chromosomes are generated by suppressing COs in a heterozygote of choice. Due to an absence of CO and consequent aneuploidy, most of the gametes are unviable. Viable gametes having balanced chromosomes are generated and further grown into DH lines at a very low frequency. The complementary DH lines can be identified to generate starting heterozygotes of choice (Dirks et al., 2009; Wijnker et al., 2012). The low efficiency of reverse breeding due to aneuploidy can be overcome using two approaches.

In the first one, COs are partially suppressed by knocking down the expression of genes such as *MSH4* or *MSH5* (Higgins et al., 2008) which are involved in pathway I (MSH4-MSH5 dependent pathway) of the CO recombination (Kohl and Sekelsky, 2013). The knocking down of such genes, however could affect the expression of other phenotypes. Disruption of *stpp1* gene, an *MSH4* homolog has led to the formation of a sporeless fruiting body in *Pleurotus pulmonarius* (Okuda et al., 2013).

In the second one, residual COs are directed to the distal ends of the chromosomes, resulting in gametes that reveal the segregation of alleles only at chromosome ends. If only the alleles at the ends of the chromosomes are important for the breeders, the resulting gametes are not considered useful. The potential use of CDKA-1, a cell cycle regulator, has been reported to induce CO to distal chromosome ends in *Arabidopsis* (Wijnker, 2013).

In *A. bisporus* var. *bisporus*, COs occur naturally at distal chromosome ends. If gene(s) that regulate the localization of COs at the ends of the chromosome in this variety are identified, not only mushroom breeding but the second approach of reverse breeding can also benefit from the knowledge generated. Meiosis can be manipulated towards COs distributed across genome (minimize linkage drag) or restrict CO localization (preserve desired allele combination). Although we are still at the outset of identifying mechanism(s), underllying MRL of the *A. bisporus*, but two distinct *A. bisporus* MRLs promise to give us a better understanding of the factors regulating specific localization of CO in the future.

Further challenges in meiotic recombination manipulation

The use of homokaryons derived from *bisporus* and *burnettii* varieties in conventional breeding techniques such as cross breeding offers, in principle, an effective method for improving strain. In this way, both desirable and undesirable traits are introduced into a newly obtained strain. The undesirable traits are difficult to get rid of due to the restriction of recombination at the ends of the chromosomes in the *bisporus* variety (linkage drag). As acknowledged in this thesis, understanding the mechanism(s) behind two MRLs of *A. bisporus* can be of great help to mushroom breeders. Identifying factor(s) contributing to the MRL of *A. bisporus* is a complex challenge in itself. In this challenge, QTL analysis must be done at offspring levels and the offspring must be genotyped in more detail in order to gain a reliable phenotypic score. If we discover the genes involved, we might bring these under inducible promotors using genome editing tools. That enables a breeder to either switch to COs at chromosome ends (to preserve desirable traits) or distribute COs over the entire chromosome (to minimize linkage drag or introduce new traits).

The genome editing tool, CRISPR/Cas9, enables targeted changes in DNA guided by a small noncoding RNA. A change in the genome occurs by both non-homologous end joining and homology-directed repair mechanisms (Cong et al., 2013). Recently, by deleting a number of base pairs of the family of genes involved in the browning of mushroom using CRISPR/Cas9 system, the common white button mushroom has been modified to resist browning (Waltz, 2016). The CRISPR edited-mushroom is not subjected to a US ruling for genetically modified organism (GMOs) and can be commercialized. A CRISPR engineered crop is still regarded as

GMO under European regulations and the decision on whether genome-edited plants should be regulated as if they were GMO is not expected before 2018 (Abbott, 2015). Indeed, in CRISPR engineered crops, a genome is edited by itself and it is even difficult to identify whether a crop has been edited via CRISPR or mutated naturally. The desirable allele combination can be created by CRISPR technology as an alternative to classical breeding. However, additional studies are required to achieve the full potential of this technology. At the same time scientists should continue pushing and introducing the safety and value of gene editing to the public. To sum it all up, a schematic overview of the various steps needed to identify factor(s) contributing to different MRL in *A. bisporus* and how this knowledge can be integrated into a breeding programme is depicted in Figure 3.

Pre-breeding/ Research			Introduction in breeding pipeline
Multi-parental mapping population (4 parents) - Two mapping populations - Intercrossing between individuals of two mapping populations - Generating second generation hybrids - Progeny isolation from second generation hybrids	QTL discovery & identification of candidate genes	QTL and candidate gene(s) validation	**Conventional and molecular mushroom/plant breeding** Hybridisation and using genome editing tools to: - direct recombination across genome to generate new allele combination or reduce linkage drag - direct recombination at chromosome ends to preserve favourable alleles
Phenotyping MRL (Genotyping progenies from second generation hybrids) Techniques such as genotyping-by-sequencing - To assess genome-wide CO recombination - To reduce complexity			**Reverse breeding** - Direct residual recombination to chromosome ends - Generating gametes that show segregation of alleles only at chromosome ends - Use generated gametes to make double haploids and heterozygotes of choice
Cytogenetic study (in parallel) - Observations on homologous chromosome pairing and synapsis in two varieties of *A. bisporus*			

Figure 3. Schematic overview of potential next steps towards QTL identification of MRL of *A. bisporus* and its application in mushroom and plant breeding.

In the context of our research study, interest in factor(s) contributing to MRL of *A. bisporus*, a multi-parental (four-way cross) mapping population instead of a bi-parental mapping population and genotyping the individuals derived from segregating populations (phenotyping MRL) using techniques such as GBS could advance the accuracy and the usefulness of the findings. Following QTL detection and the identification of candidate gene(s) linked to different MRLs, further analysis is needed for its validation. In parallel, a good cytological system needs to be optimized to visualize *A. bisporus* meiosis which could also help to identify

candidate genes clarifying the different MRL in *A. bisporus* after we have fine mapped the QTL regions. While fine mapping studies in plants, several thousand offspring plants are required to enable us to look for recombinants which are a huge investment in time as well as resources. Isolating large amounts of offspring is relatively easy in *A. bisporus*. Associating candidate gene(s) linked with CO localization can be further screened in a broader panel of mushroom genotypes and also in other germplasm. Finally, the gene(s) involved in MRL of *A. bisporus* can be used not only in mushroom but also in plant breeding, because I assume that the mechanisms are very similar, and can modify CO recombination using genome editing tools. Although modifying CO recombination is still in its infancy, the MRL of *A. bisporus* promises that we will start understanding how CO localization at the ends of the chromosomes is controlled in future. As discussed, this knowledge will help mushroom breeders to generate new mushroom cultivars without considerable linkage drag. It would also help for improving reverse breeding efficiency by directing CO recombination at the extreme ends of the chromosomes.

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Summary

Acknowledgments

About the author

Education Certificate

Summary

The button mushroom, *Agaricus bisporus* var. *bisporus*, is one of the most cultivated mushrooms worldwide. Even though wild isolates of this variety have a broad genetic variation, the traditional and present-day hybrids only have a very narrow genetic base. The button mushroom has a typical meiotic recombination landscape (MRL) in which crossover (CO) events are predominantly restricted to the extreme ends of the chromosomes. This has been one of the main obstacles for mushroom breeders in improving or generating new mushroom hybrids due to a considerable linkage drag. In addition, the secondary homothallic life cycle of the *bisporus* variety leads to the formation of mainly having two-spored basidia, each containing non-sister post-meiotic nuclei which makes them unsuitable for breeding. Screening spores to identify the homokaryotic spores produced by the rarely formed four spored basidia, creates an additional bottleneck for breeders. A wild variety of *A. bisporus*, i.e., *burnettii* appeared to have CO spread more evenly across the genome. This variety is mainly heterothallic and produces haploid offspring in large frequencies. The existence of two extremely different MRLs in two compatible *A. bisporus* varieties offers an excellent opportunity to study the genetic basis for positioning CO in meiosis. The main objective of the research presented in this thesis initially was to examine meiosis of the var. *burnettii* in more detail and subsequently to identify genomic regions revealing the difference in MRL of the two *A. bisporus* varieties.

Over the past few years, genotyping has no longer been considered a major bottleneck in molecular breeding and this also holds true for button mushroom. The availability of genome sequences in the *bisporus* variety has produced many more informative markers such as SNP. **Chapter 2** aimed to *de novo* sequence one of the haplotypes of a heterokaryotic strain of the *burnettii* variety using the PacBio sequencing technique and resequencing the other haplotype using Illumina HiSeq. In parallel to this, we used Genotyping by Sequencing (GBS) to construct the first linkage map of the *burnettii* variety, showing a more or less even distribution of COs across the genome. The constructed linkage map has also proved to be a useful tool for *de novo* assembly of the *burnettii* variety genome sequence. Genome sequencing the haplotype of the *burnettii* variety resulted in a telomere to telomere genome sequence of almost all thirteen chromosomes. In addition, we performed comparative genome sequence studies between the *burnettii* variety and the previously sequenced genomes of two of the *bisporus* variety homokaryons, indicating high levels of collinearity between all three genomes. The only chromosomal rearrangement to be found was on chromosome 10, where an inversion of ~ 800 kb in the *burnettii* variety was detected compared to the var. *bisporus* genomes.

As a starting point for unravelling the genetic basis underlying MRL in the *A. bisporus*, we performed quantitative trait loci (QTL) analysis using *bisporus* and *burnettii* varieties. In this thesis we used two heterokaryotic strains, i.e., Horst U1 strain (*bisporus* variety) and Bisp119/9 (*burnettii* variety). In **Chapter 3**, an inter-varietal population was developed from a cross between a constituent nucleus of the *bisporus* and the *burnettii* variety. This population contains 178 haploid progenies which were genotyped by 210 SNP markers to construct a genetic linkage map, which proves to be a solid foundation for exploring the genetic control of MRL of *A. bisporus*. In addition, we performed a comparative genetic mapping study using the genetic maps of the *bisporus* variety Horst U1 (Sonnenberg et al. 2016), the *burnettii* variety

Bisp119/9 (**Chapter 2**) and the inter-varietal hybrid (**Chapter 3**) by selecting markers having similar positions in these three maps. In contrast to the *bisporus* variety where CO events are mainly restricted to chromosome ends, the *burnettii* variety shows a more or less equal distribution of CO events across the entire genome. The recombination landscape of the inter-varietal hybrid shows an intermediate pattern to that of both varieties.

The MRL trait is expressed as a CO event in the offspring of each individual of the inter-varietal mapping population presented in **Chapter 3**. For this reason, the individuals of the inter-varietal mapping population were intercrossed and outcrossed to generate three types of second generation hybrids (**Chapter 4**). Two compatible tester homokaryons derived from the *bisporus* and *burnettii* varieties were used for outcrossing. Subsequently, the haploid progenies from each type of second generation hybrids were isolated to generate three types of segregating populations. The haploid progenies from segregating populations were genotyped with SNP markers covering the whole length of all the chromosomes. Recombination frequencies were determined at distal ends and elsewhere on the chromosomes and used to compare recombination frequencies between chromosomes within each population as well as between segregating populations across all chromosomes. A prerequisite for successful QTL mapping the MRL is to select segregating populations in which the segregation of MRL is clear. We observed that segregating populations outcrossed with the *bisporus* tester homokaryon were the most useful populations to generate haploid offspring in which COs are assessed for further QTL study of MRL at the time when this research was carried out.

To map genomic regions involved in the different MRLs of *A. bisporus*, 71 homokaryotic offspring of the inter-varietal hybrid were outcrossed with an unrelated tester homokaryon of the *bisporus* variety. Subsequently, the haploid progenies were isolated from each hybrid and genotyped with SNP markers. Marker pairs were generated for the end regions of chromosomes to assess CO there or anywhere else on the chromosomes for each segregating population. QTL mapping analysis revealed two QTLs located on chromosome 1 and three others located on chromosomes IV, VI and VII. The QTLs identified span large parts of their respective chromosomes; therefore further strategies are needed for a more precise assessment and localisation of MRL.

Overall, in **Chapter 6** the results provided in this thesis are summarized and put into a broader context and consideration has been given as to how the results presented in this thesis may contribute in furthering our understanding of the MRL of *A. bisporus,* its application in breeding programmes and also the future researches required to determine the factors underlying the MRL of *A. bisporus*.

Acknowledgments

It's true when they say "life is what happens" when you are completing your dissertation! One of the joys of completion is to look back on this PhD journey and acknowledge all the people who were directly or indirectly involved along the way. I would like to convey my gratitude to all those who gave me lots of help to the build-up of my scientific personality, made this dissertation possible and also to those who have come along my way and brightened up my life-journey.

First of all, I would like to thank my supervisor and co-promotor Dr. Anton Sonnenberg who gave me the opportunity to join his group and start the PhD journey. While working with you, I felt sometimes I was communicating to an extra-terrestrial, and perhaps that feeling has sometimes been mutual. Nevertheless, you inspired me to challenge myself for scientific thinking and discussed with me to find constructive solutions for many subjects. Our styles are vastly different. Saying 'I don't believe you' would help some people build confidence and prove arguments while for others results in to discouragements. However, I became a stronger scientist and person and more independent and aware that to get ahead as a researcher you not only need good skills in the lab but also good management and communication skills.

I extend my utmost gratitude to my promotor, Prof. Richard Visser for his leadership, constant support, encouragement and positive words on my work. You gave me new confidence in myself and my ability. Your fast response, availability under such a busy schedule and comments on the thesis made it possible to produce this work. Almost to my surprise, you also gave me all kind of supports in my personal life whenever I needed. From the bottom of my heart, thank you for believing in me, all your help and support.

I would like to thank all the members of the Mushroom group for their friendly support and discussions at group meetings. Although some of you have already left, you will always be remembered. Brian, thank you for being a technical assistant in my PhD project, without your help it would have taken me longer to produce this work. You were also a great friend! You have given me valuable advice not only at work but also in my personal life. You gave me helping hands and listening ears when I needed. I greatly appreciate your kindness and hope to see you and Bertus again somewhere in this world. You both are more than welcome to visit my country 'Iran' and it would be my pleasure to host you both. I would especially like to thank Patrick for his scientific input and his friendship. Dear Patrick, I was greatly impressed by your scientific advices and creativity. I really appreciated you spending your valuable time with me to discuss my project. It is also unforgettable when you helped me to solve my housing issue. I cannot thank enough for all your help. Dear Wei, thank you for being a great friend of mine. You were in the last year of your PhD when I started mine. That time, you had taken the main path of the PhD journey that I am taking today. We shared experiences on our work and you gave me valuable advice in this regard based on your experience. Jose and Marcel, I would like to thank you for all your help in the spawn lab. Without your support, it would have been impossible to perform all the cell tests on schedule. Ed, I really appreciate all your support during all cell tests for mushroom growing and harvesting. Those cell tests were the solid foundation of all the data I achieved in my project. Dear Karin, thanks for inspiring happiness

and comfort with willingness to support and help. Thanks for all your smiles and laughs making us feel happier.

My greatest gratitude is also to my students/assistants who also became my friends: Sabine, Jill, Dirk, Riina, Peter and Lujun. Working with you guys was a pleasure. Thanks for your time, hard work and patience. Thanks for challenging me into explaining things as clear and simple as possible.

This dissertation would have been impossible without the fruitful collaboration with Rijk Zwaan and Limgroup. Our meetings allowed me to make important progress at critical times of my project. Your collaboration gave me the chance to discuss ideas and problems whenever needed. Dear Evert Gutteling and Ron van der Hulst, I would like to thank you for the welcoming feeling I got every time I was confused and had questions.

I spend five years in the same office with people coming and going; now it's my turn. I had/have two wonderful friends more than officemates and had lots of laughs; Marine and Anne. I will always cherish the fun atmosphere in the office. Marine and Anne, I am thankful for all your kindness and friendship. We always had interesting conversation together. Thank you for encouraging me to get over challenges and struggles. I wish you both the utmost success in your future endeavours.

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My sincere appreciation is extended to my Iranian-family in Wageningen. Nafiseh and Arman, there is a saying 'The greatest gift of life is friendship', and I have received it from both of you. I am so fortunate to have friends like you and I wish that our friendship should go a long way. You not only gave me your shoulder to cry on, but you patiently, and without judgment, listened to me ramble on and on as I tried to sort things out. Shaghayegh, Jalal and my little friend Taher, our friendship meant a great deal to me, and I feel very lucky to have your love and support through my life. Robab and Gerard I would also like to thank you for sharing my hard times and my happy times. Your friendship and caring make each of them better. When I met you, I met a friend for life. I would also like to thank all my friends back home for all the joy and laughter they provided from afar.

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It was long ago that I was getting into my wedding dress and I intended to keep it forever. Amir you were one of my best friends and you were by my side and promising to stand by me until the end of time. However, it feels like an eternity has passed since then. It's been a whirlwind of trying to understand what happened - of trying to make sense of why it had to end. You always encouraged me to continue with my study and become a proof that women can be successful researchers even when they are in foreign countries. I would thank you for our good moments and for the times you stood by and protected me. Thank you for the memories we created in our long time as a couple and our even longer friendship. Past years have made me a stronger person and helped me realize that maybe some things aren't meant to last forever. Thank you for being a big part of my life for a big slice of time.

My special gratitude goes to my beloved friend Mehdi whom helped me face the world on my own while I'm still young, while my ambition and desire is still strong. You helped me see the world in a new light. You taught me to be more careful with my heart in the future. You loved me in spite of many circumstances and for that, I'm grateful. You were patient and you believed, and for that, I thank you. I am eternally grateful that you're in my life today. Wherever the road takes us, I'm glad that I got to walk down that road with you, creating so many beautiful memories along the way. At the end of the day, "doing life" with someone that you love and trust is a wonderful feeling, and I know that through thick and thin, I can count on you. I can truly say that before anything else, you are my best friend, and I think that our strong foundation is what I cherish the most.

My deepest gratitude goes to my family without whom I have nothing. This dissertation would have simply impossible without your constant support. Daddy, thank you for being my DAD! You've taught me to be fair, humble, kind, independent, and to be open minded. In you I see the gift of inherit and impeccable intelligence. I know you to be a philosopher of sorts, a writer, an intellectual, a critic, a thinker of deep thoughts and a speaker. You will always be my best friend, my greatest mentor, my motivation and my deepest roots. It is an honour to be your daughter and carry your name. Words cannot describe my gratitude to my mother whose love, understanding and endless patience were with me every step of the way. My mere expression of thanks does not suffice. I love you both beyond words.

At Last, many thanks to everyone for playing a part in my time at Wageningen. I may have missed your names here, but you are always remembered and appreciated.


Thank you

About the author

Narges Sedaghat Telgerd was born on May 4th, 1985 in Tehran, Iran. In 2003, Narges started to study at Tehran University, where she graduated with distinction and obtained BSc degree in Plant Protection. In the last two years of bachelor study, Narges focused on Plant disease and further raised her interest in Mycology and biocontrol of different plant diseases. Therefore, Narges continued her master's study in "Plant Pathology" in the same University after obtaining the second grade among 1200 participants in entrance exam of all universities across Iran. During her master's study, she focused on controlling aflatoxin in Pistachio using biocontrol agents. After two years of study, she decided to study abroad and further deepen her knowledge. In 2010, Narges started to work as a guest researcher at the laboratory of Plant Breeding of Wageningen University & Research in the Netherlands under the supervision of Dr. Anton Sonnenberg. In 2012, she was offered an opportunity to pursue doctor degree under supervision of Dr. Anton Sonnenberg and Prof. Richard Visser at the same laboratory. She started her PhD study on unravelling the genetic base of the meiotic recombination landscapes in two varieties of the button mushroom. In this thesis. The results obtained during her PhD period are presented.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Narges Sedaghat Telgerd
Date: 25 October 2017
Group: Laboratory of Plant Breeding
University: Wageningen University & Research
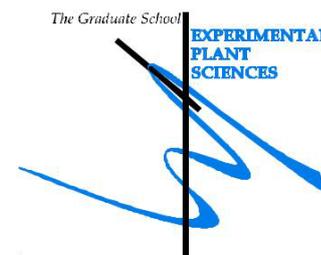
1) Start-up phase	*date*
► **First presentation of your project**	
Title: Synaptic balanced chromosome segregation in *Agaricus bisporus*	03 Jun 2013
► **Writing or rewriting a project proposal**	28-29 Jan 2016
Title: Synaptic balanced chromosome segregation in *Agaricus bisporus*	Dec 2012
► **Writing a review or book chapter**	07 Dec 2012
► **MSc courses**	24 Jan 2014
► **Laboratory use of isotopes**	03 Dec 2014
Subtotal Start-up Phase	*1.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day, Leiden, NL	29 Nov 2013
EPS PhD student day (Get2Gether), Soest, NL	28-29 Jan 2016
► **EPS theme symposia**	
EPS Theme 4 'Genome Biology' , Nijmegen, NL	07 Dec 2012
EPS Theme 1 'Developmental Biology of Plants ',Wageningen, NL	24 Jan 2014
EPS Theme 4 'Genome Biology', Wageningen, NL	03 Dec 2014
► **National meetings (e.g. Lunteren days) and other national platforms**	
Symposium '100 Years Plant Breeding', Wageningen, NL	31 Aug 2012
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	22-23 Apr 2013
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	14-15 Apr 2014
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	13-14 Apr 2015
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	10-11 Apr 2017
► **Seminars (series), workshops and symposia**	
STW anuual congress, including workshop 'how to deal with supervisors', Utrecht, NL	03 Oct 2012
Seminar Tabaré Abadie "Statistical Genetics"	22 Nov 2012
Seminar Tom Mitchell "olds strong selection on the genes controlling complex tarit in complex environment"	10 Dec 2012
Seminar Ralph Panstruga "Comparative pathogenomics of powdery mildew fungi: chasing the molecular secrets of obligate biotrophy and fungal pathogenesis"	12 Dec 2012
Writing for high impact journals by Andrew Sugden	08 Feb 2013
Seminar Gabino Sanchez Perez "Is your Research becoming Digital? Time to call the BioinforMagician"	12 Mar 2013
STW meiosis projects meeting, Fijnaart, NL	04 Nov 2013
Seminar Eric Schranz "Whole genome duplications as drivers of evolutionary innovations and radiations"	21 Nov 2013
WGS PhD Workshop Carousel	02 Jun 2014
Networking Event of STW annual meeting meiosis 2014	13 Nov 2014
Symposium 'Omics Advances for Academia and Industry - Towards True Molecular Plant Breeding'	11 Dec 2014
Seminar Jennifer Doudna 'Rewriting our Gene'	30 Sep 2016

Networking Event of STW annual meeting meiosis 2016	14 Nov 2016
Career day, Wageningen, NL	07 Feb 2017
BCF Career day, Utrecht, NL	11 May 2017
► **Seminar plus**	
► **International symposia and congresses**	
Conference 'Next Generation Plant Breeding', Ede, NL	11-14 Nov 2012
1st Iranian Mycological Congress, Rasht, Iran	03-05 Sep 2013
12th European Fungal Genetics Conference,Seville, Spain	22-27 Mar 2014
European Neuropsora meeting, Seville, Spain	23 Mar 2014
2nd Iranian Mycological Congress, Karaj, Iran	23-25 Aug 2015
13th European Fungal Genetics Conference, Paris, France	03-06 Apr 2016
International Society for Mushroom Science (ISMS) Conference, Amsterdam, NL	29 May-02 Jun 2016
► **Presentations**	
Talk: 1st Iranian Mycological Congress, Rasht, Iran	03-05 Sep 2013
Poster: 12th European Fungal Genetics Conference,Seville, Spain	22-27 Mar 2014
Talk: Mycology fall meeting, Utrecht, NL	28 Nov 2014
Talk: 2nd Iranian Mycological Congress, Karaj, Iran	23-25 Aug 2015
Poster: 13th European Fungal Genetics Conference, Paris, France	03-06 Apr 2016
► **IAB interview**	
► **Excursions**	
Visit Sylvan company, Hooymans Compost company and some mushroom growers	11 Dec 2012
Subtotal Scientific Exposure	*20.0 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Genetic linkage mapping course, Kyazma, Wageningen, NL	22-24 Mar 2013
QTL analysis course, Kyazma, Wageningen, NL	26-28 Mar 2013
Postgraduate course 'Transcription Factors and Transcriptional Regulation', Wageningen, NL	17-19 Dec 2013
Bioinformatics: A Users Approach (a practical course), Wageningen, NL	25-29 Aug, 2014
Postgraduate course 'Genome assembly', Wageningen, NL	28-29 Apr 2015
► **Journal club**	
Participation in a literature discussion group at PBR	2012-2016
► **Individual research training**	
Subtotal In-Depth Studies	*7.9 credits**

4) Personal development	*date*
► **Skill training courses**	
ExPectationS (EPS Career Day)	13 Jun 2012
PhD Competence Assessment	22 Jan 2013
Techniques for writing and presenting a scientific paper	10-13 Dec2013
Start Efficient Writing Strategies	11 Mar 2015
► **Organisation of PhD students day, course or conference**	
Monday Morning Seminars for Plant Breeding Department in 2013-2014	Jun 2013-2014
Organizing ISMS Conference, Amsterdam, The Netherlands	29 May-02 Jun 2016
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*5.1 credits**
TOTAL NUMBER OF CREDIT POINTS*	**34.5**

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.