UNIVERSITY OF BIRMINGHAM

"USING CYTOLOGICAL TECHNIQUES TO UNCOVER THE INTROGRESSION POTENTIAL OF CONSTITUENT GENOMES INTO THE ECONOMICALLY IMPORTANT CROP *BRASSICA JUNCEA*"

A thesis submitted by

Zeeshan Shamim

ID: 1392244

As part of the requirement for the degree of

Doctor of Philosophy

01/03/2018

This project is carried out under the supervision of Dr Susan J. Armstrong and Dr Eugenio Sanchez-Moran at the University of Birmingham.

Abstract

Polyploid crops (containing more than one genomes) have been included in our daily-life food since ages and are used as raw and processed products (wheat, coffee, banana, cotton etc.). Amongst these, allopolyploids are a diverse group which contain at least two different genomes. Owing to a range of options for meiotic pairing, they have evolved a strictly regulated mechanism which inhibits intergenomic pairing and enhances intragenomic interactions equally. This is a dominant phenomenon in natural populations. Brassica is one of the multigenomic genera where diploid and polyploid are linked to each other in the well-known U's triangle which presents a rich source for genetic exploitation.

Meiosis holds the key in sustaining the ploidy intact during reproduction and it produces novel allelic combinations through its conserved set of standards. Of this, homologous pairing and genetic recombination make the meiosis important as it triggers the variation and novelty in subsequent generations. To counter the food demand because of an ever-increasing population, attempts are underway to understand the genetic basis of this mechanism in order to explore and manipulate the unlocked genetic potential in crops.

Current studies are aimed at translating knowledge gained from the model plant *Arabidopsis thaliana* to its closely related crop species of the Brassica genus. Using the molecular cytogenetic tools developed for *A. thaliana*, I have been able to develop a partial karyotype of one of the research-neglected species in Brassica, i.e., *Brassica nigra*. The Genome *In Situ*

Hybridization (GISH) technique performed has enabled me to trace the constituent genomes in a complex polyploid background. The recombination frequencies and their pattern in diploid were quite different from those in the allopolyploid species. For example, this has revealed the contrasting feature in the polyploid with crossovers (COs) localised at the termini of the chromosomes as compared to a non-terminal distribution in *B. nigra*. In addition, I have also been able to study heterologue (homoeologue) pairing between distinct genomes in a hybrid allodiploid plant (AB). The introgression potential across these species could pave the way towards improving Brassica allopolyploid crop species.

Acknowledgement

I would like to thank my supervisor Sue J Armstrong and Eugenio Sanchez-Moran for providing me with the opportunity to work on the project and for their support and advice throughout my PhD. I would also like to thank Prof Chris Franklin for his valuable comments during my studies and for the guidance.

Nothing would have been possible without the good support from the lab members, especially Elaine Howell, Kim Osman, Maria Caucos , Stefen, Chris Morgan and others. I am grateful to Steve Price and Karen Staple for providing technical helps which matters a lot.

I would like to dedicate this PhD to my mom and late Father Muhammad Shamim who had been the source of inspiration for me throughout my academic life. My wife was very cooperative and helpful during this period which really boosted my confidence and helped me in achieving this milestone. My brother, Rizwan Shamim and sisters were there every time I felt discouraged and they backed my up through-out my academic career right from the beginning.

I would like to thank my friends Salman, Muhammad and Sami lala, my cousin Kamran Zaheer & his family and all other family members who supported me through out and encouraged me while achieving my goals. Special thanks to Amar Nasim for being so caring and kind who inspired me to pursue higher education from a reputed institute.

I would especially like to thank my family for their love and encouragement.

Table of Content

Table of Figures

Table of Tables

1 Introduction

1.1 Brassica

The Brassicaceae family includes a wide range of mustard species in the Brassica genus having diverse phytochemical and morphological profiles, hence making it one of the important plant families in agriculture which is currently feeding millions worldwide (Arias *et al.,* 2014). Brassica includes 37 distinct species (Gomez Campo, 1980) used to fulfil several human needs. Owing to a greater diversity and potential in the brassica morphotypes, it has been used for several purposes from condiments to vegetables at different times worldwide (Gupta and Pratap, 2007; Cheng *et al.,* 2014). The origin of Brassica (mustard) cultivation can be traced back to Asia from where it started its evolutionary course through several countries in Europe and Canada to augment allotetraploid species in the genus. Nearly 70% of global rape production was contributed in 1952 by Asia which declined gradually to a point in 2005 when more than 50% was produced in Europe and Canada together overtaking the Asian monopoly (Gupta and Pratap, 2007). Nigra and Oleracea are two of the eight clades in the Brassica genus identified from molecular phylogeny (reviewed in Arias *et al.*, 2014). The Nigra lineage comprises only one of the three diploid U triangle constituents, i.e. *Brassica nigra* whereas the Oleraceae/ rapa lineage includes two of the U triangle parents, *Brassica oleracea* and *Brassica rapa* (which are widely used as vegetables in different forms all over the world). The well known U triangle of Brassica (U, 1935) encompasses three diploid and three amphidiploid species derived from the interspecific crosses in these two clades. The distant clades in triangle had limited breeding compatibility inspite of having a common ancestor to yield interspecific

hybrids (Coyne and Orr, 1989; Foltz, 1997) which ensued into stable allotetraploids by the combination of diploids in all three probable combinations (Lysak, 2005).

The cytogenetic relationship of the six economically important species in Brassica genus had been depicted in the U triangle (UN 1935; Fig. 1-1) where *B. nigra (L.) Koch* (N= 8), *B. oleracea L.* (N= 9), and *B. rapa L.* (N= 10) represent the diploid species in the vertices, whose respective hybridized amphidiploid forms are represented by *B. carinata (*N=17), *B. juncea* (N= 18), and *B. napus* (N=19), recommending interbreeding and hybridization had taken place in past (reviewed in Gupta and Pratap, 2007). These diploid and amphidiploid species are a close relative of the model plant *A. thaliana* which make them interesting candidate species for the translation of tools adopted for the model plant to study genetic relation. Two of three mainly cultivated diploid species and one allopolyploid of the known U's triangle are the main focus of this studies (shown in Figure 1-1).

Three diploid species in U triangle are represented by different genomes, viz. Genome A was attributed to *B. rapa L.* (previously *B. campestris*), the genome-B to *B. nigra L.* and genome-C to *B. oleracea L.* (Branca and Cartea, 2011). Among the agriculturally important species of this genus, *B. rapa L.* and *B. napus L.* are categorized as rapeseed (Khan and Munir 1986). Whereas *B. juncea* has been used for producing vegetable as well as oil for human consumption and is equally essential for cattle feed.

1.1.1 Brassica Genetics

Archaeological evidence revealed that *B. rapa* (turnip rape) and *B. nigra* (black mustard) were the first domesticated diploid species in this genus besides the amphidiploid *B. juncea* (Indian mustard) that originated from the cross in between them (Navabi *et al.*, 2013). Cytogenetic

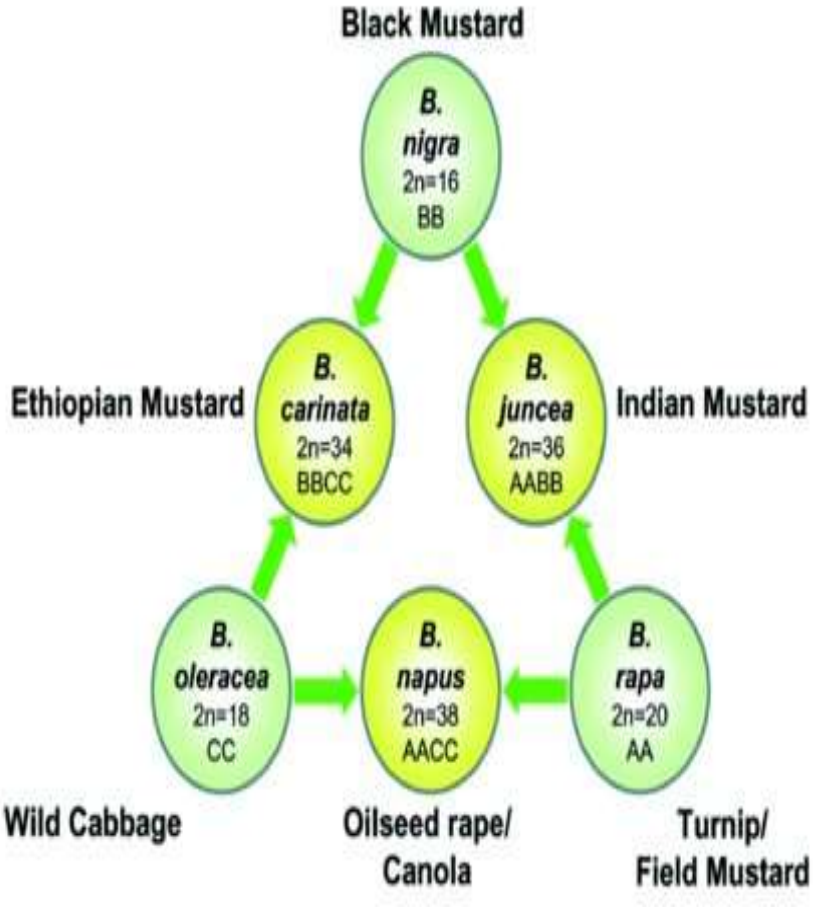


Figure 1-1 Brassica genotypes in U's triangle

and molecular studies reveal diphyletic origin which succeeded after the triplication of the ancestral genome, Proto-Celepine Karyotype (tPCK, N=7) that subsequently had been

branched into two distinct lineages, firstly the *B. nigra* lineage and the second *B. oleracea/rapa* lineage (Namai, 1976; Prakash and Hinata, 1980; Pradhan *et al.*, 1992; Palmer *et al.*, 1983; Song *et al.*,1990; Warwick and Black, 1991). The genetics modification renders distinct morphotypes in each Brassica species which helped them in the speciation process and eventually barring them from interspecific crosses (Cheng *et al.*, 2014).

1.1.2 Genome Diversity In Brassica

Genomic episodes like inversions, duplications, insertions and deletions at a molecular level had been potential causes of the observed breaks in the co-linearity of Brassica genomes. Inversions had a significant impact on plant evolution and would have barred the reproduction among individuals during the speciation drive (Garcia-Mas *et al.*, 2012). At the same time genome fractionation, deletion and transposable elements could also have led to the inactivation of genes; hence these are the evolutionary drives in the speciation process. The highest percentage of gene fractionation (the losses of genome part as a result of adopted duplication following whole genome duplication via excision of chromosomal segments) appears to have occurred prior to species evolution as gene loss is fairly universal among all three diploid genomes. Furthermore, gene content and its order in the diploids also show strong homology despite their reproductive isolation and preferences (Eilam *et al.*, 2010).

B. nigra, which does not establish effective pairing and subsequent structures (i.e. chiasma and bivalent) with the A and C genomes, possibly have had discrepant shuffling at the micro-level. This signifies the fact that chromosomal rearrangement events (such as the observed inversion)

would have been a potential factor in the earlier evolution and the distinct speciation of the B genome than the A and C genomes (Navabi *et al.,* 2013). The homology between A & C genome has been extended phenotypically in addition to chromosomal structures, where both share a similar structure of roots and leaf (reviewed in Cheng *et al.,* 2011).

B. nigra (BB) was replaced by one of its derivatives allotetraploid *B. juncea* (AABB). During the 1950s in Asia the use of this mustard crop hampered efforts to improve this vital B genome. While the other two diploids (*B. rapa* and *B. oleracea*) were a subject of focus in the genetic and genomic arena which kept *B. nigra* as an unexploited and research-suppressed genetic resource (Chauhan *et al.,* 2011). Recent studies show that *B. nigra* is an important reservoir of genes which could help crops in combating the disease as well as abiotic stress in the field, therefore an improved understanding of this important genome holds promise for the introgression potential of valuable genes in economically important brassicas (reviewed in Navabi *et al.,* 2013).

The diploid members of A and C genomes have already been sequenced which reveals the conservation of the basic genomic blocks in different orders and useful information can be extracted for a better understanding of the biological system. Sequencing of the allotetraploid, *B. juncea* reveals that the constituent genome A is different from the A in *B. napus* yet possesses conserved ancestral blocks (Yang *et al.,* 2016). The detailed analysis of all diploids in U's triangle will help us uncover outstanding questions such as diploidization phenomenon, association between nonhomologous chromosomes in the allotetraploid background and the discrimination between the chromosomes in U's triangle.

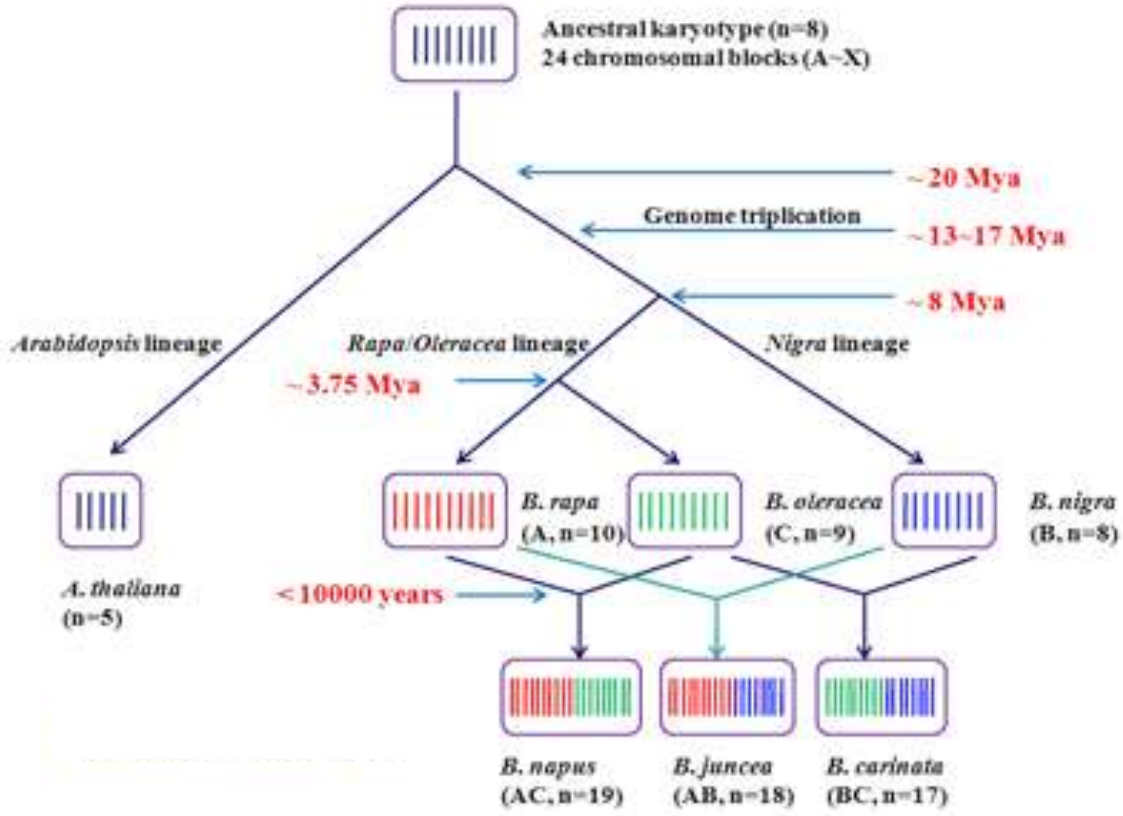


Figure 1-2 Evolutionary pathway for Brassica diploid and allotetraploid species (Adopted from Zhao, 2017)

B. rapa is mostly used as vegetable these days and *B. napus* is known for its oil potential whereas *B. juncea* is used for both vegetable as well as for oil production, therefore understanding its genome structure and organization would further our understanding of evolution and its course. Polyploid brassicas possess evolved agronomically important traits and experience the genome dominance effect which would have been helpful in the selection of certain genes during stabilization. Series of the triplication and diploidization events remain the key determinants in polyploidy and in Brassica specifically as it involves substantial gene

loss and the reshuffling at genome level (reviewed in (Wang *et al.,* 2011; Mun *et al.,* 2009). It has been found that the A subgenome in *B. juncea* shares less conserved region/ synteny with that of *B. rapa* ancestor against the *B. rapa* and the *B. napus*. Furthermore, *B. juncea* had undergone more gene loss from *B. rapa* ancestor (A genome) as compared to what was found in *B. napus,* but the ratio between *B. rapa* and *B. nigra* genome loss is comparable in *B. juncea* itself (Yang *et al.,* 2016). Such revelation prompted for further analysis using the neighbour joining tree method to study phylogeny. Single Nucleotide Polypmorphism (SNPs) was assayed to create a phylogenetic tree, for neighbour joining tree, for A subgenome which confirmed its divergence in *B. juncea* and *B. napus* and also, the A subgenome in *B. juncea* was derived from the species found in Asia while that of *B. napus* found its roots in European origin thereby confirming the independent geographical lineages (Yang *et al.,* 2016). Sequencing of *B. juncea* has unveiled that 16.2% genes show homoeologous expression and out of which 8.2% are contributed from B sub-genome which also bases the phenotypic plasticity in the allopolyploid (Yang *et al.,* 2016).

Structural changes in the chromosomes are indispensable in polyploids and are present in much higher rate than expected. The depleted genomic region is compensated with the homoeologous exchanges (HEs) (revealed from *B. napus* sequencing) and is dependent on the collinearity of telomeres which helps in bringing the chromosomes close enough for a switch, confined to certain region along the chromosomes at random. The regions next to telomeres are involved in recognition and pairing of the chromosomes. Sequence based approaches (mRNA seq) can be used to find HEs in the genome as well as trace the dominance effect of a genome in a polyploid but the copy number of the silenced genome would be hard to analyse (He *et al.,*

2017). The transcription display tile plot is very effective in analysing genome dominance effect in the allopolyploids (Harper *et al.,* 2016). Moreover, it has been found that the subgenomes in *B. juncea* have less collinearity than those constituting *B. napus* and therefore the distribution and the frequency of HEs are different and less than the *B. napus.* The HEs are more confined to sub telomeric region in the chromosomes (He *et al.,* 2017).

1.2 Breeding Perspective

Chromosomal pairing and structural modifications lay basis for the variation and evolution in heredity material, which would be used in plant breeding for several objectives, including enhancing crop production and resistance against biotic and abiotic factors. New allelic combinations could potentially combat future food insecurity, resulting from climatic as well as population growth (Osman *et al.,* 2011) by manipulating indigenous genomic content in cultivated crops for the more significant interest of humanity.

The significant advances in comparative genetics revealed collinearity in the genomic blocks (GBs) across the Brassica species which shares its conservation with the model plant *A. thaliana*. Further, 21 out of 24 labelled blocks are common in between *A. thaliana* and *B. napus* (Parkin et al. 2005). Genome sequencing of *B. rapa* (genome size = 485 Mb) and *B. oleracea* (genome size = 630 Mb) prompted prospects into the study of triplicated ancestral genome structure in detail concerning their composition and organization. This quest for exploring Brassica genus is underway for the understanding of the rich morphotypes and its plasticity.

Comparative genomic analysis revealed the syntenic relationship between *A. thaliana* and *B. rapa* genome based on greater number of copies present in Brassica genomes.

1.3 Wide Hybridization

Coalescing two different genomes with a varying number of chromosomes into a single nucleus is referred to as Distant or Wide hybridization. It could involve one or two genera (intra- or inter-genic) for combining two different genomes together, nevertheless a smart approach of transferring valuable genes from one species to another for the sake of genetic improvement (Liu *et al.,* 2013; Lelivelt *et al.,* 1993). It overcomes the species barrier concept for gene flow and results in the variation of genotypic as well as at the phenotypic level.

Genetic diversity could be achieved by incorporating alien chromosomes either as an addition, substitution or translocation into a genome for beneficial purposes (Qi *et al.,* 2007). It could also lead to so-called chromosomal engineering in improving crop diversity. An F1 hybrid between distant parents provides a broad-spectrum genetic pool to be used for breeding purposes as it holds promise for producing double haploid generation afterwards (Chan, 2010, Liu *et al.,* 2013). Haploid lines could be treated with certain chemicals (e.g. colchicine) for the sake of doubling chromosomes by inhibiting spindle fibres to induce polyploidy in breeding programmes (Forster *et al.,* 2007). One constituent genome could also be eliminated from allopolyploids and be used for haploid production.

Different genomes in brassicas combine with low frequency together in the hybridization process and are very true for the AA and BB cross (Sheng *et al.,* 2012; Srivastava *et al.,* 2004; Attia and Röbbelen, 1986). In the chromosome structure, a Histone H3 variant in centromere trades conventional H3 to disrupt connection with spindle fibre during cellular division leading to discerning chromosome elimination in distant hybrid (Henikoff and Dalal, 2005; Mochida *et al.,* 2004; Jin *et al.,* 2004; Kim *et al.,* 2002).

A foreign nuclear genome faces incompatibility issues with the female cytoplasm to produce any embryo. Changes in chromosome structure could be anticipated to counter nuclear-cytoplasm interaction, and they could be evident in a hybrid of Brassica B and A/C genomes (Pradhan *et al.,* 1992; Warwick and Black, 1991; Yanagino *et al.,* 1987; Palmer *et al.,* 1983). To overcome certain barriers during hybrid development embryo rescue is suggested after controlled pollination where conventional emasculation is followed by transfer of pollens from a different male parent. Embryos after 65 days old, when siliques starting to develop are selected aseptically to grow on media in the laboratory to counter incompatibility (Ghani *et al.,* 2014; Rahman, 2004; Zenkteler, 2000).

1.4 Meiosis in polyploids

Organisms having more than two sets of chromosomes as a result of Whole Genome Duplication (WGD) are termed as polyploid which is considered as an evolutionary dead end and is prevalent in the plant kingdom. The duplication (WGD), itself is believed to occur because of abnormal cell division as a result of which chromosomes are unable to segregate

faithfully and end up with doubled chromosomes (reviewed in Yant and Bomblies, 2015). Several epigenetic and genomic changes occur in response to the abrupt fluctuations in chromosomal configuration and numbers to alter the phenotype, linked directly with the adoptability and the stability (Yang *et al.,* 2014; Vergara *et al.,* 2016). The innovation and modification in polyploid render higher chances of habituation in the native and different environmental conditions (Ramsey, 2011). The forces behind such successful naturalization and adaptation are under continuous efforts to get insight into the mechanisms for a better understanding of the evolutionary basis.

Polyploidy is a widely spread phenomenon in plants, which features rounds of duplication episodes in the evolutionary past. Based on genome content, polyploids are branched into auto- and allo-polyploids. In autopolyploid, the single genome is believed to have undergone WGD event whereas in allo-form hybridization is an integral factor for bringing related genomes together besides WGD. The evolved gene content and genome network pose stability issue in the form of fertility for the continuation of generations in the nascent polyploids in addition to various other challenges (Comai, 2005; Yant and Bomblies, 2015). To overcome the challenges and for the propagation stabilized meiosis is vital and crucial. This has multiple options for the pairing of chromosomes and eventually segregation.

In autopolyploids, during meiosis alignment and CO could occur between chromosomes without any preferences for pairing and therefore it features multivalent formation between more than two homologues during pairing stage that would potentially compromise regular chromosomes segregation (Ramsey and Schemske, 1998; Comai, 2005). Such observations are

frequently observed in synthetic polyploids where chromosomes are involved in entanglements and multivalent formation to compromise with segregation and thus causes irregularities during meiosis (Yant, *et al.,* 2013). Established autopolyploids have evolved diploidized meiosis featuring bivalents with reduced mean chiasma frequency and a shift toward chromosomal terminals (in contrary to the progenitors) as a mechanism to keep the integrity (Yant *et al.,* 2013; Bomblies *et al.,* 2016). The designation and reduction of CO along the chromosomes are believed to ensure faithful segregation for normal meiosis (Bomblies *et al.,* 2016).

In allopolyploids, chromosomes find preferences for their homologues despite related chromosomes are present in the nucleus. Nevertheless, chromosomes could potentially adopt multivalent configurations between homologue and the non-homologue (homoeologue) that would give rise to aneuploid upon mis-segregation and prove lethal to a cell. The chromosomes are framed into bivalent structures exclusively intra-genomically for the disomic inheritance. Such robust adaptation in allopolyploid can be defined by two potential mechanisms for preferential pairing, i.e. system of genome demarcation and the genetic control.

Structural differences and the sequence dissimilarity of the chromosomes are critical determinants in recognising homologue before developing synapsis and promising dissociations of the bivalent for the ploidy reduction (Levy and Feldman, 2001). Such differentiation at the chromosomal level are not reported to confer stability in the polyploids and further insight would be interesting to substantiate the theory. Various attempts have been made in different species to study the genetics involved in the stabilization of the polyploid that

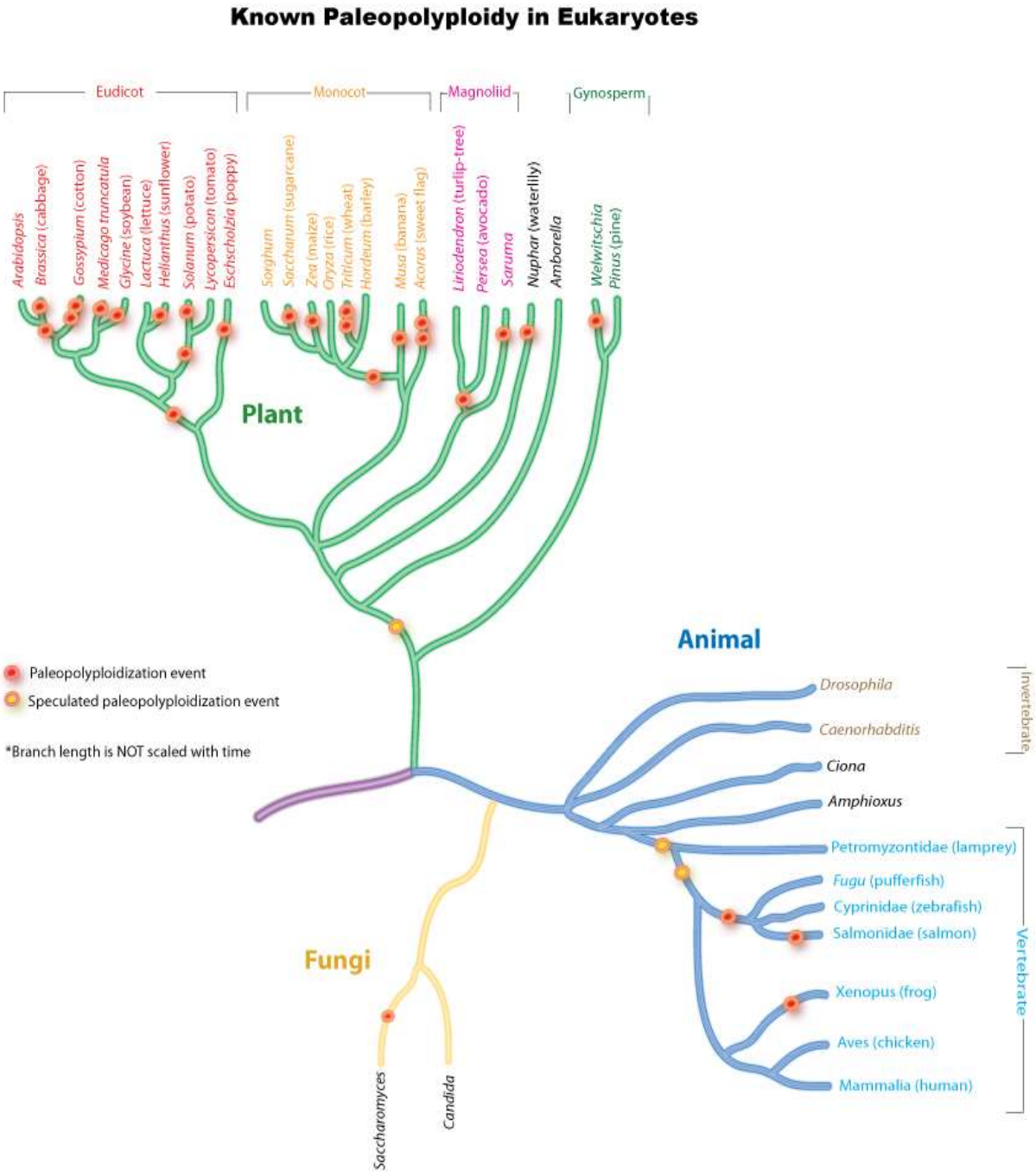


Figure 1-3 Evolutionary tree depicting life after genome duplications at different times during evolution (from www.wikipedia.org)

would discourage homoeologous pairing and promote pairing among the homologues exclusively (Poggio and Greizerstein, 2016). The genetic control of allopolyploid meiosis has been investigated in various organisms with varying success, where cytogenetic studies proved a powerful and robust tool in analysing the pairing and bivalent configuration (Jenczewski and Alix, 2004). It has been reported that certain segments of chromosomes are responsible for the inhibition of homoeologous association in two well-studied allopolyploids, *Ph1* in wheat and *PrBn* in oilseed rape (Riley and Chapman, 1958; Jenczewski *et al.,* 2003).

Two approaches are usually practised for assessing the genetic control in the allopolyploids, through comparison of a natural allopolyploid with the synthetic neopolyploid and the analysis of the mutants. In the former approach, an artificial allopolyploid is analysed at meiosis against the evolved meiotic behaviour in established ones to reveal any specific meiotic irregularity. CO frequency and distribution are the critical measures of homologues during meiosis which could lead to unravelling the genetic basis (Jenczewski and Alix, 2004). In the second approach, mutants are used which might lack one or more chromosomes that would be helpful in understanding the nature of meiosis, as was used to characterize *Ph1* locus in wheat (Riley and Chapman, 1958; Riley *et al.*, 1959). While in oilseed rape, different approach was adapted, and the natural variations of a varying degree were examined to understand the meiotic control (Jenczewski, *et al.,* 2003). In these cases, pairing occurs exclusively between the homologues and is restricted from developing between the homoeologues.

In allohexaploid wheat, one chromosome was believed to have functional characterization for the suppression of homoeologous pairing (Riley and Chapman, 1958) before it was discovered

that a specific locus on chromosome 5B was actually responsible and referred as *Pairing of homoeologous 1* (*Ph1*) (Riley and Chapman, 1971). It is now known that a cluster of defective CDK genes on chromosome 5B render conformational changes to the chromatin which is crucial in the faithful pairing between the homologues. While in its absence the chromatin undergoes asynchronous remodelling to allow the association between the homoeologues. Recent findings demonstrate that *Ph1* has no significant role during bouquet formation for developing synapsis between the homologues, rather it is developed in late zygotene after bouquet dissolution (Martin *et al.,* 2017). Furthermore, it promotes pairing between the homologues than suppressing the development of synapsis between homoeologues to ensure the CO maturation and segregation.

1.4.1 Meiosis in Brassica napus

B. napus (Oilseed rape) is an allotetraploid in Brassicaceae family and is one of the important crops sown for the oil use. The relationship between brassicas was discovered by Morinaga in 1934 and defined by U Nagahary (1935) in a widely known U's triangle. *B. napus* is a close relative of *A. thaliana* and represents an excellent model of allopolyploidy for studies to understand the evolution and meiosis happening in this species. The stability of meiosis in an allopolyploid can be explained from two different perspectives, genetically and cytologically, where it exhibits disomic inheritance and forms bivalents exclusively at meiosis respectively.

During leptotene, transient bouquet brings the chromosomes together like in *A. thaliana* before impartial synapsis can occur between the homoeologues to give rise to quadrivalents. The synaptic partners are frequently tangled in quadrivalent where four related chromosomes are

linked to each other providing opportunity for pairing to illegitimate partners equally. The lower number of quadrivalents, as compared to the bivalents, demonstrates that the choice of preferential pairing between the homologue does exist which organized the homologous chromosomes together and distances the non-homologues.

The tendency of CO markers in immunolocalization assay goes down from the leptotene to late pachytene where it would be confined to chiasma site. In synthetic *B. napus,* allosyndetic association hints at the absence of any genetic system present in diploid progenitors that could confer legitimate association and suppress homoeologous association. Therefore, in case of allotetraploids, they have evolved a mechanism that controls and regulates the meiosis for aligning and pairing chromosomes in a correct orientation. Such preferential pairing does not regulate CO formation between the homoeologous pairs in the presence of homologues and gives a variety of chromosomal association and configuration, i.e. univalent, bivalent, multivalent.

1.4.2 Pairing Regulator in B. napus, PrBn

The frequency of univalents in a segregating population helped in concluding the diallelic separation of a gene which confers homologous association that works in an integrated network of genes (Liu *et al.*, 2006). The parental pairing configuration is inherited to the offspring suggesting a genetic control for the specific association. It is believed now that there is more than one gene responsible for the stabilization of meiosis in brassica allopolyploid background.

Among many QTLs for regulating meiosis in *B. napus*, *PrBn* is a gene on chromosome 9 which has a profound effect to determine the CO designation. It is a single major gene which controls CO formation, but its mode of action remains to be elucidated despite the precedent of the characterization of *Ph1* locus in wheat. Moreover, it shows polymorphism in the natural population which was absent in case of *Ph1* locus. Such a mechanism might work in *B. juncea* where two distant genomes are present and in-depth research would help us understand the complex nature of diploidization in polyploids.

Two events, Hybridization and Whole Genome Duplication (WGD) have been vital in the evolution of an allotetraploid ancestry. In Brassicaceae the allotetraploid, *B. napus* is a young polyploid and provides grounds for deciphering meiosis with respect to preferential pairing. Despite the divergence among Brassica species, all of three diploids possess conserved chromosomal blocks which would help in pairing homoeologous association. Though the collinearity is not very evenly spread in the whole genome rather frequently it is interrupted by HEs (Lloyd *et al.*, 2017).

1.5 Diploidization in Polyploids

Whole genome duplication (WGD), a crucial phase in evolution, has played a significant role towards the speciation in eukaryotes. The majority of the angiosperm taxa, up to 80%, are believed to have experienced one or more rounds of polyploidization during their life history in contrast to 95% in the pteridophytes (Grant, 1981; Bowers, 2003). It ushered sustainability in several newly evolved species in the changing environmental and climatic fluctuations. Due to

the greater flexibility in polyploid as compared to their progenitors, they absorbed genomic shock apart from fitness issues like meiotic irregularities and reduced fertility (Ramsey and Schemske, 1998). Natural selection favoured the mechanism to adapt back a diploid-like meiotic behaviour for the survival and development.

Cytological diploid organisms are characterised by the formation of bivalent structures of chromosomes in metaphase I during meiosis to ensure symmetrical segregation during gamete formation. In the case of the autopolyploid there are equal chances of pairing for all copies of the duplicated genomes whereas in the allopolyploid, the concept of pairing preferences between homologous chromosomes over homeologous chromosomes prevail. A much known example is of wheat where a particular locus *Ph1* promotes homologous pairing for normalized meiosis thereby discouraging pairing between related (non-homologue) chromosomes to facilitate an equal halving of the complement for viable gametes (Lukaszewski and Kopecky, 2010). This could result in new chromosomal rearrangements and the effects such as, non-functionalization (a mutation where genes are transformed from functional to pseudo-form due to mutation), neofunctionalization (a mutation in genes where an evolved gene attains new behaviour other than its ancestral origin) or subfunctionalization (a mutation in genes where an evolved gene retains a subset of its ancestral function) confer genetic diploidization by inducing preferences for chromosomes to pair with identical chromosomes instead of related ones. This reduces the chances of multivalent formation successfully (Ohta, 2000).

Comparative genomics had helped to reveal the dramatic reconstruction at the genome level observed in neo-polyploid. It is evident from chromosome mapping that the reconstruction

occurs at a much higher rate in polyploid than its progenitor diploids (Soltis and Soltis, 1999). Nuclear-cytoplasmic interaction is equally vital in stabilising synthetic allopolyploids. A chromosome encompasses heterochromatin and euchromatin and is subjected to various elements out there in the nucleus which plays a critical role in the regulation of genes and the maintenance of its structure. Epigenetic effects (i.e., methylation and acetylation) are the landmarks in a chromosome structure and are responsible for the different states (active or inactive) accessible for gene expression. Acetylation promotes gene expression by relaxing the chromatin while methylation compresses the chromatin and induces inactivity in the genome. Cysteine methylation is detected abundantly in polyploids as compared to their diploid parents and is involved in gene silencing to ease the way for the polyploid to adopt a diploidized pattern during metaphase I (Matzke and Matzke, 1998).

1.6 Agriculture

As the world population is anticipated to reach 9.1 billion numbers by 2050, sustainable efforts need to be taken to address the ever-increasing population graph by making sure our food is safe and risk-free using the genetic diversity available (The Royal Society 2009; Harrison *et al.*, 2010). The genetic diversity can be unlocked to enhance the yield and disease resistance in agriculture by exploiting meiotic-silent (cold-spot) region in the genome. The plant kingdom offers an exciting platform for understanding this process in detail because of greater variability at genomic levels (Cai and Xu, 2007). Basic research has revealed interesting facts governing meiosis in the model plant *A. thaliana* which needs to be transferred to economically important crop species in breeding programs for food security measures. Desirable traits can be reshuffled

across different varieties and species by modulating the distribution of recombination frequency. This is an important goal in most of the breeding programmes.

Meiosis studies in plant sciences hold promise for combating food insecurity by unlocking genetic diversity and also enabling crops to withstand abiotic stress for improved agricultural practices (Baulcombe *et al.,* 2009). The focus of current studies is to understand the complexity of this process in different species with wider applications in crop improvement programmes by generating novel lines.

1.7 Molecular overview of Meiosis

All eukaryotic organisms exhibit a special mode of progeny production which halves the chromosomes number following single DNA replication; this is known as meiosis. Such division differs from mitosis (happening in somatic cells), where true to type, or two identical cells, are produced as an end product. In meiosis the scenario is entirely different, and four cells are produced which are not alike. A diploid (2N) cell will give rise to two diploid (2N) cells in mitosis while four haploid (N) cells are produced from the same diploid cell in meiosis to give gametes. Hence the genetic material is halved in gametes before it can be restored back in the fertilization process (Ma, 2006). Nevertheless, meiosis is essential for the survival of sexually reproducing organisms and circumvents aberrations for producing viable generations one after the other. It plays an indispensable role in maintaining and restoring the ploidy level of eukaryotes via gamete production with half of the genetic content (Hamant *et al.,* 2006). The significant role of meiosis in the evolution and the speciation across several kingdoms is

undeniable where enhanced genetic variability and stability by altered allelic combinations are ensured and maintained (Barton and Charlesworth, 1998).

A single round of DNA replication is followed by two rounds of division in standard meiosis, where several physical, chemical and biological processes coordinate with each other to accomplish viable gametes. During this division, it undergoes several chromosomal conformational changes. First it decondenses to form chromatin thread and then gradually condenses back to shape the characteristic form of a chromosome. Its landmark events are: Double-strand break (DSBs) formation, Synapsis, Inter Homologue (IH) bias, Recombination, Segregation and Haploid gametes with reduced chromosomal complement (Grelon, 2016; Schwarzacher, 2003). The typical DNA replication occurs during interphase which precedes two phases, namely Meiosis-I, the reductional division where homologous pair is disintegrated and chromosomes are pulled toward the opposite poles and Meiosis-II, the equational division similar to mitosis where sister chromatids are separated to culminate four daughter haploid cells ultimately developing into gametes. Meiosis consists of meiosis-I, and Meiosis-II and each of which has sub-stages like those in mitosis, i.e. Prophase, Metaphase, Anaphase and Telophase, but Prophase-I in Meiosis-I is comparatively longer than others and is of utmost importance because of the characteristic events which take place during this phase. It is further divided into following sub-phases; Leptotene, Zygotene, Pachytene, Diplotene and Diakinesis (Stack and Anderson, 2001; Zickler and Kleckner, 1999).

The checkpoint concept was evolved during the study of budding yeast in an X-ray irradiation experiment when it halted transition from G2 to M phase of a cell cycle which concluded that

certain genes were responsible for the delay to give time for the adaptation (Putnam *et al.*, 2009). This mechanism works on sensors, and the information is transduced from the site to the effectors for appropriate actions. When DNA is replicated during S phase, it is prone to errors arising in the form of incorrect bases, minor insertions or deletions which are potentially amended during DNA proofreading by excision mechanism to enhance its fidelity. In addition, a conserved mechanism, DNA mismatch repair (MMR), also exists as a quality control for revising all these errors because genomes are continually under the influence of internal and external sources which are potential threats to the precise working and maintaining of the genetic integrity. It could identify and rectify the mis-paired bases in addition to the ones which were not recognized during DNA proofreading (Reyes *et al.*, 2015). The efficiency goes as high as three times of the DNA replication with the MMR systems in operation.

1.7.1 Mis-Match Repair system

During the MMR system, MutS behaves like a sliding clamp in an ATP dependent manner to harmonize mismatches that has occurred during replication, a fascinating role during meiotic recombination. During the MMR system, it executes its charge with the helpers like, MutL and MutH in bacteria (Manhart and Alani, 2016), whereas MutH has not been reported yet in eukaryotes and the MutS (MSH4/MSH5) and MutL (MLH1/MLH3) have orthologs to accomplish the meiosis-specific task (Groothuizen and Sixma, 2016). During meiotic recombination, MSH4/MSH5 form a heterodimeric clamp that embraces duplex in dHJ conformation for the sake of stabilization (Snowdon *et al.*, 2004). Its integral role is evidenced in the mutated form where 85% fewer CO-I are observed than the control (Higgins *et al.*, 2004).

A helicase, MER3 promotes newly formed heteroduplex D-loop extension before capturing the second end which regulates the class-I CO formation (Mazina *et al.*, 2004). A mutated form of MER3 does not abolish COs entirely gesturing the existence of other mechanisms for COs which would be interference independent (Mercier *et al.,* 2005). The dHJ formed by the invasion is resolved by the activity of endonuclease activity of MMR protein, MutL (MLH1-MLH3) in *S. cerevisiae* (Zakharyevich *et al.*, 2012). Similar functional proteins exist in plants which are detected cytologically as class-I CO marks in immunocytological (MLH1 and MLH3) studies. Both form a heterodimer complex that resolves the dHJ into a CO via endonuclease activity (Manhart and Alani, 2016), and are detected as discrete foci at the site of CO (Jackson *et al.,* 2006). Up to 50% reduction in COs are observed in mutated form, and in some cases, MLH1 does not even mark CO between the homologue rather the sister chromatids (Storlazzi *et al.,* 2010).

In yeasts and other eukaryotes, MutS acts as homodimer which senses errors in the newly formed strand before engaging MutL homodimer to form "ternary complex". It then recruits MutH to discriminate newly formed strand based on methylation marks on the GATC site and makes necessary nicking for repairing (Jiricny, 2006; Friedhoff *et al.*, 2016). Since deoxyadenosine methylase is trailing behind the replication fork, the newly strand is un-methylated momentarily and could easily be distinguished from the parental methylated strand which is a critical step in MMR pathway (Jiricny, 2006). Later DNA is unwound by the action of a helicase which acts on the site of a nick, and the mismatched fragment is excised by the exonuclease activity before polymerization can replenish the fragment (Groothuizen and Sixma, 2016).

The nicking function of MutH is performed by the endonuclease activity of MutLα and MutLγ while MutLβ remains irrelevant with no such ability. MutL contains two diverse functional domains at either end of the polypeptide chain in prokaryotes and eukaryotes where N terminal domain is well conserved and possess ATPases activity than the less conserved C terminal domain which regulates the dimerization (reviewed in Reyes *et al.*, 2015).

1.7.2 Cohesin Complex aides in the reduction phase

The sister chromatids, produced as a result of DNA replication are held together by the multiprotein complex, the cohesion, before the start of M phase of a cell cycle in the interphase (Nasmyth and Haering, 2005; Lee and Orr-Weaver, 2001). It keeps the sister chromatids together throughout the first part of meiosis and helps the chromosomal pairs to halve the numbers faithfully in anaphase-I until it reaches anaphase II where centromeres divide like mitosis warranting haploid gametes capable of restoring the specific number upon fertilization (Cavalier-Smith, 2002; Wilkins and Holliday, 2009). The cohesion complex, a tripartite structure composed of SMC1, SMC3 and SCC1, is disintegrated by the proteolytic cleavage of SCC1 subunit, along the chromosomal arms only during Meiosis-I to allow the chromatids to move freely but remains intact at the centromere which holds them joined and as a result the chromosomal complements is reduced to half; hence this is called the reductional phase of meiosis. The complex is split apart during Meiosis-II on the centromere which grants them the morphology similar to mitosis and hence called the equational phase of meiosis. During the reductional phase in mid-prophase, chromosomes develop physical connections between the homologue forming the characteristic bivalent structures, a critical mediator for the

characteristic genetic recombination to reshuffling genes (Ohkura, 2015). After alignment on an equatorial plate, the conformation of chromosomes offers a site for kinetochore to attach on only one side to help divide the chromosomes symmetrically into two halves. Each half then undergoes conformational changes similar to mitosis and kinetochores are attached on either side of the chromosomes this time versus single sided on the homologue pairs in the first phase, and the cohesin complex is cleaved at the centromeres which halves the chromatids between the poles to conclude with individual chromatid at either end. In this way, the number is maintained from the prophase until the telophase in Meiosis-II which is a characteristic measure and standard of a normal mitosis. The cohesin complex is disintegrated in steps during meiosis, first from the arms only and then from the centromere in the second half which contradicts from the mitosis where this disintegration is achieved in one step (Nasmyth and Haering, 2005).

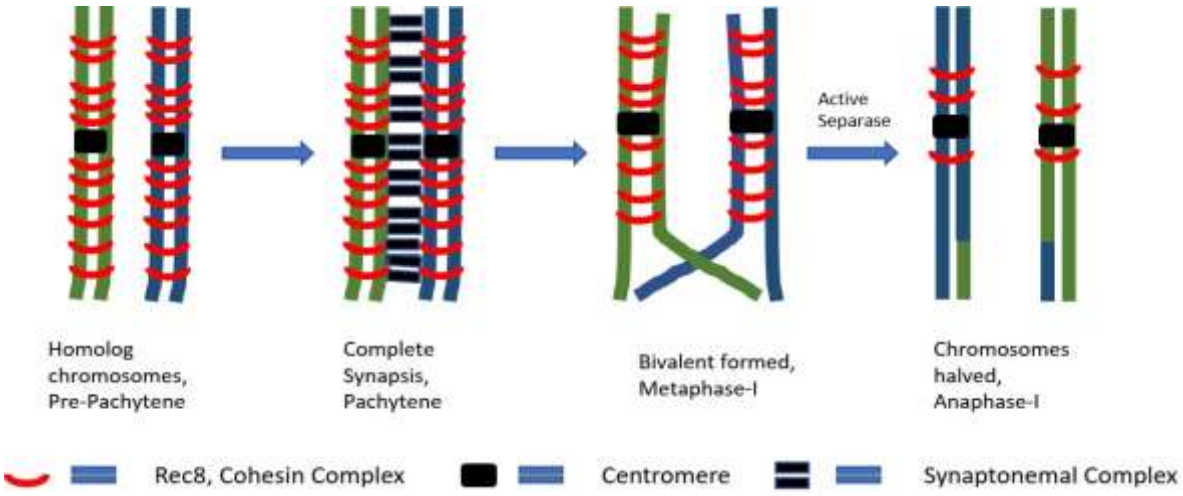


Figure 1-4 Behaviour of Cohesin complex during Meosis-I

The Cohesin keeps sister chromatids bound until anaphase-I where separase cleaves the complex along the chromosomes arms only and duplicated chromosomes move toward the opposite poles.

1.7.3 Progression into Meiosis

At the onset of meiosis, chromosomes are indistinguishable thin threads and adopt a round spherical conformation where the centromeres are scattered all around the nucleus and the ends of chromosomes called telomeres are inclined to the periphery of the nucleolus in *A. thaliana* (Armstrong *et al.,* 2001; Armstrong and Jones, 2003; Da Ines *et al.,* 2012; Ross *et al.,* 1996). Chromosomes condense progressively to find and juxtapose with their counterpart in zygotene in a dynamic fashion until they find homologue to synapse completely in the pachytene stage by Synaptonemal Complex (SC) polymerization which helps to exchange certain genetic fragments at the molecular level (Cai and Xu, 2007). SC tends to depolymerize gradually in diplotene before homologues can be seen as visible entities forming a bivalent structure in diakinesis. The chiasma formed during diplotene between the homologue are the site of genetic reshuffling and help to produce the genetic variants which potentially ensure better survival owing to the altered allelic combination to withstand unfavourable conditions in changing environment (Solari 2002; Wilkins and Holiday, 2009). The bipolar orientation of the bivalent on an equatorial plate in metaphase-I ensures the peculiar reduction of the chromosomal number in anaphase I (Chan, 2003; Winey *et al.,* 1995). Any disparity in this characteristic sub-stage at any level could compromise the quality of gametes produced, or a complete meiotic arrest with unviable pollens and ovary leading to sterility in an organism (Roeder and Bailis, 2000; Egozcue *et al.,* 2005). Therefore, a strong correlation exists between abnormal meiotic events and prevalence of infertility (Vendrell *et al.,* 1999).

1.7.4 Telomere Clustering

The telomere structure is a well conserved phenomenon across the species and comprises of the tandem DNA repeats consisting of 30 guanines single-stranded overhang, referred as a G-tail. MRX complex creates the telomere overhangs in a similar fashion to DSBs end resection which leaves 3' end capable of initiating the strand invasion (Longhese *et al.,* 2010). Mutual coordination among the early events is essential to accomplish the characteristic cross over through reshuffling between the homologues to generate recombinant haploids (Pawlowski and Cande, 2005).

The telomeres are clustered on one pole of the nucleus forming a bouquet whereas centromeres are aligned on the opposite side to make a meiosis like structure /configuration (Tiang *et al.,* 2012). It is believed that the chromatin state e.g. decondensation is provoked by an epigenetic mark such as methylation. This affects the chromosomes configuration during early meiosis and triggers chromosomal affinity and the nuclear conformation ultimately (Santos and Shaw, 2004). This also implies that homology is potentially driven by the DNA base sequence as well as the chromatin state. Homology-based pairing is a sophisticated process which orchestrates the identical chromosomes to form bivalent for proper segregation to maintain the ploidy level constant over generations (Tiang *et al.,* 2012).

Initial steps in meiosis are discussed as follows;

1.7.5 Double Strand Breaks (DSBs)

A meiotic cell is determined and well capable of thwarting any damage being caused in interphase despite various endogenous and exogenous influences which continuously affect genomic DNA quality and bring back DNA conformation for proper functioning after that. Among several forms of DNA damages, Double Strand Breaks (DSBs) are programmed DNA lesions which are destined to bring genetic novelty by allelic recombination (reviewed in Mehta and Haber, 2014) and could prove lethal if not repaired properly.

The history of this crucial homologous recombination formation goes back to 1964, when Holliday, for the first time proposed a model to illustrate the recombination process through strand exchange in the homologues. He suggested that Single-Strand Breaks (SSBs) were generated to exchange partners between the homologue forming four stranded mediators for the recombination. He further elaborated the model that a longitudinal cut of the crossed strands would give strands like those of parents while transverse cut would give recombinants with the altered allelic combinations. Later in 1975, Meselson modified this SSBs induced model and suggested the strand invasion mechanism where the double-stranded molecule is invaded by one of the strands of partner homologue to induce a Displacement loop (D loop) before offering two choices for resolving the D loop. He thought that some enzymes were responsible for the unwinding and annealing of DNA strand after the invasion. Salient features of this model were SSB formation, Holliday Junction (HJ), and the concept of Cross over (CO) and Non-Cross Over (NCO) (Jack W. Szostak *et al.*, 1983).

The model was widely accepted until 1983, when Szostak reframed the model by including Double Strand Breaks (DSBs) and double Holliday Junction (dHJ). This was a significant breakthrough which explained the nature of CO and NCO resulting from dHJ and was validated with transformation experiments of budding yeast in the lab. Much progress has been made in DSBs biology which has unveiled several enzymes responsible for its generation and repairing afterwards. The DSBs generated are very critical and toxic as it could lead to the loss of the genetic information if left unrepaired which eventually destabilize the cell and individual ultimately. Repairing of these lesions restores the genetic integrity and depends on subsequent end resection gateways:

- End resection independent pathway
- End resection depeneent pathway

1.7.5.1 DSBs End Joining/repairing

In an independent pathway, it does not undergo end resection in any of the DSBs strands and is termed as classical Non-Homologous End Joining (C-NHEJ). It ligates two dissimilar end breaks and is usually a more error-prone mechanism with lethal consequences for the cell. On the other hand in the end resection dependent pathway, DSBs have three options to anneal with the homologue based on the sequence similarity. The most prevalent form of repairing is Homologous Recombination (HR) which is based on the strand invasion and is more precise than others, while the other two pathways are Alternative End Joining (alt-EJ) and Single-Strand Annealing (SSA) which are less adopted in cell and cause insertion and deletion in the

genome (Reviewed in Ceccaldi, *et al.*, 2016). Both SSA and alt-EJ tend to harm the genomic integrity and the fidelity owing to intrinsic mutagenic nature.

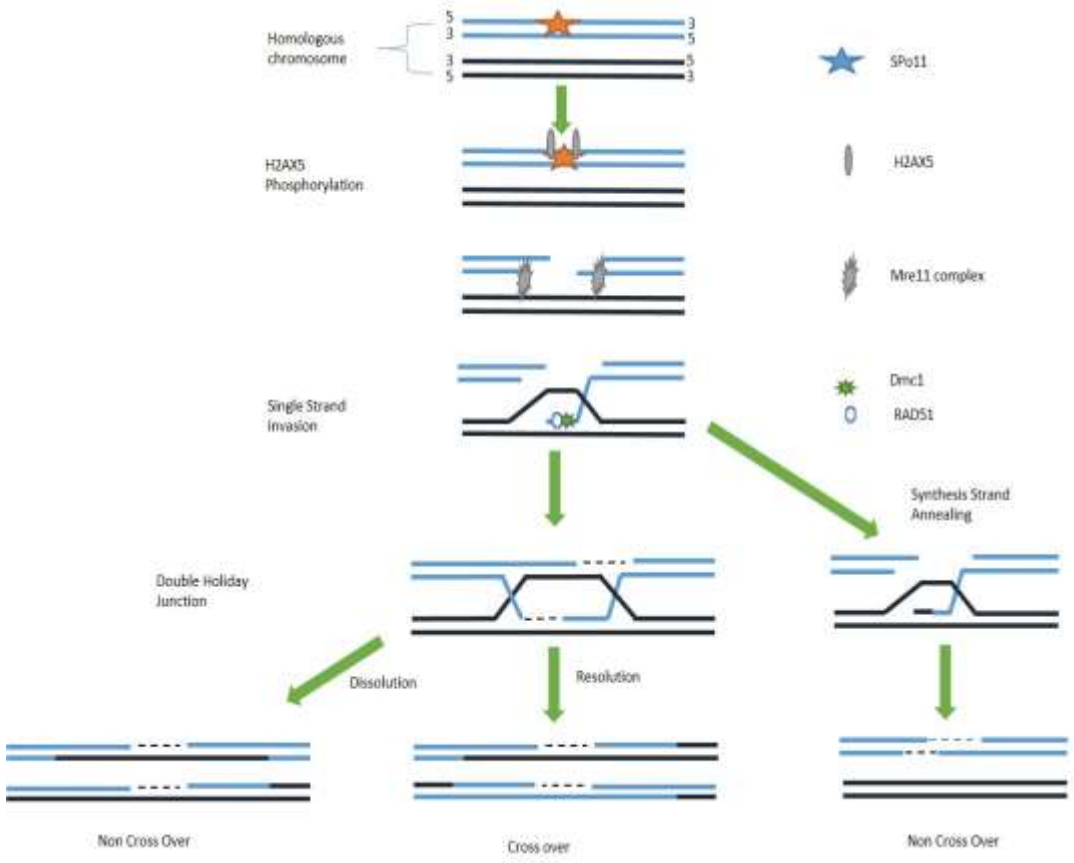


Figure 1-5 Homologous recombination during prophase-I in meiosis-I (Adapted from Osman *et al.*, 2011)

Different organisms have evolved their preferences for genetic repairing, and in budding yeast most of the times, HR is the leading repair system against NHEJ in mammals (e.g. in human) (Chang *et al.*, 2017; Lieber, 2010). The focus of the study will be HR pathways as it is the main source of variation than NHEJ in plants and will be discussed in detail. One of the critical

features of HR that differentiate it from NHEJ is its ability to develop synapsis, and which is absent in NHEJ even in the presence of a mediator protein complex, ku70/ku80 complex.

1.7.5.2 *SPO11*, Endonuclease

The gene SPO11 was discovered in the 1990s in the *S. cerevisiae* mutant, which was essential for sporulation (Esposito and Esposito, 1969) also had a role in DNA double-strand breaks (Robert *et al.,* 2016). It showed similarity to the A subunit of Topoisomerase VI found in *Archea* that had a catalytic function for creating nicks in double-stranded DNA. TOPOVI is a tetrameric complex composed of two A and two B components, while the SPO11, in eukaryotes, had a similar function to the A subunit of Topoisomerase VI. It (SPO11) has three copies in plants out of which two work in meiosis and one in mitotic (somatic) cells. In *A. thaliana* there are three SPO11 orthologs present, and the DSBs are produced by the action of two SPO11 orthologs (SPO11-1 and SPO11-2) which form a heterodimer in addition to three more proteins (PRD1, PRD2 and PRD3) for inducing breaks in a double strand of the DNA (Edlinger and Schlogelhofer, 2011). The third SPO11-3 is not involved in meiosis and works in somatic cells only. Recent work has demonstrated that component B of the TOPOVI has a critical role in DSBs formation as it arbitrates the formation of a SPO11 heterodimer. Yeast-to-hybrid assay has revealed that the C terminus bearing transducer domain of TOPOVIb interacts with the N terminus (with Winged-Helix Domain) of the SPO11 heterodimer. Such an association is not fully exploited yet and needs to be elucidated to show the mode of action (Robert *et al.,* 2016; Vrielynck *et al.,* 2016).

With the chromatin immunoprecipitation (CHIP) assay it has been demonstrated that SPO11 colocalizes with the Rec8 cohesin component on the chromosomes axis in yeast whereas DSBs are found in the loop other than the axis through the tethered mechanism. Furthermore, SPO11 is first loaded on centromeric region before moving to the arms of the chromosome (Kugou *et al.,* 2009) In budding yeast, there are nine more proteins which coordinate with SPO11 to generate DSBs (Keeney, 2001), in addition to epigenetic factors (Henderson *et al.,* 2006; Ogino *et al.,* 2006) whose functional conservation in other organisms has not been recognised and reported yet (De Muyt *et al.,* 2007). While in a fission yeast, there are six proteins to work with SPO11 for the DSBs formation.

Moreover, it has been found that their orthologs have evolved different functions despite some sequence homology-like RAD50, MRE11 and XRE2 are not required for DSBs formation in fission yeast, *A. thaliana* and mammals rather they process the DSBs toward stabilization of genome integrity which is not the case in budding yeast. The DSBs are exclusively produced by the catalytic action of the tyrosine-containing domain in SPO11 which leaves the 5', and 3' ends open of duplex DNA fragments for further processing. DNA repair is a quite complicated process and undergoes the following steps;

i- DSBs formation

ii- 5' end resection

iii- Strand invasion

iv- DNA synthesis

v- Branch migration

vi- dHJ resolution

When SPO11 forms DSBs, the chromatin changes modify the histone epigenetic mark so that H2AX is transformed by the action of ataxia-telangiectasia mutated (ATM)/ataxia-telangiectasia and Rad3-related (ATR) to γH2AX. It serves as an early signal for DNA damage and DSB processing and facilitates the recombination process, although it is not universally involved in the recombination process essentially and is dispensible in nature, therefore exploration of its role could further our understanding of DSBs processing (Yuan and Chen, 2010). A mutation in the phosphorylation state of H2AX has milder genomic instability effect in contrast to the changes in some other essentially functional genes (ATR, MRN complex, RAD51 etc.) temporally and spatially (Celeste, *et al.,* 2003). In phosphorylated form it transduces the signal to accumulate other related proteins in the periphery for the regulation of the repair system, hence not possessing any central role in controlling the repair process. Nevertheless we can categorise two pathways, one which relies on γH2AX and it amplifies the DNA damage signal for effective cellular response which involves several other proteins as well and the other pathway, which is independent of it, where MRN directly detects the lesions and repair the damaged DNA by HR (Yuan and Chen, 2010).

DSBs generated are processed in following order:

- DNA damage sensors
- Activation of kinases to lead phosphorylation of the substrates including H2AX,
- Initial recruitment of MRX complex does not depend on phosphorylated H2AX

The blunt ends produced are the result of the catalytic action of SPO11 on both strands of DNA during early leptotene. The repairing process begins with the resection of 5’ end by the concerted action of recombinases to leave the 3’ end free for invasion purpose to find a new partner for the sake of diversity.

1.7.6 Strand Invasion

The strand invasion and exchange are facilitated by Rec A family recombinases which form nucleoprotein filaments capable of invading the homologue partner in subsequent steps; (it is Rec A in bacteria and Archaea while it is RAD51 in eukaryotes). Strand homology search is a very critical step where Rec A (or Rad 51) nucleofilament searches a strand by sequence homology to generate Holliday Junctions. The invasion starts at the local site of homology on dsDNA in a paranemic joint which denatures first and allows the extension of the invading strand before conversion to the plectonemic structure. Paranemic joint refers to the un-intertwined structure when strands are parallel while plectonemic refers to the intertwined strands of DNA duplex.

Once chromosomes are engaged in HR pathway for repairing the DSBs to maintain genomic integrity, it undergoes end resection which precedes the genetic reshuffling between the homologues. Meiotic recombination, one of the aspects of DNA metabolism in plants is initiated, as it does in yeast, where DSBs are created by the catalytic function of transesterase SPO11 which provides a potential site for a strand exchange. SPO11 remained covalently attached at the tyrosine residue to the DSBs until (Borde and de Massy, 2013) it is recognized

and replaced by MRX complex for 5' end resection. MRX is a heterotrimeric complex of MRE11, RAD50 and XRE2 (or NBS1) which executes its role with the help of SAE2 in the early "dirty end" resection by its catalytic activity before it diminishes upon acetylation (Mehta and Haber, 2014). The walking domains of Rad50, ATP bound N and carboxylic termini with motifs for DNA binding interact with the two MRE11 forming the head of the MRX complex molecules while the intervening sequence of RAD50 is being looped out to form a coiled-coil which helps in DNA tethering. The conformational changes occur upon hydrolysis activity of a 150 kDa protein, RAD50 which bestows functional activity to MRE11 during end resection. In ATP bound form RAD50 negatively controls MRE11 by masking the active sites of MRE11, but its hydrolysis renders changes in the nucleotides in the processing core of MRX complex which expose DNA to the active MRE11 site (Lim *et al.,* 2011; Mockel *et al.,* 2012). Further, enhancing ATP hydrolysis capacity of RAD50 would upsurge the end resection by MRE11 which validates the inactive nature of the complex in ATP bound form during early DSB recognition. It has been reported that end resection is a slow process confirming the ATP bound state of RAD50 in most of the times and might require some accessory proteins for processing.

MRE11 is a 70-90 kDa protein possessing DNA binding activity with high affinity for DNA duplex for endonuclease and exonuclease processing. It is the central core of the complex which also suppresses NHEJ for the recombination purpose. To generate 3' end tail overhang, it exerts its 3'-5' exonuclease activity on dsDNA while SAE2 stimulates its endonuclease activity. ssDNA nicks are produced by its endonuclease activity whereas exonuclease activity removes the SPO11 bound fragment, and as a result, the 3'end tail is exposed which invades an intact DNA in subsequent steps for the D loop formation. The five phosphodiester motif in the N

terminal of the polypeptide chain endows it the nuclease activity, and mutation in this region would result in cells lacking any recombination as 3'end cannot be generated. Non-catalytic component of MRX, XRE2 act as a chaperone (Tsukamoto *et al.*, 2005) and proficiently translocate the MRE11 component in the nucleus (Borde *et al.,* 2004). This complex, in short, carries out three primary functions; first as a DSBs sensor, second coactivator of the checkpoint signals, and third the DSBs effector. It is also found to be associated with the telomeres where it maintains its integrity.

Following 5'end resection, 3'end is left over hanged and recognized by Replication Protein A (RPA) well before the essential meiotic steps of strand invasion, and D loop formation. RPA is a heterotrimeric protein which shields the exposed tail from forming any secondary structures and further degradation by the MRX complex and is replaced by the recombinases RAD51 and DMC1 for the homology search in the DSB repair process (Symington, 2002). RAD51and DMC1 are the homologue of REC A protein found in bacteria and are essential for the DSBs repair. RAD51 is active in mitosis as well as in meiosis whereas DMC1 is a meiosis-specific only and promotes Inter-Homologue (IH) bias. Based on the presence of RAD 51 and DMC1, organisms can be divided into two categories; first category includes *Drosophila melanogaster*, *Caenorhabditis elegans*, and the fungus *Sordaria macrospora* with only RAD51 recombinase while in the second category contains both RAD51 and DMC1 for recombination purpose like in yeast (budding & fission), plants and mammals (Holsclaw *et al.*, 2016).

The RAD51 protein interacts at the interface of the single stranded and double stranded DNA in an ATP dependent fashion with the help of RPA to the ssDNA 3' end tail in yeast which

initiates the recombination process subsequently. The timing of RPA loading on ssDNA is critical as it would compete with RAD51 and if it loads at the same time as RAD51 does, it will result in meiosis irregularities. In such case, RAD52 mediates the loading of RAD51 on ssDNA for displacing the RPA (Sugiyama and Kowalczykowski, 2002; Sung, 1997a). Some other RAD family members like RAD55 & RAD57 also play their respective roles in the stabilization of RAD51 nucleoprotein for the strand exchange in vitro (Sung, 1997b). Whereas its negative regulator is also present in the cell to prevent nucleoprotein formation in the form of SRS2 helicase (Symington *et al.,* 2014). RAD51, being a dominant player carries mitotic recombination on its own where it invades a sister chromatid for repairing. In meiosis it is DMC1 in a DSB mediated pathway which plays a central role and RAD51 only works as an accessory protein (Bishop 1994 from Bhalla 2008). RAD51 nucleates with the DMC1 in a slow process to form a presynaptic nucleofilament for homology search. Any defects in the RAD51 lead to a decreased interhomologue and intersister chromatid recombination while DMC1 mutant affects interhomologue activity to a greater extent deducing that meiotic recombination is heavily dependent on the activity of DMC1 (Sansam and Pezza,, 2015). When RAD51 or DMC1 is mutated in *A. thaliana*, the plant undergoes normal somatic growth but is sterile. RAD51 mutated plant experiences extensive DNA fragmentation whereas a DMC1 mutated plant has intact univalents with no bivalents. This signifies its role in interhomologue strand invasion and also the role of RAD51 in DSBs repair (Li *et al.,* 2004). Further, it has been found that DMC1 mediate centromeric association between the chromosomes whereas RAD51 is responsible for the association between the chromosome arms. Nevertheless, both RAD51 and

DMC1 are required for recombination as RAD51 extends the synapsis after interhomologue bias by DMC1 (Da Ines *et al.,* 2012).

The number of RAD51 or DMC1 foci is generally much higher than the actual COs in a cell detected under microscope, e.g. 40 times more than the actual COs in Allium species while in mouse it is 10 times and in *A. thaliana* it tends to be 25 times more than the actual COs (Anderson and Stack, 2005, Mezard *et al.,* 2007). More DMC1 foci are detected in early zygotene than the late zygotene which gradually decreases further in early pachytene (Hansen *et al.,* 2016). The Single End Invasion (SEI) intermediates formed by the recombinases is extended into D loop which is resolved further to give COs and NCOs or dissolved merely to give NCOs (Hunter and Kleckner, 2001; Mercier *et al.,* 2015; Lambing, 2017). SEI could produce NCOs by synthesis-dependent strand annealing while second end capture would mature double Holliday Junctions (dHJs) with the help of ZMM proteins (4.1.1 in the next chapter). Apart from the COs produced by the resolution of dHJ, there exists another mechanism which moves out before D loop formation, and DSBs were processed by MUS81 protein in ZMM independent pathway.

1.7.7 Holliday junction

The DNA strand extension is followed from the nucleofilament 3’ overhang invasion on homologue through D loop by the action of DNA polymerase in a process termed as Single End Invasion (SEI) (Hunter and Kleckner, 2001). Recombination fate is determined at this time whether SEI will choose a gene conversion or reciprocal exchange between the homologues of

a characteristic four-armed DNA molecule called Holliday junction. Recapturing of the second end by nick ligation would give rise to double Holliday Junction (dHJ) (Fig. 1-3). This is an intermediate transitory configuration in recombination process capable of maturing into COs and NCOs for proper segregation (Kaur *et al.,* 2015). NCOs are also formed independently of the dHJ pathway through Synthesis-Dependent Strand Annealing (SDSA) in yeast where D loop is disassembled by the action of helicase-topoisomerase complex leading to gene conversion only (Nimonkar *et al.,* 2009). The two processing pathways for dHJ are Dissolution and Resolution; dHJ is dissolved by the action of DNA helicase and topoisomerase to give NCO products only (Manthei and Keck 2014) while the resolution of dHJ is more promising to yield COs mediated by endonucleases (Topo3a and RMI1) (Hartung *et al.*, 2008).

Robin Holliday in 1964 proposed a` transient four-way structure highlighting three main events in meiosis of crossing over, gene conversion and the chromosomal segregation. The crux of the model was that the breaks mediated association between the homologue after DNA replication to develop into a transitory pairing four-stranded structure which later became known as Holliday Junction and provide an opportunity for a reshuffling of genetic material. For the resolution of dHJ into CO, resolvase possess following salient features;

- High affinity toward negatively charged DNA,
- Possess divalent metal ion necessary for the DNA cleavage,
- Dimeric molecules with active sites capable of coordinated incisions in dHJ
- Selectivity and prefer to work on four-stranded DNA molecule.

It has been shown that the incisions of resolvase are executed in a coordinated order that, one cut follows the other in quick succession rather than the simultaneous nature. The first incision tends to relax the dHJ structure before second would be done in an expedited fashion (Wyatt and West, 2014).

1.7.8 Synaptonemal Complex

As meiosis proceeds, the sister chromatids develop meiotic-specific structure comprising axial elements (Hop1 and Red1 in budding yeast and ASY1/SYN1/ASY3 in plants) which upon juxtaposition with their respective homologue, become sealed with a zipper like transverse elements of ZIP in budding yeast and ZYP1 in plants to complete Synaptonemal Complex (SC) structure for synapsis (Higgin *et al.*, 2005). The complex was first reported six decades ago in 1956 by Fawcett and Moses who identified it in mosses and regarded it as discrete substage in the prophase. The SC scaffold is a meiosis-specific and links homologues necessary for the bivalent conformation of chromosomes in the metaphase stage which prepares the chromosomes for a typical reduction (Heyting, 1996).

The specialized complex is an evolutionarily conserved tripartite structure comprising of two Lateral Elements (LEs) and one Central Element (CE) which heralds recombination in some organisms whereas some are independent of SC formation for achieving genetic recombination; even though both SC formation and segregation are considered unique for the meiosis. SC starts its installation in leptonema and progresses through the zygotene stage until the pachytene when homologue fully synapse along the chromosomal length (Zickler and Kleckner, 2015). After

that stage, SC disassembles, and chromosomes are linked only at the point of chiasma in the diplotene stage. Contrary to the canonical meiosis, SC is absent in fission yeast where meiotic pairing could be either recombination-dependent or independent. In male *Drosophila melanogaster* pairing and SC formation occurs independent of genetic recombination and the dedicated connections in spite of synapsis ensure proper segregation of haploids. Hence alternative pathways also exist to warrant proper meiosis in some organisms (reviewed in Tsai and McKee, 2011; Lake and Hawley, 2012). In addition to the SC assembly between homologues, the SC also develop between the non-homologues in mutant yeast and maize mutant (Leu *et al.*, 1998; Pawlowski *et al.*, 2004). The LEs gets in a close contact in pachytene where the spatial isolation is reduced to 60-100 nm so that transverse filament can bridge the two LEs during synapsis.

The transitory pairing starts at many particular sites (centres) along the chromosomes as the synapsis starts (Fung *et al.*, 2004) and matures into far fewer COs on an individual chromosome through the conserved action of MER2 in fungi and PRD1/PAIR1 in plants which mediate the inter-homologue spatial juxtaposition and induces chromosomes compaction (Tesse *et al.*, 2017). Cellular machinery keeps stringent control over CO maturation through CO homeostasis (i.e. maintenance of COs) , CO assurance (i.e. the obligate CO between homologue necessary for faithful segregation) and CO interference (i.e. the inhibtion zone of CO along the chromosome arm) which is unresolved vex yet and it is thought that it could be different aspects of one phenotype rather than separate phenomenon. The COs formed are also under continued influence of anti CO factors (e.g. RECQ4, FANCM, FIGL1) which inhibit such physical connection across homologues while in their mutated forms (individual and combined as well)

boost CO along the chromosomes (reviewed in Wang and Copenhaver, 2018). Functional conservation of SC is even more dominant and observed across different life kingdoms than the actual sequences at the molecular level (Mercier *et al.*, 2015). Formation of the Synaptonemal complex during synapsis is an extremely coordinated process in the prophase to orchestrate with other events in cell and avoid unfaithful synapsis (Armstrong *et al.,* 2002). In plants, the number of COs are relatively low than the SC initiation sites (Higgin *et al.*, 2004), but in budding yeast, COs correspond well to the SC initiation sites (Fungal *et al.*, 2004).

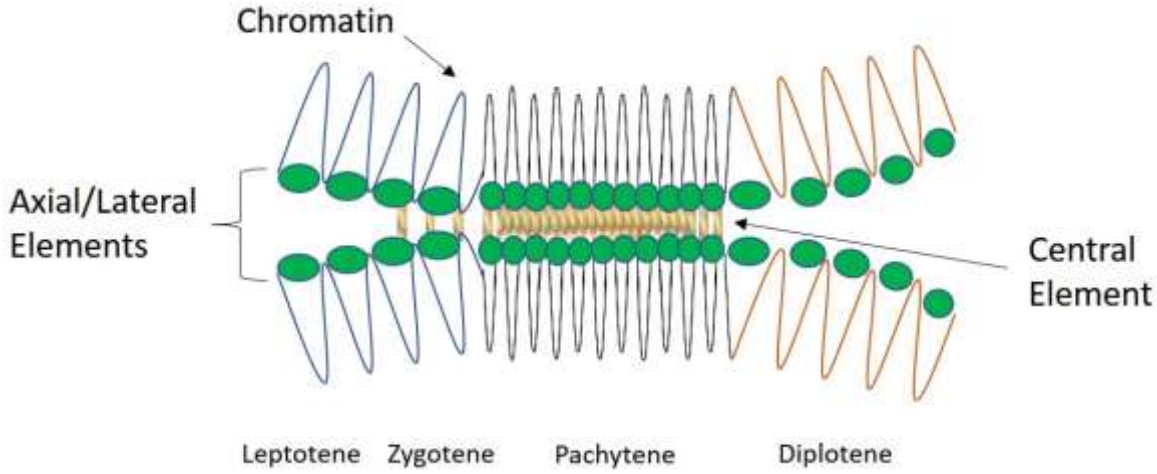


Figure 1-6 Synaptonemal complex during early prophase-I

During early prophase axis is developed which is supplemented with the transverse element of the ZYP1 in pachytene stage which depolymerizes soon it enters into diplotene.

1.7.9 Recombination

The CO, central feature of meiosis, formed by the reciprocal exchange falls into two classes, Class-I and Class-II. The ones which are mediated through the MSH4-MSH5 complex form

class-I COs and are under the influence of an already existing CO so that no more CO could form in a close domain, a phenomenon known as CO interference (Chua and Roeder *et al.,* 1997). Earlier it was thought to be due to mechanical stress (in Stress-Stress relief model) which is distributed along the chromosome to prevent any other CO to occur. The model was supplemented with the mathematical explanation (Beam-Film system) for quantitative analysis on CO probabilities along the chromosomes. According to which CO designation is determined by stress and is relieved once CO is formed and is redistributed again in the vicinity which corresponds well with the stress release model (Zhang *et al.,* 2014). While the other class is mediated by heterodimer MUS81-MMS4 (EME1 in fission yeast) complex which does not restrict developing a CO in the proximity of an existed CO and hence are non-interfering CO (Whitby *et al.,* 2003). The concept of two classes in organism came from studies on *S. Pombe* and *E. elegant* where either of the class is present predominantly. Fission yeast (*S. pombe*) lacks MSH4-MSH5 complex and form the CO through MUS81-EME1 resolvase predominantly (Boddy *et al.,* 2001; Smith *et al.,* 2003) but in budding yeast (*S. cerevisiae*) only a subset of the total COs are attributed to MUS81-MMS4 complex (MMS4 is an EME1 ortholog in budding yeast) (Argueso *et al.,* 2004). While in *C. Elegans,* all of the COs show strong interference effect and are mediated through MSH4-MSH5 complex (Meneely *et al.,* 2002) suggesting two different pathways in two organisms with a contrasting effect on COs.

Moreover, not all the COs in *S. cerevisiae* and *A. thaliana* fit under these two known pathways as double mutant organisms MSH4/MUS81 are not devoid of COs completely and hint toward an additional mechanism during meiosis which recombines two homologues (de Los Santos *et al.,* 2003). Interestingly in the absence of MSH4-MSH5 proteins in Drosophila, most of the

time COs have a profound interference effect on each other according to the mathematical modelling, thereby indicating the presence of other mechanisms which execute the same effect like MSH4-MSH5 proteins do in *A. thaliana* (Copenhaver *et al.,* 2002; Sekelsky *et al.,* 2000). Looking at the principal roots of these two pathway proteins reveals that one is pro crossover resolvase while the other is anti-crossover. MSH4-MSH5 have acquired antagonistic role while favouring COs during class-I, where it blocks untwining of the anti-crossover helicase SGS1 in yeast (an ortholog of BLM of *S. cerevisiae*) while MUS81-MMS4 resolve dHJ to promote COs.

In principle, CO during the first half of the meiosis executes a profound effect on precise segregation of chromosomes by bringing them in bivalent configuration and equatorial orientation. MSH4-MSH5 binds to the dHJ and D loop playing a critical role in the execution of class-I CO which restricts any further CO to occur along the chromosomes. Its mutated form had a drastic effect on the CO frequency than the wild type in *A. thaliana* implying its major contribution toward genome stabilization and rehabilitation process (Osman *et al.,* 2011).

The current studies will focus on such changes at the crop level to address food security in the longer run in an effort to maximize the genetic diversity to counterfeit potential environmental threats in future times. Significant diversity could be a potential solution for broadening the crop genetic base to maximize the yield even under adverse conditions being poised by global warming. New varieties developed by combining different genomes would be helpful in studying evolution process and the diploidization adaptation.

1.8 Aim

My present studies will be a continuation of the preceding attempts which had insubstantial success, for transferring traits into *B. oleracea* and *B. rapa* from *B. nigra* (Struss *et al.,* 1991; Plieske *et al.,* 1998; Saal and Strauss, 2005; Schelfhout *et al.,* 2006; Navabi *et al.,* 2010; Navabi *et al.,* 2011). Since meiosis in crops has not been explored fully to understand the evolutionary basis and stabilizing forces, the fundamental approach will be applied to get insight into *B. nigra*, a closely related organism to model plant *A. thaliana* by developing its karyotype. The information will be useful in downstream research for the analysis of chromosomes identified and physical mapping purposes. The preliminary information about the genome organization will be extended to decipher the complexity of the genome using cytogenetics and immunochemistry tool developed on model plant *A. thaliana*. In light of the precedent progress of genome sequencing in *B. rapa*, *B. oleracea* and *B. juncea*, an improved and thorough understanding of the genome structure and gene composition of the B nigra genome would help us better understand its relationship with the other lineage (i.e. *B. rapa*/*B. B. oleracea*) and could potentially ease the exploitation of this vital source of the genes of economic importance.

The procedures were also developed to exploit the useful source of novel agronomical traits of the B genome present in *B. nigra* (BB) which could be introgressed into related species of this genus (e.g. *B. juncea* [4X: AABB]) in crop improvement programmes (Struss *et al.,* 1991; Navabi *et al.,* 2010). This project is aimed to build an improved understanding of this genome structure, organization and behaviour during meiotic recombination. The cytogenetical knowledge gained in the model plant *A. thaliana* to trace meiotic progression would be applied

in *B. nigra* (BB), *B. rapa* (AA), hybrids between both diploid species (AB), the allopolyploid *B. juncea* (AABB) and de novo neo-allopolyploids (AABB) which will be useful in deciphering the control of B genome introgression at different ploidy level.

1.9 Objectives

To understand the genome organization and the diploidized meiosis in allopolyploid in crop species, fundamental knowledge will be acquired using cytological and immunochemistry approaches in Brassica diploid species. The manual crossing will be adopted to get the wide hybrid between different genomes (*B. nigra* X *B. rapa*) to compare it with the (existing) stable allotetraploid (*B. juncea*) to get insight into the stabilization factor toward fertility. The karyotype of *B. nigra* will be first developed under following design;

Characterization of the diploid B genome

- Develop ideotype to identify the 8 pairs of chromosomes in *B. nigra* using ribosomal DNA markers.
- Development of Genome *In situ* hybridization (GISH) technique for studying diploid genomes in the allotetraploid background.

Cytological Characterisation of meiosis in diploid and allotetraploid brassica

- Fluorescence *in situ* hybridization with a telomere probe to analyse bouquet formation.
- Immunolocalisation of several meiotic proteins to trace the meiotic progression of diploid and allotetraploid species.
- Analysis of the chiasma frequency in brassica diploid and allotetraploid species. Exploring the artificial manipulation of homoeologous recombination in *B. juncea* by Okadaic acid treatment.

Characterisation of the B genome in artificial hybrids

- Obtaining interspecific crosses:

- *B. rapa x B. nigra (AB)*
- *B. nigra x B. juncea (ABB)*

- Analysis of the chiasma frequency and genome introgression in the hybrids.
- Immunolocalisation of several meiotic proteins to trace meiotic progression in the hybrids.
- Induced polyploidization hybrid allodiploid *AB* plant using colchicine.

2 . Materials and methods

2.1 Plant material

Current studies were aimed at elucidating meiosis in diploid and polyploid brassica species to transform knowledge from model to crop species under the umbrella of crop improvement. The seeds were planted in soil-based compost in a glasshouse to get the inflorescence buds for analysing meiosis under 16 hours light, 8 hours cycles.

The genotypes used in the studies were as follows;

Table 2-1 Brassica species and origin of the collection

Serial Number	Species	Genotypes	Origin
1	*B. nigra*	B150, B1196, B1198	Agricultural University Peshawar Pakistan
2	*B. oleracea*	DH A12	University of Birmingham UK
3	*B. rapa*	R-O-18	University of Birmingham UK
4	*B. juncea*	Accession # 4289	HRI, University of Warwick UK
5	*B. carinata*	195923.3.2_01DH	University of Geissen Germany

2.2 Karyotyping

Root tips were chosen for nicely spread of mitotic metaphases, where chromosomes are finely separated from each other, than the anthers in the inflorescence due to the abundance of somatic cells in meristematic tissue (Osalou *et al.*, 2013).

The seeds of *B. nigra* (B150) were surfaced sterilized in 70% ethanol followed by bleach treatment (10 times diluted household chlorine bleach/sodium hypochlorite) for 10-15 min. The procedure was repeated twice and washed with large amount of sterile distilled water (SDW). The seeds were planted onto agar enriched Murashige & Skoog (MS) media Petri plates in a controlled environment at the 25^{o}C room.

2.3 Root fixation

The primary roots of young seedling after 4-5 days of planting were chosen for the analysis of mitotic metaphase stage. The roots were washed with water before keeping at 4^{0}C for 24 hours to get a good spread of chromosomes before fixation. The roots were kept in freshly made fixative (3 Ethanol: 1 Glacial Acetic Acid) for overnight.

2.4 Chromosome spreads

After 16-20 hours the fixative was replaced once more before washing with 10mM citrate buffer, pH 4.5 to wash away any traces that could potentially affect enzyme efficiency in subsequent steps. The milky white tips of the root containing meristematic cells were finely cut

apart in fixative under a binocular microscope and separated from the rest of the debris. The rest of roots were discarded, and the tips were washed three times with citrate buffer (10 mM, pH 4.5) for five minutes each. The washed tips were then incubated in diluted enzyme mixture 0.3% (w/v) cellulose and 0.3% (w/v) pectinase) (Sigma) in 10 mM citrate buffer pH 4.5 for 1 hour in a moist chamber at 37^0C. The digested root tips were taken out at room temperature. The tips were squashed with the needle tip on a frosted glass slide in 15 µl of 60% acetic acid and were placed on a hot block at 45^0C for 45 seconds. Ice cold freshly made fixative was poured around the acetic acid drop on the slide so that a blob was formed and it was then mixed thoroughly. The slides were tilted and air dried before looking at it under a phase contrast microscope. Slides with appropriate chromosomes spreads were selected for further analysis of FISH to develop karyotype of *B. nigra*.

2.5 Image capturing

The selected slides were washed with 70% ethanol to wash away any debris before staining with 8µL DAPI (4',6-diamidino-2-phenylindole) vectashield antifade mounting medium. The images were captured with SmartCapture software on Olympus fluorescence microscope.

2.6 SmartType Karyotyper

Good spreads of mitotic chromosomes were selected for developing an ideotype on SmartType Karyotyper. The images were imported in a SmartType Case folder as Bright Field in the drop-down menu to classify chromosomes into pairs. It brought the selected image to the working

window panel before arranging all the chromosomes into a Karyotyper window panel just next to it. The chromosomes boundaries were coloured in blue, and they were well separated from the background noise based on the colour intensities. The separation and sorting of the chromosomes were done following the software rich toolbox. Once all the selected images were karyotyped (see appendix), the length of each chromosomes pair was measured on the Image-J and analysed using a spreadsheet. The relative chromosomal length (% of haploid complement) was determined using the total length of the genome complement. The centromeric index (which is the ratio of the length of the short arm to the total length of the chromosomes) was calculated on the Microsoft Excel spreadsheet by measuring the short arm length and the total chromosomal length.

2.7 Fluorescence *in situ* hybridization (FISH)

Mitotic chromosomal spreads were further subjected to Fluorescence *in situ* hybridization (FISH) analysis to determine the chromosomes order in the karyotype. The two ribosomal DNA probes were used to recognize the 5S and 45S rDNA loci in the following respective karyotype.

The two following DNA probes were used:

1- For 45S rDNA clone pTa71 (Gerlach and Bedbrook, 1979) with a 9kb *Eco*RI fragment of *Triticum aestivum* L. entailing 18S-5.8S-25S rRNA genes and the non-transcribed intergenic spacer region was used on the slide.
2- For 5S rDNA clone, pCT 4.2 having a 500 bp insert from *A. thaliana*

The plasmids were grown and DNA extracted by the method developed by Sambrook *et al.,* in 1989. The probe was labelled by nick translation as per manufacturer's instructions. (Boehringer Mannheim).

The slides were washed with a 2X SSC solution to remove the coverslip and to dissolve the DAPI vectashield following the protocol adopted by (Howell *et al.,* 2002). The method was described initially by (Fransz *et al.,* 1996) and modified slightly for Brassica species. The slides were dehydrated by alcohol series of 70%, 85% and 100% each for two minutes each in a row. Following steps were followed for Fluorescence *in situ* hybridization,

i) The slides were washed in 2xSSC for 10 minutes in a Coplan jar to change the ionic strength for the better hybridization of the probe.

ii) The pepsin solution was prepared and kept at 37^0C for an hour before digestion can be done.

iii) The mounted cells on the slide were digested with the pepsin solution for 90 seconds in a jar at room temperature. This would digest cytoplasm and help in accessing the target sequences in the genome for the probe.

iv) Two times washing was done in 2xSSC solution afterwards for 5 minutes each to remove any debris left on the slide.

v) The slides were fixed in 4% paraformaldehyde at room temperature for 10 minutes under fume hood which helps in improving cross-linking ability in the cell.

vi) Washed the slides with sterile distilled water (SDW) three times.

vii) The slides were dehydrated in ethanol serial (i.e. 70%, 85% and 100%) each for two minutes before they were left for drying for at least half an hour. It would prevent the DNA from shrinkage and allow it to denature in subsequent stages.

viii) The probe mixture was prepared as follows:

45S rDNA Dig labelled	3 µl
5S rDNA Biotin labelled	3 µl
Master mix	14 µl
Total volume	20 µl

ix) A total of 20 µl of a probe containing probe mix was used for single slide and covered with a coverslip.

x) Vulcanizing rubber solution was used to seal the coverslip as it was placed on a hotplate at 75^0C for 4 minutes.

xi) The slides were kept in a moist box at 37^0C for overnight before it was washed the next day.

xii) The water bath was set at 45^0C with the wash jar containing 50% formamide solution in 2X SSC in it the next morning before the rubber and coverslips were carefully removed from the slides.

xiii) Washing of the slide was done in three jars of formamide solution for 5 minutes each.

xiv) The slides were washed after that in 2xSSC for 5 minutes and also in 4xSSC solution at 45^0C.

xv) The slides were transferred to 4x SSC, 0.05% Tween 20 solution at the room temperature before the application of secondary antibodies on it as described below.

xvi) DIG block was defrosted only while Milk block was thawed and centrifuged to pellet down the milk solid to leave the supernatant for use.

xvii) Anti-Digoxygenin FITC was used in a ratio of 1 µl in 50 µl of DIG block while anti-Biotin CY3 was used as one µl in 200 µl of Milk block.

xviii) A solution of 100 µl for the single slide was prepared and applied on the slide with the help of parafilm coverslip.

xix) Each secondary antibody was incubated separately for 30 minutes at 37^0C and washed with the 4xSSC solution thrice in the dark for 5 minutes each.

xx) The slides were stained with antifade medium DAPI vectashield to maintain the integrity of chromosomes.

2.7.1 BAC-FISH

Several BACs were obtained from Isobel Parkin, a researcher in Canada, for developing physical map of *B. nigra*. The detail is given in the following table.

Table 2-2 List of BAC used for developing physical map in *in situ* hybridization

BAC	CHR	CHR_ length	CHR_ start	CHR_ end	BAC_ start	BAC_end	FPC_CTG_ length	# genes	#genes/10Kb
BNi.E050.H12	B1	42219745	89110	176072	1	131073	135169	18	2.0699
BNi.E070.B20	B1	42219745	2633244	2733043	4097	155649	303105	16	1.6032
BNi.E045.B11	B1	42219745	38411055	38574847	69633	253953	376833	28	1.7095
BNi.E058.G23	B1	42219745	38827807	38933555	425985	540673	811009	24	2.2695
BNi.E080.D12	B2	52654246	5660685	5755213	172033	303105	577537	18	1.9042
BNi.E057.M21	B2	52654246	42419002	42579213	12289	212993	278529	31	1.9349
BNi.E077.L04	B2	52654246	50108664	50237698	8193	176129	417793	27	2.0925
BNi.E052.K20	B3	46823113	3324282	3459706	311297	479233	884737	29	2.1414
BNi.E023.F23	B3	46823113	42089980	42212275	212993	356353	532481	14	1.1448
BNi.E025.M12	B4	43385499	163761	298446	32769	184321	516097	23	1.7077
BNi.E043.L24	B4	43385499	4568088	4672987	1433601	1597441	1720321	21	2.0019
BNi.E018.H02	B4	43385499	40956801	41040266	45057	131073	163841	11	1.3179
BNi.E051.A24	B5	51746375	772624	885735	4097	155649	155649	24	2.1218
BNi.E079.I14	B5	51746375	51147223	51252475	1	172033	417793	18	1.7102
BNi.E074.C05	B6	36665641	119358	219958	1	139265	139265	28	2.7833
BNi.E079.E08	B6	36665641	32227117	32346097	512001	749569	749569	23	1.9331
BNi.E019.G20	B7	41623772	537945	592332	262145	335873	335873	17	3.1257
BNi.E062.O13	B7	41623772	41102774	41213982	1	122881	139265	no_ match	
BNi.E083.O02	B8	54183509	556574	634598	1	118785	131073	16	2.0507
BNi.E081.C11	B8	54183509	52741397	52839939	45057	147457	155649	21	2.1311

2.8 Meiotic atlas development

Since meiosis is a complex biological system, it has been divided into sub-phases according to the spatiotemporal chromosomal configuration. Pollen mother cells were selected for developing a meiotic atlas of Brassica species as described below.

2.8.1 Slide preparation

Primary inflorescences were harvested, and individual buds were arranged in a line by size from small to medium on a moist filter paper. A single bud was carefully dissected under a binocular microscope, and one anther out of six was squashed in lacto-proprionic orcein for observing meiotic stage under a stereomicroscope. The anthers of the appropriate stages were fixed in freshly prepared ice-cold 3:1 fixative (ethanol: acetic acid) for 18-24 hours. It followed the same standard steps explained earlier in heading 2.4, except that the enzyme used was relatively concentrated, three times for what was used for roots meristematic tissues. The duration of incubation was extended to 90 min for diploid species (i.e. *B. nigra* and *B. rapa*) and 120 minutes for the allopolyploid (i.e. *B. juncea*). The hot block duration at 45^0C for spreading was increased to 60 seconds while developing meiotic atlases.

2.9 Telomere probe preparation- Nested PCR

2.9.1 Primary PCR

A secondary PCR approach was used to synthesize repeat probe (TTAGG) from the primers of telomere (Ijdo *et al.,* 1991). The primers used for this reaction were;

- Primer-1 5'-TTTAGGGTTTAGGGTTTAGGGTTTAGGGTTTAGGG-3'
- Primer-2 5'-CCCTAAACCCTAAACCCTAAACCCTAAACCCTAAA-3'

This approach was different from standard PCR reaction as no DNA template was used in the reaction and primers served as template for the extension. The primers were used in higher concentration of 5 µM in the final volume of 50 µl reaction. The recipe of the reaction is as follows:

2.9.2 Primary PCR recipe

PCR Mix	Volume	Concentration
10 x Buffer	5 µl	50 mM
50 mM $MgCl_2$	2 µl	2 mM
10 mM d-ATP	1 µl	0.2 mM
10 mM d-GTP	1 µl	0.2 mM
10 mM d-CTP	1 µl	0.2 mM
10 mM d-TTP	1 µl	0.2 mM

Primer-1	3 µl	0.3 mM
Primer-2	3 µl	0.3 mM
Taq Polymerase	0.5 µl	2.5 units
H_2O	32.5 µl	
Total	50 µl	

Table 2-3 Primary PCR Cycling profile

Temperature (^{0}C)	**Duration** (Seconds)	**Cycle**
95	60	8
55	30	
72	60	
94	60	24
60	30	
72	60	
72	300	1

2.9.3 Secondary PCR

Once primary amplicon is ready, 3 µl of this product was mixed with 23.5 µl of sterile distilled water (SDW) and was denatured at 95^0C for 5 minutes. This denatured probe is kept on ice and mixed with the following PCR master mix:

2.9.4 Secondary PCR recipe

PCR Mix	Volume	Concentration
10 x Buffer	5 µl	50 mM
50 mM $MgCl_2$	2 µl	2 mM
10 mM d-ATP	1 µl	0.2 mM
10 mM d-GTP	1 µl	0.2 mM
10 mM d-CTP	1 µl	0.2 mM
10 mM Dig-11-dUTP	1 µl	0.2 mM
Primer-1	3 µl	0.3 mM
Primer-2	3 µl	0.3 mM
Taq Polymerase	0.5 µl	2.5 units
H_2O	6 µl	
Total	23.5 µl	

Table 2-4 Secondary PCR Cycling profile

Temperature (^{0}C)	**Duration** (Seconds)	**Cycle**
95	60	25
60	30	
72	60	
72	120	1

2.9.5 Wide Hybridization and induced polyploidy

Crossing two distant genotypes to produce hybrid is referred as Wide Hybridization since U's triangle consisted of established three diploid and three allotetraploid species, it provided me with an opportunity to create synthetic allotetraploid and analyse meiosis taking place in the newly formed plant. The diploids were closely related species but with different genome sizes, i.e. *B. nigra* (2n=16), *B. oleracea* (2n=18) and *B. rapa* (2n=20). The diploids were crossed in different combinations to get a wide hybrid (combination of two different genomes), bearing in mind the potential chromosomal barrier between the species. The manual crossing was carried out by emasculating one plant and using the other as a pollen source and vice versa. The hybrid

achieved was treated with colchicine to double the genome complement for synthesizing allotetraploid.

Unopened buds of *B. rapa* (AA) were manually emasculated to make it a female receptor and then pollinated with *B. nigra* (BB) pollen to get a hybrid (AB) to use for synthetic *B. juncea* (AABB). Manual crosses were made in different combinations in an attempt to combine two genomes together. The seed of the hybrid was plated on the M&S media enriched plate for mitotic analysis and then transplanted to glass house under standard conditions for analysing the meiosis in young buds.

Clonal propagation was done by cuttings to increase the number of plants for the analysis and to generate a synthetic allotetraploid. A young branch of 10-12 inches was cut under water and inserted into rooting media for one minute before planting into a pot containing soil rich medium (compost).

Colchicine was used to double the chromosomes complement in two ways, i.e. on buds as described in (Santos *et al.,* 2003) and with roots (Lionneton *et al.*, 2001). A concentration of 3.4g/L colchicine was used as described (Lionneton *et al.*, 2001) for 90 min treatment before the plant was returned into the pot. Visual difference was spotted when the buds were enlarged and swollen which was further analysed for meiosis.

2.10 Genomic *in situ* hybridization

2.10.1 Probe preparation

For GISH, total genomic DNA of both diploid genomes (*B. nigra* and *B. rapa*) was used to detect respective subgenome in the allotetraploid *B. juncea*. Genomic DNA was extracted from the young leaf of *B. nigra* and *B rapa* using a DNA extraction kit Phytopure according to manufacturer instructions. After treatment with RNases and quantification using nanodrop, DNA was diluted and sheared by autoclaving for 5 minutes twice to obtain fragments of 100-500 bp. DNA was one sub-genome (*B rapa*) was labelled with FITC and other sub-genome (*B nigra*) with biotin-16-dUTP by nick translation (Roche) according to manufacturer's instruction.

Slide pre-treatment, chromosome and probe denaturation, hybridization and post-hybridization were followed in the same fashion as described (in section 2.7) similar to the method of FISH except that the denaturation in GISH at 75ºC was reduced to 3 min 30 sec (section 2.7 x). Nearly 50-100 ng of the genomic DNA of each subgenome was used per slide for GISH to hybridize with respective chromosomes. Probes used were as follows in the recipe;

Biotin labelled *B. nigra*	3 µl
Dig labelled *B. rapa*	3 µl
Master mix	14 µl
Total	20 µl

The images were captured and analysed using the SmartCapture software on Olympus fluorescent microscope.

2.11 Immunolocalization

2.11.1 Slide preparation

Young buds from inflorescence were dissected on a moist filter paper under a binocular microscope. Zygotene and pachytene stage were identified under a stereo microscope before digesting the rest of anthers on the slide in 10μL digestion mix (0.4 % cytohelicase, 1.5% sucrose, and 1% polyvinylpyrrolidone). The anthers were squashed and tapped gently with a brass rod for 1 minute. An extra 5μL of digestion mix was added to the slide, and the slide was incubated in a moist chamber at 37ºC for 4 minutes. The anthers became swollen, and debris was removed with the help of a pair of forceps pair. 1.5% lipsol was added with a volume equal cell sap on the slide, nearly 10 μl and was mixed & spread thoroughly with a mounted needle. This will allow cells to move away from each other to give nicely spread cells. 20 μl of 4% paraformaldehyde was used to fix the cell on the slide immediately and was left to dry under the fume hood for nearly 3 hours. Once the slides were dried they were incubated for 30 minutes at 37ºC with blocking solution (0.1 x phosphate buffered saline (PBS), 0.01% Triton X-100, 0.1% Bovine Serum Albumin (BSA)) The slides were then ready to be mounted with an antibody you want to hybridize with.

Each slide was added nearly 70 μl of each primary antibody and was incubated with a parafilm solution. Following primary antibodies were used in diluted form as follows;

- Anti-ASY1 rabbit 1 µl in 500 µl of blocking solution
- Anti-ZYP1 rat 1 µl in 500 µl of blocking solution
- Anti-Mlh1 rabbit 1 µl in 200 µl of blocking solution

The slides were incubated for 48-72 hours at 4ºC.

The slides were rinsed three times following the incubation in a washing solution (1 x PBS, 0.1% Triton X-100) for five minutes each. 70µL of secondary antibody diluted in the blocking buffer was then added onto the slide using a parafilm coverslip, and the slide was incubated in a moist chamber at 37ºC for 30 minutes. The antibodies used were diluted as under;

- Anti-rat/ rabbit FITC 1 µl in 50 µl of blocking solution
- Anti-rat/rabbit CY3 1 µl in 200 µl of blocking solution

The slides were washed thrice after incubation in the washing solution for 5 minutes each and before can be mounted with 10 µl DAPI in Vectashield. Different combination of two and three antibodies were tested on the hybrid allodiploid (AB).

2.12 Sequential Fluorescence *in situ* hybridization (FISH)

The slides were washed with ethanol to remove any oil from the slides. They were kept in 4T solution (100ml 20xSSC, 400ml SDW, 250ul Tween20) for overnight at room temperature. Next day they were washed with 4xSSC+0.1% Triton X-100 three times for one minute each followed by one time in 2xSSC. The slides were placed on the hot plate at 73 ºC for 4 minutes

to denature the previous hybridization bonding. Ethanol series of 70%, 85% and 100% was done to dehydrate the slide which made the slide ready for rehybridization. Standard practices were followed for rehybridization with the new probe.

2.13 Time course analysis

To induce homoeologous recombination, I needed to know the time course analysis on *B. juncea* -how long did it take for *B. juncea* to reach in the meiotic metaphase-I stage where chromosomes could be seen in a desired configuration. I used the EdU technique for labelling DNA with thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU) to find out whether chemicals could be taken up by the roots via transpiration pull while the plant was in reproductive stage. During DNA replication new bases are continuously used in the semi-conservative replication fork. The thymidine analogue became incorporated and could easily be identified later using click chemistry.

2.13.1 The Edu (5-Ethynyl-2-deoxyuridine) pulse and slide preparation

The protocol was followed as detailed in (Armstrong, 2013). The branch bearing buds were cut under water to avoid any air in the upstream through the xylem. The branch was then placed in the bottle containing EdU for two hours for it to be pulled up. Following the EdU treatment, it was immersed in water containing small bottles for different time scales; 24 hours, 38 hours and 40 hours in a glass house temperature. After the specified time, inflorescences were fixed, and slides were made according to the standard method for chromosome spreads. The slides

were washed with 0.1% Triton X-100 in PBS for 15 min at room temperature. It was followed by two times washes with PBS only before click reaction could happen. The Click solution was prepared according to the instructions in EdU detection kit (Click-it® Edu Alexa Fluor 488 HCS Assay) from the following components;

Reaction component	Volume (µl)
Alexa Fluor® azide	0.13
$CuSO_4$	2.02
1X Click-it® EdU buffer additive	5.04
1X Click-it® EdU reaction buffer	42.81
Total	50.0

A total of 50 µl was applied on a single slide and covered with parafilm under the dark condition for 30 minutes. After washing in PBS for a few seconds, it was stained with DAPI Vectashield. The images were captured using Software Digital Scientific under the Olympus fluorescence microscope.

2.13.2 Induced Homoeologous association

1% okadaic acid, phosphatase inhibitor was used to induce homoeologous association between different genomes by altering Cdk2 activity (Knight *et al.,* 2010). Branches bearing inflorescence were cut in water before putting them 1% okadaic acid for different hours to see the post-translational modification effect on chromosome condensation. Inflorescences were fixed, and chromosomal spreads were prepared following standard procedure.

3 . Karyotyping and Genome *in situ* hybridization (GISH) of the species in U's triangle

3.1 Introduction

3.1.1 Karyotype

The chromosomal organization succeeding multiple rounds of polyploidization during evolutionary course has posed complications in analysing brassica genomes in detail. Various attempts had been made to decipher obscurities using molecular cytogenetics to ease the way for better and sustained agricultural research practices (Fukui *et al.,* 1998; Snowdon, 2007). The advances have led us identifying heritable entities (chromosome), their number and structure which are characteristic features of species in the phylogenetic tree. Such a fundamental framework is referred as a karyotype which encompasses the information about the number, size, and shape of chromosomes in a single cell and is of great importance for the downstream sequencing projects to reveal genetic information for multiple purposes. The karyotyping has been difficult because of the complex nature of chromosomes in this genus comprising a blend of diploid and polyploid species (Xiong and Pires, 2010) which have undergone paleopolyploidization during their evolution. The two genomes (*B. rapa* and *B. oleracea*) out of the three diploids in the U's triangle have been studied extensively and sequenced fully to reveal important information about the syntenic relationship between them and with the model plant, *A. thaliana*. The third diploid, in the U's triangle, is *B. nigra*, was overlooked due to its poor yield potential and was therefore exploited quite less than the other diploid species in U's triangle.

I have used cytogenetic tools during my studies to explore *B. nigra* and analyse its behaviour in one of its derived polyploid *B. juncea*. Species-specific chromosome number has been a critical milestone in cytogenetics and had extensively been exploited studying genetics and taxonomy. FISH was employed to study the chromosomes in *B. nigra,* and it identified four chromosomes out of eight which aided in developing an ideotype like that of *B. oleracea* (Armstrong *et al.*, 1998).

A karyotype sets the framework for the chromosome and provides ground for subsequent research to be used for mapping and chromosomal identification. It elucidates the characteristic shape and dimension of every chromosome in individual species. The discovery of constant species-specific chromosomes was considered to be an important landmark in the field of cytogenetics and would be helpful in studying the relationship between chromosomal interaction and designing hierarchy of different species in taxonomy (Gianfranco *et al.,* 2008). It is essentially a basic set of information not for the genome project only but also opens new vistas for evolutionary, phylogenetic and taxonomic studies (Fukui *et al.,* 1998). Individuals in a genus can be classified and categorised based on karyotype which might have evolved in the past as result of slight variation in DNA content or its rearrangement in the cell nucleus (Stebbins, 1966). For the systematic arrangement of individuals in taxonomic order, knowledge derived from cytogenetics proves way better than the ecological or morphological data (Coutinho, 1952; Hanelt and Metetin, 1964; Dewey, 1984). Therefore, the chromosomal structure serves as a diagnostic tool as well. Cytogenetics established recognition with the hallmark credit of 46 chromosomes in a human cell (Tjio and Levan, 1956) which rectified the

three decades old concept of basic 48 chromosomal complements in human (Painter, 1923) paving the way for more research in this area.

We define several types of chromosomal categories based on centromere position, metacentric, sub metacentric, acrocentric and telocentric chromosomes (Hanelt and Mettin, 1989).

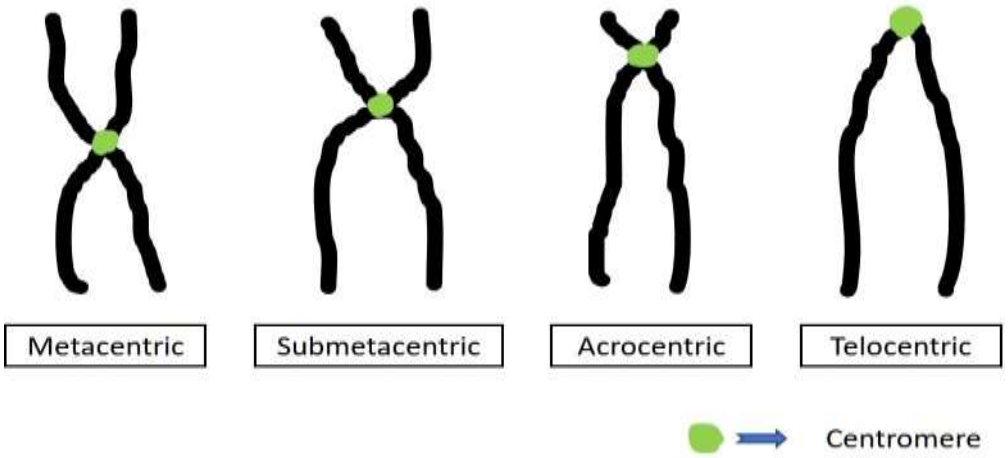


Figure 3-1 Chromosomal classification in a karyotype

3.1.2 Fluorescence In Situ Hybridization (FISH)

The study of chromosomes was inspired and stimulated by the discovery of DNA structure (Watson and Crick, 1953) which unveiled its duplex nature with each strands running anti-parallel to the other. The compatibility among nitrogenous bases was exploited further to account for the complementarity and hybridization potential for genome mapping by *in situ* hybridization (ISH) (O'Connor, 2008). The fluorophore discovery revolutionized this exciting

field of hybridization to Fluorescent *in situ* hybridization (FISH) which has applications ranging from karyotyping to gene localization (Guerra 2004).

According to the fundamental principle of FISH in plants for meiotic studies, probe and targets must have been denatured first before the annealing of complementary sequences could happen. The target cells are permeabilized well before incubating with the probe to achieve stable hybridization and specificity. Even in the presence of high through-put era, it is still a cornerstone in genetic labs because of its simplicity, specificity and sensitivity. For the study of structural and functional analysis, FISH has been used extensively in all domains of research from plant sciences to personalized human medicine (Hu *et al.*, 2014). It has wide application in physical mapping of the sequencing projects. To develop an ideotype of *B. nigra*, FISH with ribosomal DNA was performed on its mitotic and meiotic cells which identified several chromosomes and helped me in categorizing them in order to be compared with different lineages.

3.1.3 Genome In Situ Hybridization (GISH)

The hybridization in a controlled environment on cytological slides where DNA-DNA association is achieved had revolutionized the field of cytogenetics and had identified specific sequence/segments in the targets. Such advances revealed detailed information about the chromosomal structure which was hard to analyse with the orthodox methods. Until early 1970 classical/conventional cytogenetics tools were only effective to reveal chromosomal morphology, their arm lengths and centromeric position using standard staining procedure to

differentiate euchromatin and heterochromatin (Silva and Souza, 2013). With the invention of DNA-DNA hybridization, it revolutionized this field to decipher specific segments of interest on chromosomes which proved to be a provenance for several analysis in living organisms (Pardue and Gall, 1969).

Genome *In Situ* Hybridization (GISH) is a particular type of hybridization where the whole genome is used as a probe to identify the subgenomes and to study the evolutionary basis in organisms and plants in particular. It helps in discriminating more than one genome in the hybrid and allopolyploid in crops breeding programmes, therefore it is a valuable tool to adopt for early selection breeding in the hybrid production industry. It can help us investigate evolutionary roots and karyotypic alteration in various economically important crops. The progenitors in case of an allopolyploid are identified with the easy and simple approach and robust output image service (Wang *et al.,* 2009; Soltis and Soltis, 2000). The results are significantly reliable and highly reproducible thus enhancing its credibility and usability than any other methods. In GISH, blocking probe is also used when the constitutive genomes are very similar to increase the specificity of staining to the unique segment hence minimizing cross-hybridization between related genitors in a hybrid (Anamthawat-Jónsson *et al.,* 1990). The technique is so accurate and fast that two very closely related genomes, with 95% homology can be distinguished from each other (Parokonny *et al.,* 1997).

As most of the present-day crops are believed to be paleopolyploid, their ancestors could easily be analysed using modern day cytogenetics tools. Meiosis occurring between different genomes can be visualized by labelling individual genome separately. During this studies, GISH was

used exclusively while analysing the allotetraploid genome as well as the hybrid between two diploids (for wide hybrid see Chapter 5). It unveiled the inter-genomic relationship in the polyploid background in mitotic and meiotic cells.

3.1.4 Physical Mapping

Understanding complex organization of a genome has been a dilemma in research community since ages. Since DNA constitutes the genomes of eukaryotes, and carries information across generations, their exact pattern and sequence will help us understand evolution as well as favour the marker assisted selection. It gives detail information about the genome and chromosomes as compared to the genetic map which gives an overview of the genome. Physical maps are important because it gives information based on nucleotide sequence and more accurate information about the markers which can be used for further analysis in human genetics, animal and plant sciences (O'Rourke, 2001). This map complements the information in genetic map by giving a detailed picture of the genome organization. It explicates the outlines being delivered by genetic map for sake of assessing genes straightforwardly. Such explanation is very valuable to breeders in marker assisted breeding programmes to focus on targeted genes of interest (Zhang *et al.,* 2010). Several crops (rice, chickpea, few Brassica species) have been sequenced which has expedited research toward improvement of the genetic base.

Physical mapping is essential to define certain region on chromosomes with known DNA sequences, and for this purpose to aid in sequencing project certain BACs would be tested on the chromosomes to identify their specific location on *B. nigra* genome and the structure of the

chromosome. For this purpose, the pachytene stage was preferred for hybridization, and out of 8 linkage groups, only one was promising which showed stable hybridization.

3.1.5 Lepidium campstre

My supervisor Dr Sue Armstrong was approached by Zeratsion Abera Desta of the Agricultural University of Sweden for help with his karyotyping for his study on Lepidium field cress chromosomes. *Lepidium campestre* is a wild species belonging to brassicacea family, and not yet domesticated normally. The average height of the plant is 50 to 60 centimetres. They are deeply self-pollinated with white flower petal colour and rarely cross pollinated by insect and wind. The research work was carried out in an attempt to integrate cytogenetic and genetic map. The purpose of the work was to include mapping of the field cress genome using integrative approaches in genetic, cytogenetic, and comparative map analysis.

3.2 Results

3.2.1 Fluorescence In Situ Hybridization (FISH) in *Brassica nigra*

Chromosomal spreads from meristematic root tissues were used for developing partial karyotype which laid the basis for the identification and discrimination of the chromosomes in the haploid genome of *B. nigra* (2N = 2X = 16). The respective length of chromosomes was measured using Image-J rich toolbox followed by determining the centromeric index which helped in numbering the chromosomes. Fluorescence *In situ* hybridization (FISH) with ribosomal DNA (45S rDNA & 5S rDNA) on chromosomes paved the way for structuring a haploid ideotype (Armstrong *et al.*, 1998). The chromosomes are organised based on the length gradation measured on the Image J software (Figure 3-3).

High-quality mitotic metaphases were selected to identify chromosomes in the genome. Ribosomal DNA (45S rDNA and 5S rDNA) were used as markers for the cytological study of the genome that paved the basis for subsequent analysis. 45S rDNA probe hybridized efficiently giving green signals on six different chromosomes, to make three pairs whereas 5S rDNA could associate to two chromosomes (to make a single pair) and detected by red signals. The intensity of signals for 45S could be distinguished easily where two chromosomes gave relatively brighter signals than others at the termini of the short arms of chromosomes (Figure 3-2). On the other hand, 5S rDNA signals were detected in the interstitial region near the centromeric constriction and away from the telomeric region under the microscope.

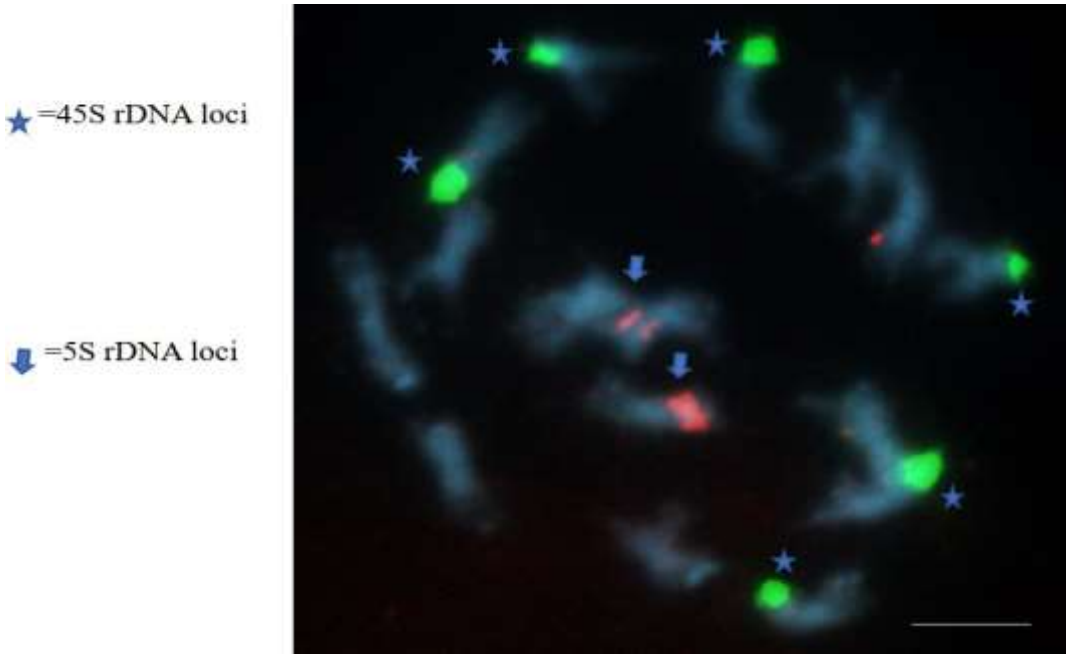


Figure 3-2 ***In Situ*** **hybridization of the chromosomes in** ***B. nigra,***

Mitotic metaphase stained with DAPI showing three pairs of loci for 45S rDNA detected with FITC and 5S with CY3 under a fluorescent microscope. Bar =10 µm

3.2.2 Karyotyping and developing an ideotype

A threshold was determined and defined on SmartType Karyotyper software to discriminate and distinguish chromosomes from the background noise based on colour intensity for accurate downstream chromosomal analysis. Using a wide range of editing tools available in a working panel of Smart Typer, individual and overlapped chromosomes were relocated to enhance their visual appearance in term of to their shape and morphology. Auto cut and manual free/poly cut options were used for resolving complex overlap to extricate chromosomes from the underlying ones into individual entities before all the 16 chromosomes were well separated and grouped subsequently into legitimate pairs in the overview panel (Figure 3-2). The classification was

concluded based on the visual observation (against the auto option) based on length gradients and shape. Several individually karyotyped cells were aligned together in a composite karyotype to corroborate chromosome pair and their corresponding order.

The initial version of karyotype was developed for *B. nigra* L based on the measurement done on Image-J of 20 well spread mitotic metaphases (Table 3-1). The relative length of chromosomes was calculated and has been depicted in (Figure 3-3) showing chromosomes in a gradient of length where the largest chromosome is 1.5 times the length of the shortest chromosome, and the rest of chromosomes are within the extreme ranges. Fluorescence *In Situ* Hybridization (FISH) with 45S and 5S rDNA helped us identifying four out of 8 chromosome pairs (Figure 3-1) with confidence and certainty for setting reference benchmark for the future work and analysis. From the ideotype we can deduce three chromosomes, 3, 4 & 6 had 45S on the shorter arm of the chromosome while 5S rDNA is on a different chromosome and lies close to centromeric heterochromatin region and are shown accordingly in a preliminary model.

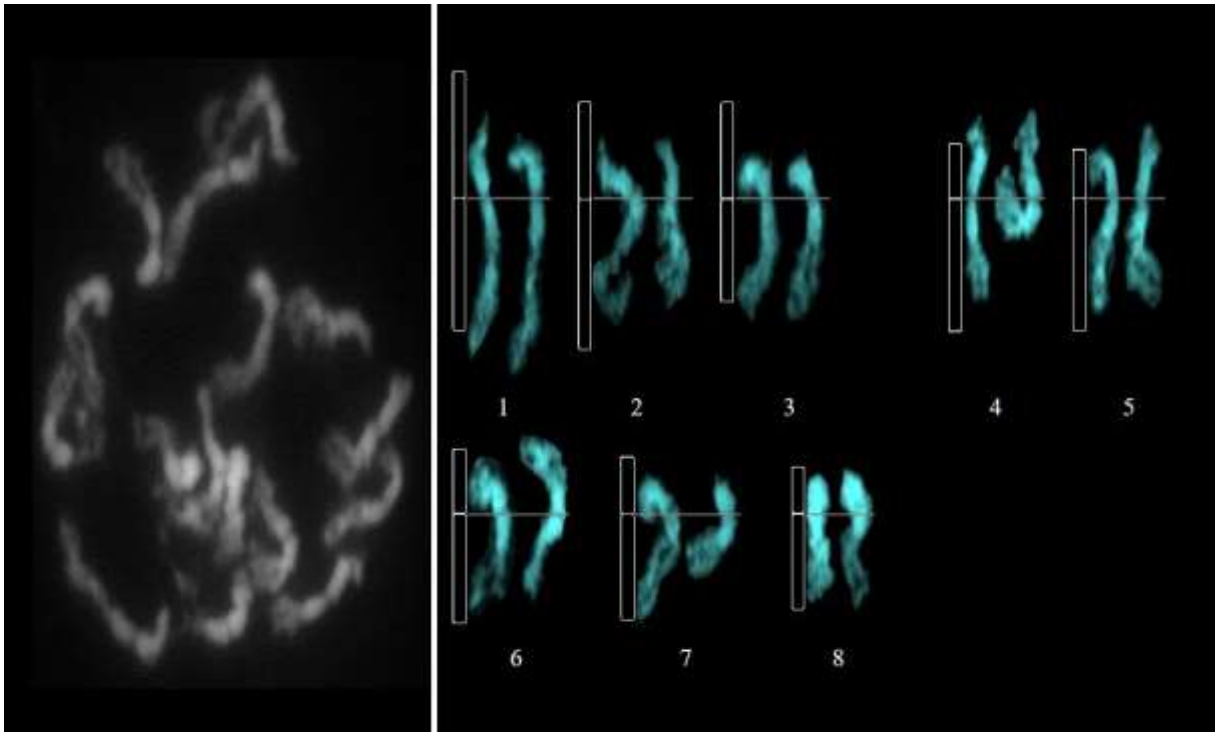


Figure 3-3 A Karyotype of *B. nigra* based on Smart Karyotyper software in Smart Capture package.

Table 3-1 Mean relative lengths and centromere indices from mitotic cells (n=20) of *B. nigra* accession B150

Chromosome	Relative Length (%)	Centromeric Index
1	15.69 ± 0.24	37.62 ± 2.17
2	14.39 ± 0.13	32.39 ± 2.50
3	13.54 ± 0.12	35.81 ± 2.27
4	12.82 ± 0.12	40.63 ± 2.33
5	11.97 ± 0.10	35.14 ± 2.46
6	11.32 ± 0.10	34.53 ± 1.87
7	10.58 ± 0.12	36.41 ± 2.42
8	9.69 ± 0.15	40.51 ± 1.91

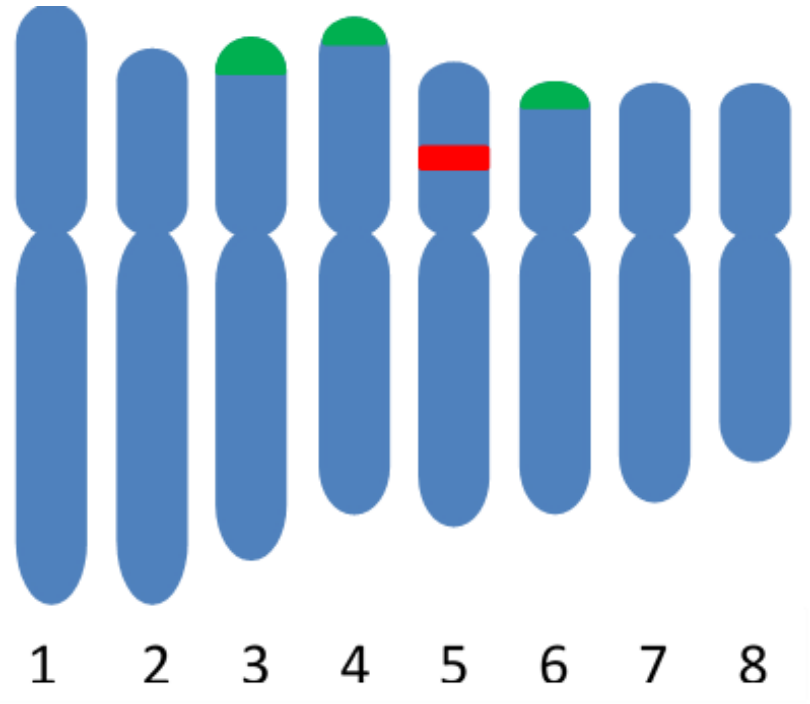


Figure 3-4 Haploid ideogram of *B. nigra*, accession B150 showing the position of 45S rDNA green and 5S Red while the depression shows the centromere.

3.2.3 Fluorescence In Situ Hybridization in Brassica rapa

High-quality meiotic metaphase-I from pollen mother cells of *B. rapa* (2N = 2X = 20) were chosen for *in situ* hybridization to identify chromosomes in the genome and to compare the cytological markers in the haploid genome.

The repetitive sequences markers, 45S & 5S rDNA successfully identified 5 pairs of chromosomes in the genome. Out of these, three chromosomes had both 45S rDNA and 5S rDNA on it while one separate pair for each of the single probe was found. Therefore, 45S

identified four chromosomes, and 5S rDNA also was able to hybridize with four pairs of chromosomes. The signal intensity for 45S rDNA was variable, and I could easily distinguish two darker signals from two faint foci. Whereas 5S rDNA was quite bright on three pairs of loci as compared to one faint signal.

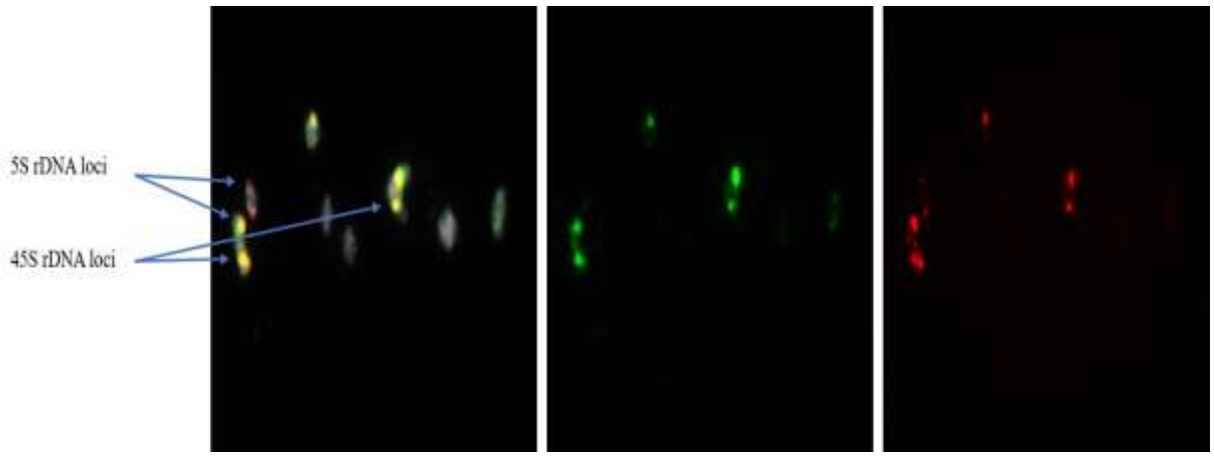


Figure 3-5 ***In Situ*** **hybridization to the chromosomes of** ***B. rapa***

Meiotic M-I stained with DAPI showing four pairs of loci for 45S rDNA detected as Green and four pairs of 5S detected as Red under a fluorescent microscope.

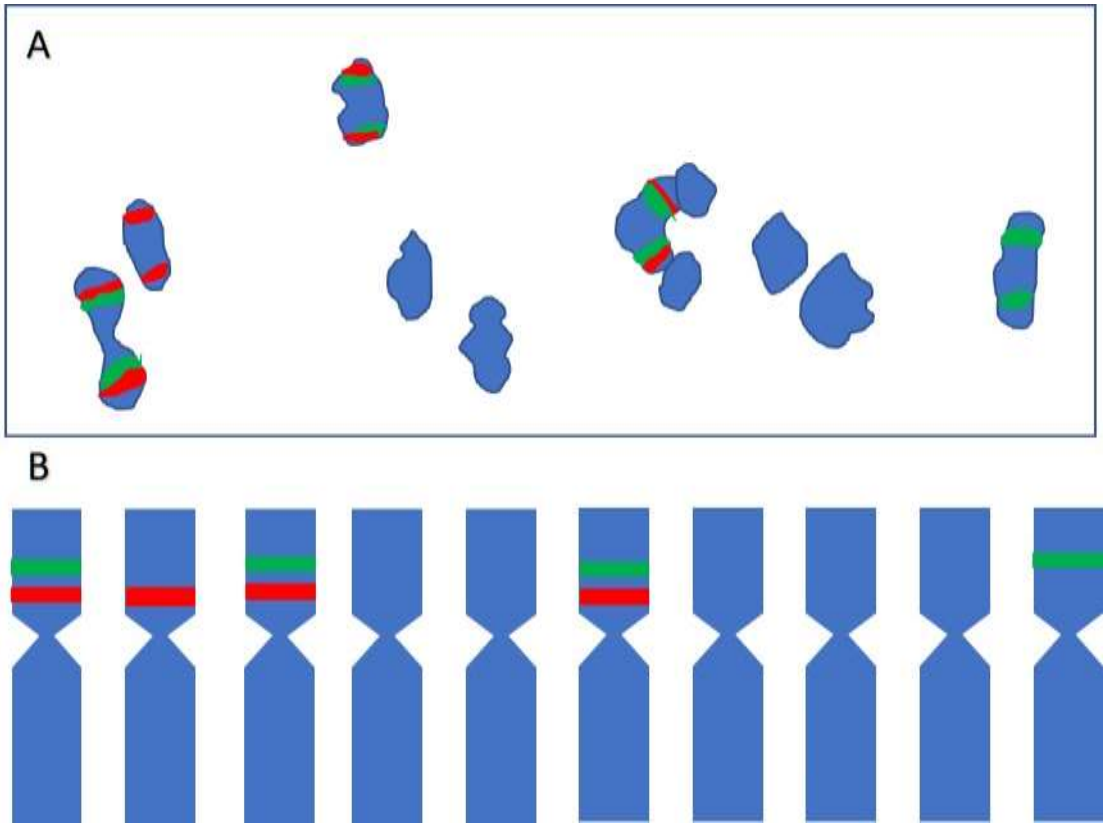


Figure 3-6 General representation of the chromosomes during meiotic M-I stage of *B. rapa* showing loci for 45S and 5S rDNA along with the chromosomal arm in green and red colour respectively.

3.2.4 FISH in Brassica juncea

In U's triangle, *B. juncea* (2N = 4X = 36) represents an allotetraploid which is a hybrid containing *B. nigra* and *B. rapa* genomes together in a cell. Repetitive sequence density was analysed in this polyploid species to compare chromosomal behaviour in somatic as well as in pollen mother cells (for detail of meiosis see Chapter 4). For the analysis of FISH with 45S and 5S rDNA, mitotic cells were chosen (from the roots) which identified six pairs of chromosomes out of 18 (*B. nigra* = 08 & *B. rapa* = 10). Among them, 3 pairs with 45S showed relatively

brighter signals as compared to 2 faint ones while 2 pairs were detected for 5S rDNA along the chromosome arms (Figure 3-6). One pair of chromosomes had overlapping 45S & 5S foci on the short arm of the chromosome whereas the other 5S signals were observed separately on a chromosome. The sharpness of signal intensities and number of chromosomes were not concordant with the cumulative strength of the ancestral genomes together as less 5S rDNA signals were recorded in *B. juncea* than 4 and 1 in its parental lines (*B. rapa* & *B. nigra*) and a similar trend was true for 45S rDNA.

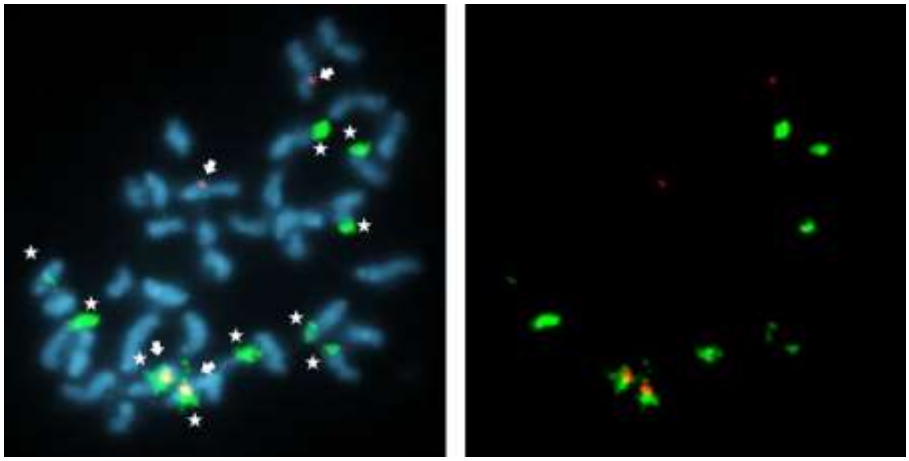


Figure 3-7 ***In Situ*** **Hybridization to the chromosomes of *B. juncea,***

Mitotic metaphase stained with DAPI showing five pairs of loci for 45S rDNA (white asterisk) detected by FITC and two loci of 5S (white arrows) by CY3 under a fluorescent microscope. Scale bar 10 µm.

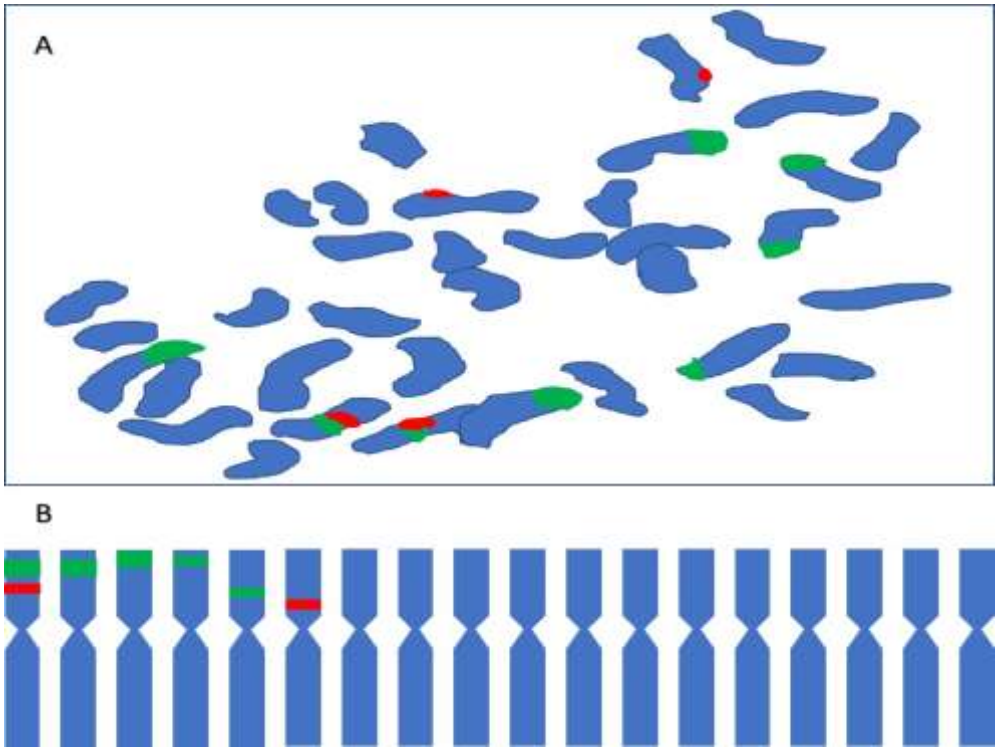


Figure 3-8 General representation of the chromosomes (not karyotyped ones) based on the mitotic metaphase stage of *B. juncea* showing loci for 45S and 5S rDNA along with the chromosomal arm in green and red colour respectively

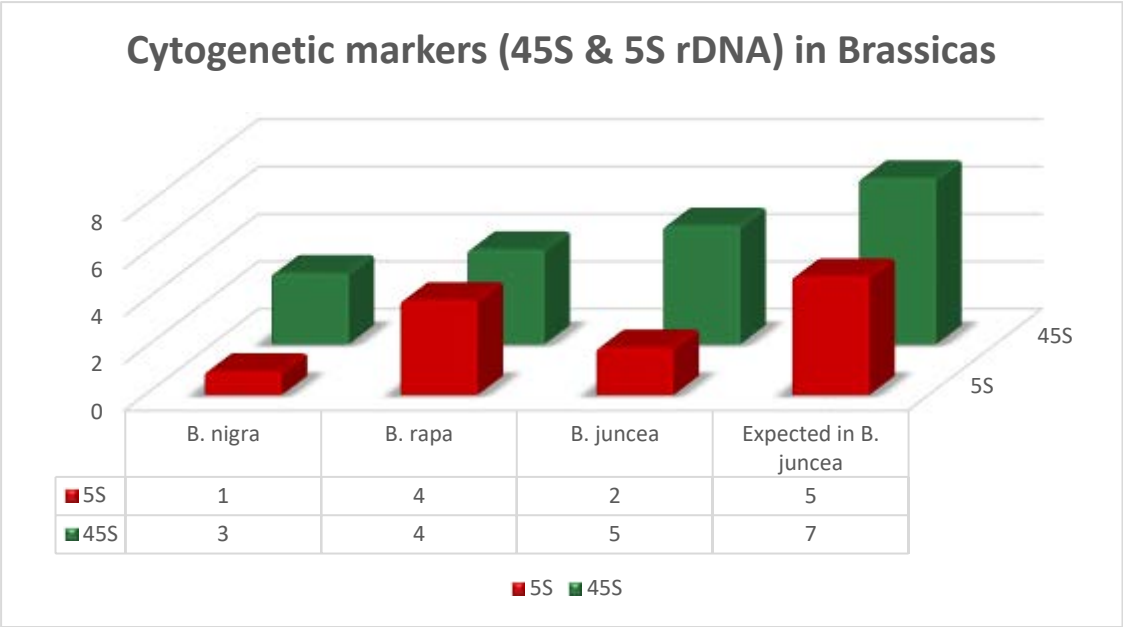


Figure 3-9 Ribosomal DNA signals in Brassica diploid and tetraploid species in U's triangle, (Number of cells, n=20)

Comparison between diploid and allotetraploid revealed some interesting facts about repetitive sequences as more 5S signals were detected in *B. rapa* genome, 4 as compared to 1 in its counterpart diploid *B. nigra*. Moreover, the allotetraploid derivative contained only 2 pairs of chromosomes with the 5S rDNA which is half those found in *B. rapa* and twice those found in *B. nigra*. Further analysis revealed a fact that 4 chromosomes were detected having ribosomes in *B. nigra*, 5 in *B. rapa* but only 6 chromosomes in *B. juncea,* which otherwise should have 9 in total after the hybridization of two distinct genomes involved (Figure 3-9).

Table 3-2 Ribosomal DNA hybridization in Brassica diploid and tetraploid species of U's triangle.

	Haploid	Mbp	5S foci	45S foci	# of chr	5S per Mbp	45S per Mbp
B. nigra	8	632	1	3	4	632.00	210.67
B. rapa	10	529	4	4	5	132.25	132.25
B. juncea	18	1068	2	5	6	534.00	213.60
Expected B. juncea	18	1161	5	7	9	232.20	165.86

3.3 Genome *In Situ* Hybridization (GISH)

3.3.1 Probe preparation for GISH

The DNA extracted from both parental genomes, i.e. *B. nigra* and *B. rapa* was quantified on Nanodrop to tweak an appropriate concentration for the nick translation. DNA was sheared mechanically in an autoclave to get short fragments to be used as probes which were labelled with Biotin and FITC separately through nick translation. Around 70-140 ng/µl of DNA was used as a probe from each subgenome in GISH analysis on a single slide.

3.3.2 Self-Genome In Situ Hybridization with Brassica nigra and Brassica rapa

To identify individual constituent subgenome in an allotetraploid, I carried out self-genome GISH on the same parental line first to validate the probing fidelity. Being widely used in polyploid research, GISH identifies genomes discriminately in mitosis and meiotic cells for several purposes. According to the principle and prospect, BB would hybridize on the *B. nigra* slides only and AA should be biased toward *B. rapa* only, and this was true for 16 red signals of *B. nigra* with no green signal for *B. rapa* on *B. nigra* slide (Figure 3-10). Similar behaviour for *B. rapa* was spotted, and it detected all of the chromosome indicating perfect intra-genomic hybridization (Figure 3-10). The total number of foci on *B. rapa* slides were counted to be 20, which is the diploid state of *B. rapa* and could not hybridize on *B. nigra* slides which served as a control in this case.

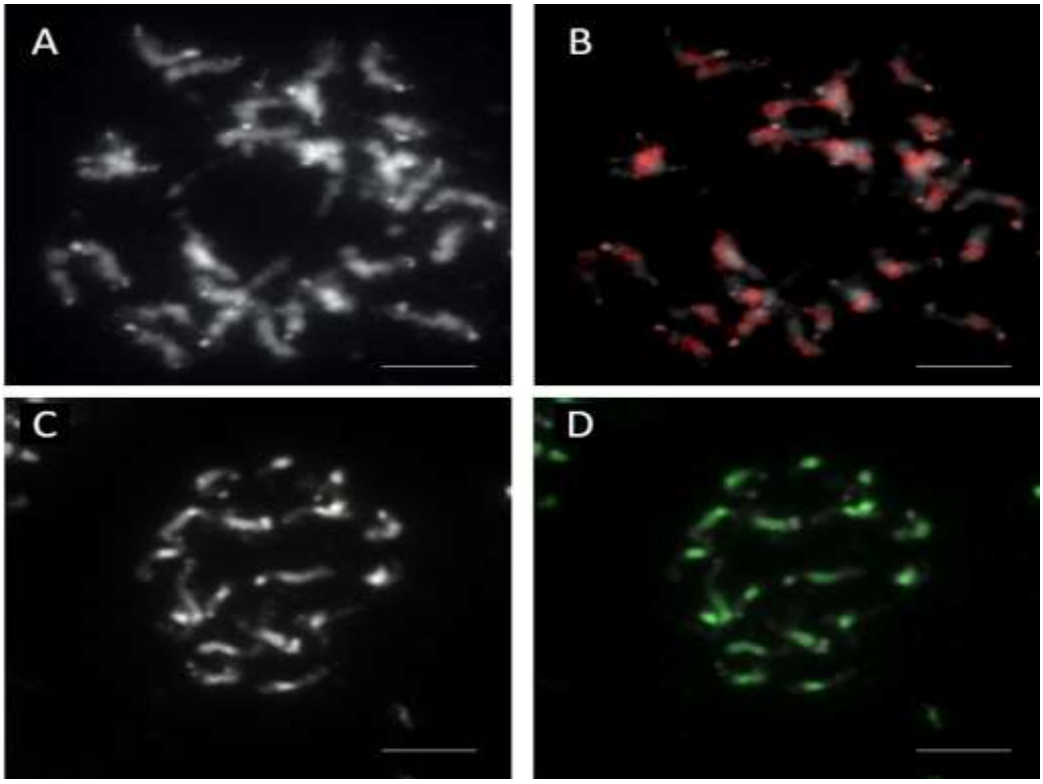


Figure 3-10 Self GISH analysis in Brassica diploids

(A) *B. nigra.* DAPI spread of mitotic spread, (B) Self GISH with *B. nigra* probe, (C) *B. rapa.* DAPI spread of mitotic spread, (D) Self GISH with *B. rapa* probe Scale bar 10 µm.

3.3.3 In Situ Hybridization with lineage-specific centromere probe

Since *B.rapa* and *B. nigra* belong to different lineages, FISH was performed with a lineage-specific probe to help us understand the divergence between the two diploids in U's triangle. Mitotic metaphase from both genomes was then subjected to FISH with the centromeric probe (61G14) to identify any shared sequences at the centromere or heterochromatin region. The probe gave 16 signals in *B. rapa* genome showing localization at the heterochromatin

constriction sites against 20 signals from GISH to zero in *B. nigra* genome. To address the curiosity, it was reprobed with 45S rDNA, *B. rapa* genomic DNA in GISH analysis. Some fascinating evidence was unveiled upon re-hybridization for both of these two probes as follows:

- With 45S rDNA I got eight signals, out of which six were considerably stronger than the two (Figure 3-11 A, C).
- When reprobed with *B. rapa* genome in subsequent GISH, I observed the expected 20 signals are corroborating the presence of the *B. rapa* genome in it (Figure 3-11 B, D).

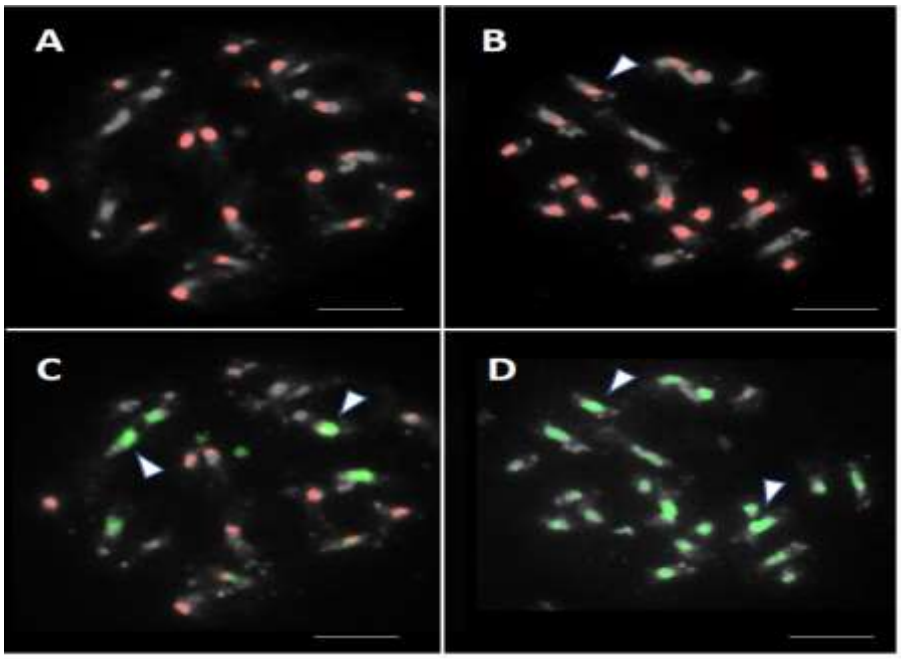


Figure 3-11 *In Situ* Hybridization of mitotic metaphases of *B. rapa* (AA)

(A, B) FISH with Centromeric probe 61G14 (Red fluorescence), (C) Sequential FISH, rehybridized with 45S rDNA (Green fluorescence), (D) GISH; reprobed with *B. rapa* genomic DNA (Green fluorescence). Bar =10 µm

3.3.4 Genome In Situ Hybridization (GISH) in allopolyploid Brassica juncea

Various stages of mitosis and meiosis were examined with GISH to study the behaviour of progenitors in the allotetraploid *B. juncea* (AABB), which contains AA genome of *B. rapa* and BB genome of *B. nigra*. Chromosomal spreads from both anther and root meristematic tissues were used for this purpose which helped to identify individual sub-genomes in the polyploid background. The meiotic atlas was also developed for *B. juncea* to assess the behaviour of chromosomes in a reproductive phase where preferential pairing occurs between identical chromosomes.

Slides featuring well spread meiotic metaphase-I and mitotic metaphase were subjected to GISH where FITC-labelled *B. rapa* DNA hybridised selectively to its corresponding AA part of the genome discriminating it from the BB counterpart subgenome of *B. nigra*. This polyploid species, *B. juncea,* contains 36 chromosomes altogether and were counted under a microscope (Figure 3-12). *B. rapa* chromosomes could be distinguished spatially from 8 pairs of *B. nigra* chromosomes based on different colours detected with a microscope in mitotic cells and during the metaphase-I stage of Meiosis-1 where respective homologues paired with each other and formed bivalents with observable signatures of intergenomic translocation between *B. nigra* and *B. rapa* genomes (Figure 3-12). The evidence was substantiated by the number of signals detected for *B. nigra* and *B. rapa,* where nine pairs of signals were detected against usual eight for *B. nigra* and archetypal ten for *B. rapa* (Figure 3-12).

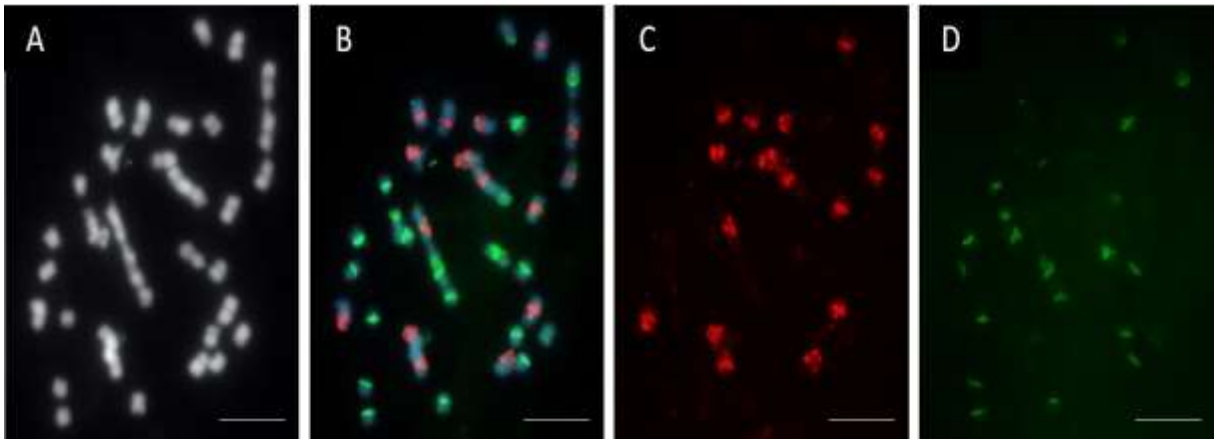


Figure 3-12 *In Situ* Hybridization of mitotic metaphase of *B. juncea* (AABB) species:

(A) DAPI spread of *B. juncea* chromosomes from root tip (B) GISH with total genomic DNA from the *B nigra* (Red fluorescence) and *B rapa* (Green fluorescence) (C) *B. nigra* biotin label

During metaphase-I, normal bivalents were observed between like typical homologues except one bivalent which contained both genomes together (n=15) (Figure 3-13A). The preferential pairing was observed in the rest of 17 bivalents accounting for 95% normal pairing in the genome, with one bivalent presenting both genomes (Figure 3-13). According to the results, it seemed that *B. nigra* has introgressed into *B. rapa* genome so to answer if *B. nigra* has the potential for introgression or the *B. rapa* was more susceptible, I selected *B. carinata* for single genome hybridization to see if *B. nigra* has more signals than expected or it had the same typical eight pairs.

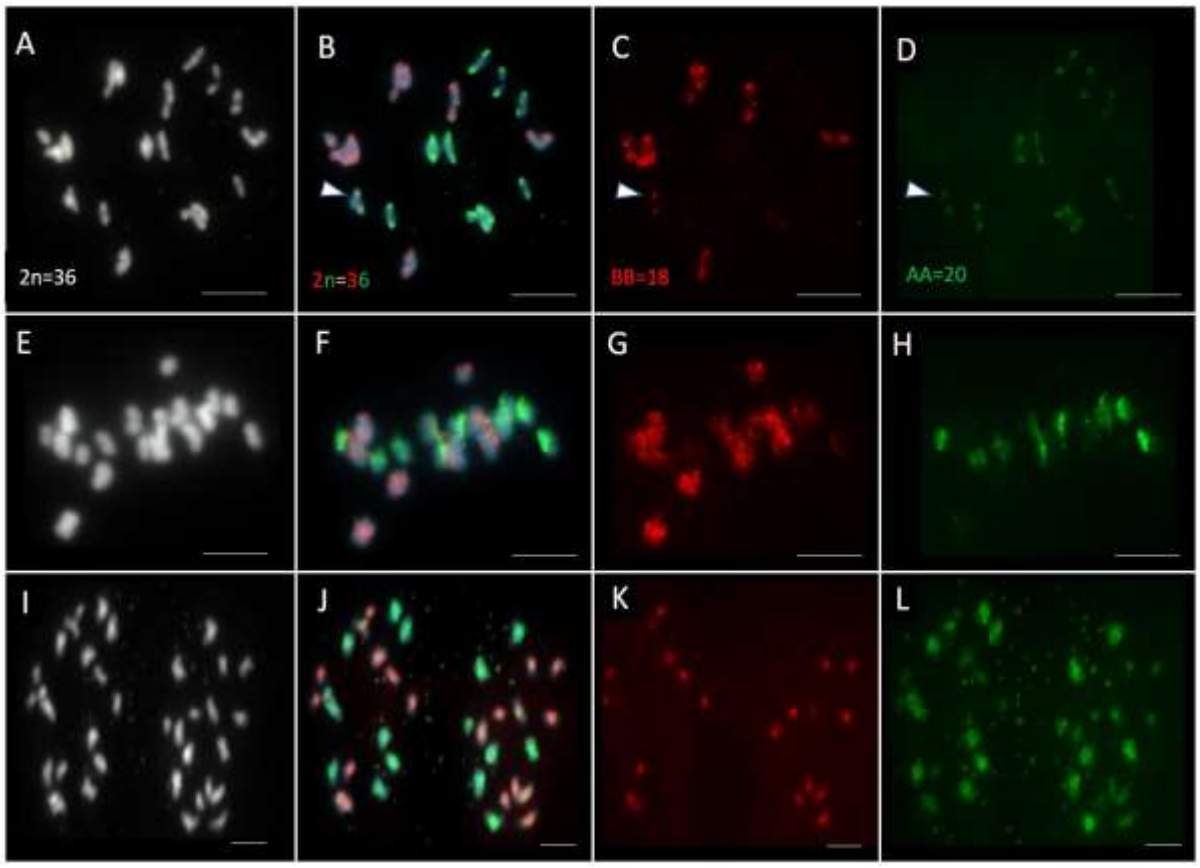


Figure 3-13 ***In Situ*** **Hybridization of meiotic metaphases of** ***B. juncea*** **(AABB) species**

(A, E,I) DAPI spread of meiotic cell at M-I and telophase-I stage (B,F,J), GISH with total genomic DNA from the ***B nigra*** **(Red fluorescence) and** ***B rapa*** **(Green fluorescence) (C,G,K)** ***B. nigra*** **biotin labelled detected red (D,H,L)** ***B. rapa*** **FITC labelled detected green under the fluorescent microscope. White arrow heads show two signals on the same chromosome. Bar =10 µm.**

3.3.5 Genome In Situ Hybridization (GISH) with Brassica carinata

For the validation of previous results obtained in *B. juncea,* mitosis and meiosis were examined in *B. carinata* with single genome GISH to study the behaviour of progenitor in the allotetraploid *Brassica carinata* (BBCC), which contains BB genome of *B. nigra* and CC genome of *B. oleracea*. Chromosomal spreads from both anther and root meristematic tissues were used for this purpose which identified *B. nigra* subgenome in this allotetraploid background. Meiotic atlas was developed for *B. carinata* to assess the behaviour of chromosomes in meiosis. Slides featuring nice images of meiotic Metaphase-I and mitotic Metaphase were subjected to GISH where Biotin-labelled *B. nigra* DNA hybridized preferentially to its corresponding BB part of the genome discriminating it from the unstained CC genome of the *B. oleracea*. The haploid genome size of this polyploid species was 17 (Figure 3-14). *B. nigra* chromosomes could be distinguished spatially from 9 pairs of *B. oleracea* chromosomes based on different wavelengths of colour under the microscope in mitotic cells where respective homologue pairs were differentiated during bivalent formation stage of Meiosis-1 as there was no concrete evidence of translocation between different genomes. Only 16 chromosomes were detected against 18 unstained chromosomes, which are from the other parental line. Lack of any extra signal from *B. nigra* showed that it was *B. rapa* which was more susceptible to introgression than the ability of the BB genome to introgress into others (Figure 3-14). Because in *B. carinata* expected number of signals were observed and no additional foci were observed for *B. nigra*.

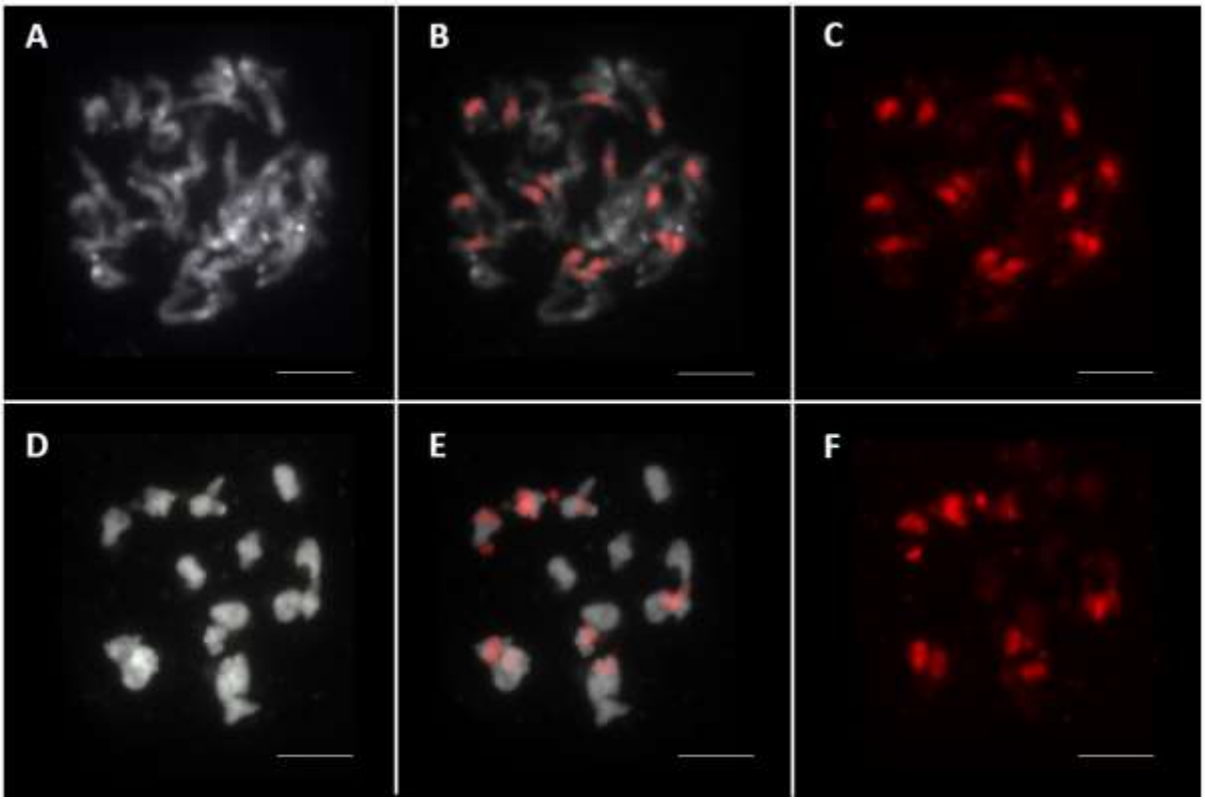


Figure 3-14 GISH in *B. carinata* showing *B. nigra* genome in red only

(A,D) Mitotic metaphase and Meiotic diakinesis showing bivalent with DAPI only, (B, E) GISH with *B. nigra* genome in red colour, (C,F) *B. nigra* genome signals. Bar =10 µm.

3.4 BAC-FISH analysis

Several BAC probes (Table 2-2) were used in this study to develop physical map of *B. nigra* but the probes did not work very well for most of the chromosomes in the genome. Only probe for chromosomes 5 gave promising results with a bright signal of hybridization as shown in (Figure 3-15).

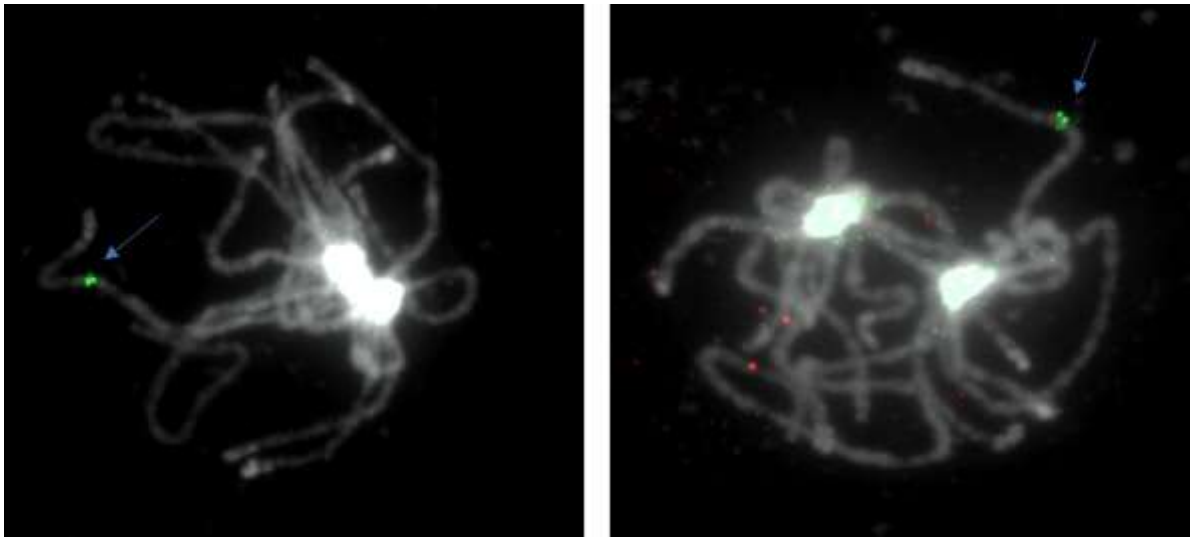


Figure 3-15 BAC-FISH images of pachytene in *B. nigra*.

Blue arrow shows hybridization of BAC on the arm clearly shown in images.

3.5 Karyotyping of *Lepidium campestre*

The chromosomes were obtained from roots as well as PMC to identify the chromosomes and integrate it with the genetic map. Using 45S & 5S rDNA two chromosomes were identified (Figure 3-16)which were different from each other which were karyotyped subsequently based on the relative length of chromosomal complement. 45S rDNA was present on either side of the centromere, and it was quite extended segments as compared to the *A. thaliana, B. nigra, B. rapa* and *B. juncea*. Whereas 5S rDNA was probed on the interstitial region of the

chromosomes on the short arm. The haploid ideotype was developed (Fig-2) which was further integrated with a genetic map for in-depth analysis of the chromosomes.

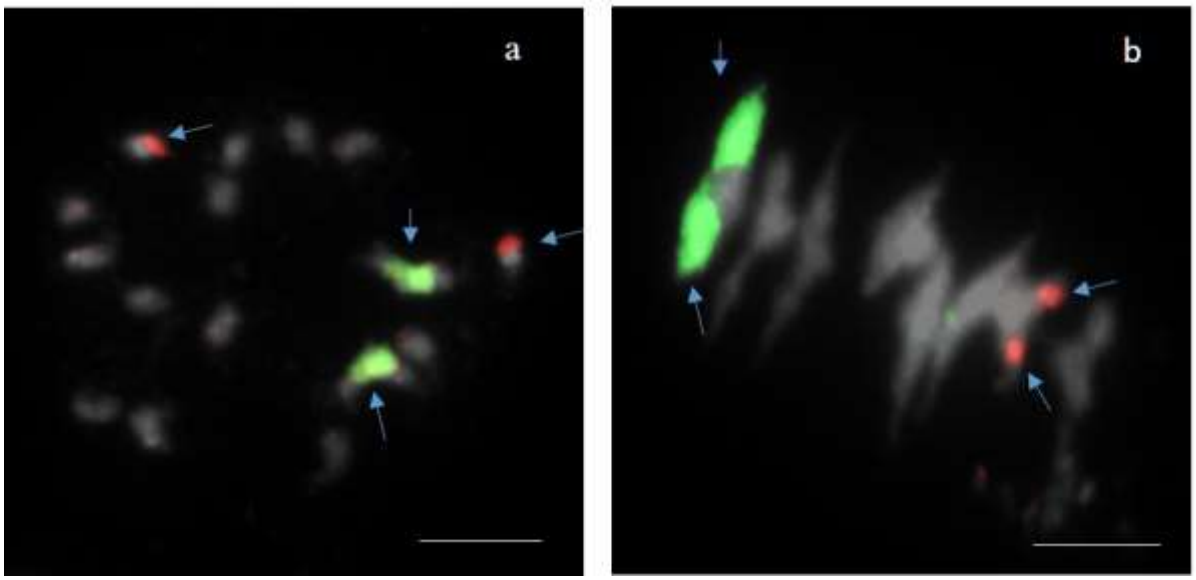


Figure 3-16 Localization of the 45S rDNA on chromosomes shown in green colour and 5S rDNA in red colour in *Lepidium* Chromosomes.

(a) Mitotic cell complement during metaphase and (b) meiotic chromosomes adopt a bivalent structure of *L. campestre*. Bar represents 10 µm

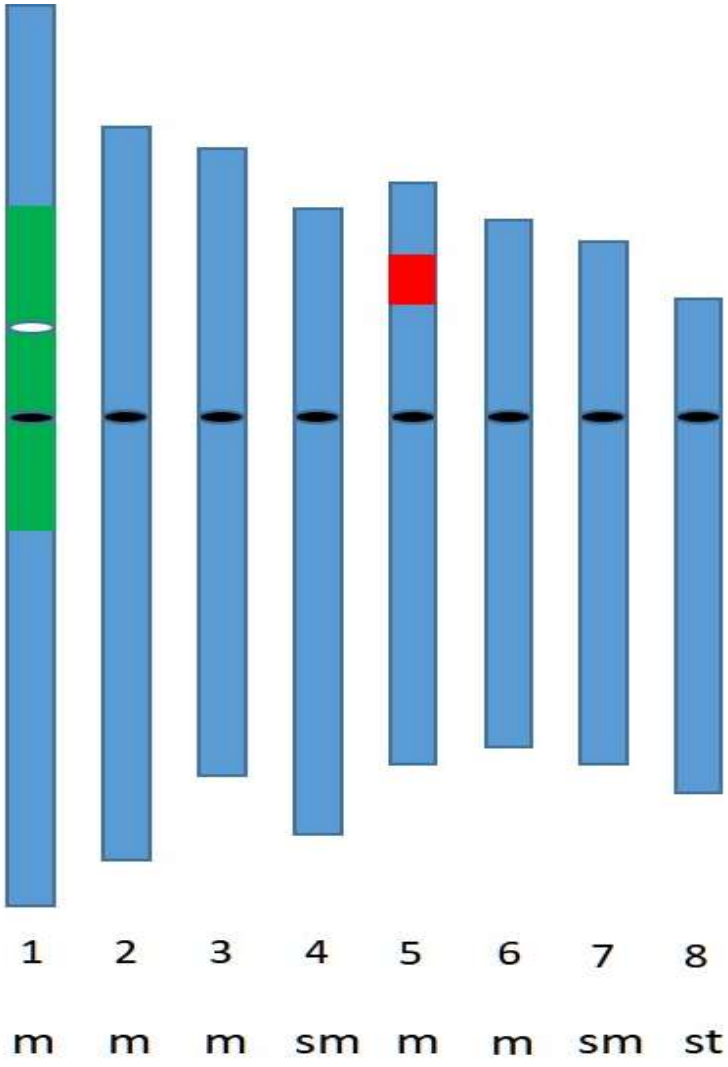


Figure 3-17 Haploid ideogram representing *L. campestre*.

The location of the 45S rDNA (green) and the 5SrDNA(red) is indicated in chromosome 1 and 5, respectively. A black line represents the centromeres, whereas a white line symbolizes the secondary constriction. Abbreviations: m: metacentric chromosome; sm: sub-metacentric chromosome; st: sub-telocentric chromosome.

3.6 Discussion

3.6.1 Karyotyping

Since no reference data set existed for *B. nigra*, an ideotype was developed as a preliminary approach based on certain repeat sequences, ribosomal DNA (essential for driving molecular functioning of a cell). Nearly seven decades ago, *B. juncea* (AABB) had replaced *B. nigra* (BB) as mustard crop due to higher yield and productivity, and thereafter very limited attention was paid to improve BB genome in brassica despite the fact it was believed to be a repository of some desirable genes against biotic and abiotic stress (Navabi *et al.,* 2013). To gain insight into the whole genome in detail, it was needed to develop its physical map for localizing gene content using cytological approaches which would help us in comprehending its genomic organization. *B. nigra* is believed to be a paleo-hexaploid degenerate that evolved from the same ancestral genome Proto-Celepine Karyotype (tPCK, N=7) which shared the roots for the model plant, *A. thaliana*. The phylogenetic tree reveals that *B. nigra* evolved separately from other diploids *B. rapa* and *B. oleracea* of U's triangle (Figure 1-2).

The chromosomes order was determined based on the relative length of chromosomes, and the classification was made as the longest at number 1 and the shortest at the lowest in order. The chromosomal length measured on Image J was supplemented with a centromeric index which helped us defining chromosomes present in the genome (Armstrong *et al.,* 1998). The identification of individual chromosomes in the complement was augmented using FISH analysis which has been a vital tool in the cytogenetics even after the discovery of DNA-DNA

hybridization nearly five decades ago (Gall and Pardue, 1969). It has been practised for multiple purposes since then to refine better understanding of the genome structure both in plants and animal kingdoms.

Roots tips were preferred to get well-spread chromosomes in the mitotic metaphase against floral buds where individual chromosomes could be separated appropriately (Osalou *et al.*, 2013). Metaphase (staged) cells were further subjected to *in situ* hybridization, where a tandem array of genomic loci 45S & 5S rDNA (Song, 1987; Volkov *et al.*, 2017)) hybridized to unveil vital information about the constituent chromosomes in the genome.

Although the majority of the repetitive sequences have an unknown function in the cellular metabolism yet 45S and 5S rDNA have a well defined and essential role in the assembly of the ribosome (the molecular machinery for protein synthesis) and thus contribute to the most of total RNA in the cell (Xue and Barna, 2012). Ribosomal DNA exists in both active and inactive form in the Nucleolar Organizing Regions (NOR) and gives rise to nucleoli during interphase, in addition to non-active rDNA. 45S rDNA is often located in the NOR region at the end of short arms while 5SrDNA could be found in interstitial regions with relatively low copy number. The numbers of the satellite were reported to be three previously in B genome (Maćkowiak and Heneen, 1999) while in the present studies it shows two chromosomes exhibiting secondary constriction with NOR region.

Several chromosomal features, like length, rDNA marks, and identical morphology were taken into account for grouping homologues together which would be used for downstream research

analysis. Total chromosomal length of the whole genome was measured on the Image-J software before the relative length of individual chromosomes (percentage of haploid complement) and their respective centromeric index (short arm/total chromosome length times 100) could be determined as defined by Armstrong *et al.,* 1998.

Karyotyping analysis revealed that chromosomes exhibited a considerable range of length between the shortest and the longest chromosome. To make it clearer I divided the range into three categories; large, medium and small. The threshold for larger chromosomes was selected as > 14 µm, and two chromosomes fell in this class which lacked any signal for 45S/5S rDNA. The second category ranged between 11 µm and 14 µm, and I noticed that the chromosomes in this class showed 45S/5S rDNA signals and were significantly important for developing an ideotype of *B. nigra*. Further, the rest two chromosomes were measured <11 µm which did not have any foci for 45S/5S rDNA. All the chromosomes were defined either sub-metacentric (chromosomes 1, 3 & 4) or acrocentric (chromosomes 2, 5, 6, 7 & 8) (Figure 3-3). It was not in line with the previous finding in *B. oleracea* by Armstrong *et al.,* (1998), which also featured metacentric and telocentric chromosomes, *B. nigra* genome does not seem to have other categories of chromosomes.

For *B. oleracea* (a diploid relative of *B. nigra*), Armstrong *et al.,* 1998 found that 45S rDNA hybridized successfully on three different chromosomes and were in line with the result obtained (Fukui *et al.,* 1998) whereas 5S rDNA gave one pair of the signal. While in the case of present studies, in *B. nigra* the signal density lies in parallel with an altered pattern where each signal has been detected on separate chromosomes and show the signature of

heterogeneity. In *B. oleracea* one chromosome had both 45S, and a 5S signal which did not appear in this genotype, therefore, could help in identifying four chromosomes in the complement whereas only three could be detected in *B. oleracea*. The pattern for 45S was not uniform as one brighter signal on the short arm could easily be discriminated from the rest of two relatively weaker signals on the short arms of other chromosomes and thus highlight copy number variation. Faint signals were detected on chromosome four and six while darker signal illustrated chromosome 3 short arm in the genome. While 5S rDNA was detected in the interstitial region just off the centromere in the shorter arm against the longer arm in *B. oleracea* (Armstrong *et al.,* 1998). Comparing the density with that of *B. rapa* disclosed that five chromosomes could be identified with the ribosomal makers where three chromosomes shared both loci for 45S & 5S rDNA and one chromosome had one signal apiece only.

The allopolyploid, *B. juncea* which is a hybrid of *B. nigra* and *B. rapa* genome had five chromosomes with 45S rDNA that was less than the aggregate of its sub-genomes (i.e., 3+4=7). The result trend line agreed with previous findings (Hasterok *et al.,* 2001) who discovered less ribosomal site in allopolyploid than its progenitors. Contrary to the present finding three chromosomes were detected with both signals colocalizing on the same chromosome in *B. juncea* against single chromosomes which share site for both markers. Further, it was found that seven chromosomes were detected with 45S and 5S rDNA in present studies against ten chromosomes showing hybridization with the 5S and 25S rDNA (Kulak *et al.,* 2002) and 16 chromosomes for 45S rDNA in *B. juncea* (Xu *et al.,* 2016). Less number of marker sites were not as useful in identifying more chromosomes in a complex background which needs more discrimination and characterization. Nevertheless, the reduction in the numbers could have

evolved as a manifest of genomic restructuring and stabilization events (Volkov *et al.*, 2007). The signal density was not even in all three species, which shows great variation of copy number and heterogeneity on diploid and allotetraploid species on certain chromosomes.

45S rDNA is transcribed by the polymerase I which yields components of both larger (60S) and smaller (40S) subunits, while 5S rDNA is transcribed in the nucleus and contribute to larger subunit (60S) only. I observed for every 632 Mbp in *B. nigra* there is only one copy of 5S rDNA present versus 1 copy after every 132 Mbp in *B. rapa* which is nearly 5 times higher than the *B. nigra* genome. Whereas in the allotetraploid the scenario was not significantly different from *B. nigra* and had one copy of 5S rDNA per 534 Mbp in *B. juncea*, which would be 232.2 Mbp had both genomes contributed equally in the genome composition and organization. When comparison was made for 45S rDNA, it revealed that one 45S rDNA is detected for every 210.67 Mbp in *B. nigra* genome while in *B. rapa* one locus is present at every 132.25 Mbp of the genetic map. Again, in allotetraploid, the density was like *B. nigra* where each locus is present every 213.60 Mbp of the genetic map against 165.86 Mbp in the expected *B. juncea*.

This cytogenetic difference hints at a biased genomic loss in the evolutionary pathway (Liu *et al.*, 2017) which could be one of the probable cause of instability and sterility in the synthetic allotetraploid production as we cannot regulate the extent and biasedness of the genomic loss. This begs concerted efforts to elucidate the genomic losses in polyploidy evolution and could potentially open new avenues for crop improvement programmes.

Phenotypic analysis revealed larger seed size as well as leaf size in *B. rapa* than diploid *B. nigra* & allotetraploid *B. juncea* which might have happened because of efficient processing at the ribosomal level because former had more copies of 5S rDNA observed under a microscope. Moreover, based on the cytological result I can conclude that *B. nigra* and *B. juncea* had a smaller copy number of 5S rDNA. The enhanced understanding of constituent genomes would help us understand the derived polyploid nature; this has been a subject of research since the last century. Since *B. rapa* and *B. nigra* have more chromosomes with cytological markers on them, it was easier to identify individual chromosomes than those in allopolyploid.

Since further work is needed to enhance our understanding of the chromosomes in *B. nigra* this gave a less clear picture of the physical mapping. Different methods of FISH were used, on separated pachytene staged cell and on the same pachytene cell with rehybridization procedure. Since the working conditions were the same for all of the probes, there might exist some problems with the probe fidelity and integrity. It would be quite interesting, given all probes work well, to determine the actual chromosomes number and it would be useful in further research. Such information is supplemented with the genetic map for a better understanding of the genome and it could guide toward the sequencing project.

3.6.2 Genomic In Situ Hybridization

Genomic *In Situ* Hybridization (GISH) is a one of the derived version of a standard Fluorescence *In situ* hybridization (FISH) where the whole nuclear genome is used (on microscopic slides) as a probe to analyze the chromosome structure of constituent sub-genomes

in a multiploid (Shwarzacher, 1989). It is based on the customary hybridization principle visualized as fluorescence and involves user intervention to analyze the interrogated genomes in the subject. Owing to the complex nature and duplicated genomes in higher plants it has widely been used for better understanding by deciphering the evolutionary roots and basis. Because of the widely accepted belief, that 70-80% of angiosperms had undergone genome duplication events before accomplishing present day stable form, this technique has applications in the study of natural evolution in polyploid plants. The phenomenon (of polyploidy) is more widespread in plant kingdom than any other kingdom, therefore more attention needs to be paid for a better understanding of the complex genomes.

I used it for dissimilar objectives in this study on different ploidy levels in Brassica species and found significantly revealing facts about the sub-genomes in the polyploid. Four of the six species in U's triangle (two diploids and two polyploid species) were used for this particular experiment and were subjected to GISH analysis. The diploid genomes (*B. nigra* and *B. rapa*) were assessed through distribution pattern of chromosomes for self-GISH to substantiate the authentication of the procedure before extending it to the allotetraploid derivatives to differentiate indistinct homologous and homoeologous marks in the evolution. Self-GISH gave me the opportunity to analyse the repetitive sequence on the chromosomes at the centromeric region which has seldom been detected toward the telomere. The signals detected on every individual chromosome paved the way for the component genome analysis in the polyploid background. Each of the chromosomes showed signals in *B. nigra* and *B. rapa* genome upon selfed-GISH analysis. The signal strength of selfed-GISH along the chromosomes was similar to that found in rice, sorghum and maize (She *et al.*, 2007). The heterochromatin is widely

distributed around the centromeric region as well as in NOR which is denser than the euchromatin. The signal intensity is sharper in the dense region than the light euchromatin containing a region in the hybridization assay.

The interstitial region which is believed to be the site of euchromatin in the genome was devoid of GISH signal in the experiment endorsing the earlier stated fact. The signal strength was also non-uniform across all the chromosomes indicating random distribution of heterochromatin and in *B. rapa,* I observed 5 pairs of chromosomes showing relatively stronger signal than the rest of 5 pairs like observed in *Alstroemeria aurea*, *Aegilops ventrocosa*, *A. thaliana* and two Medicago species (Kuipers *et al.,* 1997; Bardsley *et al.,* 1999; Zoller *et al.,* 2001 and Falistocco *et al.,* 2002). The repetitive elements in the genome vary from medium to highly repetitive sequences that constitute a significant portion of genomes in higher order complex plants (Chaowen She *et al.,* 2007). The signal density in *B. rapa* was more intense than those found in *B. nigra* showing a scattered pattern along the entire length of chromosomes.

Lineage-specific centromeric probes were also used to validate phylogenetic distance which showed the highly conserved region in *B. rapa* and did not hybridize at *B. nigra* chromosomes. Although the diploid brassicas are believed to be the predecessor of the same ancient genome that underwent various structural modification before ending into stable diploid forms. The *B. nigra* lineage diverged around 3.7Mya compared to the evolution of *B. rapa* and *B. oleracea* (Figure 1-2) and conservation footprints were different at centromeric regions which indicates the different genomic structure in these closely related diploids in the U's triangle. *B. nigra* gave a negative result when hybridized with the centromeric probe, but *B. rapa* showed the

promising result with eight signals out of ten chromosomes while two pairs remained unstained. The slides were subjected to re-hybridization with 45S rDNA to understand the distinguishing chromosomes, and their number was very normal, enough to confirm the *B. rapa* genome. Rehybridization was also done for GISH with *B. rapa* genome, and interestingly all the chromosomes stained with centromere probes showed promising results including the two pairs which were unstained with centromere probe.

The model organism, *A. thaliana* genome evolved as a result of three genome multiplication events (gamma, beta & alpha events) which bifurcated along the way following WGD to give rise to two Brassicaceae lineages, one *B. nigra* lineage, and the other *B. rapa*/*B. oleracea* lineage. The two lineages had significant affinity despite the evolutionary gap, toward each other to hybridize with each other forming triangle, known as U's triangle after the discovery of this relationship by the Japenese scientist in 1935. The allotetraploid species along with diploid species in U's triangle present an interesting scenario for deciphering the evolution dilemma. *B. nigra* evolved 6.2Mya (Navabi *et al.,* 2013) against 3.7Mya evolution of *B. rapa*/*B. oleracea* lineage (Lysak *et al.,* 2007) and had different genomic and phenotypic characteristics. It is noteworthy that both *B. nigra* and *B. rapa* genome did not show any evidence of cross-species hybridization during selfed GISH limiting any introgression potential.

For exploitation of the complex genomes, a robust tool of cytogenetics, GISH was adopted to reveal facts about dissimilar genomes in hybrids and many other composite backgrounds where rounds of duplication and multiplication might have happened in ancient times (reviewed in Silva and Souza 2012). It was first used in 1989 to study chromosomal behaviour of *Hordeum*

chilense (2N = 2X = 14) and *Secale africanum* (2N = 2X= 14). As chromosomes in *B. juncea* were difficult to distinguish between the subgenomes, GISH was developed to identify sub genomes using large-scale genomic algorithms based on different colours.

GISH had successfully been used in hybrid crops where more than one genome is present in a complex combination to separate one genome from the other (reviewed in Silva and Souza, 2012). As both subgenomes belong to different lineages in *B. juncea*, they were distant enough from each other, and no blocking DNA was needed as per requirement in a standard GISH for the sake of specification and discrimination (Tang *et al.*, 2011). Being a powerful procedure in the toolbox of evolutionary biology, it effectively differentiated *B. rapa* chromosomes from *B. nigra* genome when hybridized simultaneously, and revealed more than expected signals for *B. nigra*, i.e. more than the expected 16 signals in mitotic cells against the normally expected 20 signals for *B. rapa* in the complement (Figure 3-12). To verify the perspective of the introgression potential of *B. nigra* genome, GISH was also analysed in meiotic cells which yielded the same result for an increased number of signals for *B. nigra* and normal for *B. rapa* (Figure 3-13). This urges a question, either it is *B. nigra* which has the potential for introgression or *B. rapa* is more susceptible for any cross-species recombination.

To address this query, GISH was performed on the *B. nigra* derivative *B. carinata* (which contains *B. oleracea* as well) to see the behaviour of *B. nigra* in the mitotic and meiotic cell. The analysis revealed that in this allotetraploid *B. nigra* gave only normal 16 signals in the complement (Figure 3-14) and even during the meiosis. Based on the above mentioned cytological results, I can say that *B. rapa* is more prone to the addition/introgression than the

B. nigra's potential to introgress into another genome. In that case, it should also show more signals in *B. carinata* like it did in *B. juncea*. The *B. juncea* presents a useful crop species which have the natural signature of homoeologous recombination and thus pose great potential for improvement.

Results for *Lepidium campestre*

Linkage groups of *L. compestre* were coordinated with the haploid ideotype, and it revealed that the linkage group had collinearity with the chromosome bearing 45S rDNA. Furthermore, it was revealed that this chromosome had a syntenic region with the chromosome 2 of the *A. thaliana*. The bright signal of 45S rDNA present on chromosome could be correlated with the larger gap on linkage group 7 which is devoid of any markers on it. Chromosome 1 in the ideotype was quite large chromosome as compared to the rest of the complement and could be seen in a meiotic configuration forming a bivalent. The linkage groups were assigned chromosome number based on morphological features as well as the genome was also aligned against *A. thaliana* genome for the syntenic association.

3.6.3 Summary

Due to extensive cultivation of polyploid in Brassica species, *B. nigra* was sidelined for the last seven-decades, and limited literature is available to exploit its genetic potential. Based on such grounds, present studies were designed to develop an ideotype of the species which could be extended further for physical mapping studies. Classical *in situ* hybridization technique was used to determine chromosomes order in the complement and compare the ribosomal loci with other diploid genome and tetraploid genome in U's triangle. Further, an extended form of FISH was developed during the studies for analyzing the behaviour of the diploids in allotetraploid *B. juncea* which helped me to identify individual genome in addition to exciting homoeologous evidence which could potentially be used in crop improvement programs after further research. The subgenomes preferentially paired with their homologue discriminating non-identical chromosomes hinting for some regulatory system operating in the allopolyploid which favours only homologue pairing somewhat like happening in wheat. In *B. napus* (AACC) *PrBn* on genome CC is held responsible for the strict pairing among the homologues and similar control might be working in the *B. juncea* (AABB) where either AA or BB is control centre of such regulation, still remains a big question mark in evolutionary biology.

4 . Meiosis in diploid and allotetraploid species

4.1 Meiosis, a specialized cellular division in sexually reproducing organisms

The characteristic feature of meiosis (a specialized attribute is the reduction of a cell) includes pairing and genetic recombination which not only distinguishes it from the standard equational division, mitosis but brings novelty in subsequent generations. The hallmark is achieved in multiple steps starting from the nicks to the recombinant strands of DNA. The outstanding effect of recombination begins with the formation of DSBs which mature by the end of prophase-I to align in bivalents exposing single kinetochore of the pair to the opposing poles for the division purposes in contrast to biorientation of kinetochore in the equational division, i.e., mitosis and Meiosis-II. The chromosomes align along their length, and the juxtaposition plays a critical role in halving the ploidy at the end of meiosis-I (Zickler and Kleckner, 1999).

Critically speaking, there exist three main steps in meiosis which are of paramount importance; (1) Homology search, (2) Synaptonemal Complex (SC) installation and (3) Crossing Over or Recombination which exchanges the genetic material between the homologous chromosomes.

The premature chromosomes interaction during leptotene/zygotene mediated by the telomere association results in homology encounters which serves as a homology testing mechanism (Bass *et al.,* 2000; Bolcun-Filas and Schementi, 2012). It also resolves the interlocks between the chromosomes to ease the segregation and the reduction at the end. Nearly 50% of the homologue pairs in normal pachytene stage would have had developed association before the onset of prophase-I through telomere clustering. The extended interstitial pairing before

prophase-I ensues due to the non-catalytic SPO11 which anchors telomere to nuclear envelope through SUN1 protein for the transient homologue association (Boateng *et al.,* 2013).

The SUN1 protein plays a significant role tethering the chromosomes to the nuclear envelope and the KASH protein in the cytoplasm which oscillates the nucleus to facilitate chromosomal alignment for the homologue juxtaposition and synapsis subsequently (Scherthan *et al.,* 1996; Hiraoka and Dernburg, 2009).

4.1.1 Bouquet formation

The chromosomes tethering, homology search and recombination establish the core concepts of the meiosis which source variation through the recombination of parental alleles (reviewed in Da Ines *et al.,* 2014). The chromosomes undergo rapid and fast movement in early prophase-I before global pairing is achieved in pachytene which is characterised by relatively slower movement of chromosomes (Ronceret and Pawlowski, 2010). The telomere clustering on NE during zygotene is referred to as telomere bouquet and this brings the chromosomes close-by for the potential pairing through its rapid oscillation. Chromosomal reorganization and movement during early prophase-I translocate the nucleolus from centre to the peripheral zone with the passage of time & meiotic progression. This leads to the clustering of telomeres; a characteristic bouquet configuration in many species including maize, wheat & rye from plant kingdom (Harper *et al.,* 2004). In an extensively studied model plant *A. thaliana*, a canonical bouquet is absent and telomeres cluster around the nucleolus in a loose orientation through to

the leptotene and associate transiently to the NE in late zygotene (Armstrong *et al.,* 2001; Roberts *et al.,* 2009).

Axis associated protein HOP1 in *S. cerevisiae* plays a vital role during DSBs formation and its repair as it facilitates inter-homologue association than the sister chromatids and its ortholog ASY1 is present in plants for the same objective (Armstrong *et al.,* 2002; reviewed in de Massy, 2013). The characteristic HORMA domain of the HOP1 binds to the chromatin involved in DSBs and serves as a moderator for engaging other proteins for the subsequent processing (Woltering *et al.,* 2000). While in plants, ASY1 protein contains HORMA domain with no effect on DSBs formation but have a profound effect in its processing (reviewed in de Massy, 2013).

Biochemical nature of the class-I COs reveals that several proteins (ZMM proteins) are involved in maturing the interference dependent COs. The ortholog for Zip1 in *A. thaliana* is represented by ZYP1 which plays a vital role in the polymerization of the synaptonemal complex (SC) and is the central element of its tripartite structure. Its mutated form had a lowered chiasma frequency indicating its crucial part in promoting CO. In barley the situation is different and had reduced chiasma frequency, but the CO fidelity was maintained intact (Barakate *et al.* 2014).

4.1.2 Meiosis in polyploids

Since the plant kingdom includes a vast majority of polyploids which are divided into autopolyploid and allopolyploids; the appropriate pairing seems even more challenging and

inspiring in the presence of multiple choices for every chromosome during pairing (Tiang *et al.*, 2012). Studies in wheat and *B. napus*, an allohexaploid and an allotetraploid, have identified a genetic control system exists in a locus which limits pairing between non-homologous chromosomes and facilitates discrimination based on chromatin conformation between the homologues and non-homologues (Moore and Shaw, 2009; Jenczewski *et al.*, 2003) The locus known as *Ph1* (in wheat) consists of a cluster of Cdk (Cyclin-dependent kinases) genes which regulate the meiosis progression and parameters homoeologous pairing, notably in allohexaploid wheat (Griffiths *et al.*, 2006). Recent studies (Rey *et al.*, 2017) reveal that ZIP4 within *Ph1* locus on chromosome 5 in wheat suppresses homoeologous recombination and promotes CO formation between the homologues. Intriguingly, chromatin state is also a crucial regulatory measure of homology bias as H1 phosphorylation in histone protein has a profound effect on pairing dynamics and has been exclusively studied using application of okadaic acid which exerts a similar effect for enhancing recombination (Knight *et al.*, 2010). It is a specific and potent inhibitor of protein phosphatases which interact with C-termini of the proteins to block the dephosphorylation and thereby, enhances phosphorylation of proteins (Cohen *et al.*, 1990).

4.2 Okadaic Acid

Okadaic acid was originated isolated from a marine sponge *Halichondria okadaii*, hence the name refers to the species and is known as a potent phosphatase inhibitor, composed of polyether fatty acid (Cohen *et al.*, 1990). Kinases and phosphatases are phosphotransferases that catalyse addition and removal of a phosphate group to/from protein substrate. Kinases

transfer a phosphate group from ATP to the substrate while phosphatases transfer a phosphate group to the water molecule from the substrate (Cheng, *et al.*, 2011). Three major types of phosphatases that play a crucial role in plants are PP1, PP2A and PP2C which dephosphorylate serine and threonine residue on most of the occasions in polypeptide chain in a plant cell. Okadaic acid is a potent and specific inhibitor which could safeguard proteins from the denaturation and degradation at different concentrations. PP2A could be inhibited by a smaller concentration of Okadaic acid while PP1 would only be withstand a higher concentration (Cohen *et al.*, 1990). Therefore, 1 µM concentration was used in the experiment.

Treatment of plantlets with Okadaic acid showed increased interaction as were observed in wheat-rye hybrids which mimicked *Ph1* locus inactivation (Knight *et al.*, 2010). It is an essential component which keeps the genome integrity in wheat during stable meiosis by suppressing cross-species interactions during homology search and pairing. However, at the same time, it also barred new allelic combination to form for the transfer of a gene from one genome to the other in allopolyploid species (Crismani *et al.*, 2013). The locus, *Ph1*, is believed to keep control over proper de-condensation of heterochromatin and phosphorylation at histone H1 which helps in distinguishing different genomes for pairing (Prieto *et al.*, 2005; Greer *et al.*, 2012).

4.2.1 Induced homoeologous association

Even in the presence of two different genomes in established *B. juncea*, chromosomes find their homologues and undergo preferential pairing, therefore, provide limited opportunity to evolve

novel allelic combination. Allopolyploid like *B. juncea* provides an equal opportunity for genomes to recombine with another genome in the nucleus to enhance their genetic base which could outperform the wildtypes. Limited traces of cross-genome translocation were detected in established *B. juncea* where two signals of *B. nigra* were detected on *B. rapa* chromosomes indicating compatibility and complementarity among the chromosomes despite the fact that pairing strictly follows homology determined association and alignment between the chromosomes. So, exploiting that opportunity for enhancing interaction between the different genomes, *B. juncea* plants were treated with Okadaic acid at different concentrations which inhibited phosphatases and enhanced homoeologous interaction between *B. nigra* and *B. rapa*. Different conditions were tested to see chromosomal conformation by Okadaic acid after treating plant for a different time, and it was found that 2 hours treatment of 1 µM Okadaic acid yields homoeologous association, which was corroborated by GISH analysis as well. Plant branches were submerged in 1% Okadaic acid for two hours before keeping it in water for another 38-40 hours. To find out the exact stage, the time course was also done with the wild-type to confirm the timing for M-I, and it was found that Edu could be detected in replicated chromosomes at an M-I stage after 42 hours.

4.2.2 Results

To evaluate the chromosomal behaviour in meiosis, a series of different approaches including chromosomal spreading, *In Situ* Hybridization and immunolocalization were employed for analysing the meiotic hallmarks in *B. nigra*, *B. rapa* and *B. juncea*. The key markers such as chromosome bouquet formation, synapsis and bivalent stages during meiosis were studied by the telomeric FISH, immunochemistry of axis protein and chromosome spreading respectively. The early events featuring thin threads or filament of chromatin in leptotene are analysed cytologically using FISH with the telomeric probe (Figure 4-1).

The meiotic atlases were developed for both diploid and allotetraploid species before selecting appropriate stages to proceed with telomeric FISH. The leptotene stage marks the entry point of meiosis for a cell and can be distinguished from the mitotic cell with the characteristic configuration of chromatids. The terminals of chromosomes were investigated in all three brassica species with telomere probes and the signals observed were categorised into two distinct classes, i.e. double and quadruple signals. The double foci were light, tiny marks (Figure 4-1) and were formed by two chromatids and could be detected under the microscope whereas the quadruple signals were relatively more prominent than the medium sized foci of the double and were formed of four chromatids of a homologue pair and could easily be distinguished from the doubles. The double signals (asterisk marked in figure 4-1) were observed extensively at the beginning of prophase-I when individual chromosomes were scattered well apart in the nucleus. Later in meiosis, leptotene stage chromosomes start pairing with their homologue, and the signal strength gets intensified to quadruple (arrows shown in

figure 4-1) range foci which were easily detected in zygotene stage. The telomeres could be seen as clustered around nucleolus and are bigger than those of leptotene signals. The condensation of chromosomes caused the signals to intensify further into prominent marks which seem to be formed of four chromatids together at full synapsis stage and were easily distinguished from un synapsed chromosomes.

Based on telomere FISH of PMCs (Figure 4-2) a downward trend existed in prophase-I where high numbers were observed in earlier prophase-I than the later stages when chromosomes found partners and undergo synapsis. At the beginning of leptotene, double signals from the sister chromatids were significantly high before the onset of synapsis which converged to quadruples where chromosomes were in close association (Figure 4-1). During early prophase double signals dominated and were distributed all around the nucleus. In diploids *B. nigra* and *B. rapa,* an average of 32 and 37 signals were observed respectively in leptotene, and most of the foci fell in the first category, the doubles as compared to few quadruple signals. The number of foci during leptotene in *B. nigra* varied from 31-36 while in *B. rapa* it ranged from 36-40 (Table 4-1).

Whereas in allopolyploid *B. juncea* an average of 54 foci were detected in leptotene and the proportion of the double to quadruples was relatively lower than its progenitor diploids. The number of foci ranged from 48-60 where signals were detected scattering across the nucleus. The total number of the double signals dominated in leptotene and were reduced in the zygotene stage where synapsis began to develop between the homologues. In the zygotene stage, the signals trend skewed toward quadruples where telomere clustered in a meiosis like

configuration around nucleolus or toward one side of the nucleus which might have helped in homology search. In *B. nigra* and *B. rapa* telomere numbers were reduced to 27 and 22 in zygotene stage where the mixture of double and quadruple signals were observed, and the double signals were more dominant than the quadruples because most of the chromosomes have started pairing with counterparts. The number of foci ranged from 24 to 28 and 20 to 24 in *B. nigra* and *B. rapa* respectively during the zygotene stage. The telomere could be seen polarized and in a cluster like conformation against the classical bouquet form of telomere which facilitates homologue pairing in rice and other organisms (Zhang *et al.*, 2017). In the allotetraploid *B. juncea*, the number was reduced to an average of 46 during zygotene where quadruple dominated over the double signals. The signal frequency was between 40 and 48 where signals were detected for the double and quadruple nature. The clustering was present in allotetraploid as well as its progenitors, but here quadruple frequency and ratio were higher than those found in the diploid parents. The chromosomes forms pairs which will undergo recombination in between them for the exchange of genetic material in subsequent stages. The SC starts polymerization during zygotene and was validated with immuno assay; this is explained in the next section. The telomere behaviour in pachytene revealed that the number of foci were reduced further to an average of 16 and 20 in *B. nigra* and *B. rapa* respectively while their ranges were 14 to 17 in *B. nigra* and 20 to 22 in *B. rapa* accordingly. Most of the foci seemed to be formed of four chromatids while some detected as double which are either overlapping on each other or very close to each other. More quadruples were observed in *B. juncea* during the pachytene stage and the average foci detected were 36 with a range of 27 signals to 39. A detailed analysis of telomere foci has been summarised in Table 4-1.

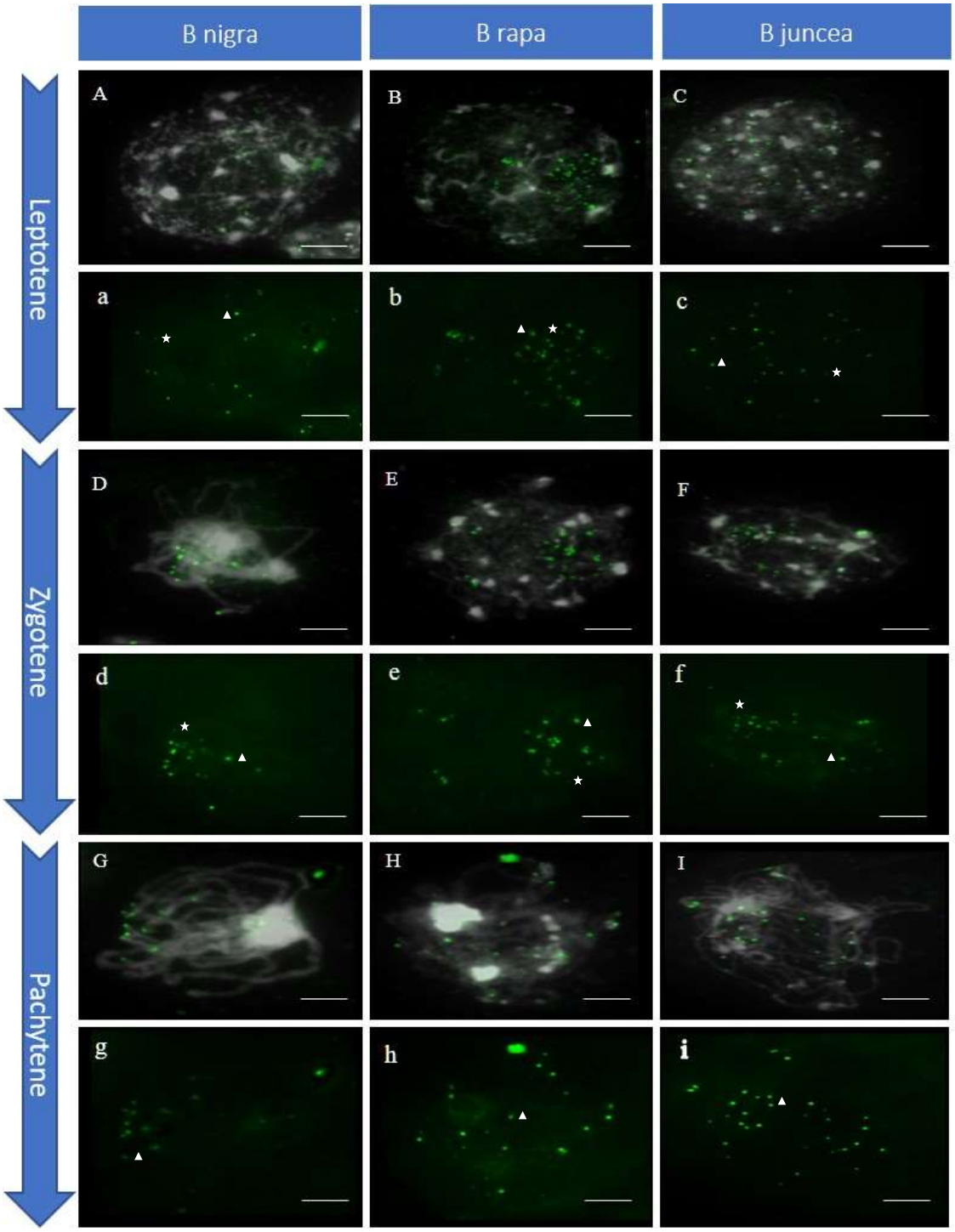


Figure 4-1 FISH with telomeric probes on *B. nigra*, *B. rapa*, and *B. juncea* under a fluorescence microscope. An asterisk shows doubles while an arrow head shows quadruples.

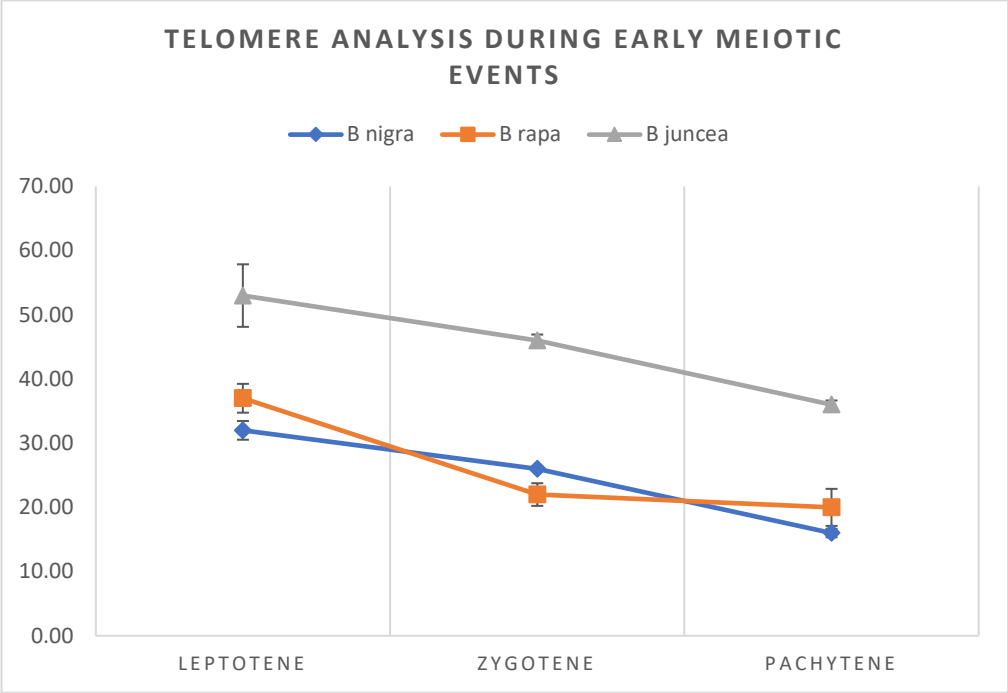


Figure 4-2 Telomere frequency in early events of meiosis in *B. nigra*, *B. rapa* and *B. juncea* under a fluorescence microscope. (n=30 for all the species)

Table 4-1 Analysis of telomere foci based on Fluorescence *In situ* hybridization of Brassica species.

	B. nigra	*B. rapa*	*B. juncea*
Leptotene	32 ± 1.46	37 ± 2.25	54 ± 4.87
St Dev	6.85	5.50	9.74
Range	29-32	36-40	48-60
Zygotene	27 ± 0.33	22 ± 1.76	46 ± 0.92
St Dev	2.53	3.06	3.46
Range	24-28	20-24	40-48
Pachytene	16 ± 0.66	20 ± 2.89	36 ± 0.66
St Dev	5.04	5.77	3.44
Range	14-17	20-22	27-39

4.2.3 Immunolocalization – Synapsis initiation

When cells embark into the zygotene stage of meiosis, the chromosomes tend to juxtapose along their homologue to initiate pairing and subsequently synapsis is achieved in the pachytene stage. Molecular analysis reveals that certain proteins play their conserved roles across species from yeast to human; one of these proteins feature Horma domain which has a vital role in meiotic recombination process (Muniyappa *et al.,* 2014). These were discovered in budding yeast for the first time where three proteins shared a common domain for interacting with the chromatin to execute a profound effect on meiotic recombination, and it also recruits other related proteins to accomplish landmark events of recombination among the homologues (Aravind and Koonin, 1998).

The domain was named Horma after its discovery in three proteins, HOP1, REV7 and MAD1. The homologue in plants was discovered in the form of asynaptic gene, *ASY1*, which possesses domain similar to that of *HOP1* and is a chromosome axis associated protein that forms the lateral elements of the SC (Armstrong *et al.,* 2002). Two lateral elements are linked through transverse ZYP1 during pachytene in prophase-I when chromosomes are closely juxtaposed (Higgin *et al.,* 2005).

The axis protein, ASY1 was observed as discrete foci at the beginning of the meiosis in pre-leptotene stage and develop into a linear signal in early zygotene. The signals persisted until full synapsis could occur between chromosomes and ZYP1 gets more prominent suppressing ASY1 signals which were faded by that stage under a microscope (Armstrong *et al.,* 2002;

Sanchez-Moran *et al.*, 2007). ASY1 is depleted by the action of PCH2 protein which localizes to the axis remodelling sites. In its absence, homologues compromise polymerization of the SC complex and cause meiotic abnormalities (Lambing *et al.*, 2015). An antagonistic trend was observed for polymerization between ASY1 and ZYP1, but this ASY1 does not go depleted if PCH2 is mutated. ASY1 diminishes in pachytene stage while ZYP1 starts to polymerize in zygotene and appeared as discrete foci indicating the origin of the polymerization which transformed into a linear axis signal in pachytene endorsing the complete synapsis. ASY1 intensity was brighter in the absence of synapsis among the homologues or had interlocking in between until ZYP1 is loaded and could be detected as linear axis upon complete synapsis and ASY1 is swapped completely (Figure 4-3).

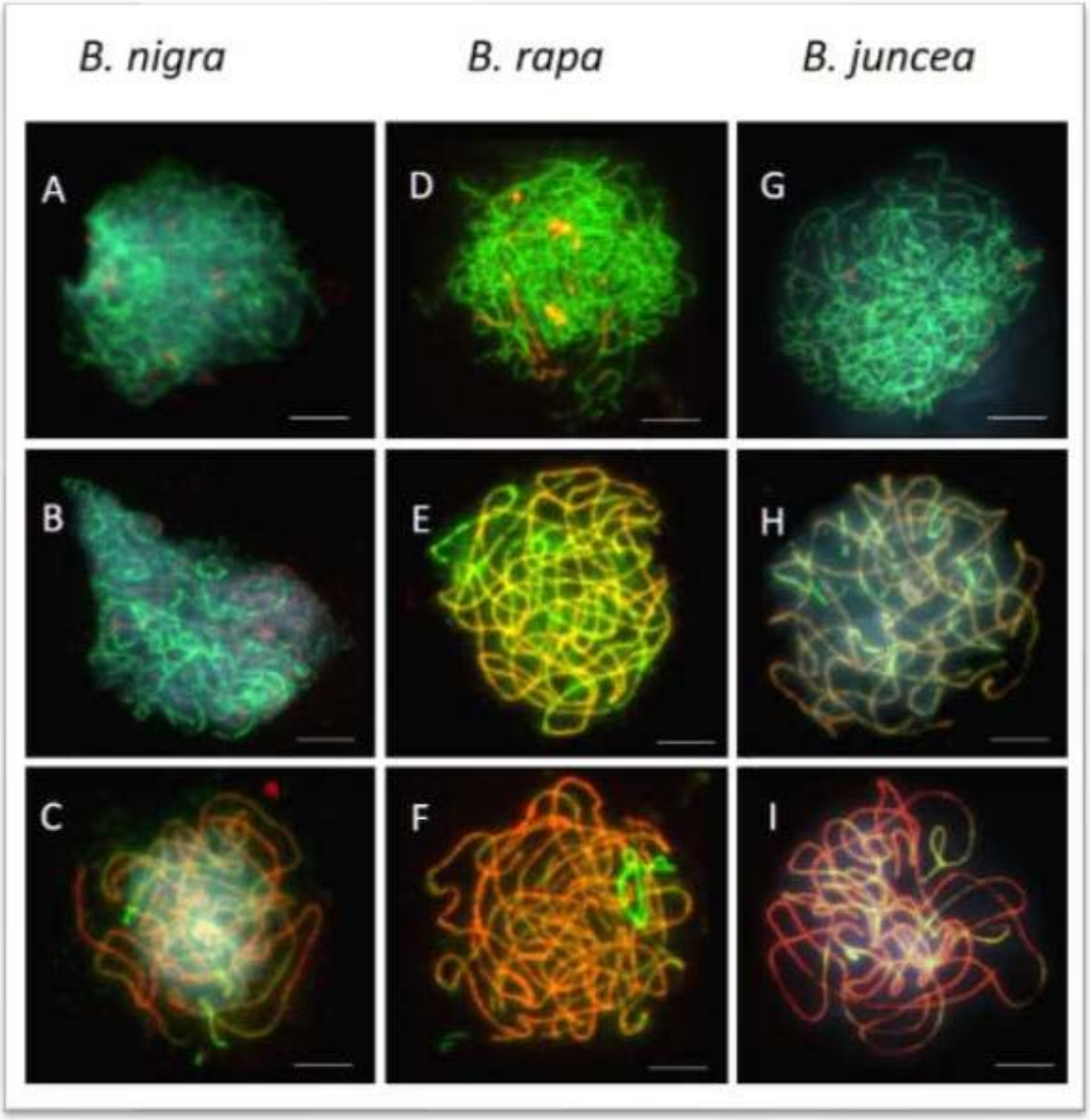


Figure 4-3 Immunolocalization of *B. nigra*, *B. rapa*, *B. juncea* PMCs

meiotic chromosome axis protein ASY1 (green) shows lateral elements of the synaptonemal complex while transverse filament protein ZYP1 is shown in (red). Early Zygotene (A, D, G), Mid-Zygotene (B, E, H) Pachytene (C, F, I). Bar =10 µm (n=20 each for the species)

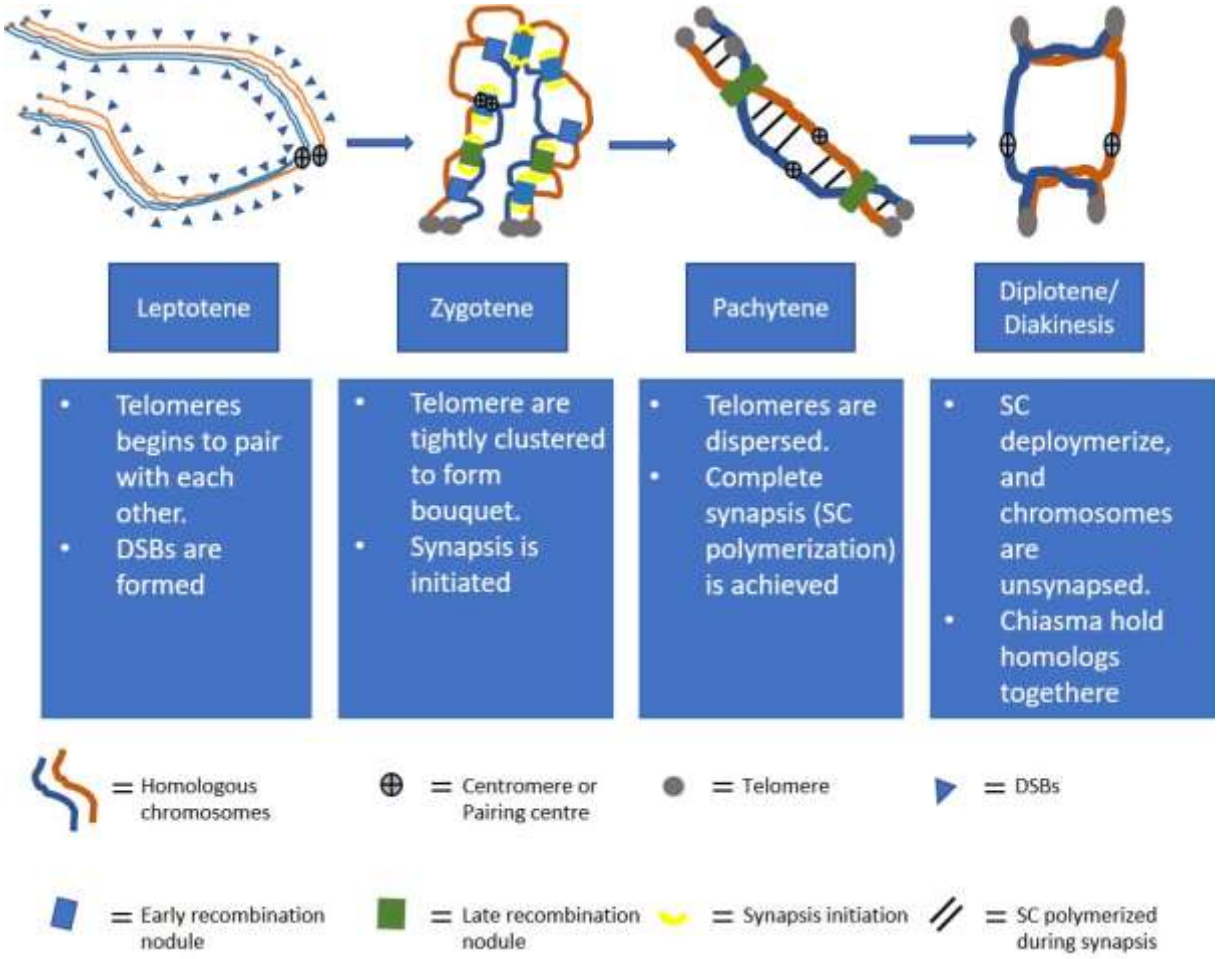


Figure 4-4 Schematic representation of early events in prophase-I,

Chromatin undergoes the catalytic action of SPO11 during leptotene to yield DSBs which are then repaired by the series of steps to make COs, a characteristic feature of meiosis.

Immunolocalization of the meiocytes in both diploids (*B. nigra* and *B. rapa*) and allotetraploid (*B. juncea*) with ASY1 and ZYP1 revealed polymerization of the proteins in the early phase of meiosis which implied the existence of structures necessary for the synapsis between the chromosomes. FITC labelled ASY1 antibody gave green signals under a fluorescent

microscope at the start of zygotene and characterized as an entangled ball of thin threads which transformed into a less complicated conformation in pachytene when chromosomes are relatively condensed and paired with homologues. ZYP1 replaced ASY1 in red, which started as foci in the early zygotene for the localized pairing among the homologues. In late zygotene, there is partial synapsis where cells were visualized as green and red filaments intermingled to indicate complete pairing among the homologues in the pachytene stage of full synapsis (Figure 4-3). Since SC is tripartite complex made up of two lateral and one central element; length of ZYP1 during pachytene was measured using the Image-J rich toolbox to analyse SC in PMCs. The results showed a considerable difference between diploid and allotetraploid brassicas which tests the trend line of genomic sizes of the three-species studied (Figure 4-5).

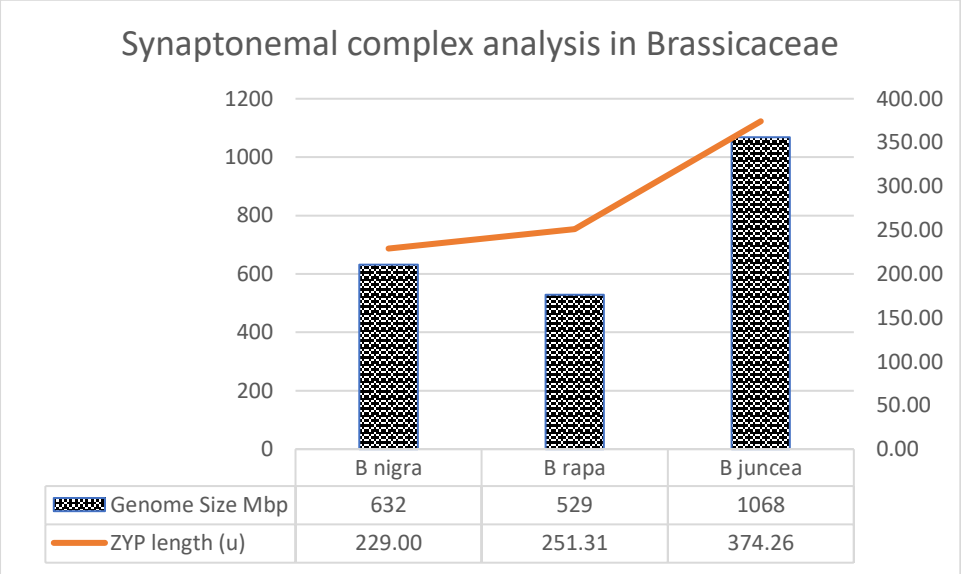


Figure 4-5 Synaptonemal complex analysis in Brassica diploid (*B. nigra* and *B. rapa*) and allotetraploid (*B. juncea*)as measured on the Image-J is plotted against their respective genome sizes. (n=20)

4.2.4 Slide preparation for the analysis of class-I CO

Medium flower buds were selected for the analysis of the mid meiotic events of pairing and the crossing over between the homologue chromosomes. The young inflorescence were dissected on a moist filter paper under a binocular microscope to examine the late pachytene to diplotene meiotic stages under phase contrast stereo microscope. As meiosis progresses, Synaptonemal Complex (SC) disintegrates leaving the homologue chromosomes attached at the site of chiasma which can be visualized during immunolocalization analysis as MLH1 foci. The slide was prepared as described in section 4.1.2.

4.2.4.1 Genetic recombination viewed as Cross Over (CO-I)

The pairing of homologous chromosomes is followed by the genetic recombination which is characterized by the physical exchange of DNA segments between non-sister chromatids. The swapping of the genetic material results in Cross Overs (COs) and Non-Cross Overs (NCOs) in the ZMM pathway which involves polymerization of ZYP1 protein. The physical connection between the chromatids is marked by the accumulation of MLH1 protein and evaluated as discrete foci under a microscope. Co-immunolocalization of ZYP1 spreads with MLH1 antibodies were used to identify the late recombination nodule (/CO) frequency in the nuclei of Brassica species. MLH1 is loaded before double Holliday Junction (dHJ) is resolved, and the CO site is visualized as a bright mark in cytological preparations.

The number of MLH1 counts was higher in polyploid species than the diploids and are well in accordance with their SC filament length. (Figure 4-8). The frequency of MLH1 for *B. nigra*,

B. rapa and *B. juncea* was observed in late pachytene stage when the SC filaments were nicely spread and separated (Figure 4-6). The MLH1 foci were spaced away from each other along the SC filament showing a strong interference phenomenon governing in both diploid and allotetraploid species. In diploids, the signals were observed in the interstitial region, whereas in the allotetraploid it exhibits a different pattern of the signals which were observed at the end of SC in the telomeric region. The frequency is listed in Table 4-2 which details the analysis between SC and MLH1 foci. To validate the telomeric site for MLH1 foci in *B. juncea* immuno cum FISH was performed to determine the telomeres and MLH1 foci on the same cell. The signals detected confirmed the co-localization of both MLH1 and telomere foci at the end of SC filament which endorses that the site for cross over in the telomeric/sub-telomeric region in the allopolyploid (Figure 4-7).

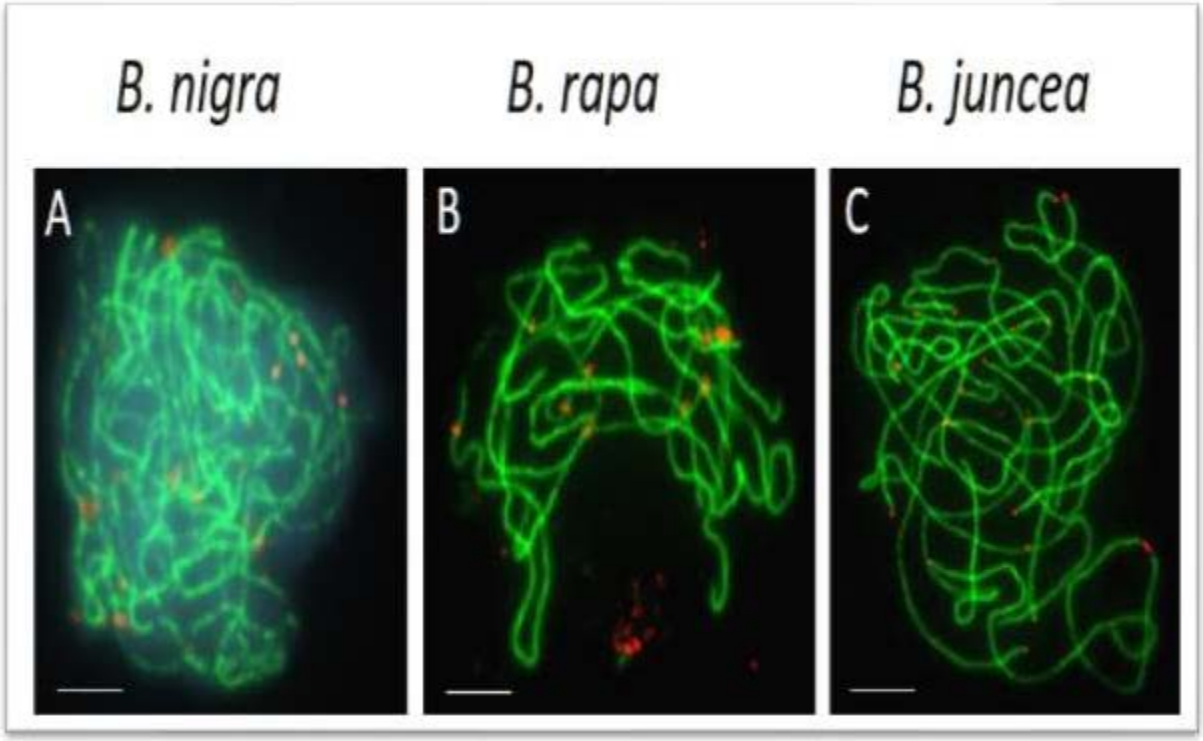


Figure 4-6 Immunolocalization of *B. nigra*, *B. rapa*, *B. juncea* PMCs

Meiosis-specific chromosomal axis protein ZYP1 (green) for SC polymerization and MLH1 (red) to mark COs in *B. nigra* (A), *B. rapa* (B) and *B. juncea* (C). The foci can be seen in the interstitial region in diploids but are present at the end in allotetraploid. Bar =10 μm

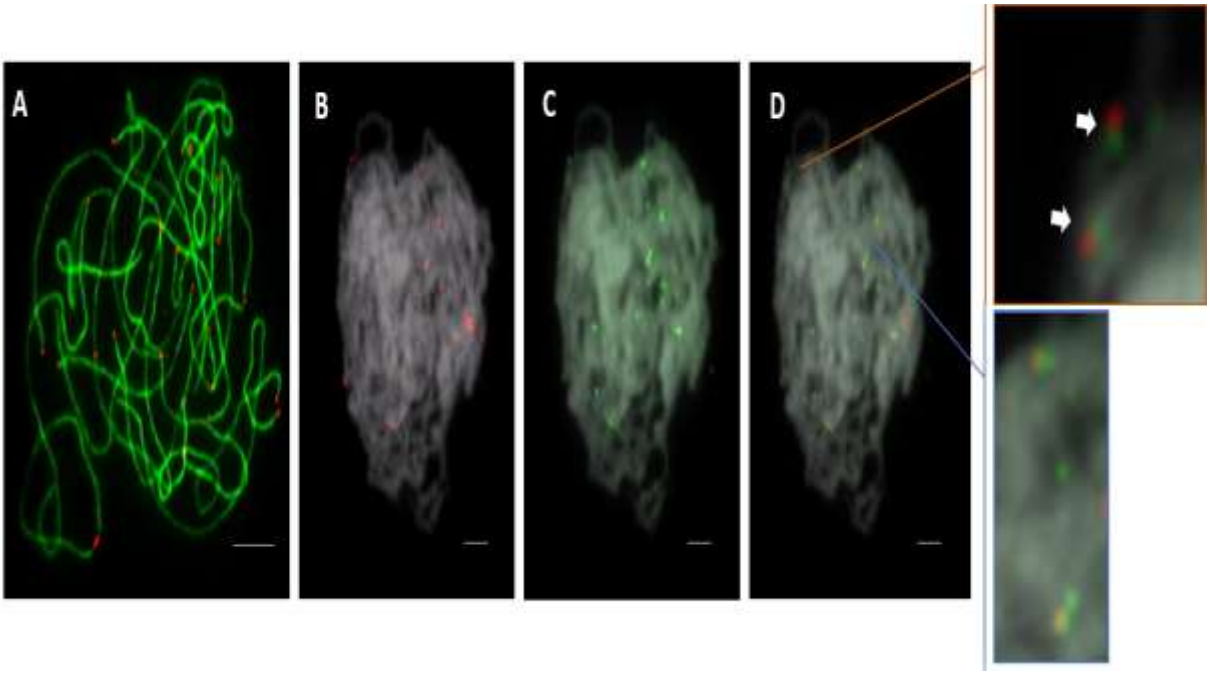


Figure 4-7 Immunolocalization and reprobing *B. juncea* with the telomere probe

(A) Immunolocalization of *B. juncea* pachytene showing ZYP1 in green and MLH1 in red (B) Immunolocalization DAPI shown in while and MLH1 foci in red, and (C) FISH, DAPI shown as white and telomere foci in green, (D) Merged Immuno cum FISH image showing green telomere foci with red MLH1 foci in PMC. White arrow shows the colocalization of telomere with MLH1 signal. Bar =10 µm

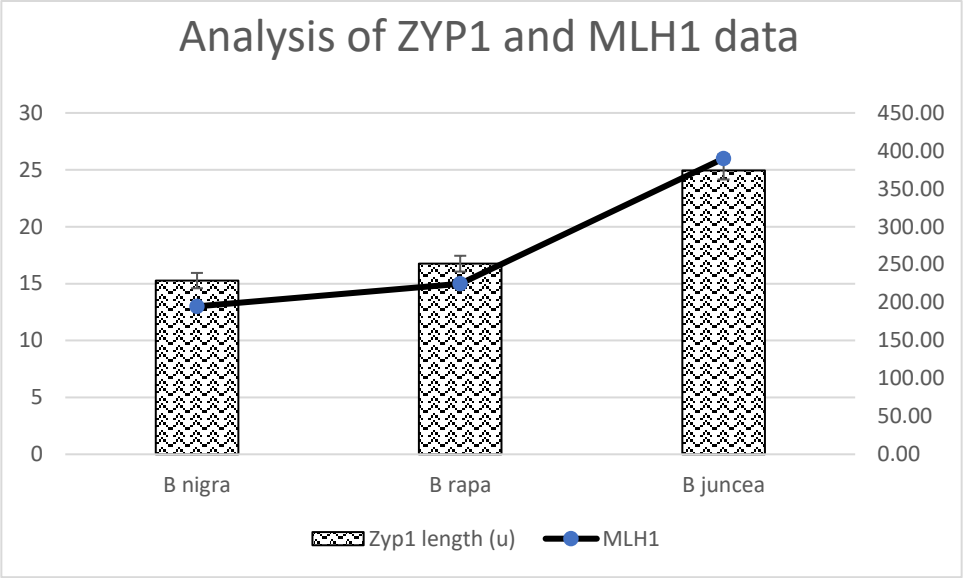


Figure 4-8 MLH1 frequency in different Brassica species represent Class-I CO frequency along with SC filament length measured on the image J.

When compared with the SC filament, CO follows the same upward trend on the graph showing somewhat collinearity between SC and CO frequency, but when compared according to the ratio of MLH1 foci to SC length, the trend line for polyploid genotype dropped against the respective rise (Figure 4-9). In diploids average CO lies every 15.91µm and 15.08µm of the SC filament, but allotetraploid had an average CO per 13.14 µm of the SC filament. The data (table 4.2) showed a higher density of CO per SC length as compared to a weaker CO density. This also correlates with the interference phenomenon that in diploid the CO interference was strong enough not to let another CO to occur nearby versus a weaker interference in allotetraploid where two CO are found close to each other than would occur in the diploids.

Table 4-2 Frequency of MLH1 signal in Brassica species (n=25) for each species

	Genomic size (Mbp)	ZYP1		MLH1		Range		Av MLH1 per SC filament
		Filament length (µm)	St. Err	Foci	St. Err.	Min	Max	
B. nigra	529	229	10.11	13	0.17	8	22	17.62
B. rapa	632	251.31	10.42	15	0.33	10	24	16.75
B. juncea	1068	374.26	11.93	26	0.37	23	36	14.39

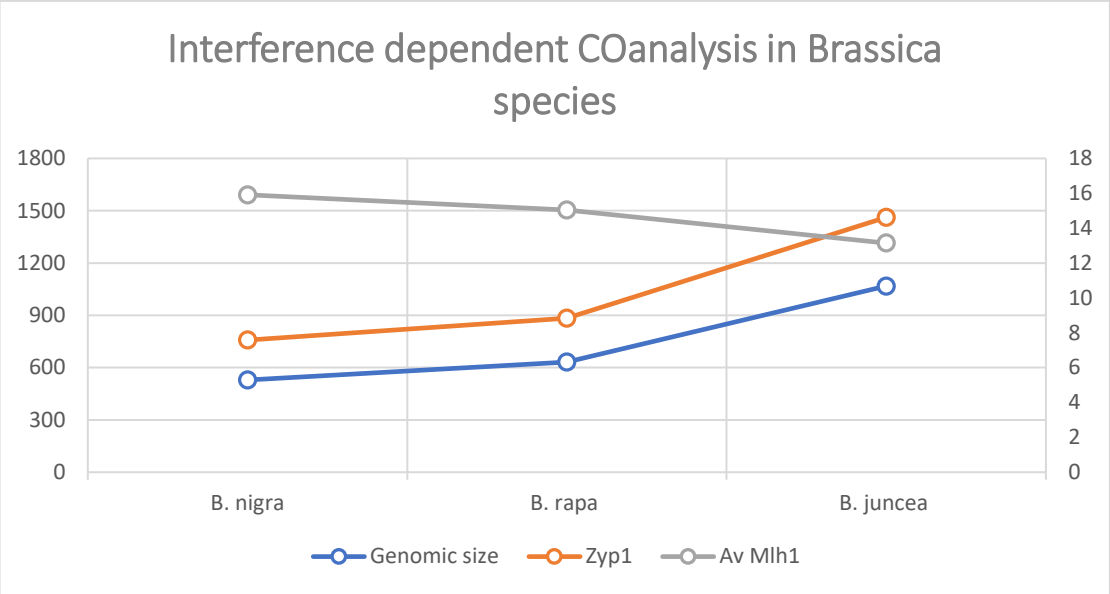


Figure 4-9 Analysis of Brassica species on the basis of Synaptonemal Complex (SC) and MutL (MLH1) immunochemistry to show the interference dependent Class-I COs.

4.3 Cytological analysis of the homologue chromosomes

The characteristic feature of the homologues pairing in meiosis is essentially vital to halve the chromosomes between the opposite poles of the cell at the end of the first part of the cell division. The pairing precedes alignment and bouquet-like clustering of telomeres in zygotene, and full-length synapsis is achieved in the pachytene stage where the central element of SC is polymerized between lateral elements to tether the homologues spatially together. The stage is better studied using immunochemistry with ASY1 and ZYP1 antibodies before SC starts to degrade in diplotene where homologues are linked physically only via chiasma to certain points; the site where genetic reshuffling could occur. The linkage helps chromosomes to adopt a bivalent configuration for aligning chromosomes at the equator of the cell during metaphase, a

pre-requisite for faithful disjunction of the complement in gametes. Failure of such a typical configuration gives rise to the asymmetric division between the poles prompting aneuploidy in the subsequent generations. The meiotic atlas of all brassica species was developed to compare the chromosomal behaviour and configuration at this important phase of meiosis which differentiates it from mitosis. During metaphase-I, the chromosomes are oriented in a way to expose single kinetochore of the bivalent for spindle fibre attachment before disjunction can reduce the number to half (Zhang *et al.*, 2014). The observed bivalents are classified into two categories in established brassicas, i.e. ring and rod-shaped bivalents (Figure 4-10). Analysis of chiasma counts in different brassicas has been summarised in Table 4-3 which reveals the fact that there was the nearly equal distribution of ring and rod-shaped bivalent in *B. nigra*, *B. rapa* and *B. juncea* and are biased to 50% in a cell.

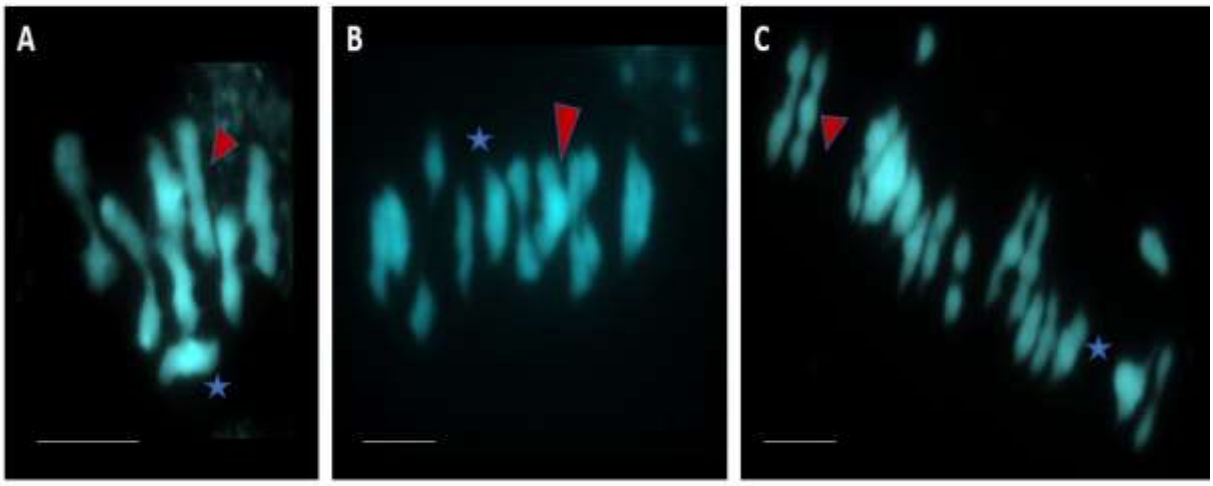


Figure 4-10 Metaphase-I stage of *B. nigra* (A), *B. rapa* (B) and *B. juncea* (C), red arrow head shows rod bivalent while a blue asterisk marks ring bivalent configuration. Scale bar 10 µm.

Table 4-3 Chiasma counts in brassica genotypes in M-I

	n	Chiasma	St. Err	Ring	Rod
B. nigra	27	14.37	1.74	3.85	4.15
B. rapa	31	17.77	1.31	4.73	5.30
B. juncea	28	28.36	1.34	8.29	9.71

The data was plotted against COs frequency, and SC filament length which revealed the trend skewed toward the increasing complexity of the organism and higher chromosomes numbered will have more chiasma (Figure 4-11). The number of chiasma were recorded higher than the COs obtained by immunolocalization analysis which shows other alternate pathways operating for the chromosomal exchanges via cross-over, i.e. Class-II COs or others which are characterized as interference independent and does allow new cross overs to happen nearby.

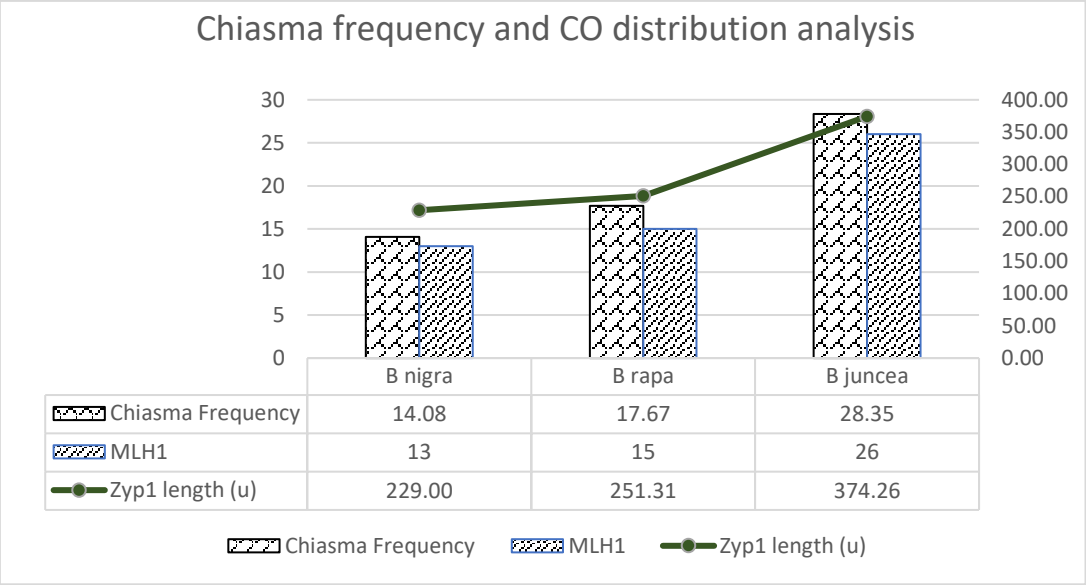


Figure 4-11 Correlation among Chiasma, Cross over (CO) and SC filament in Brassica species (n=25 for each species)

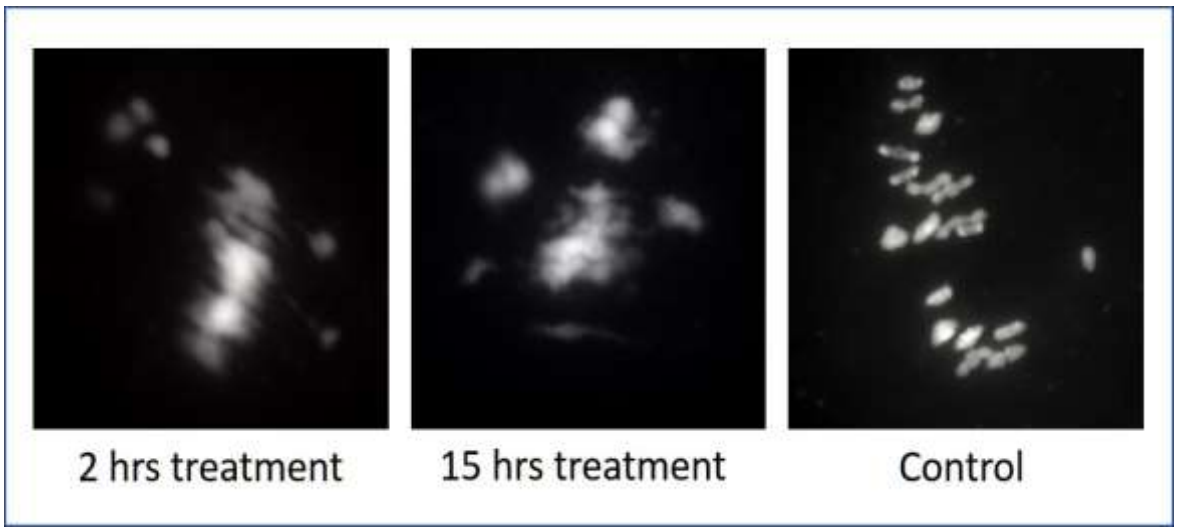


Figure 4-12 Metaphase-I of *B. juncea* under different conditions of Okadaic Acid treatment shows different chromosome configuration which happened due to different condensation pattern.

4.3.1 Epigenetic effect following okadaic acid treatment

During the diakinesis stage of prophase-I, genomic destabilization was observed when multivalents were formed between more than two chromosomes. They seemed to have entangled across each other in addition to the presence of univalent (Figure 4-13). There has been an association between different genomes which was not observed in wild-type. Okadaic acid had caused premature condensation of chromosomes as could be seen at M-I stage, where chromosomes had different condensation pattern than the control (i.e. *B. juncea*) (Figure 4-12). Later in the anaphase-I, the chromosome could be seen linked with each other by the anaphase bridges which kept the chromosomes in contact resembling some mutants in meiotic genes (like Dmc1 and MRE11-mutant plants (Siaud *et al.*, 2004; Puizina *et al.*, 2004). The chromosomes were seen linked with the other chromosome even in telophase-I which opened an opportunity in introgression potential within the genome. The possible explanation for the formation of dicentric chromosomes (Figure 4-13) could be the NHEJ repair system which combines two chromosomes together (Bertuch, 2002)

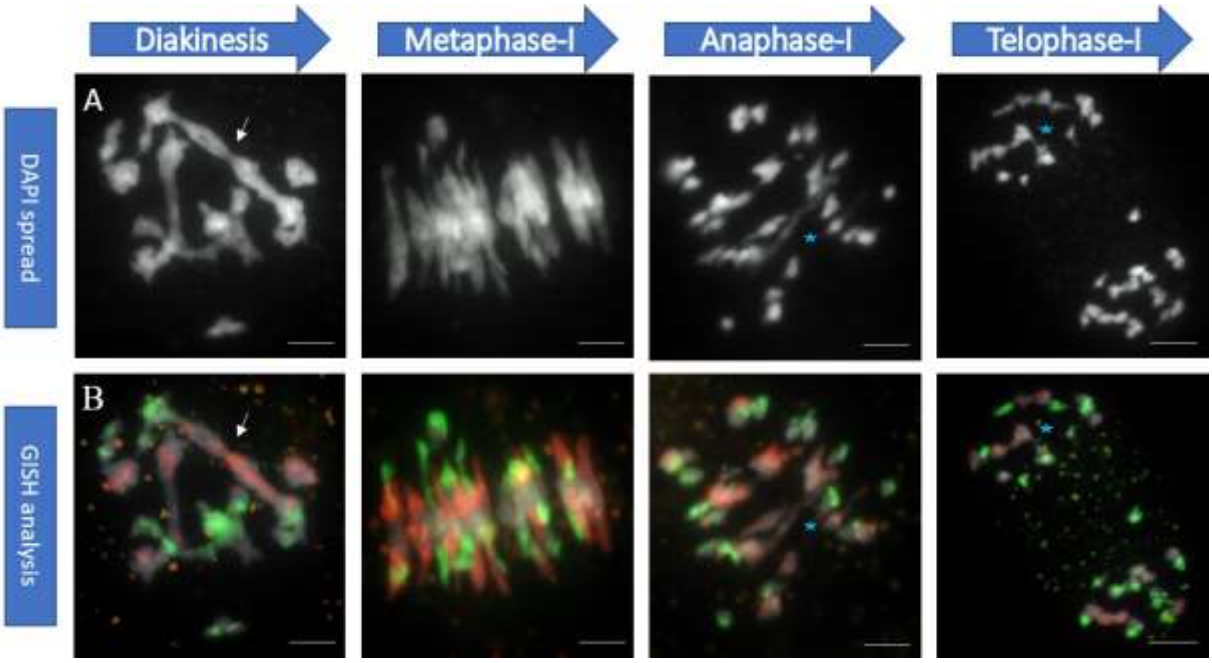


Figure 4-13 GISH analysis of Okadaic acid treated *B. juncea*

showing evidence of multivalent and homoeologous association where red represent *B. nigra* and green show *B. rapa* genome. (A) Showing Dapi stained only, (B) Showing GISH with *B. nigra* in red and *B. rapa* in green. Arrows show multivalent while asterisk represents anaphase bridges among the chromosomes. Scale bar 10 µm.

4.4 Discussion

Polyploidy imparts a significant share in our daily life in different prospects thus making it an important subject to understand at the molecular level. Nearly 80% of the angiosperms are believed to have polyploidy nature, therefore, it is important to decipher the genetic basis of this important natural phenomenon for improving crop breeding strategies. An attempt was made during this study to get insight into meiotic progression in the brassica diploid and tetraploid species using several experiments to transfer knowledge from the model plant *A. thaliana* to the crop species, like brassica species, wheat, rice etc .

Two well-known forms of the polyploids are auto polyploid and allopolyploids. Allopolyploids are different (from the autopolyploid) as they contain more than one intact genome (/set of chromosomes) in a cell, therefore, it also involves the concept of hybridization in addition to whole genome duplication (WGD) during its evolution. The Brassica genus contains important crops for its various domestic uses and *B. juncea* is grown worldwide for vegetable and oil production and for fodder use (while *B. rapa* is cultivated mostly for vegetable use and *B. napus* for oil production). Interestingly Brassica has numerous ecotypes which provide ample opportunities for the improvement at the genetic level and to enrich novelty. During this study, three species were selected for the analysis of meiosis in two diploids and one allopolyploid which is derived from both diploid parents.

The survival of a species (eukaryotes, sexually producing organisms) and its propagation depend largely on the faithful cellular division which helps in proliferation in addition to

creating variations in subsequent generations via recombinant gametes while maintaining the genome integrity is balanced and intact. The special kind of cell division for reproduction called meiosis bases novelty by virtue of the intricate process of pairing and recombining alleles between homologues during the first part of meiosis pathway. The chromosomes undergo a dynamic movement during telomere pairing and clustering which make this step of vital importance. Telomeres are the GC rich segments of chromosomes and are present at the ends of chromosomes. The region plays a crucial role in the process of (homologous) pairing which starts by telomere coupling followed by the clustering, which is better known as bouquet during zygotene (Loidl *et al.*, 2012; Armstrong *et al.*, 2001).

When analyzed with the telomeric probe this early phase of meiosis revealed *B. nigra* behaves differently to *B. rapa* (Figure 4-1), the telomeres are scattered around the nucleus against the clustering in *B. rapa*. The telomere helps in the early association in chromosomes which precede synapsis and recombination. The typical telomeres clustering was absent in leptotene where it leads to bouquet formation to facilitate the homology search. The *B. nigra* chromosomes develop telomere clustering during the zygotene when telomeres are polarized to one end of the cell as been reported in *A. thaliana* (Roberts *et al.*, 2009). The progression through prophase is perceived by the gradual decrease in telomere numbers which attained stability at their respective genomic numbers after mid zygotene to pachytene stage (Armstrong *et al.*, 2001).

The chromosomes start to cluster together around the nucleolus to form a bouquet like configuration (Figure 4-1) near the nuclear envelope (Tiang *et al.*, 2012). The bouquet oscillates

under the influence of cytoplasmic dynein-driven protein KASH which is itself connected through the nuclear membrane to SUN protein in the nucleoplasm. The telomeres get attached to the SUN proteins in the nucleoplasm and undergo vigorous movement before finding the corresponding partners (Varas *et al.,* 2015). The configuration is common in some plants (e.g. rice) and animals (e.g. mouse), but a slight modification of structure is observed in brassica where telomeres are loosely clustered around nucleolus similar to what was found in *A. thaliana* (Roberts *et al.,* 2009; Zhang *et al.,* 2014). It has been postulated that there might exist a supplementary genetic system which brings the homologue together in the absence of the bouquet. Molecular analysis in rice has revealed a gene, *ZYGO1* is believed to have an influence on the pairing of the homologue via telomere association and their clustering during prophase (Zhang *et al.,* 2014) and could be exploited in the Brassica genus in further studies for such control mechanism during early leptotene as a true bouquet is missing her. It would be interesting to analyse its behaviour in the allotetraploid background for its role in preferential pairing and discriminating the non-homologues. Allelilic variation could be responsible for bouquet configuration in different living systems.

Since both diploids were evolved from the separate lineages, they differed in their genomic sizes with *B. nigra* having a large genomic size than the *B. rapa* diploid genotype. As the meiosis progresses, the chromosome develop axes for the sister chromatids to align against their homologue by the deposition of ASY1 and other accessory proteins (ASY3 and ASY4 which are present throughout prophase-I and their absence could hamper proper synapsis (Chambon *et al.,* 2018)), which play a crucial role in determining the fate of a DSB intermediate. This axis facilitates inter-homologue bias between the chromosomes for the progression through synapsis

in meiosis (Morgan *et al.,* 2017). Once homologue partners are sorted they develop transverse elements of SC which precede CO for the genetic exchanges. In allotetraploid preferential pairing was observed as strictly confined between the homologue despite the presence of dissimilar homoeologues in the vicinity. The SC length was recorded longer than the constitutive diploids and is parallel with the increased genomic size of *B. juncea*. The paired chromosomes undergo recombination in subsequent stages which subsequently links chromosomes via physical points. This fits well in line the finding (He *et al.,* 2017) where they showed in *B. juncea* that the pairing is interstitial compared to distal only COs in *B. napus*. This linkage results in CO formation during the dHJ resolution process.

The COs had a varying nature and based on different biochemical pathways adopted after DSBs formation in meiocytes; these can be classified into two categories; Class-I cross over (CO-I), and Class-II cross over (CO-II). Class-I refers to the interference dependent COs where MLH1 is involved while class-II are interference independent COs and involves MUS81 instead. Since class-I COs are more prevalent and are mediated by ZMM pathways than the class-II in plants (Mezard *et al.,* 2007; Martin *et al.,* 2014), our focus of studies in Brassica diploid and polyploid species was on class-I COs which inhibit any further CO happening in the vicinity of an already existing CO. Based on the results, it could be inferred that interference effect was weaker in polyploid with multiple sets of chromosomes than its diploid parents (Table 4-2) as second CO was observed close to the first one.

Furthermore, the CO frequency was also less in allopolypolyploid as observed for autopolyploid *A. arenosa* which is deemed to be one of the reasons behind genomic stability in

polyploidy (Yant *et al.,* 2013). The distribution pattern of CO marks observed were also different in allotetraploid than its progenitors at the terminal of the chromosome which could happen due to allelic changes in polyploidy cell. Similar findings were observed in *A. arenosa* where COs were evidenced at the terminal of chromosomes (Morgan Chris, 2016). In *B. juncea* there were two different genomes, so in the light of the observations made in *A. arenosa*, it could be postulated that the observed CO shifting might be the cause of stabilization of the allotetraploid as well. Nevertheless, analysing alleles in allopolyploid needs to be explored and would be interesting to see the homology principle with different alleles and how it helps in diploidization of polyploids. Further, genome dominance also exerts its effect in the phenotypic plasticity of the allopolyploid in enhance performance, therefore in *B. juncea* further attempts are needed to endorse this dominance phenomenon acting in the presence of competitive alleles. Moreover, a current studies (He *et al,* 2017) has revealed that *B. juncea* has less HEs than *B. napus* which is a young polyploid and show more phenotypic plasticity. Structural variants in chromosomes are excellent subject while studying evolution in polyploid and synthetic allotetraploid. Since HEs abundance in *B. juncea* is different from that of *B. napus*, detailed analysis of constituent genomes will help us understand the early events toward stabilization.

Cytological observations were in line with the linkage map developed with help of AFLP and RFLP markers for first time as minor groups were reported (Cheung *et al.,* 1997) in addition to 18 linkage groups (Pradhan *et al.,* 2003). The linkage map also revealed that the diploid ancestors were unchanged in the amphidiploid *B. juncea* and the subgenomes retained their integrity against the widely accepted belief of rapid changes that occur during the polyploidization process (Song *et al.,* 1995). Synteny analysis demonstrated that certain number

of genes show homoelogous expression and most of the genes are biased toward the *B. nigra* subgenome in *B. juncea* (Yang *et al.,* 2016). Fewer recombination events than the parental ancestors could have happened due to the chromosomal rearrangements at a large scale in subgenomes or due to the presence of some genetic control which suppresses the intergenomic pairing (Axelsson *et al.,* 2000). Further, it was discovered that the subgenomes retained conserved blocks from their parents albeit scattered along the chromosomes with intergenic spaces sometimes and the genetic difference was much lower than across the subgenomes in the allopolyploid (Zou *et al.,* 2016). The chiasma count (Fig. 4.11) also hints at the conservation of the veracity of parental genomes. Further studies could help us unveil the conservation of genomic structure found in diploids (*B. rapa* and *B. nigra*) against the allopolyploid, *B. juncea*. Sequencing *B. nigra* and genome B containing allotetraploid will help us understand the evolutionary basis and broader genetic diversity present in U's triangle. It has been found that *B. napus* has undergone more changes than the *B. juncea* (Yang *et al.,* 2016) which is intriguing to stimulate the research and focus on an evolutionary basis which helped in speciation in the past.

Okadaic acid (OA) was used to induce interaction between different sets of chromosomes by affecting chromatin state. Due to common ancestor of both genomes in *B. juncea* (which are related to each other) in the same cell, the chromosomes had a chance of partial pairing with related chromosome, so to explore this in *B. juncea,* plants were treated with Okadaic acid to inhibit the phosphatases and induce genomic instability by disturbing Cdk2 activity (Griffiths *et al.,* 2006; Greer *et al.,* 2012). Chromosomal fragments in addition to the unusual physical connections between related chromosomes were observed which divulged the ability of

chromosomes to recombine with others by manipulating the chromatin state, i.e. premature condensation. OA causes an increase of histone H1 phosphorylation which mimics the *Ph1* control locus for promoting the homoeologous association and recombination (Knight *et al.,* 2010). Taking control of such association would have far-reaching benefits for plant breeding to generate more versatility in crops. Novel association and combination along the chromosomes could bring variation and help in the speciation process.

During diakinesis (Figure 4-13) where bivalents are usually visible, multivalents were observed in the treated plant suggesting repairing would have occurred via Non-Homologous End Joining (NHEJ) between different genomes. After DSBs formation, they might have repaired though adjacent ends which developed into a multichromosomal structure containing both genomes connected indiscriminately to form dicentric chromosomes. The genomic stability was also observed in subsequent stages where univalent was also observed during metaphase-I in addition to the altered pattern of chromatin condensation which might happen due to the effect happened similar to that in *Ph1* locus absence where Cdk's over-expression had caused early condensation (Knight *et al.,* 2010). Following M-I, anaphase bridges were observed which showed improper segregation of the chromosomes. In telophase-I bridges were seen among different chromosomes which confirmed the effect of okadaic acid of non-homologous association. In addition to genomic instabilities, micronuclei and mitotic instabilities were also observed where alternate segments of the chromosomes could be detected with both genomes.

4.4.1 Summary

The capability of reduction and restoration of ploidy in sexually reproducing eukaryotes is a fascinating aspect of meiotic cell division which lays the basis for variation and brings diversity in the subsequent generations. Such changes occur in the early part of meiosis where chromosomes juxtapose and exchange genetic material at the molecular level (Hollingsworth *et al.,* 1995). Furthermore, the gametes then restore back the ploidy upon fertilization to maintain the genomic integrity and stability (Chelysheva *et al.,* 2012; Martin *et al.,* 2014).

Understanding the genetic basis of naturally evolved brassica species at different time will be useful. Lineages will be helpful in dissecting genetic recombination and introgression phenomena between different diploid species in the Brassica genus. As B genome is believed to have some important genes (Navabi *et al.,* 2013) which might have been silenced or mutated over the time in other genomes, so concrete efforts are needed to address this introgression which may facilitate the transfer of useful genes from B genome into extensively cultivated A/C genomes all over the world.

By manipulating the chromatin state, the non-homologous association could be achieved which could be helpful in breaking species barrier for introgression of genes across the species. Meiotically locked genetic content could be exploited by optimising post-translation modification which would be helpful in addressing the food security in longer term and enhancing the genetic base of the genotypes.

5 Wide hybridization (Distant hybridization)

5.1 Wide Hybridization

The Brassicaceae family consists of a vast range of crop species which also include three well-known diploids and their derived paleopolyploid species. The relationship between these six species was first defined by Nagaharu U. in 1935 which later became an essential concept in the evolution of Brassica species, and even today these species are recognised by U's triangle (U, 1935). The Brassica species in U's triangle provide an excellent model for studying the evolutionary basis of diploid and polyploid species. The diploids were believed to have undergone cross-hybridization between them to produce allopolyploid species during evolutionary past. The diploids evolved as a result of palaeopolyploidization events which later diverged into two lineages, namely the nigra lineage and the rapa/oleracea lineage. The two lineages are well separated reproductive units, and barely fertilize each other in field conditions unless practised under control conditions. The two lineages are believed to have been spaced by 4.25 Million years (Navabi *et al.,* 2013). All three diploids (i.e. *B. nigra, B. rapa,* and *B. oleracea*) are distinct from each other and possess different genome sizes and organization. The haploid chromosome number of *B. nigra* (genome B) is 8, 10 for *B. rapa* (genome A) and 9 for *B. oleracea* (genome C).

Owing to a common ancestor, all three diploids share subtypes of homologous genes in the form of orthologs and paralogs. The former evolves because of the speciation drive whereas genome doubling gives rise to the paralogs (Glover *et al.,* 2016). Therefore, gene content and organization are conserved to some extent across brassica diploids. Nevertheless, it does not legitimize the production of viable interspecific hybrid due to genetic & meiotic barriers

(Kitashiba and Nasrallah, 2014). Interestingly, the allotetraploid of diploid species is naturally well adopted and exhibits normal pairing and segregation to allow continuity of the generations (Glover *et al.*, 2016).

My focus is to create an allodiploid hybrid between brassica genome A and genome B and to evaluate the meiotic process taking place in it. To get synthetic *B. juncea* (AABB), an attempt has been made to generate AB hybrid plant at first between *B. nigra* (BB) with *B. rapa* (AA) in reciprocal crosses, i.e. by emasculating both *B. nigra* and using *B. rapa* pollen on it and vice versa (Figure 5-1) so that each of the species can behave as female and male respectively in a cross before doubling its genome content using chemicals.

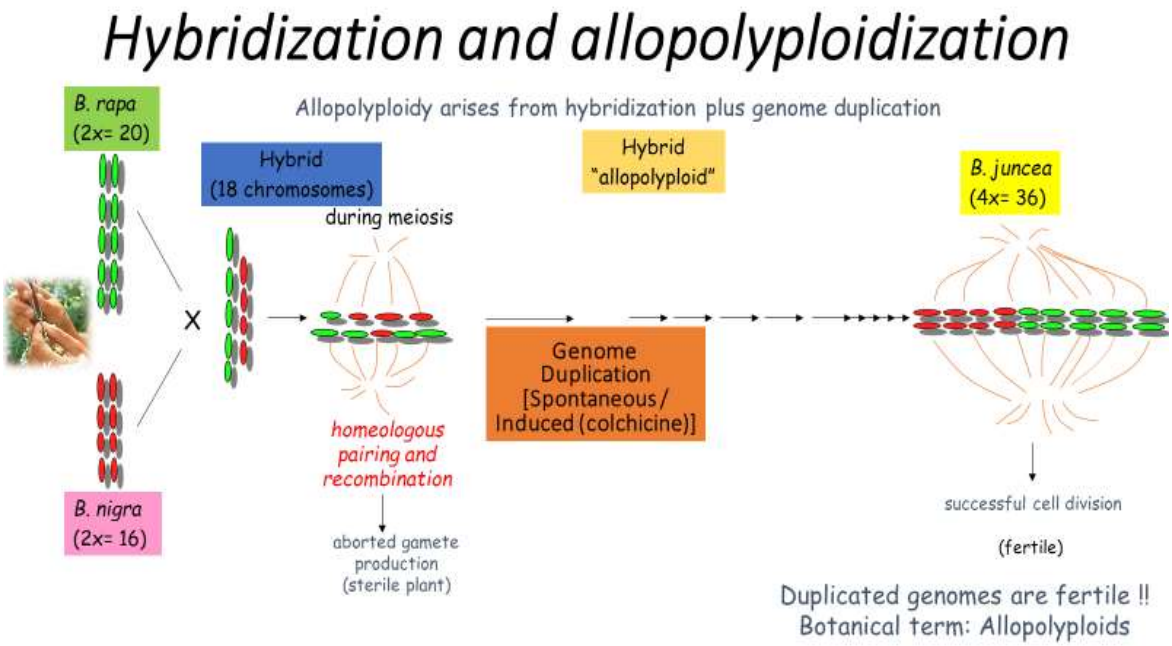


Figure 5-1 Schematic representation of producing a hybrid between *B. nigra* and *B. rapa* genomes.

A hundred interspecific crosses were made between different genotypes for each combination to bring non-identical genomes in a hybrid cell, i.e. allodiploid. The hybrid plant could be doubled to generate a tetraploid to compare it with naturally existing allotetraploid brassica. The efficiency of hybrid production was as low as 3% which was only observed when the crosses involved *B. rapa* as female (as cytoplasm donor) with *B. nigra* as male while all the crosses for its reciprocal combination (Table 5-1), i.e. *B. nigra* as female and *B. rapa* as male were unsuccessful. The hybrid seeds (AB) obtained from the crosses possess qualities of both parents, i.e. the colour of the AB seed was like *B. rapa* seed (brown and relatively large) and the size of the seed was more like *B. nigra* parent or near intermediate (Figure 5-2). Once the seeds were harvested, they were placed at 16 ^{o}C for two weeks to let the extra moisture dries before planted on MS media. The germination efficiency further reduced the viability of the seeds as only one-third of the seeds were able to grow. The root meristematic cells were selected to confirm chromosomes number and to distinguish both genomes in the hybrid seed. For this purpose, well-spread metaphases were proceeded for the GISH to identify each genome of *B. nigra* and *B. rapa* chromosomes in the nucleus. The chromosomes count was not like each of the parents and was recorded as 18 against 16 of *B. nigra* and 20 of *B. rapa* under a microscope confirming successful hybridization.

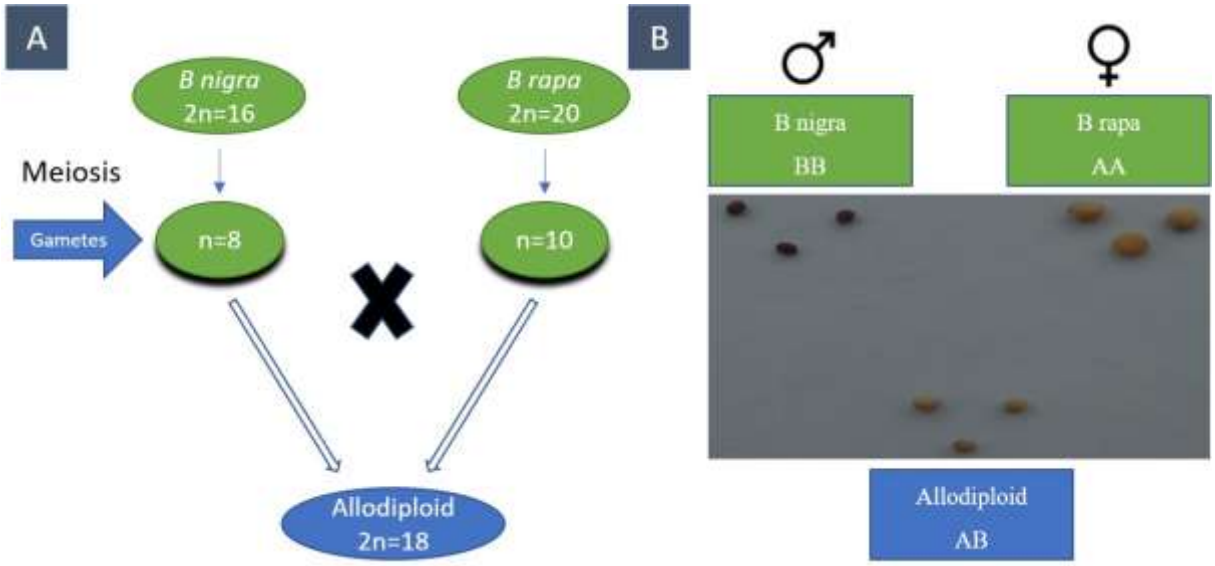


Figure 5-2 Wide hybridization between B. nigra and B. rapa

(A) Schematic representation of the cross between brassica diploid species.

(B) Allodiploid (AB) seeds are intermediate between the parental lines.

Decent quality metaphases were subjected to the GISH analysis which revealed allodiploid line consisting of two distinct genomes (see 3.1.3 for the detail about GISH). The red colour represents *B. nigra*, and the green colour represents *B. rapa* in the mitotic complement, and their counts were according to the haploid genomes of parents. Out of 18 chromosomes, eight descended from *B. nigra*, and ten were from *B. rapa* which can be seen labelled differently (Figure 5-3). The seedlings were transplanted from petri dishes to the pots in the glasshouse so that meiosis could be studied in the later stage of growth in the floral buds. Early young buds

were selected for developing the meiotic atlas and GISH analysis during pairing and disjunction phase.

Table 5-1 Wide hybridization crosses between different Brassica genomes

Brassica crosses	Number of pollinated ovaries	Siliques developed	Seed setting	Seed setting efficiency %
B. rapa X *B. nigra* 150	80	0	0	0
B. rapa X *B. nigra* 1196	70	0	0	0
B. nigra 1196 X *B. rapa*	110	25	0	0
B. nigra 1198 X *B. rapa*	100	30	0	0
B. nigra 150 X *B. rapa*	300	174	9	0.052
B. nigra 150 X *B. juncea*	50	15	0	0
B. rapa X B. oleracea	50	10	2	0.20

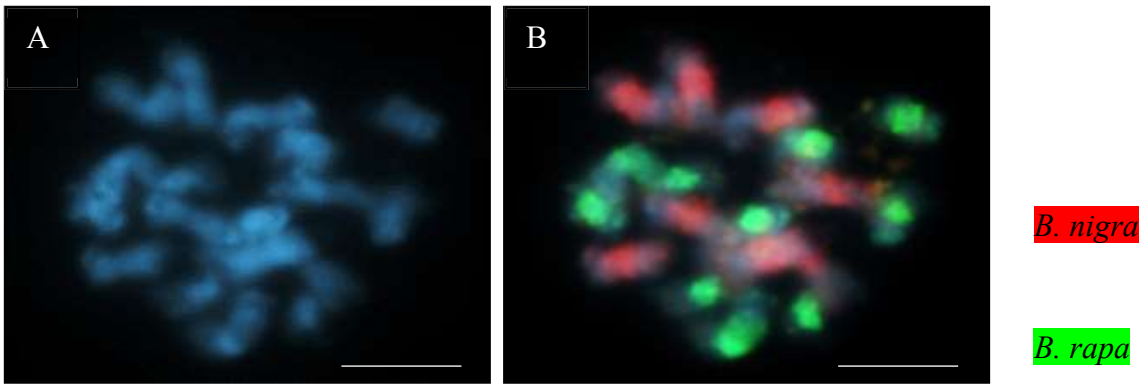


Figure 5-3 Validation of hybrid AB plant with GISH analysis

(A) Mitotic metaphase chromosomes configuration in hybrid allodiploid AB plant stained with DAPI (B) GISH analysis showing 8 *B. nigra* chromosomes in red colour and 10 for *B. rapa* in green colour. Scale bar 10 µm

5.2 Meiotic atlas and GISH analysis

To gain insight into meiosis, the meiotic atlas was created from pollen mother cells from young buds of the allodiploid (AB) plant. The chromosomal behaviour was unusual during the reductional phase as single chromosomes appeared pairing with multiple chromosomes which gave clues about homoeologous recombination occurring between *B. nigra* and *B. rapa* chromosomes (Figure 5-4). Further to analyse the pairing behaviour of chromosomes, GISH was performed on meiotic cells which labelled distinct chromosomes in the genome with red and green colour respectively. During the pachytene-like stage, at certain points, both genomes seemed synapsed with each other despite genomic incompatibilities (Figure 5-4 A). In early

diplotene stage there appeared two chromocenters one seems to be from *B. rapa* while the other bigger one contained both genomes in red and green colours (Figure 5-4 B). While in diakinesis pairing between different genomes as *B. rapa* and *B. nigra* seemed joined due to limited homology between the genomes. As the cell enters the metaphase-I stage in normal cases, bivalents are formed between homologue of the same genome, but in this allodiploid, I found that both genomes can be seen linking to each other on more than one occasion. Since the number of the chromosomes was not even and corresponding to each other, irregularities were expected during orientation. Homoeologous pairs were identified between non-identical chromosomes which possess great potential for introgression of a gene across species, especially beneficial for plant breeding in crop improvement programmes. In addition to bivalents, univalents are also observed frequently which ranged from 4 to a maximum of 10 in M-I (N=10), and interestingly *B. rapa* chromosomes were seen more often as univalent than the *B. nigra* and an average five univalents were seen against 3 for *B. nigra*. The number of COs varied in different cells and ranged from 6 to maximum 10 in a cell complement.

Further, Anaphase bridges confirmed the homoeologous evidence between different coloured genomes (Figure 5-4 E,e). At this stage, fractionation was very prominently observed and frequently 4-5 small fragments of chromosomes were found near chromosomes, which persisted till the end of meiosis (Figure 5-4).

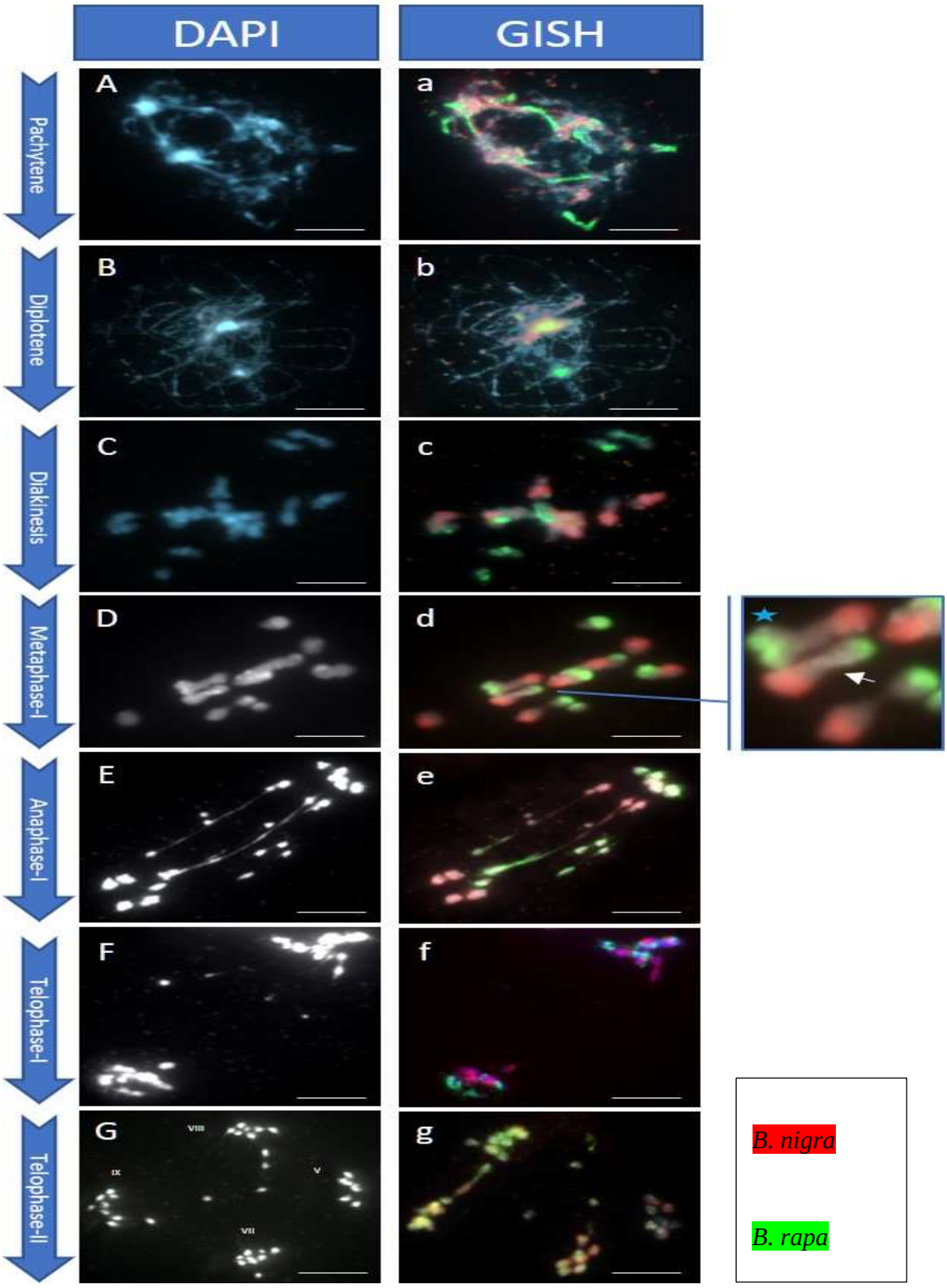

DAPI
GISH
Pachytene
Diplotene
Diakinesis
Metaphase-I
Anaphase-I
Telophase-I
Telophase-II
A
a
B
b
C
c
D
d
E
e
F
f
G
g
VIII
IX
V
VII
B. nigra
B. rapa

Figure 5-4 Meiotic atlas of allodiploid AB plant

(A,B,C,D,E,F,G) DAPI stained PMCs in allodiploid AB plant (n=10) (a,b,c,d,e,f,g) GISH analysis showing *B. nigra* and *B. rapa* chromosomes in red and green colour respectively for respective stages. (d) The autosyndetic association is seen as a blue star along with the allosyndetic (homoeologous pairing) shown by the white arrow between different genomes, i.e. *B. nigra* and *B. rapa*. Scale bar 10 µm

5.3 Partner switching during pachytene

Looking closely at the pachytene stage revealed that chromosomes lacked proper alignment and pairing Figure 5-5 with dissimilar counterpart chromosomes from the other genome. When compared with its progenitors where pairing was strictly between the like chromosomes, the hybrid showed abnormal behaviour and found pairing with more than one partner during this stage. The chromosomes did not have global pairing rather were just confined to limited section or fragments with others. The chromosomes could be seen separated from each other during post zygotene stage, which is supposed to be pachytene like stage during which switching partners were seen frequently; this happens because *B. rapa* genome could not find its partner to pair and thereby transient pairing was more prevalent. In addition to this, some loops could also be seen which likely be the associations happening within the genome. In contrast, in its progenitors *B. nigra* and *B. rapa*, the pairing was observed between similar chromosomes, and full synapsis was detected in immunostaining with anti ZYP1.

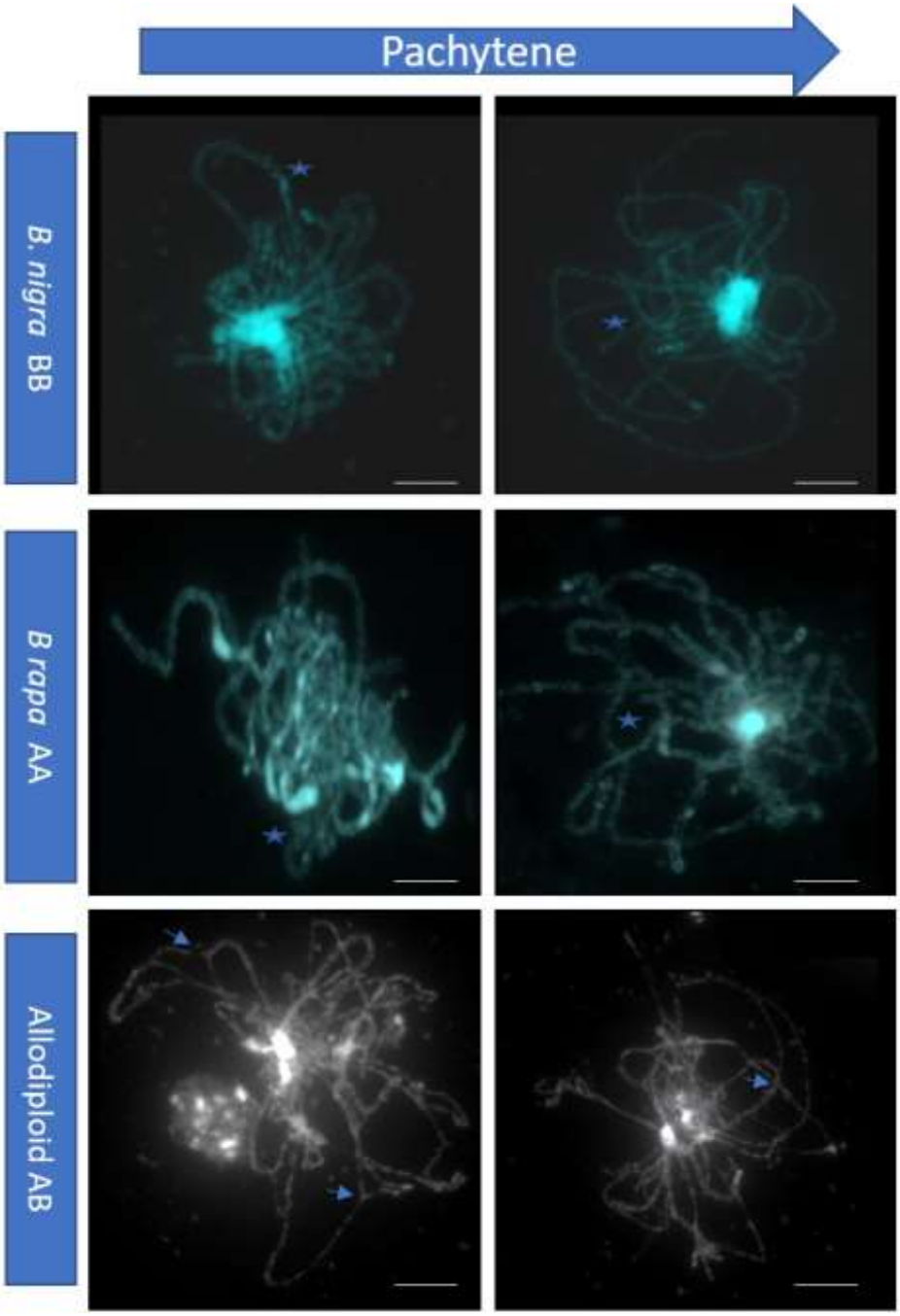


Figure 5-5 Pachytene stage analysis of *B. nigra*, *B. rapa* and pachytene-like stage in allodiploid AB plant

Asterisk marks proper pairing of the chromosomes where as arrow head points to the partner shifting between different chromosomes. Scale bar 10 µm.

Later in the diplotene stage when chromatin starts condensing and adopts the bivalent form, multi chromo-centres were observed, and upon probing through GISH, it was discovered that certain regions contain both genomes colocalized and might have undergone homoeologous association while some remain unpaired and could be found isolated from others (Figure 5-6).

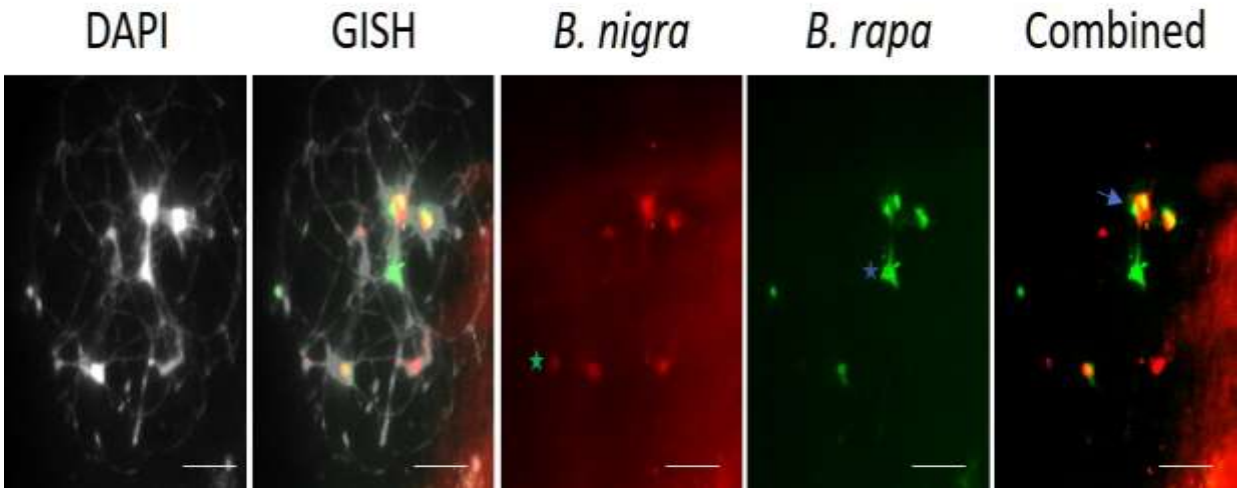


Figure 5-6 GISH analysis of diplotene stage in allodiploid AB plant

Diplotene stage showing both genomes colocalizing at a certain point (blue arrow) in green and red colours indicating pairing and found isolated either green or red (blue and green star mark) showing unpairing in an allodiploid plant. Scale bar 10 µm.

5.4 Sequential FISH analysis

Since meiotic irregularities were observed from the very start till the end, a striking deviation was observed during post metaphase stage in the anaphase-I when chromosomes are pulled to the opposite poles to reduce the chromosome numbers to half. The chromosomes were attached

to each other via anaphase bridges along with some fragments lying next to them. To understand the nature of fragmented chromosomes, the cells were subjected to rehybridization with 45S rDNA following GISH to analyse if the lost fragments contained any NOR, which is essential molecular machinery of the cell and required for the optimal functioning. The hybridization results confirmed that the fragments were coming from the region other than the NOR and possibly from interstitial regions which might have lost connection during the DSB repair (Acilan *et al.,* 2007). From the GISH analysis, it appeared that *B. nigra* genome is more fragile and prone to error as more fragments were labelled with red colour for *B. nigra* probe. There was an asymmetric disjunction between respective genomes, and as a result, more *B. rapa* chromosomes were seen on one pole and prevalent against more *B. nigra* chromosomes on the opposite pole (Figure 5-7).

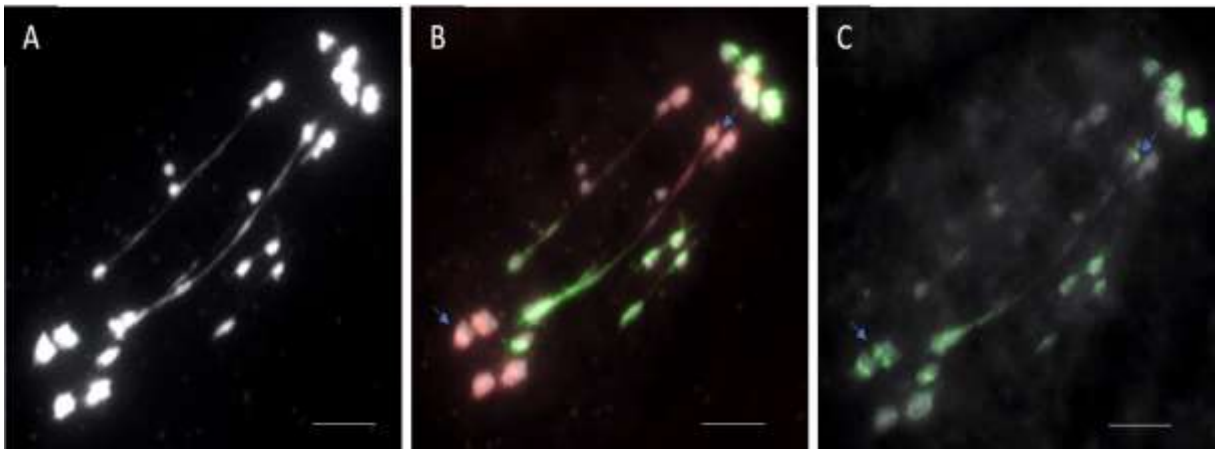


Figure 5-7 Sequential FISH with 45S rDNA of anaphase bridge in artificially generated allodiploid AB plant

(A) Dapi spread of anaphase-I in allodiploid AB, (B) GISH showing *B. rapa* in green and *B. nigra* in red (C) Sequential FISH with 45S rDNA in green indicated by blue arrows in (B) and (C) to determine if the ribosomal segments are found during fragmentation. Scale bar 10 µm

5.5 Telomere analysis in hybrid plant

The homoeologous behaviour and the anomalous pattern of chromosomes prompted me to analyse the chromosomes pairing which is a crucial component of meiosis and responsible for facilitating the recombination. For this purpose, the FISH experiment was designed with a telomere probe to evaluate the chromosomal termini during the initial stages of prophase-I. Early stages of prophase-I were subjected to telomere probe hybridization, and it was found that telomeres were seen isolated and scattered around the nucleus with some clustering at a common point. The number of foci counted during the leptotene stage was 36 on average (n=20) which decreased gradually in subsequent stages until the pachytene like stage is achieved. Nevertheless, the transient pairing brought chromosomes together, and the number of telomere foci was dropped to 31 where half of the signals were relatively brighter than the unpaired chromosomes. The brighter signals were from paired telomeres while relatively faint signals are from unpaired chromosomes (Figure 5-8, Table 5-2).

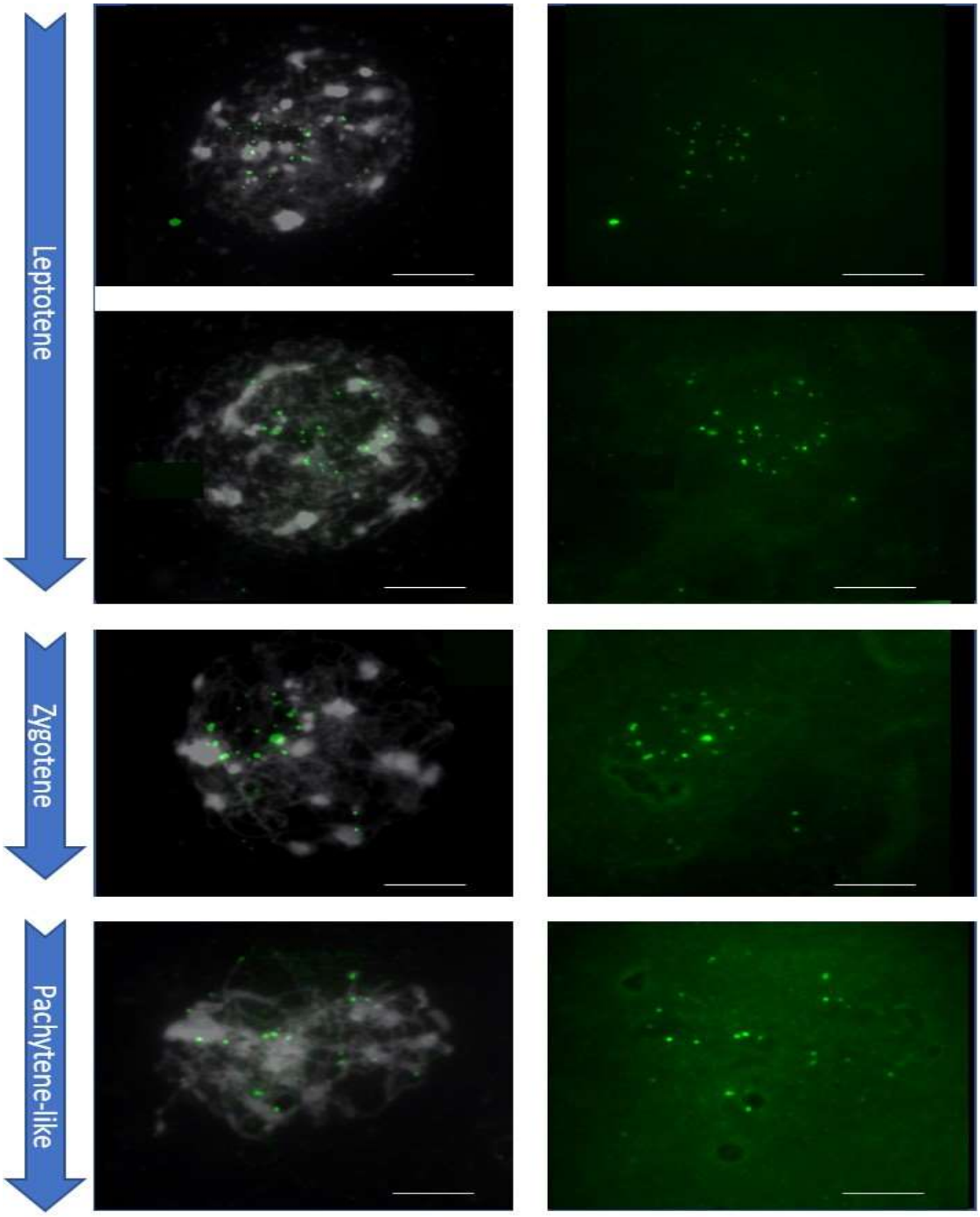


Figure 5-8 FISH analysis with telomere probe (in green) in Hybrid AB plant showing terminals of the chromosomes clustering and pairing.

Scale bar 10 µm.

Table 5-2 Telomere foci analysis in allodiploid (AB) plant

		Range	
Prophase-I (sub-stages)	Telomere foci counts	Min	Max
Leptotene	34	30	36
Zygotene	28	24	32
Pachytene	25	21	27

Figure 5-9 Wide hybrid (AB) obtained from crossing Brassica diploid species,

Manually crossed *B. rapa* plants, (B) Allodiploid (AB) flowering bud, (C) Allodiploid plant, (D) Empty siliques of allodiploid, (E) Leaf morphology of *B. nigra* and allodiploid plant, (F) Colchicine treated allodiploid plant, (AABB) (G) Swollen floral bud of synthetic allotetraploid *B. juncea* (AABB).

5.6 Axial and central elements of the Synaptonemal Complex in hybrid AB

Owing to extensively diverged meiosis and especially in pachytene in the hybrid plant, I used immunochemistry techniques to analyse SC polymerization between the chromosomes. Therefore immunolocalization of ASY1 and ZYP1 protein was done on young buds which exposed the incomplete pairing between chromosomes. In a typical pachytene stage where homologues pair with each other and full synapsis is witnessed, in the allodiploid AB plant where identical chromosomes were absent, the pairing was confined to certain regions, and only a few fragments were observed under a microscope (Figure 5-10). The length of fragments was variable between single dot to a typical linear filament in the pachytene stage. The SC length was measured using Image J (n=10). Next, to find out cross over frequency among homoeologues, immunolocalization of three proteins was practised tracing ASY1, ZYP1, and MLH1 altogether on the same slide and five bright foci were observed for MLH1 indicating class-I CO (Figure 5-11).

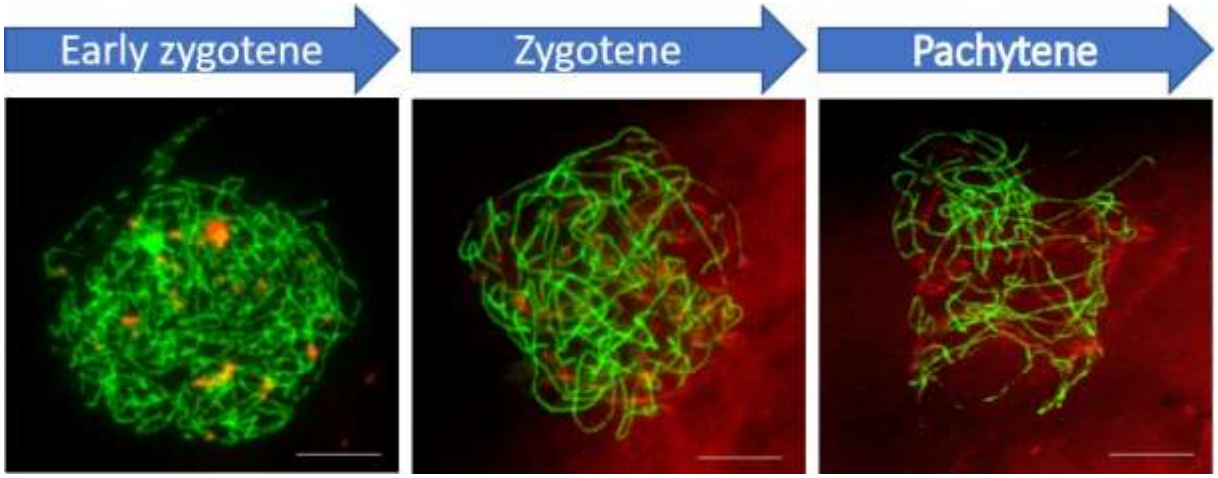


Figure 5-10 Immunochemistry of hybrid AB plant with ASY1 and ZYP1

(A) Represents the late leptotene stage in prophase-I (B) represents zygotene and (C) shows pachytene with ASY1 and ZYP1 in green and red colour respectively. Scale bar 10 µm.

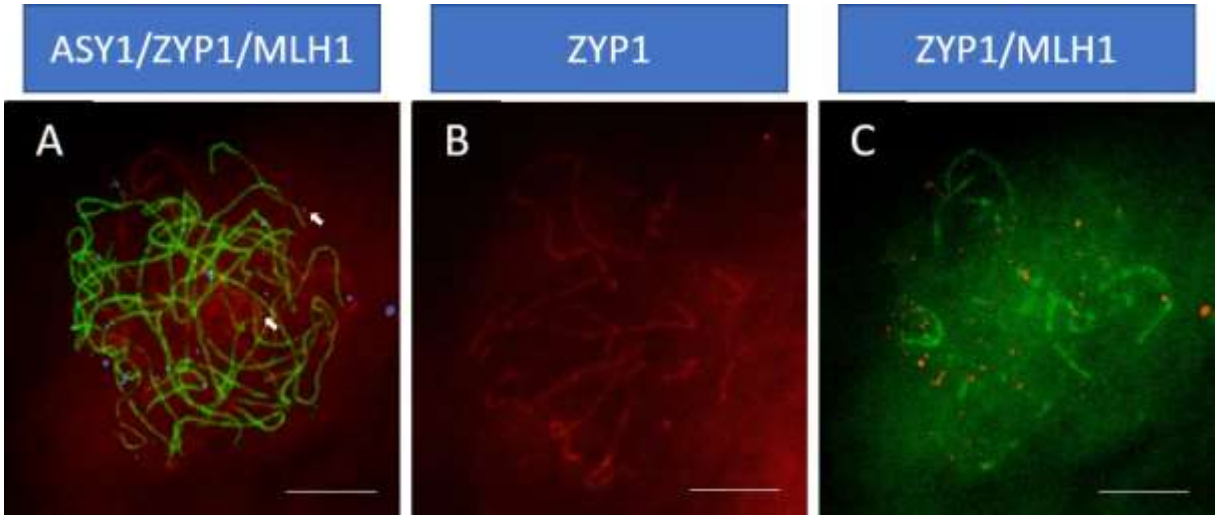


Figure 5-11 Immunolocalization of Allodiploid plant AB with ASY1/ZYP1/MLH1

(A) Pachytene stage showing three antibodies lateral element of synaptonemal complex by ASY1 in green, the central transverse element by ZYP1 in red and MLH1 for class-I Cross Over (CO) shown in while arrows in blue colour (B) Showing fragmented central element by ZYP1 in red, and (C) ZYP1 with the MLH1 foci in green and red colour respectively. Scale bar 10 µm.

5.7 Induced polyploidy

Since the plant was sterile and did not produce any seed, more crosses were made and propagated through cuttings afterwards. A slanting cut on 8-10 cm long branch was immersed in rooting media for 1 minute and then planted into the pot. Clones of the hybrid plant were obtained successfully with 70% efficiency, and the new plants were subjected to colchicine treatment. Two methods were tested for the application of colchicine to double the hybrid plant complement, but one could only prove faithful with the success rate of as low as 6%. The buds treatment did not yield viable anthers; therefore, roots treatment was adopted for the hybrid plant which doubled only 1 plant out of 15 treated plantlets. After mitotic complement confirmation, the meiotic atlas was developed for synthetic *B. juncea* which had 36 chromosomes. The size of the cell was comparatively larger than its progenitors as well as the established allotetraploid *B. juncea*. During leptotene nicely spread chromatin makes a big ball like structure which condenses gradually in the later stages and finds the respective homologues (Figure 5-13). In pachytene chromosomes appeared synapsing with more than one chromosome and sometimes unpaired showing abnormal meiosis. GISH analysis revealed a mosaic of cells in a colchicine treated plant, as a mixture of diploid and tetraploid cells were observed in anthers. Nearly 50% of the cells' complement was 36 while the remaining half just like the hybrid parent. In the tetraploid plant,16 chromosomes of *B. nigra* were detected in red colour and 20 chromosomes of *B. rapa* genome by GISH (Figure 5-12).

Moreover, the flower buds were also different from the parent hybrid plant and observed as quite swollen buds (Figure 5-9 F, G). And also The plant was sterile and could not produce viable seed.

Table 5-3 Colchicine treatment of allodiploid plant (AB)

Cells analysed	Diploid like	tetraploid like	abnormal
12	4	5	3

Out of 12 mitotic cells, 4 were observed as its parent, i.e. allodiploid (AB), 5 had a double chromosomal complement of their parent, i.e. AABB, while 3 abnormal cells were observed which were not doubled properly and showed an abnormal number of chromosomes.

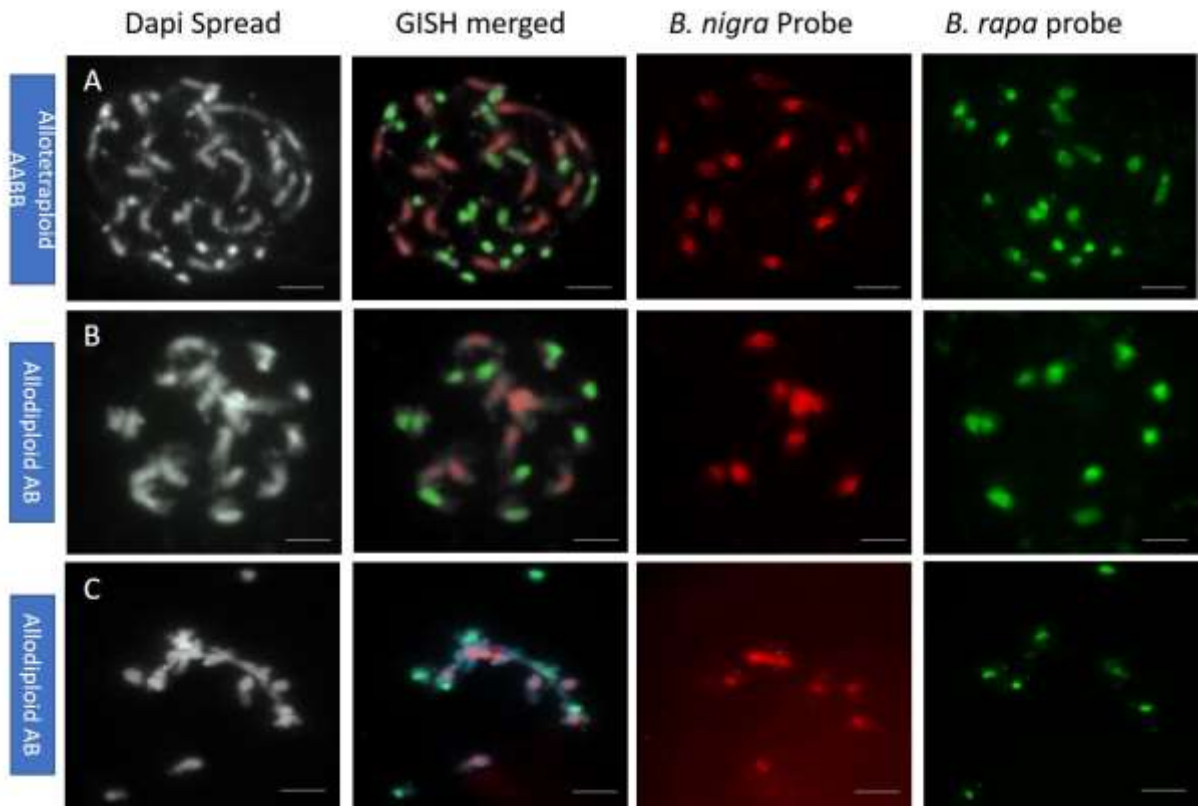


Figure 5-12 GISH analysis of synthetic *B. juncea* (AABB),

(A) Mitotic metaphase of colchicine-treated allotetraploid *B. juncea* complement (AABB), (B) Mitotic metaphase of colchicine-treated allodiploid *B. juncea* complement (AB), (C) Meiotic metaphase-I of colchicine-treated allopolyploid *B. juncea* (AABB). Scale bar 10 µm.

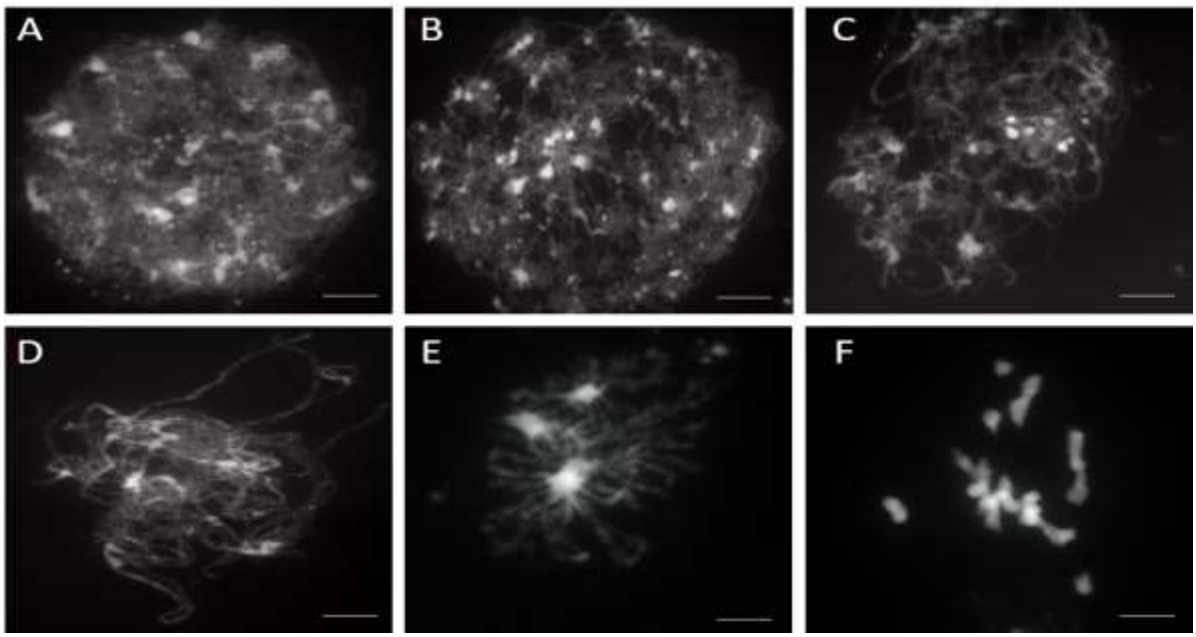


Figure 5-13 Meiotic atlas of artificially produced *B. juncea* by the application of colchicine. Early leptotene, (B) late leptotene, (C) zygotene, (D) Pachytene, (E) Diplotene and (F) Diakinesis. Scale bar 10 µm.

5.8 Immunolocalization

The synthetic polyploid plant was further studied using immunochemistry to analyse axis proteins dynamic during the early phase of meiosis. An incomplete pairing between the respective genomes was obvious from the atlas, so its synaptonemal complex polymerization was studied using ASY1 and ZYP1 protein. A linear signal for the lateral elements of the SC in the form of ASY1 was observed while ZYP1, the central elements were distorted and never had full linear polymerization between the homologues. When measured on image J the filament was far less than the established *B. juncea* which exhibited full polymerization of the transverse element of SC. The progression of meiosis was further tracked with ZYP1 and

MLH1 antibodies to analyse the cross over frequency and found only a few foci for MLH1 which showed the inference-based CO in newly synthesized polyploid. Since the pairing and recombination did not comply with the expectation nor with the naturally existing allotetraploid, there were no seeds and siliques were empty. Abnormal meiosis and the alignment of chromosomes could explain the reason behind such sterility in the newly formed polyploid plant.

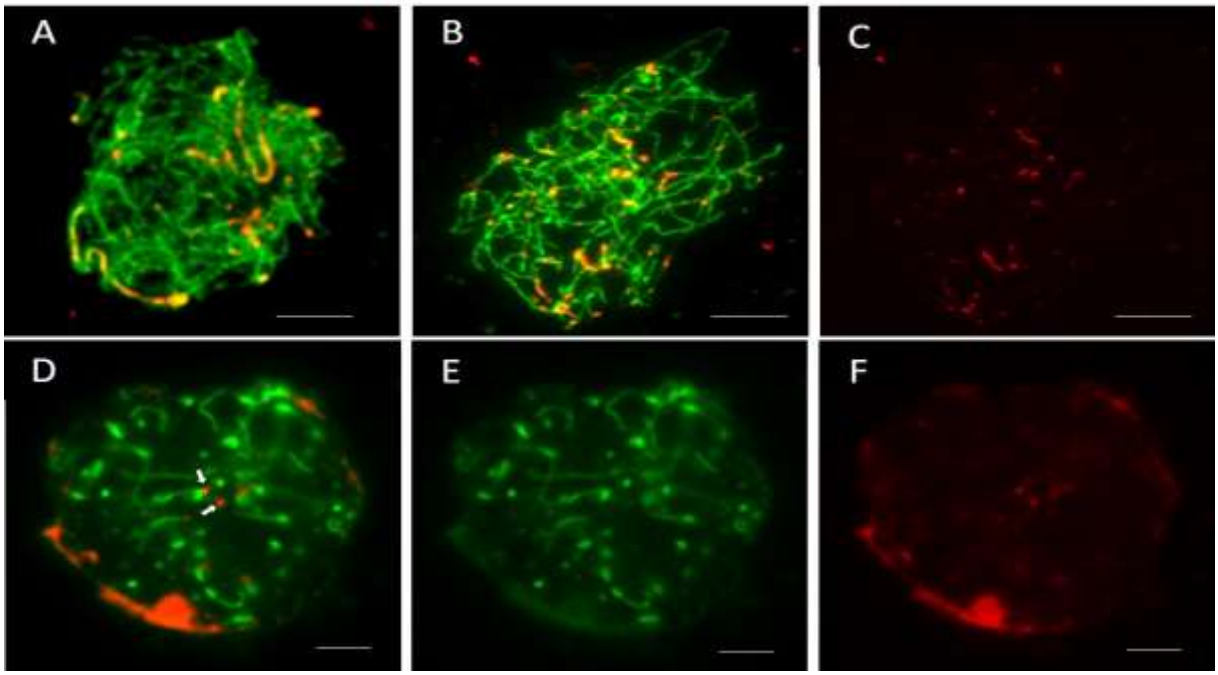


Figure 5-14 Immunolocalization of synthetic allotetraploid (AABB)

with ASY1, ZYP1 and MLH1 proteins. (A, B) Showing ASY1 and ZYP1 in zygotene in green and red respectively, (C) fragmented ZYP1 filaments in red, (D, E) showing ZYP1 and MLH1 in white arrows during pachytene in green and red respectively and (F) MLH1 foci in red. Scale bar 10 µm.

5.9 Discussion

5.9.1 Wide hybridization

Genome multiplication and hybridization had an integral role in plant speciation which increased their genetic basis in the evolutionary past. Majority of the sexually reproducing angiosperm (i.e. 80% in total) have experienced round of genome duplications during their evolution. To understand the duplication phenomenon U's triangle is quite rich in diploid (three species) and allotetraploid (three) species to help in understanding its roots in relation to the evolution and stabilization of polyploids.

Out of three diploids in U's triangle, *B. nigra* and *B. rapa* were analysed in the current studies These evolved differently in separate lineages during the ancient times with a gap of 4.24Mya in between them (Figure 1-2). The polyploidization event steered these genomes in different lineages to enable them to develop into separate diploid reproductive units with *B. nigra* (N=8) and *B. rapa* (N=10). Owing to the differences at the genomic level between the two, compatibility among them is significantly low, and a hybrid between these two does not exist in nature very often. On the other hand, both genomes contribute to existing allotetraploid where two copies of each genome co-exist to help them behave like a typical diploid during homologous pairing in meiosis-I to generate viable gametes for the continuation of successive generations.

The present study was carried out to elucidate the hybridization event occurring between two distant genomes with the idea of improving the genetic basis of economically important brassicas (Sosnowska and Cegielska-Taras, 2014). The genomes incompatibility was one of the constraints for prolific interspecific hybridization which keeps them separate during meiosis to carry on transgenerational inheritance. The constituent genomes would be compatible for within breeding with limited or restricted cross-species acceptability (Mallet, 2005).

Low seed setting was observed during reciprocal crosses between the distant *B. nigra* and the *B. rapa* genomes during this study. When *B. rapa* flower was emasculated and made to act as a female in a cross, the ovary led to the formation of viable seeds but failed to develop into seeds in another way around cross when it was used a pollen source. The failure of *B. nigra* acting as female could be attributed to the smaller size of the ovary in addition to well defined isolating mechanisms of pre- and post-zygotic obstacles. The pollen failed to fertlize with the ovule to develop embryo because of genetic incompatibilities during prezygotic aberrations (Franklin-Tong and Franklin, 2000) while in post-zygotic conditions, the embryo is formed after fertilization but cannot develop into seed due to lack of a fostering endosperm (Zenkteler, 1990). The efficiency of hybrid (AB) production between *B. rapa* (AA) and *B. nigra* (BB) was as low as 0.05% which is less considerably than the crosses between *B. rapa* and *B. oleracea*, i.e. 0.13% (Sosnowska and Cegielska-Taras, 2014). This also highlights the relatedness among the species lineages as *B. rapa* and *B. oleracea* belongs to the same lineage in comparison to *B. nigra* which evolved separately before *B. rapa* (Figure 1-2). This failure was believed to happen because of the chromosomal incompatibilities, but surprisingly it was fruitful when the ovary bearing plant (mother) had higher chromosomes numbers than its pollen bearing counterpart

(male). The behaviour seems well in line with those of the results obtained in a cross between *B. oleracea* and *B. rapa* when later with 10 chromosomes used as female (Olsson, 1960). The dead embryo in (Fig 1.14) hints that pollen succeeded in fertilizing the ovary overcoming prezygotic barrier but the malfunctioning endosperm (subtype in double fertilization) failed to nourish the embryo to develop into a viable seed during postzygotic obstacle. The number of aborted seeds were considerably higher due to postzygotic barriers in this wide hybrid (AB).

GISH analysis was employed to confirm the constituent sub-genomes in subsequent generation and their respective behaviour during cellular divisions; mitosis and meiosis (Ali *et al.*, 2004). In the mitotic cell, a number of chromosomes (i.e.18) in a cell were intermediate between their parental genomes (i.e. 16 & 20) and GISH was effective in discriminating the individual genome (Figure 5-3). The differentiation allowed precise analysis of the respective sub-genomes in meiotic pairing phase when chromosomes exchange genetic material between them. The meiotic atlas of hybrid AB was generated (Figure 5-4) and supplemented with the GISH analysis which revealed autosyndesis and allosyndesis occurring simultaneously within the genome and between the genomes respectively. Autosyndesis refers to the pairing within the genome; intragenomic while allosyndesis is the pairing between different chromosomes sets (Figure 5-4 d) so it is intergenomic interaction at the chromosomal level. In the hybrid AB plant, allosyndesis was prevalent which replaced homologous recombination instead and adopted homoeologous recombination with fewer autosyndesis and univalents which remained unpaired. Since both genomes were stemmed from the ancient triplication of basic genome, they retained a certain level of homology among the chromosomes structure and gene content which facilitated the autosyndetic pairing between them (Mason *et al.*, 2010).

It is believed that allosyndesis is affected by the genomic complexity and their preferences to combine with each other which is under genetic control. Since only haploid genomes were present in the hybrid AB plant, autosyndesis was likely to occur within individual genomes in addition to allosyndesis (Figure 5-4), and it showed that *B. rapa* had more tendency to have autosyndesis as compared to *B. nigra* (Mason *et al.*, 2010). The results were in agreement with Mason *et al.*, (2010) where autosyndesis in B genome was suppressed by A (and C genome). The frequency of allosyndesis was far higher than the autosyndesis in hybrid (Szadkowski *et al.*, 2011) since genome B did not show any sign of any autosyndesis in my GISH analysis.

The homoeologous pairing between A and B genome provides an excellent opportunity to introgress genes across the species to improve genetic basis by introducing important genes, e.g. in this case disease or drought resistant gene from *B. nigra* to *B. rapa* and other related allotetraploid crops. Such relationship between chromosomes was revealed by FISH & GISH analysis which determined individual genomes, their corresponding tendencies and associations.

Relatively fewer genomes were found involved in autosyndetic pairing in addition to univalent appearances than the allosyndesis in the hybrid plant (Mason *et al.*, 2010) in their studies. During metaphase-I, 6 allosyndetic pairs were detected between *B. nigra* and *B. rapa* genomes while 4-5 univalents are also observed which contributed to the non-disjunction and asymmetric division of chromosomes in anaphase-I. Chromosomal configuration during metaphase-I was different from those of its predecessor as trivalents were also found which revealed auto- and allosyndetic association in the same structure where *B. rapa* chromosomes developed

autosyndetic pairing with the chromosome of another subgenome, *B. nigra* chromosome via allosyndetic pairing. Allosyndetic was more dominant than the autosyndetic which was observed for *B. rapa* only in the hybrid plant and 1-2 autosyndesis for *B. rapa* per cell in hybrid vs *B. nigra*, as observed (Mason *et al.,* 2010)

Hybridization has a significant role in the evolutionary course as it brings back ortholog genes to the homeologues in the alloploid complement. Orthologs are the genes which diverged as a consequence of speciation event in the past (Glover *et al.,* 2016). Since two different genomes are involved, the meiotic aberration is more likely to occur during the pairing phase owing to limited conservation of the gene content and the structure between the two. The incompatibility of the subgenomes in the hybrid had readily been observed during pachytene stage where chromosomes were involved in limited synapsis confined to shorter regions in contrast to a complete pairing in its progenitors. Owing to limited complementarity, only a few chromosomes could find an affinity to each other and develop transient pairing while some of the chromosomes were seen unpaired in pachytene stage, which would form univalent hereafter. With immunolocalization analysis, it was further revealed that subgenomes developed transitory pairing between them and the transverse element of the SC was also fragmented and found bulging at certain points where SC had just started to polymerize.

Late in metaphase-I, an average of 4 bivalents were observed in a hybrid between the subgenomes, the rest of the chromosomes were unpaired and proceeded as univalents. The behaviour of chromosomes contributed to the improper orientation at metaphase which yielded asymmetric segregation of chromosomes causing aneuploidy and consequently sterility in the

allodiploid (Baptista-Giacomelli *et al.*, 2000). During the disjunction of chromosomes at anaphase-I, nearly 4 fragments on average were observed which persisted till the end of meiosis-II. The fragments were likely to be a remnant of unrepaired DSB, and perhaps the loose connections left away from the polar aggregate at the end of meiosis-I (Acilan *et al.*, 2007).

The sequential FISH revealed that the fragments were not repetitive sequences of the nucleolar region by its nature which could be expressed constitutively for the synthesis of the ribosome as it failed to hybridize with 45S rDNA probe. During leptotene, chromatin undergoes extensive breakage due to the action of SPO11 yielding DSBs which are then repaired in subsequent stages through different pathways to produce characteristic COs and NCOs. Since there were no homologous sequences available for repairing correctly, chromosomes developed transient loose connection which could not withstand the kinetochore pull during the post-metaphase-I stage.

The findings prompted me to investigate the behaviour of telomere at the start of the process to analysis the pairing initiation. The FISH analysis with telomere probe showed the unpaired chromosomes which were scattered in the nucleus in leptotene and came close to each other in zygotene where an incomplete bouquet configuration was observed, though pairing was not perfect like the diploid parents but restricted to few chromosomes. In pachytene-like-stage the number of the telomere foci were 25 rather than 18 as would be expected assuming that all of the telomeres are paired up in the AB hybrid suggesting incomplete pairing between the chromosomes and the possible cause of univalent in metaphase-I and asymmetric disjunction

afterwards. Because of the meiotic irregularities, allodiploid (AB) failed to produce any viable seed to give subsequent generations.

When evaluated with the immunolocalization of the proteins ASY1 and ZYP1 in prophase-I, the plant exhibited normal axis development for the lateral component of the SC in the form of ASY1 filament during leptotene. In later stages during zygotene indication of limited synapsis was witnessed by the ZYP1 installation which polymerized randomly between the chromosomes. The chromosomes failed to find their corresponding partners to juxtapose completely as observed in the parental lines. The ZYP1 polymerization terminated and was recorded as fragmented ZYP1 filaments which ranged from discrete foci to few microns when measured on the Image J. The intensity of the ZYP1 filament was not uniform rather bulging at certain points aggregating meiotic problems. Further CO analysis was done with MLH1 protein which is thought to resolve double Holliday Junction (dHJ) to yield CO, and only a few loci were detected in the allodiploid.

Wide hybridization has played a significant role in plant speciation (Oleszczuk and Lukaszewski, 2014) which involved bringing distant parents into a single cell. Several attempts have been made to synthesize a neo-polyploid which has been failed due to incompatibilities among chromosomes and meiotic mis segregation. The plant was then treated with a chemical, colchicine, to induce genome doubling by inhibiting spindle formation during the first half of the meiosis. The efficiency of producing synthetic allotetraploid was quite low, and only one plant survived out of 15 treated plants (i.e. 6.67%). Two different treatment methods were tested, and only root-treated plants could produce the synthetic tetraploid (Lionneton *et al.*,

2001) while bud treatment was unsuccessful in contrast to the faithful doubling in Arabidopsis (Santos *et al.*, 2003). The doubling effect of the cell was confirmed on mitotic cell initially followed by GISH analysis of both mitotic and meiotic cell which revealed twice as many chromosomes found in the hybrid. A mosaic of allodiploid and allotetraploid configuration was observed with a ratio of 1: 5, hinting that some tissues could not take up the colchicine via roots while most of the tissues got doubled up. When buds were dissected to see the meiosis progression in the treated plant, it revealed irregularities involving improper pairing between the chromosomes suggesting that chromosomes failed to find their corresponding partners. Chromosomes were seen unpaired in the pachytene stage and were further corroborated by the univalent presence during metaphase-I which lacked proper bivalent structures (Ahirwar and Verma, 2015). A mixture of multivalent and univalents were witnessed during later stages which potentially caused sterility in the plant. The abnormal structures of chromosomes resulted in the asymmetric division to produce no seed for the next generation. The genomic and epigenetic effect might have played a role in the stabilising the newly formed allotetraploid in nature.

In my plant, the chromosomes arms are seen switching their partners and therefore associate with more than one chromosome at one time which deregulate the normal meiotic progression to have faithful disjunction of chromosomes at the end of meiosis. The chromosomes adopted an unusual configuration for segregation where univalents and multivalents were also observed in addition to chromosomal fragments. The phenotype has a resemblance with what found in FIGL mutant in rice (Zhang *et al.*, 2017) and FANCM in Arabidopsis (Crismani *et al.*, 2012).

Several genomic and epigenetic factors are held responsible for facilitating such a transition during evolution which is yet to be exploited in detail (Renny-Byfield and Wendel, 2014). Since brassica polyploid species have distinct genomes, it features inter genomic cross over occurring among related chromosomes in addition to the standard intragenomic recombination, which stimulates certain evolved phenotypic expression than the parents (Lloyd *et al.*, 2017). This leads to a plethora of questions about the loss and replacement of genomic fragments and its subsequent effect on the transcriptome, which is poorly understood in allopolyploids. Sub functionalization of the duplicated genome along with epigenetic marks (DNA methylation, histone acetylation) are crucial in adopting new role in cellular machinery of the polyploids. A more radiated transcriptome has been reported to correlate with the greater rearrangements in the genome (Adams, 2007; Adams and Wendel, 2005).

5.9.1.1 Summary

During my studies, I have shown cross-species hybridization occurring between distant genomes which are difficult to get into the natural environment. Some facts were revealed about homoeologous recombination happening between different genomes. Chromosomes incline to find pairing with more than one homologues and exchange partners frequently to attain stability for equational division. In the hybrid plant, I observed that *B. rapa* genome experienced more autosyndesis which could be one of the likely factors in the instability in wide hybrids. Whereas the recombination between different genomes opens opportunities for the introgression of a gene across species during allosyndesis.

6 General Discussion

A set of experiments were designed during this study to evaluate meiosis in Brassica diploids and allotetraploids in an attempt to transfer knowledge from model plant *A. thaliana* to crop species. Meiosis is a core phenomenon in plant breeding which renders variation and novelty in subsequent generations via genetic recombination. Some facts were unveiled about allotetraploid species *B. juncea* which is an amphidiploid of diploid progenitors (*B. nigra* and *B. rapa*) and had very strict pairing control mechanism operating under normal conditions. Polyploids are often associated with increased vigour of the crops as compared to their progenitors (Comai, 2005) and contribute significantly in different forms in our daily life, e.g. three brassica amphidiploids species are used for oil and vegetable sources and others are found in the production of coffee, cotton, fruits, cereals etc. Polyploids are believed to form as a result of chromosomes rearrangement (inversion or addition) which changes genes expression and associated phenotype. Brassica diploids evolved by extensive triplication of their ancestral genome and possess 24 conserved blocks in different orders (Cai *et al.*, 2014).

Brassica is an important oilseed crop second to Soybean with annual production of 68.52 million metric tons worldwide (Brassica.info, 2018). Several attempts had been made to improve and enhance the genetic base of this important genus with varying success. The genus is a very interesting candidate in the field of genetics because it encompasses diploids as well as allotetraploid species interlinked in a model known as U's triangle.

Due to extensive cultivation of polyploid and some diploids, *B. rapa* and *B. oleracea*, its relative diploid, *B. nigra* was studied at a lower level and as a consequence incomplete database exists for this species (Navabi *et al.,* 2013). Realising its importance in an economically important crop because of its ability to withstand biotic and abiotic stress, attempts are in progress to sequence this genome and exploit the genetic potential in crop improvement programmes. Although all three diploid genomes had been evolved from the common ancestor, but *B. nigra* diverged along the evolutionary course and adopted a distinct lineage for itself leaving *B. rapa* and *B. oleracea* together in the other lineage. Therefore, due to greater similarity in their sequences higher homology exists between these genomes than the *B. nigra*. To pursue its importance in the genus, a preliminary data were obtained during this attempt which enabled me to identify the chromosomal complement of the genome using *in situ* hybridization. The ideotype developed showed a gradient of length across them and had revealed that 4 out of the total 8 chromosomes were identified with ribosomal DNA markers. In comparison with the other diploid genomes, *B. nigra* had 5S rDNA on a separate chromosome while in other two genomes, 5S rDNA (which is supposed to be more conserved marker than the 45S rDNA) always shares its loci on the same chromosome carrying a 45S rDNA as also been observed in *S.cerevisiae* (Hasterok *et al.,* 2001). The organization was quite different from the rest of its family including *A. thaliana* where 5S rDNA was found with 45S rDNA on the same chromosome. Since both markers are concerned with the biogenesis of ribosome which translates mRNA into a polypeptide chain in the cytoplasm, the copy number has a direct influence on the total proteome of the cell, but if the spatial organization has any known effect, it is still elusive and debatable. Comparing genomic size revealed that *B. nigra* genome was

more compact than the *B. rapa* and *B. juncea* genome. On average 79 Mbp per chromosome was estimated for *B. nigra* against 52.9 Mbp for *B. rapa* and 59.33 Mbp per chromosome which might have any role in the expression profile of the genome and the access to translation machinery, although no concrete evidence exists to date. Interestingly 50% of the genome was identified in the diploids but in the allotetraploid which might have undergone genome restructuring and reorganization have fewer chromosomes with the rDNA makers in our studies. This begs serious attention and efforts to be done for unravelling the hidden facts in the organization of the polyploid genome.

For the evaluation of polyploid, I needed to know the behaviour of each subgenome, therefore GISH was developed using the nuclear genome after necessary processing. The hybridization reinforced the constituent subgenomes which was later used to identify an association between different genomes during meiosis. In *B. juncea,* two distant genomes paired up strictly with their homologue for proper segregation for giving viable gametes, however some traces of homoelogous recombination were observed during GISH analysis for one chromosome as a result more than expected signals were detected for *B. nigra* in *B. juncea* background while *B. rapa* has not deviated from its expected numbers. The observation leads us to postulate that introgression of the gene into *B. rapa* genome is a more feasible option than into *B. nigra* genome for crop improvement programmes as *B. carinata* showed a number of foci parallel to its progenitors. Increased phenotypic expression was evidence of increase hybrid vigour in the allopolyploid (Marfil *et al.*, 2018) which would motivate us in addressing food security issues in the near future by raising synthetic allopolyploid plants to increase the heterogeneity. Moreover, an even distribution of repetitive sequence families was detected for the A

subgenome of *B. juncea* in contrast to the scattered one in the B genome which is not uniform along the chromosome like that obtained by Maluszynska and Hasterok, 2005. The distribution of repetitive sequence could be one of the explanations for the more compact structure of *B. nigra* chromosomes as compared to *B. rapa* genome. The density of chromatin is usually correlated with the methylated state which is often silenced part of the genome and could be detected as showing strong hybridization during FISH as compared to euchromatin which is gene-rich region. Gene editing technology, like CRISPR, could be deployed to demethylate the region and relax the compaction in the genome to see the effect on overall phenotype and the gene expression.

The spatial chromatin configuration follows a hierarchical fashion where chromosomes undergo dynamic movement in addition to the molecular processing to align and find homologue chromosomes for the exchange of genetic material before its one-sided orientation of kinetochore could facilitate the canonical division. The organization and configuration of genomic DNA after DSBs induction into the nucleus relies on the regulatory layer of coordinated pathways to govern the fate of chromosomes. The significant changes chromatin undergoes from the prophase till the telophase operates under the genetic influence and associative factors for keeping the genome integrity intact. Alteration in genome organization would prove lethal for cell fate, and survival of species could be compromised. Any disruption during chromosomal alignment would compromise the meiosis to cause aneuploidy and arrest the cell potential.

The homologue pairing, a hallmark of meiosis, is requisite for the equal halving of chromosomes by the end of the first part of meiosis. It is initiated during the leptotene stage of the prophase-I where chromosome terminals are joined together in the process of telomere pairing before telomere clustering could be formed in an organized bouquet format. Like *A.thaliana*, a canonical bouquet is short lived in brassica species and a loose clustering of paired telomere could be visualized surrounding nucleolus (Armstrong *et al.,* 2001). The chromosomes are attached via SUN protein in the inner nuclear membrane to the KASH domain in the cytoplasm which oscillates the chromosomes facilitating in homology search. The replicated sister chromatids (of S phase) during this phase are aligned together with cohesion complex which is key regulator of segregation during the first part of meiosis. The cohesion complex is retained along the centromeric region while it is dissolved along with the arms which plays a crucial role in splitting the complement during anaphase-I (Zhang *et al.,* 2014).

The pairing between chromosomes was confirmed with immunochemistry during pachytene stage of meiosis where homologue juxtaposed with each other and exhibited complete synapsis from end to end by showing polymerization of the transverse element of Synaptonemal Complex (SC). The chromosomes are linked physically to their homologue during recombination via double Holliday Junction (dHJ) before it could resolve or dissolve to direct CO and NCO formation respectively. One-way exchange of genetic material is referred as NCO and causes gene conversion only while the two-way exchange is termed as a CO and creates reciprocal changes in DNA. There are two well-defined forms of CO happening in the genome, one which is dependent on the existence of a CO while the other is independent and could occur regardless of an already existing CO. The chromosomes condense gradually in subsequent

stages and during diplotene stage traces of mismatch protein MLH1 could be traced as discrete foci along the SC filament.

Interestingly in the diploids (*B. nigra* and *B. rapa*), the marks for CO were observed in the interstitial region while the distribution pattern was different in allotetraploid (*B. juncea*) where the CO was seen at the terminals of the chromosomes. The pairing time between the homologues could potentially be responsible for this change in the distribution pattern which occurs in the allopolyploid (*B. juncea*) where early recognition of chromosomes undergo pairing near telomeres. A similar explanation has been postulated where alleles of axis associated protein ASY1 might confer distal localization of CO in autopolyploid species (Chris Morgan thesis 2016). Moreover, the number of CO was also less in the allotetraploids than its progenitors collectively. The reduction and shifting of CO toward the end of chromosomes might have played a role in the stabilization of polyploid during evolution, but concrete efforts are needed to reinforce this hypothesis (Yant *et al.*, 2013) by studying more generations in the synthetic *B. juncea* One of the motives behind this hypothesis was the reason that the synthetic polyploids are often not stabilized while nature had adapted the system which has perfect normalization and diploidization in meiosis to yield stable gametes (Dorone, 2013). Insight into the stabilization of meiosis and its diploidization would be way forward in creating novelty and synthetic crop industry to cope with food security issues in the coming years.

Since allopolyploids represent a unique group of plants with more than one set of chromosomes in a cellular space, there exists an exciting and intriguing control centre that restricts pairing between homoeologous chromosomes and favours only interaction between identical

chromosomes (from the same set of chromosomes) as found in *B. napus* in the form of *PrBn* (Jenczewski *et al.,* 2003). Another analysis of such control has already been exploited in allopolyploid wheat by Moore *et al.,* 2012, who unveiled the locus responsible for such control, called Pairing Homoelogous 1 (*Ph1*). When the locus was deleted, the interaction between the homoelogous chromosomes had been increased which was coupled with chromosomes rearrangement enough for inducing infertility (Feldman and Levy, 2005). It was believed that the reason behind enhanced homoeologous pairing could be increased level of Cdk which had also effect on the replication of DNA and premature chromosomes condensation.

Parallel to such genetic control in meiosis had been exploited in allotetraploid Arabidopsis where a major QTL, referred to as Boy Named Sue (BNS) was found responsible for stable meiosis and control pairing between homologue. Similarly, in its close relative allotetraploid *B. napus*, *PrBn* was found responsible for the suppression of pairing among homoeologous chromosomes to ensure proper segregation which works in a complex manner (Jenczewski *et al.,* 2003) in *B. napus* this control was present on C genome so in *B. juncea* it would be interesting to decipher which subgenome confers this selective pairing and inhibit homoeologous recombination. Such a mechanism needs to be exploited further for taking control in polyploid for improving crop sciences to feed the human numbers.

Homology search relies heavily on chromosomes conformation and condensation pattern among different chromosomes (Prieto *et al.,* 2004). To understand and confirm the Cdk like activity which disturbs condensation in chromosomes, I used OA to mimic the absence of a locus, similar to *Ph1*, and to enhance Cdk activity. OA treatment is also interesting in the

context that it could turn on and turn off the control of homologous and homoeologous recombination that could potentially produce higher genetic diversity, a desirable feat in plant breeding.

The chromosomes in allotetraploid, despite the presence of related chromosomes paired strictly with homologues instead which lies parallel with the genetic control of wheat and *B. napus*. Treatment with okadaic acid caused genomic instability in allopolyploid species and instigated altered condensation which might have occurred because of inhibiting phosphatases thereby, enhancing the phosphorylation of certain amino acid (serine and threonine) of polypeptides. Future studies in this direction would help us in inducing homoeologous recombination and understanding the nature of preferential pairing. Different genetic approaches to screen for homoeologous recombination would also be beneficial in deciphering the mechanism which suppresses homoeologous recombination in *B. juncea* to help us understand more fully the stabilization factor in allopolyploids.

Distant hybridization was performed between different genomes to study the meiosis and for the synthetic allopolyploid. Contrary to normal meiosis in parents, the hybrid exhibited different chromosomal behaviour at leptotene and zygotene where unpaired telomeres were detected. Pairing was also not complete, and immunochemistry revealed fragmented SC filament during post-zygotene phase (near pachytene stage as it lacked synapsis). Although partner switching was observed with DAPI, GISH analysis unveiled the autosyndetic and the allosyndetic association of chromosomes in allodiploid AB plant. The homoeologous association was evidenced in M-I which is promising from a plant breeding point of view that

cross-species association could create a novel allelic combination in addition to introgression of certain genes. Anaphase-I showed traces of chromosomes as fragments which hints at improper repairing of DSBs formed earlier in prophase-I. The phenotype was similar to some meiotic mutant like in figl, recq4 and fancm in *A. thaliana* (Fernandes *et al.,* 2018). One of the explanations of such kind of failure in DSBs repair could be an allelic variation that did not work appropriately to recombine, and unrepaired DSBs were left out when cohesins along the arms were released. Moreover, the condensation pattern of different genomes in the allotetraploid background renders unrepaired segments which would be visualized as lesions and are lethal to cells. Subtracting subgenomes and evaluating them individually would help us answer some of the questions regarding allelic expression and the repair of the DSBs.

Due to meagre success ratio of the crosses between these distant genomes, studies were confined to limited experiments, but with plentiful plants, further studies could potentially elucidate homeolog pairing which would improve breeding strategies. Doubling hybrid genomes would also be interesting for producing high yield synthetic polyploids which are often related with high yield in the field. High level of the homoelogous recombination was observed in synthetic *B. juncea* which might lack strict genetic control over homologue pairing only. In addition to the crop improvement programs, hybrids also provide an opportunity to study the meiotic pairing and segregation. To study the recombination in the mutant form in polyploid, CENH3 technology would be deployed to reduce the genome to half first and then doubling will give a homozygous form for studying pairing in the synthetic. Comparison of the synthetic with the natural will be a valuable source of information for elucidating and uncovering steps involved in successful meiosis.

Newly formed polyploids also feature a higher frequency of multivalents during meiosis (Yant *et al.,* 2013). The complex structure is further correlated with mis-segregation and reduced fertility, and in my studies, it did not produce any seeds which were mainly because of abnormal meiosis which could not give viable pollen to yield any seed at the end.

Owing to the economic importance of polyploid crops, understanding of meiosis would be of great interest to the breeders and researcher. In conclusion, using a wide range of experiments enabled me to identify and develop partial karyotype of *B. nigra* species which would prove useful for subsequent research. Using the GISH technique, homoeologous traces of *B. nigra* into *B. rapa* were detected which pave the way for further research for the transfer of useful genes (like drought & insect resistant). The pattern of CO was also observed different in complex genomes which could be studied to understand the difference between the alleles functions. Homoeologous recombination is useful for transferring desirable traits across species and increases heterogeneity which may have a greater market value and improve farmer economy. A detailed analysis could unleash facts about the preferential pairing control of homologue pairing in allotetraploid brassicas which would have high impact and implementation in plant breeding.

7 References

Able, J. A., Crismani W. and Boden S. A. 2009. Understanding meiosis and the implications for crop improvement. Funct Pl Bio, 36: 575-588.

Acilan, C., Potter, D. M. and Saunders, W. S. 2007. DNA repair pathways involved in anaphase bridge formation. Gen Chrom Canc, 46: 522-531.

Adams, K. L. 2007. Evolution of duplicate gene expression in polyploid and hybrid plants. J Hered, 98: 136-41.

Adams, K. L. and Wendel, J. F. 2005. Polyploidy and genome evolution in plants. Curr Opin Plant Biol, 8: 135-41.

Ahirwar, R. and Verma, R. C., 2015. Colchicine induced asynaptic chromosomal behavior at meiosis in Allium cepa L. The Nucleus, 58: 47-51.

Ali, H. B., Lysak, M. A. and Schubert, I. 2004. Genomic in situ hybridization in plants with small genomes is feasible and elucidates the chromosomal parentage in interspecific Arabidopsis hybrids. Genome, 47: 954-960.

Anamthawat-Jónsson, K., Schwarzacher, T., Leitch, A. R., Bennett, M. D. and Heslop-Harrison, J. S. 1990. Discrimination between closely related Triticeae species using genomic DNA as a probe. Theor Appl Genet, 79: 721-728.

Anderson, L. K. and Stack, S. M., 2005. Recombination nodules in plants. Cytogenet Genome Res, 109: 198-204.

Anderson, L.K., Hooker, K.D. and Stack, S.M. 2001. The distribution of early recombination nodules on zygotene bivalents from plants. Genetics, 159(3) : 1259-1269.

Aravind, L. and Koonin, E. V. 1998. The HORMA domain: a common structural denominator in mitotic checkpoints, chromosome synapsis and DNA repair. Trends in Biochem Sci, 23: 284-286.

Arias, T., Beilstein, M.A., Tang, M., McKain, M.R. and Pires, J.C. 2014. Diversification times among Brassica (Brassicaceae) crops suggest hybrid formation after 20 million years of divergence. American J of Bot, 101(1) : 86-91.

Armstrong S.J.,, Caryl A.P., Jones G.H. and Franklin F.C.H. 2002 ASY1, a protein required for meiotic chromosome synapsis, localizes to axis-associated chromatin in Arabidopsis and Brassica. J of Cell Sci, 115:3645-3655.

Armstrong, S. J. 2013. A time course for the analysis of meiotic progression in Arabidopsis thaliana, Humana Press, Totowa, NJ.

Armstrong, S. J., Franklin, F. C. H. and Jones, G. H. 2001. Nucleolus-associated telomere clustering and pairing precede meiotic chromosome synapsis in Arabidopsis thaliana. J of Cell Sci, 114: 4207-4217.

Armstrong, S. J., Fransz, , Marshall, D. F. and Jones, G. H. 1998. Physical mapping of DNA repetitive sequences to mitotic and meiotic chromosomes of Brassica oleracea var. alboglabra by fluorescence in situ hybridization. Hered, 81: 666-673.

Armstrong, S.J., Caryl, A.P., Jones, G.H. and Franklin, F.C.H., 2002. ASY1, a protein required for meiotic chromosome synapsis, localizes to axis-associated chromatin in Arabidopsis and Brassica. J of Cell Sci, 115(18) : 3645-3655.

Armstrong, S.J., Franklin, F.C.H. and Jones, G.H., 2001. Nucleolus-associated telomere clustering and pairing precede meiotic chromosome synapsis in Arabidopsis thaliana. J of Cell Sci, 114(23) : 4207-4217.

Attia, T. and Röbbelen, G., 1986. Cytogenetic relationship within cultivated Brassica analyzed in amphihaploids from the three diploid ancestors. Canad J of Genet and Cytol, 28(3): 323-329.

Axelsson, T., Bowman, C. M., Sharpe, A. G., Lydiate, D. J. and Lagercrantz, U. 2000. Amphidiploid Brassica juncea contains conserved progenitor genomes. Genome, 43: 679-688.

Baptista-Giacomelli, F. R., Pagliarini, M. S. and De Almeida, J. L. 2000. Meiotic behavior in several Brazilian oat cultivars (Avena sativa L.). Cytologia, 65: 371-378.

Barchi, M., Roig, I., Di Giacomo, M., De Rooij, D. G., Keeney, S. and Jasin, M. 2008. ATM promotes the obligate XY crossover and both crossover control and chromosome axis integrity on autosomes. PLoS Genet, 4: e1000076.

Bardsley D, Cuadrado A, Jack P, Harrison G, Castilho A, Heslop-Harrison JS. 1999. Chromosome markers in the tetraploid wheat Aegilops ventricosa analysed by in situ hybridization. Theor Appl Genet, 99(1-2): 300−304.

Baudat, F. and de Massy, B., 2007. Regulating double-stranded DNA break repair towards crossover or non-crossover during mammalian meiosis. Chrom Research, 15(5): 565-577.

Baudat, F., Imai, Y. and De Massy, B., 2013. Meiotic recombination in mammals: localization and regulation. Nat Rev Genet, 14(11): 794-806

Bennett, M.D., Finch, R.A. and Barclay, I.R., 1976. The time rate and mechanism of chromosome elimination in Hordeum hybrids. Chromosoma, 54(2): 175-200.

Bertuch, A. A. 2002. Telomeres: the molecular events driving end-to-end fusions. Curr Biol, 12: R738-R740.

Bhalla, N. and Dernburg, A.F., 2008. Prelude to a division. Ann Rev of Cell and Develop Biol, 24: 397-424.

Boateng, K. A., Bellani, M. A., Gregoretti, I. V., Pratto, F. and Camerini-Otero, R. D. 2013. Homologous pairing preceding SPO11-mediated double-strand breaks in mice. Dev Cell, 24: 196-205.

Branca F. and Cartea E. 2011. Brassica C. Kole (ed.), Wild Crop Relatives: Genomic and Breeding Resources, Oilseeds, Springer.

Cai, X. and Xu, S.S., 2007. Meiosis-driven genome variation in plants. Curr Genom, 8(3): 151-161.

Cai, G., Yang, Q., Yi, B., Fan, C., Edwards, D., Batley, J. and Zhou, Y. 2014. A complex recombination pattern in the genome of allotetraploid Brassica napus as revealed by a high-density genetic map. PLoS One, 9: e109910.

Cavell A. C., Lydiate D. J., Parkin I. A., Dean C. and Trick M. 1998. Collinearity between a 30-centimorgan segment of Arabidopsis thaliana chromosome 4 and duplicated regions within the Brassica napus genome. Genome 41: 62–69.

Ceccaldi, R., Rondinelli, B. and D'Andrea, A.D., 2016. Repair pathway choices and consequences at the double-strand break. Trends in Cell Biol, 26(1): 52-64.

Celeste, A., Fernandez-Capetillo, O., Kruhlak, M.J., Pilch, D.R., Staudt, D.W., Lee, A., Bonner, R.F., Bonner, W.M. and Nussenzweig, A., 2003. Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. Nat Cell Biol, 5(7): 675.

Chakraborty, , Pankajam, A.V., Lin, G., Dutta, A., Krishnaprasad, G.N., Tekkedil, M.M., Shinohara, A., Steinmetz, L.M. and Nishant, K.T., 2017. Modulating crossover frequency and interference for obligate crossovers in Saccharomyces cerevisiae meiosis. G3: Genes, Genomes, Genetics, 7(5): 1511-1524.

Chambon, A., West, A., Vezon, D., Horlow, C., De Muyt, A., Chelysheva, L., Ronceret, A., Darbyshire, A., Osman, K., Heckmann, S., Franklin, F. C. H. & Grelon, M. 2018. Identification of ASYNAPTIC4, a Component of the Meiotic Chromosome Axis. Plant Physiol, 178: 233-246.

Chan, S.W., 2010. Chromosome engineering: power tools for plant genetics. Trends In Biotech, 28(12): 605-610.

Chang, H. H. Y., Pannunzio, N. R., Adachi, N. and Lieber, M. R. 2017. Non-homologous DNA end joining and alternative pathways to double-strand break repair. Nat Rev Mol Cell Biol, 18: 495-506.

Chauhan J. S., Singh K. H., Singh V. V. and Satyanshu K. 2011. Hundred years of rapeseedmustard breeding in India: accomplishments and future strategies. Ind J of Agr Sci, 81(12): 1093 – 1109.

Chelysheva, L., Vezon, D., Chambon, A., Gendrot, G., Pereira, L., Lemhemdi, A., Vrielynck, N., Le Guin, S., Novatchkova, M. and Grelon, M., 2012. The Arabidopsis HEI10 is a new ZMM protein related to Zip3. PLoS Genet, 8(7): e1002799.

Cheng, F., Liu, S., Wu, J., Fang, L., Sun, S., Liu, B., Li, P., Hua, W. and Wang, X., 2011. BRAD, the genetics and genomics database for Brassica plants. BMC Plant Biol, 11(1): p.136.

Cheng, F., Wu, J. and Wang, X., 2014. Genome triplication drove the diversification of Brassica plants. Hort Res, 1: 14024.

Cohen, , Holmes, C. F. and Tsukitani, Y. 1990. Okadaic acid: a new probe for the study of cellular regulation. Trends Biochem Sci, 15: 98-102.

Coutinho L.A., 1952 Possibilitadades taxonomicas da citogenetica. Gen. Iber., 4: 21-42.

Crismani, W., Girard, C. and Mercier, R. 2013. Tinkering with meiosis. J Exp Bot, 64: 55-65.

Culligan, K. M. and Hays, J. B. 2000. Arabidopsis MutS Homologs—AtMSH2, AtMSH3, AtMSH6, and a Novel AtMSH7—Form Three Distinct Protein Heterodimers with Different Specificities for Mismatched DNA. The Plant Cell, 12: 991-1002.

Da Ines, O., Abe, K., Goubely, C., Gallego, M. E. & White, C. I. 2012. Differing requirements for RAD51 and DMC1 in meiotic pairing of centromeres and chromosome arms in Arabidopsis thaliana. PLoS Genet, 8: e1002636.

Da Ines, O., Gallego, M. E. and White, C. I. 2014. Recombination-independent mechanisms and pairing of homologous chromosomes during meiosis in plants. Mol Plant, 7: 492-501.

De los Santos, T., Hunter, N., Lee, C., Larkin, B., Loidl, J. and Hollingsworth, N.M., 2003. The MUS81/Mms4 endonuclease acts independently of double-Holliday junction resolution to promote a distinct subset of crossovers during meiosis in budding yeast. Genetics, 164(1): 81-94.

De Massy, B., 2013. Initiation of meiotic recombination: how and where? Conservation and specificities among eukaryotes. Ann Rev of Genet, 47: 563-599.

Dernburg, A.F., McDonald, K., Moulder, G., Barstead, R., Dresser, M. and Villeneuve, A.M., 1998. Meiotic recombination in C. elegans initiates by a conserved mechanism and is dispensable for homologous chromosome synapsis. Cell, 94(3): 387-398.

Edlinger, B. and Schlogelhofer, 2011. Have a break: determinants of meiotic DNA double strand break (DSB) formation and processing in plants. J Exp Bot, 62: 1545-63.

Egozcue, J., Sarrate, Z., Codina-Pascual, M., Egozcue, S., Oliver-Bonet, M., Blanco, J., Navarro, J., Benet, J. and Vidal, F., 2005. Meiotic abnormalities in infertile males. Cytogen and Genom Res, 111(3-4): 337-342.

Eilam, T., Anikster, Y., Millet, E., Manisterski, J. and Feldman, M., 2010. Genome size in diploids, allopolyploids, and autopolyploids of Mediterranean Triticeae. J of Bot, 2010.

Erickson L. R., Straus N. A. and Beversdorf W. D. 1983. Restriction patterns reveal origins of chloroplast geno mes in Brassica amphidiploids. Theor Appl Genet, 65: 201–206.

Falistocco E, Torricelli R, Falcinelli M. Genomic relationships between Medicago murex Willd. and Medicago lesinsii E. Small. investigated by in situ hybridization. Theor Appl Genet, 2002, 105(6-7): 829−833.

Forster, B.P., Heberle-Bors, E., Kasha, K.J. and Touraev, A., 2007. The resurgence of haploids in higher plants. Trends in Plant Sci, 12(8): 368-375.

Franklin-Tong, V. E. and Franklin, F. C. H. 2000. Self-incompatibility in Brassica: The elusive pollen S gene is identified! The Plant Cell, 12: 305-308.

Fransz, F., Alonso-Blanco, C., Liharska, T. B., Peeters, A. J., Zabel, and Jong, J. H. 1996. High-resolution physical mapping in Arabidopsis thaliana and tomato by fluorescence in situ hybridization to extended DNA fibres. The Plant J, 9: 421-430.

Feldman, M. and Levy, A. A. 2005. Allopolyploidy--a shaping force in the evolution of wheat genomes. Cytogenet Genome Res, 109: 250-8.

Fernandes, J. B., Seguela-Arnaud, M., Larcheveque, C., Lloyd, A. H. and Mercier, R. 2018. Unleashing meiotic crossovers in hybrid plants. Proc Natl Acad Sci, 115(10): 2431-2436

Friedhoff, , Li, and Gotthardt, J., 2016. Protein-protein interactions in DNA mismatch repair. DNA Rep, 38: 50-57.

Fukui K., Nakayama S., Ohmido N., Yoshiaki H. and Yamabe M. 1998. Quantitative karyotyping of three diploid Brassica species by imaging methods and localization of 45s rDNA loci on the identified chromosomes. Theor Appl Genet, 96: 325—330.

Gall, J. G. and Pardue, M. L. 1969. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. P Natl Acad Sci, 63: 378-383.

Garcia-Mas J., Benjak A., Sanseverino W., Bourgeois M., Mir G., Gonzalez V. M., Henaff E., Camara F., Cozzuto L. and Lowy E. 2012. The genome of melon (Cucumis melo L.). P Natl Acad Sci, 109(29): 11872 – 11877.

Gerlach, W. L. and Bedbrook, J. R. 1979. Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucl Acids Res, 7: 1869-1885.

Gerton, J. L. and Hawley, R. S. 2005. Homologous chromosome interactions in meiosis: diversity amidst conservation. Nat Rev Genet, 6: 477-487.

Ghani, M.A., Sun, Q., Li, J., Cao, L., Rao, L., Zou, X. and Chen, L., 2014. Phenotypic and genetic variation occurred during wide hybridisation and allopolyploidisation between Brassica rapa and Brassica nigra. Scient Horticul, 176: 22-31.

Gianfranco V., Ravalli C. and Cremonini R., 2008. The karyotype as a tool to identify plant species: Vicia species species belonging to Vicia subgenus. Caryologia, 61(3): 300-319.

Glover, N. M., Redestig, H. and Dessimoz, C., 2016. Homoeologs: What Are They and How Do We Infer Them? Trends Plant Sci, 21: 609-21.

Gray, S. and Cohen, E., 2016. Control of meiotic crossovers: from double-strand break formation to designation. Ann Revof Gen, 50: 175-210.

Griffiths, S., Sharp, R., Foote, T.N., Bertin, I., Wanous, M., Reader, S., Colas, I. and Moore, G., 2006. Molecular characterization of Ph1 as a major chromosome pairing locus in polyploid wheat. Nature, 439(7077) : 749-752

Groothuizen, F. S. and Sixma, T. K. 2016. The conserved molecular machinery in DNA mismatch repair enzyme structures. DNA Repair (Amst), 38: 14-23.

Gupta, S.K. and Pratap, A., 2007. History, origin, and evolution. Adv in Bot Res, 45: 1-20.

Hanelt P. and Mettin D. 1964. Bemerkungen zur karyologie und systematik einiger sippen der gattung Vicia L. Kulturpfl anze, 77: 11-30.

HaneltP. and Mettin D. 1989 Biosistematics of the genus Vicia L. (Leguminosae). Ann Rev of Eco, and System., 20: 199-223.

Hansen, R.K., Mund, A., Poulsen, S.L., Sandoval, M., Klement, K., Tsouroula, K., Tollenaere, M.A., Räschle, M., Soria, R., Offermanns, S. and Worzfeld, T., 2016. SCAI promotes DNA double-strand break repair in distinct chromosomal contexts. Nat Cell Biol, 18(12): 1357-1366

Harper, L., Golubovskaya, I. and Cande, W.Z., 2004. A bouquet of chromosomes. Journal of Cell Sci, 117(18): 4025-4032.

Harper, A.L., Trick, M., He, Z., Clissold, L., Fellgett, A., Griffiths, S. and Bancroft, I., 2016. Genome distribution of differential homoeologue contributions to leaf gene expression in bread wheat. Plant Biotech J, 14(5): 1207-1214.

He, Z., Wang, L., Harper, A. L., Havlickova, L., Pradhan, A. K., Parkin, I. a. P. and Bancroft, I. 2017. Extensive homoeologous genome exchanges in allopolyploid crops revealed by mRNAseq-based visualization. Plant Biotech J, 15: 594-604.

Hasterok R., Jenkins G., Langdon T., Jones R. N. and Maluszynska J. 2001. Ribosomal DNA is an effective marker of Brassica chromosomes. Theor and Appl Gen, 103: 486–490.

Hemingway, J.S., 1976. Mustards: Brassica spp. and Sinapis alba (Cruciferae). Evolution of Crop Plants. NW Simmonds, ed.

Henikoff, S. and Dalal, Y., 2005. Centromeric chromatin: what makes it unique?. Curr Opin in Gen and Develop, 15(2): 177-184.

Heyting, C., 1996. Synaptonemal complexes: structure and function. Current Opin in Cell Biol, 8(3): 389-396.

Higgins, J.D., Armstrong, S.J., Franklin, F.C.H. and Jones, G.H., 2004. The Arabidopsis MutS homolog AtMSH4 functions at an early step in recombination: evidence for two classes of recombination in Arabidopsis. Genes & development, 18(20): 2557-2570.

Hiraoka, Y. and Dernburg, A. F. 2009. The SUN rises on meiotic chromosome dynamics. Dev Cell, 17: 598-605.

Hollingsworth, N. M., Ponte, L. and Halsey, C. 1995. MSH5, a novel MutS homolog, facilitates meiotic reciprocal recombination between homologs in Saccharomyces cerevisiae but not mismatch repair. Genes and Develop, 9: 1728-1739.

Holsclaw, J.K., Hatkevich, T. and Sekelsky, J., 2016. Meiotic and mitotic recombination: first in flies. In Genome Stability, : 139-154

Houben, A., Sanei, M. and Pickering, R., 2011. Barley doubled-haploid production by uniparental chromosome elimination. Plant Cell, Tissue and Organ Culture (PCTOC), 104(3): 321-327.

Howell, E. C., Barker, G. C., Jones, G. H., Kearsey, M. J., King, G. J., Kop, E. P., Ryder, C. D., Teakle, G. R., Vicente, J. G. and Armstrong, S. J. 2002. Integration of the cytogenetic and genetic linkage maps of Brassica oleracea. Genetics, 161: 1225-1234.

Hunter, N. and Kleckner, N., 2001. The single-end invasion: an asymmetric intermediate at the double-strand break to double-holliday junction transition of meiotic recombination. Cell, 106(1): 59-70.

Ijdo, J. W., Wells, R. A., Baldini, A. and Reeders, S. T. 1991. Improved telomere detection using a telomere repeat probe (TTAGGG) n generated by PCR. Nucleic Acids Res, 19: 4780.

Jackson, R.C. and Casey, J., 1982. Cytogenetic analyses of autopolyploids: models and methods for triploids to octoploids. Amer J of Bot, 69(4): 487-501.

Jin, W., Melo, J.R., Nagaki, K., Talbert, B., Henikoff, S., Dawe, R.K. and Jiang, J., 2004. Maize centromeres: organization and functional adaptation in the genetic background of oat. The Plant Cell, 16(3): 571-581.

Jiricny, J. 2006. The multifaceted mismatch-repair system. Nat Rev Mol Cell Biol, 7: 335-346.

Johnston, J. S., Pepper, A. E., Hall, A. E., Chen, Z. J., Hodnett, G., Drabek, J., Lopez, R. and Price, H. J. 2005. Evolution of genome size in Brassicaceae. Ann Bot, 95: 229-235.

Kaur, H., De Muyt, A. and Lichten, M., 2015. Top3-Rmi1 DNA single-strand decatenase is integral to the formation and resolution of meiotic recombination intermediates. Molecular cell, 57(4) : 583-594.

Keeney, S. 2001. Mechanism and control of meiotic recombination initiation. Curr Top Dev Biol, 52: 1-53.

Kim, N.S., Armstrong, K.C., Fedak, G., Ho, K. and Park, N.I., 2002. A microsatellite sequence from the rice blast fungus (Magnaporthe grisea) distinguishes between the centromeres of Hordeum vulgare and H. bulbosum in hybrid plants. Genome, 45(1): 165-174.

Kitashiba, H. and Nasrallah, J. B. 2014. Self-incompatibility in Brassicaceae crops: lessons for interspecific incompatibility. Breed Sci, 64: 23-37.

Kleckner, N. 1996. Meiosis: how could it work? Proc Natl Acad Sci, 93: 8167-74.

Knight, E., Greer, E., Draeger, T., Thole, V., Reader, S., Shaw, and Moore, G. 2010. Inducing chromosome pairing through premature condensation: analysis of wheat interspecific hybrids. Funct Integr Genomics, 10: 603-608.

Kowalczykowski, S. C. 2000. Initiation of genetic recombination and recombination-dependent replication. Trends in Biochem Sci, 25: 156-165.

Kugou, K., Fukuda, T., Yamada, S., Ito, M., Sasanuma, H., Mori, S., Katou, Y., Itoh, T., Matsumoto, K., Shibata, T., Shirahige, K. and Ohta, K. 2009. Rec8 guides canonical Spo11 distribution along yeast meiotic chromosomes. Mol Biol Cell, 20: 3064-76.

Kuipers AGJ, van Os DPM, de Jong JH, Ramanna MS. Molecular cytogenetics of Alstroemeria: identification of parental enomes in interspecific hybrids and characterization of repetitive DNA families in constitutive heterochromatin. Chromosome Res, 1997, 5(1): 31−39.

Kulak, S., Hasterok, R. and Maluszynska, J. 2002. Karyotyping of Brassica amphidiploids using 5S and 25S rDNA as chromosome markers. Hereditas, 136: 144-150.

Lamers, M. H., Perrakis, A., Enzlin, J. H., Winterwerp, H. H., De Wind, N. and Sixma, T. K. 2000. The crystal structure of DNA mismatch repair protein MutS binding to a G x T mismatch. Nature, 407: 711-717.

Lee, J.Y. and Orr-Weaver, T.L., 2001. The molecular basis of sister-chromatid cohesion. Ann Rev of Cell and Develop Biol, 17(1): 753-777.

Lelivelt, C.L.C., Leunissen, E.H.M., Frederiks, H.J., Helsper, J.P.F.G. and Krens, F.A., 1993. Transfer of resistance to the beet cyst nematode (Heterodera schachtii Schm.) from Sinapis alba L.(white mustard) to the Brassica napus L. gene pool by means of sexual and somatic hybridization. Theor Appl Genet, 85(6-7): 688-696.

Lichten, M. 2001. Meiotic recombination: breaking the genome to save it. Curr Biol, 11: R253-6.

Lieber, M. R. 2010. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annu Rev Biochem, 79: 181-211.

Lionneton, E., Beuret, W., Delaitre, C., Ochatt, S. and Rancillac, M. 2001. Improved microspore culture and doubled-haploid plant regeneration in the brown condiment mustard (Brassica juncea). Plant Cell Rep, 20: 126-130.

Liu, D., Xu, L., Geng, X., Zhou, Y., Zhang, Z., Wang, B. and Zhou, W., 2013. Distant hybridization involving different in vitro techniques. In Biotechnology of Crucifers (pp. 23-44). Springer New York.

Liu, T. Y., Huang, H. H., Wheeler, D., Xu, Y., Wells, J. A., Song, Y. S. and Wiita, A. P. 2017. Time-Resolved Proteomics Extends Ribosome Profiling-Based Measurements of Protein Synthesis Dynamics. Cell Syst, 4: 636-644 e9.

Lloyd, A., Blary, A., Charif, D., Charpentier, C., Tran, J., Balzergue, S., Delannoy, E., Rigaill, G. and Jenczewski, E. 2017. Homoeologous exchanges cause extensive dosage-

dependent gene expression changes in an allopolyploid crop. New Phytol, 217 (1): 367-377

Loidl, J., Lukaszewicz, A., Howard-Till, R. A. and Koestler, T. 2012. The Tetrahymena meiotic chromosome bouquet is organized by centromeres and promotes interhomolog recombination. J Cell Sci, 125: 5873-80.

Longhese, M. P., Bonetti, D., Manfrini, N. and Clerici, M. 2010. Mechanisms and regulation of DNA end resection. EMBO J, 29: 2864-2874.

Lukaszewski, A.J. and Kopecký, D., 2010. The Ph1 locus from wheat controls meiotic chromosome pairing in autotetraploid rye (Secale cereale L.). Cytogen and Genom Res, 129(1-3): 117-123.

Maćkowiak, M. and Heneen, W. K. 1999. Meiotic karyotypes of the B genomes of Brassica nigra and B. carinata. Hereditas, 130: 131-135.

Mallet, J. 2005. Hybridization as an invasion of the genome. Trends Ecol Evol, 20: 229-37.

Manhart, C.M. and Alani, E., 2016. Roles for mismatch repair family proteins in promoting meiotic crossing over. DNA Repair, 38: 84-93.

Martin, A. C., Shaw, , Phillips, D., Reader, S. and Moore, G. 2014. Licensing MLH1 sites for crossover during meiosis. Nat Commun, 5: 4580.

Mason, A. S., Huteau, V., Eber, F., Coriton, O., Yan, G., Nelson, M. N., Cowling, W. A. and Chevre, A. M. 2010. Genome structure affects the rate of autosyndesis and allosyndesis in AABC, BBAC and CCAB Brassica interspecific hybrids. Chromosome Res, 18: 655-66.

Matzke, M.A. and Matzke, A.J.M., 1998. Polyploidy and transposons. Trends Ecol Evol, 13(6): 241.

Mehta, A. and Haber, J. E. 2014. Sources of DNA double-strand breaks and models of recombinational DNA repair. Cold Spring Harb Perspect Biol, 6: a016428.

Mezard, C., Vignard, J., Drouaud, J. and Mercier, R., 2007. The road to crossovers: plants have their say. Trends in Genet, 23(2): 91-99.

Mochida, K., Tsujimoto, H. and Sasakuma, T., 2004. Confocal analysis of chromosome behavior in wheat× maize zygotes. Genome, 47(1): 199-205.

Moens, P.B., Kolas, N.K., Tarsounas, M., Marcon, E., Cohen, P.E. and Spyropoulos, B., 2002. The time course and chromosomal localization of recombination-related proteins at meiosis in the mouse are compatible with models that can resolve the early DNA-DNA interactions without reciprocal recombination. J of Cell Sci, 115(8): 1611-1622.

Moore, G. and Shaw, 2009. Improving the chances of finding the right partner. Curr Opin in Genet and Develop, 19: 99-104.

Morita, R., Nakane, S., Shimada, A., Inoue, M., Iino, H., Wakamatsu, T., Fukui, K., Nakagawa, N., Masui, R. and Kuramitsu, S., 2010. Molecular mechanisms of the whole DNA repair system: a comparison of bacterial and eukaryotic systems. J of Nucl Acids, 2010.

Mun, J. H., Kwon, S. J., Yang, T. J., Seol, Y. J., Jin, M., Kim, J. A., Lim, M. H., Kim, J. S., Baek, S., Choi, B. S., Yu, H. J., Kim, D. S., Kim, N., Lim, K. B., Lee, S. I., Hahn, J. H., Lim, Y. P., Bancroft, I. and Park, B. S. 2009. Genome-wide comparative analysis of the Brassica rapa gene space reveals genome shrinkage and differential loss of duplicated genes after whole genome triplication. Genome Biol, 10: R111.

Muniyappa, K., Kshirsagar, R. and Ghodke, I., 2014. The HORMA domain: an evolutionarily conserved domain discovered in chromatin-associated proteins, has unanticipated diverse functions. Gene, 545(2): 194-197.

Namai H. 1976. Cytogetic and breeding studies on transfer of economic characters by means of interspecific and intergeneric crossing in the tribe Brassiceae of Cruciferae . Mem Fac Agric Tokyo Univ Edu 22: 101–171.

Naranjo, T. 2012. Finding the correct partner: the meiotic courtship. Scientifica (Cairo), **,** 509073.

Nasmyth, K. and Haering, C.H., 2005. The structure and function of SMC and kleisin complexes. Ann Rev Biochem, 74: 595-648.

Navabi Z. K., Parkin I. A., Pires J. C., Xiong Z., Thiagarajah M. R. , Good A. G. and Rahman M. H. 2010. Introgression of B-genome chromosomes in a doubled haploid population of Brassica napus x Brassica carinata. Genome, 53(8): 619– 629.

Navabi Z. K., Stead K. E., Pires J. C., Xiong Z., Sharpe A. G. , Parkin I. A., Rahman M. H. and Good A. G. 2011. Analysis of B-genome chromosome introgression in interspecific hybrids of Brassica napus x B . carinata. Genetics, 187 (3): 659– 673.

Navabi, Z.K., Huebert, T., Sharpe, A.G., O'Neill, C.M., Bancroft, I. and Parkin, I.A., 2013. Conserved microstructure of the Brassica B Genome of Brassica nigra in relation to homologous regions of Arabidopsis thaliana, B. rapa and B. oleracea. BMC Genome, 14(1): 250.

Navabi, Z.K., Parkin, I.A.P., Pires, J.C., Xiong, Z., Thiagarajah, M.R., Good, A.G. and Rahman, M.H., 2010. Introgression of B-genome chromosomes in a doubled haploid population of Brassica napus× B. carinata. Genome, 53(8): 619-629.

Navabi, Z.K., Stead, K.E., Pires, J.C., Xiong, Z., Sharpe, A.G., Parkin, I.A., Rahman, M.H. and Good, A.G., 2011. Analysis of B-genome chromosome introgression in interspecific hybrids of Brassica napus× B. carinata. Genetics, 187(3): 659-673.

Neale, M. J. and Keeney, S. 2006. Clarifying the mechanics of DNA strand exchange in meiotic recombination. Nature, 442: 153-158.

Nimonkar, A.V., Sica, R.A. and Kowalczykowski, S.C., 2009. Rad52 promotes second-end DNA capture in double-stranded break repair to form complement-stabilized joint molecules. P Natl Acad Sci, 106(9): 3077-3082.

O'Connor, C., 2008. Fluorescence in situ hybridization (FISH). Nat Edu, 1(1): 171.

Ohkura, H. 2015. Meiosis: an overview of key differences from mitosis. Cold Spr Harb Pers Biol, 7.

Ohta, T. 2000. Evolution of gene families. Gene, 259: 45-52.

Oleszczuk, S. and Lukaszewski, A. J. 2014. The origin of unusual chromosome constitutions among newly formed allopolyploids. Am J Bot, 101: 318-26.

Olsson, G. 1960. Species crosses within the genus brassica. Hereditas, 46: 351-386.

Osalou, A. R., Rouyandezagh, S. D., Alizadeh, B., Er, C. and Sevimay, C. S. 2013. A comparison of ice cold water pretreatment and alpha-bromonaphthalene cytogenetic method for identification of Papaver species. Sci World J, 2013: 608650.

Osman K., Higgins J. D., Sanchez-Moran E., Armstrong S. J. and Franklin F. C. 2011. Pathways to meiotic recombination in Arabidopsis thaliana. New Phyto, 190: 523-544.

Paigen, K. and Petkov, 2010. Mammalian recombination hot spots: properties, control and evolution. Nat Rev Genet, 11: 221-233.

Painter, T. S. 1923. Studies in mammalian spermatogenesis. II. The spermatogenesis of man. Journal of experimental zoology part A: Eco Genet and Physiol, 37: 291-336.

Pardue, M.L. and Gall, J.G., 1969. Molecular hybridization of radioactive DNA to the DNA of cytological preparations. P Natl Acad of Sci, 64(2): 600-604.

Parkin, I.A., Gulden, S.M., Sharpe, A.G., Lukens, L., Trick, M., Osborn, T.C. and Lydiate, D.J., 2005. Segmental structure of the Brassica napus genome based on comparative analysis with Arabidopsis thaliana. Genetics.

Parokonny, A.S., Marshall, J.A., Bennett, M.D., Cocking, E.C., Davey, M.R. and Power, J.B., 1997. Homoeologous pairing and recombination in backcross derivatives of tomato somatic hybrids [Lycopersicon esculentum (+) L. peruvianum]. Theor Appl Genet, 94(6-7): 713-723.

Pawlowski, W.P. and Cande, W.Z., 2005. Coordinating the events of the meiotic prophase. Trends in Cell Biol, 15(12): 674-681.

Plieske J., Struss D. and Robbelen G. 1998. Inheritance of resistance derived from the B genome of Brassica against Phoma lingam in rapeseed and the development of molecular markers. Theor Appl Genet, 97(5–6): 929–936.

Prieto, I., Tease, C., Pezzi, N., Buesa, J. M., Ortega, S., Kremer, L., Martínez, A., Martínez-A, C., Hultén, M. A. and Barbero, J. L. 2004. Cohesin component dynamics during meiotic prophase I in mammalian oocytes. Chrom Res, 12: 197-213.

Pradhan, A. K., Gupta, V., Mukhopadhyay, A., Arumugam, N., Sodhi, Y. S. & Pental, D. 2003. A high-density linkage map in Brassica juncea (Indian mustard) using AFLP and RFLP markers. Theor Appl Genet, 106: 607-14.

Pradhan, A.K., Prakash, S., Mukhopadhyay, A. and Pental, D., 1992. Phytogeny of Brassica and allied genera based on variation in chloroplast and mitochondrial DNA patterns: molecular and taxonomic classifications are incongruous. Theor Appl Genet, 85(2-3): 331-340.

Prakash S. and Hinata K. 1980. Taxonomy, cytogenetics and origin of crop Brassicas – a review. Oper Bot, 55: 1–57.

Puizina, J., Siroky, J., Mokros, P., Schweizer, D. and Riha, K. 2004. MRE11 deficiency in Arabidopsis is associated with chromosomal instability in somatic cells and Spo11-dependent genome fragmentation during meiosis. Plant Cell, 16: 1968-78

Putnam, C.D., Jaehnig, E.J. and Kolodner, R.D., 2009. Perspectives on the DNA damage and replication checkpoint responses in Saccharomyces cerevisiae. DNA Repair, 8(9): 974-982.

Qi, L., Friebe, B., Zhang, and Gill, B.S., 2007. Homoeologous recombination, chromosome engineering and crop improvement. Chrom Res, 15(1): 3-19.

Qiu D., Muqiang G., Genyi L. and Quiro´s C 2009 Comparative sequence analysis for Brassica oleracea with similar sequences in B. rapa and Arabidopsis thaliana . Plant Cell Rep, 28: 649–661.

Rahman, M. H. 2004. Optimum age of siliques for rescue of hybrid embryos from crosses between Brassica oleracea, B. rapa and B. carinata. Canad J of Plant Sci, 84: 965-969.

Rakow, G., 2004. Species origin and economic importance of Brassica. In Brassica (pp. 3-11): Springer, Berlin, Heidelberg.

Ramsey, J. and Schemske, D.W., 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. Ann Rev of Ecol and Syst, 29(1): 467-501.

Renny-Byfield, S. and Wendel, J. F. 2014. Doubling down on genomes: polyploidy and crop plants. Am J Bot, 101: 1711-25.

Reyes, G.X., Schmidt, T.T., Kolodner, R.D. and Hombauer, H., 2015. New insights into the mechanism of DNA mismatch repair. Chromosoma, 124(4): 443-462.

Rezaei Osalou, A., Daneshvar Rouyandezagh, S., Alizadeh, B., Er, C. and Sevimay, C.S., 2013. A comparison of Ice cold water pretreatment and α-bromonaphthalene cytogenetic method for identification of Papaver species. The Sci World J, 2013.

Roberts, N. Y., Osman, K. and Armstrong, S. J. 2009. Telomere distribution and dynamics in somatic and meiotic nuclei of Arabidopsis thaliana. Cytogen Genome Res, 124: 193-201.

Robert, T., Vrielynck, N., Mezard, C., De Massy, B. and Grelon, M. 2016. A new light on the meiotic DSB catalytic complex. Semin Cell Dev Biol, 54: 165-76.

Roeder, G.S. and Bailis, J.M., 2000. The pachytene checkpoint. Trends in Genet, 16(9): 395-403.

Rogacheva, M.V., Manhart, C.M., Chen, C., Guarne, A., Surtees, J. and Alani, E., 2014. MLH1-Mlh3, a meiotic crossover and DNA mismatch repair factor, is a Msh2-Msh3-stimulated endonuclease. J of Biol Chem, 289(9): 5664-5673.

Ronceret, A. and Pawlowski, W.P., 2010. Chromosome dynamics in meiotic prophase I in plants. Cytogen and Genome Res, 129(1-3): 173-183.

Rudkin, G. T. and Stollar, B. D. 1977. High resolution detection of DNA–RNA hybrids in situ by indirect immunofluorescence. Nature, 265: 472-473.

Saal B. and Struss D. (2005). RGA- and RAPD-derived SCAR markers for a Brassica B genome introgression conferring resistance to blackleg in oilseed rape. Theor Appl Genet ,111(2): 281– 290.

Sanchez-Moran, E. and Armstrong, S.J., 2014. Meiotic chromosome synapsis and recombination in Arabidopsis thaliana: new ways of integrating cytological and molecular approaches. Chrom Res, 22(2): 179-190.

Sansam, C.L. and Pezza, R.J., 2015. Connecting by breaking and repairing: mechanisms of DNA strand exchange in meiotic recombination. The FEBS J, 282(13): 2444-2457.

Santos, A. P. and Shaw, 2004. Interphase chromosomes and the Rabl configuration: does genome size matter? J of Micr, 214: 201-206.

Santos, J. L., Alfaro, D., Sanchez-Moran, E., Armstrong, S. J., Franklin, F. C. H. and Jones, G. H. 2003. Partial diploidization of meiosis in autotetraploid Arabidopsis thaliana. Genetics, 165: 1533-1540.

Schelfhout C. J., Snowdon R., Cowling W. A. and Wroth J. M. (2006). Tracing B-genome chromatin in Brassica napus x B . juncea interspecific progeny. Genome, 49(11): 1490 – 1497.

Scherthan, H., Weich, S., Schwegler, H., Heyting, C., Härle, M. and Cremer, T. 1996. Centromere and telomere movements during early meiotic prophase of mouse and man are associated with the onset of chromosome pairing. The J of Cell Biol, 134: 1109-1125.

Schwarzacher T. (2003). Meiosis, recombination and chromosomes: a review of gene isolation and fluorescent In Situ hybridization data in plants. J of Exp Bot, 380(54): 11-23

She, C., Liu, J., Diao, Y., Hu, Z. and Song, Y. 2007. The Distribution of Repetitive DNAs Along Chromosomes in Plants Revealed by Self-genomic in situ Hybridization. J of Genet and Genom, 34: 437-448.

Sheng, X., Wen, G., Guo, Y., Yan, H., Zhao, H. and Liu, F., 2012. A semi-fertile interspecific hybrid of Brassica rapa and B. nigra and the cytogenetic analysis of its progeny. Genet Res and Crop Evo, 59(1): 73-81.

Silva, G.S. and Souza, M.M., 2013. Genomic in situ hybridization in plants. Genet Mol Res, 12(3): 2953-65.

Siaud, N., Dray, E., Gy, I., Gerard, E., Takvorian, N. and Doutriaux, M. P. 2004. Brca2 is involved in meiosis in Arabidopsis thaliana as suggested by its interaction with Dmc1. The EMBO J, 23: 1392-1401.

Soltis, S. and Soltis, D. E. 2000. The role of genetic and genomic attributes in the success of polyploids. P Natl Acad Sci, 97: 7051-7057.

Soltis, D.E. and Soltis, S., 1999. Polyploidy: recurrent formation and genome evolution. Trends in Ecol and Evol, 14(9): 348-352.

Soltis, S. and Soltis, D.E., 2000. The role of genetic and genomic attributes in the success of polyploids. P Natl Acad Sci, 97(13): 7051-7057.

Song K. M., Osborn T. C. and Williams P. H. 1990. Brassica taxonomy based on nuclear restriction fragment length polymorphism (RFLPs) 3. Genome relationship in Brassica and related genera and the origin of B. oleracea and Brassica rapa (syn. campestris). Theor Appl Genet 79: 497–506.

Sosnowska, K. and Cegielska-Taras, T. 2014. Application of in vitro pollination of opened ovaries to obtain Brassica oleracea L. x B. rapa L. hybrids. In Vitro Cell Dev Biol Plant, 50: 257-262.

Speicher, M.R. and Carter, N.P., 2005. The new cytogenetics: blurring the boundaries with molecular biology. Nat Rev Genet, 6(10): 782-792

Spies, M. and Fishel, R., 2015. Mismatch repair during homologous and homeologous recombination. Cold Spring Harb Perspect Biol, 7(3): a022657.

Srivastava, A., Mukhopadhyay, A., Arumugam, N., Gupta, V., Verma, J.K., Pental, D. and Pradhan, A.K., 2004. Resynthesis of Brassica juncea through interspecific crosses between B. rapa and B. nigra. Plant Breeding, 123(2): 204-206.

Stebbins G. L. 1966. Chromosomal variation and evolution. Science, 152: 1463–1469.

Struss D., Bellin U., Robbelen G. 1991. Development of B-genome chromosome addition lines of B. napus using different interspecific Brassica hybrids. Plant Breeding, 106(3): 209 – 214.

Sugiyama, T. and Kowalczykowski, S.C., 2002. Rad52 protein associates with replication protein A (RPA)-single-stranded DNA to accelerate RAD51-mediated displacement of RPA and presynaptic complex formation. J of Biol Chem, 277(35): 31663-31672.

Sung, , 1997a. Function of yeast Rad52 protein as a mediator between replication protein A and the RAD51 recombinase. J of Biol Chem, 272(45): 28194-28197.

Sung, , 1997b. Yeast Rad55 and Rad57 proteins form a heterodimer that functions with replication protein A to promote DNA strand exchange by RAD51 recombinase. Genes and Develop, 11(9): 1111-1121.

Symington, L.S., 2002. Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. Microb and Mol Biol Rev, 66(4): 630-670.

Symington, L.S., Rothstein, R. and Lisby, M., 2014. Mechanisms and regulation of mitotic recombination in Saccharomyces cerevisiae. Genetics, 198(3): 795-835.

Szadkowski, E., Eber, F., Huteau, V., Lode, M., Coriton, O., Jenczewski, E. and Chevre, A. M. 2011. Polyploid formation pathways have an impact on genetic rearrangements in resynthesized Brassica napus. New Phytol, 191: 884-894.

Szostak, J.W., Orr-Weaver, T.L., Rothstein, R.J. and Stahl, F.W., 1983. The double-strand-break repair model for recombination. Cell, 33(1): 25-35.

The Royal Society. 2009. Reaping the benefits: science and the sustainable intensification of global agriculture. London: The Royal Society.

Tiang, C.L., He, Y. and Pawlowski, W.P., 2012. Chromosome organization and dynamics during interphase, mitosis, and meiosis in plants. Plant Physiol, 158(1): 26-34.

Tjio, J. H. and Levan, A. 1956. The chromosome number of man. Hereditas, 42(1-2): 1-6. Hereditas, 42**,** 1-6.

Trask, B.J., 2002. Human genetics and disease: human cytogenetics: 46 chromosomes, 46 years and counting. Nat Rev Genet, 3(10): 769-778.

U, N. 1935. Genome analysis in Brassica with special reference to the experimental formation of B. napus and peculiar mode of fertilization. Jpn J Bot, 7: 389-452.

Varas, J., Graumann, K., Osman, K., Pradillo, M., Evans, D. E., Santos, J. L. and Armstrong, S. J. 2015. Absence of SUN1 and SUN2 proteins in Arabidopsis thaliana leads to a delay in meiotic progression and defects in synapsis and recombination. Plant J, 81: 329-46.

Vendrell, J.M., Garcia, F., Veiga, A., Calderon, G., Egozcue, S., Egozcue, J. and Barri, N., 1999. Meiotic abnormalities and spermatogenic parameters in severe oligoasthenozoospermia. Human Reprod, 14(2): 375-378.

Villeneuve, A. M. and Hillers, K. J. 2001. Whence meiosis? Cell, 106: 647-50.

Volkov, R. A., Panchuk, Ii, Borisjuk, N. V., Hosiawa-Baranska, M., Maluszynska, J. and Hemleben, V. 2017. Evolutional dynamics of 45S and 5S ribosomal DNA in ancient allohexaploid Atropa belladonna. BMC Plant Biol, 17: 21.

Vrielynck, N., Chambon, A., Vezon, D., Pereira, L., Chelysheva, L., De Muyt, A., Mézard, C., Mayer, C. and Grelon, M. 2016. A DNA topoisomerase VI-like complex initiates meiotic recombination. Science, 351: 939-943.

Wang, X., Wang, H., Wang, J., Sun, R., Wu, J., Liu, S., Bai, Y., Mun, J. H., Bancroft, I., Cheng, F., Huang, S., Li, X., Hua, W., Wang, J., Wang, X., Freeling, M., Pires, J. C., Paterson, A. H., Chalhoub, B., Wang, B., Hayward, A., Sharpe, A. G., Park, B. S., Weisshaar, B., Liu, B., Li, B., Liu, B., Tong, C., Song, C., Duran, C., Peng, C., Geng, C., Koh, C., Lin, C., Edwards, D., Mu, D., Shen, D., Soumpourou, E., Li, F., Fraser, F., Conant, G., Lassalle, G., King, G. J., Bonnema, G., Tang, H., Wang, H., Belcram, H., Zhou, H., Hirakawa, H., Abe, H., Guo, H., Wang, H., Jin, H., Parkin, I. A., Batley, J., Kim, J. S., Just, J., Li, J., Xu, J., Deng, J., Kim, J. A., Li, J., Yu, J., Meng, J., Wang, J., Min, J., Poulain, J., Wang, J., Hatakeyama, K., Wu, K., Wang, L., Fang, L., Trick, M., Links, M. G., Zhao, M., Jin, M., Ramchiary, N., Drou, N., Berkman, P. J., Cai, Q., Huang, Q., Li, R., Tabata, S., Cheng, S., Zhang, S., Zhang, S., Huang, S., Sato, S., Sun, S., Kwon, S. J., Choi, S. R., Lee, T. H., Fan, W., Zhao, X., Tan, X., Xu, X., Wang, Y., Qiu, Y., Yin, Y., Li, Y., et al. 2011. The genome of the mesopolyploid crop species Brassica rapa. Nat Genet, 43: 1035-9.

Wang, Y. & Copenhaver, G. P. 2018. Meiotic Recombination: Mixing It Up in Plants. Ann Rev Plant Biol, 69: 577-609.

Wang, Y., Zhi, H., Li, W., Li, H., Wang, Y., Huang, Z. and Diao, X. 2009. A novel genome of C and the first autotetraploid species in the Setaria genus identified by genomic *in situ* hybridization. Genet Res and Crop Evol, 56: 843-850.

Warwick S. I. and Black L. D. 1991. Molecular systematics of Brassica and allied genera (Subtribe Brassicinae Brassicae) chloroplast genome and cytodeme congruence. Theor Appl Genet 82: 81–92.

Warwick S. I., Francis A. and Gugel R. K. 2009. Guide to Wild Germplasm of Brassica and Allied Crops (tribe Brassiceae, Brassicaceae) 3rd edition.

Warwick, S.I. and Black, L.D., 1991. Molecular systematics of Brassica and allied genera (subtribe Brassicinae, Brassiceae)—chloroplast genome and cytodeme congruence. Theor Appl Genet, 82(1): 81-92.

Watson, J.D. and Crick, F.H., 1953. Molecular structure of nucleic acids. Nature, 171(4356): 737-738.

Woltering, D., Baumgartner, B., Bagchi, S., Larkin, B., Loidl, J., de los Santos, T. and Hollingsworth, N.M., 2000. Meiotic segregation, synapsis, and recombination checkpoint functions require physical interaction between the chromosomal proteins Red1p and Hop1p. Mol and Cell Biol, 20(18): 6646-6658.

Wyatt, H.D. and West, S.C., 2014. Holliday junction resolvases. Cold Spring Harb Perspect Biol, 6(9): a023192.

Xu, Z., Xie, B., Wu, T., Xin, X., Man, L., Tan, G. and Xiong, Z. 2016. Karyotyping and identifying all of the chromosomes of allopolyploid Brassica juncea using multicolor FISH. The Crop J, 4: 266-274.

Xue, S. and Barna, M. 2012. Specialized ribosomes: a new frontier in gene regulation and organismal biology. Nat Rev Mol Cell Biol, 13: 355-369.

Yanangino, T., Takahata, Y. and HINATA, K., 1987. Chloroplast DNA variation among diploid species in Brassica and allied genera. The Jpn J of Genet, 62(2) : 119-125.

Yang, J., Liu, D., Wang, X., Ji, C., Cheng, F., Liu, B., Hu, Z., Chen, S., Pental, D., Ju, Y., Yao, P., Li, X., Xie, K., Zhang, J., Wang, J., Liu, F., Ma, W., Shopan, J., Zheng, H., Mackenzie,

S. A. and Zhang, M. 2016. The genome sequence of allopolyploid Brassica juncea and analysis of differential homoeolog gene expression influencing selection. Nat Genet, 48: 1225-32.

Yuan, J. and Chen, J., 2010. MRE11-RAD50-NBS1 complex dictates DNA repair independent of H2AX. J of Biol Chem, 285(2): 1097-1104.

Zenkteler, M. 1990. In vitrofertilization and wide hybridization in higher plants. Crit Rev in Plant Sci, 9: 267-279.

Zenkteler, M., 2000. In vitro pollination of angiosperm ovules with gymnosperm pollen grains. In Vitro Cell and Develop Biol-Plant, 36(2): 125-127.

Zhang, L., Liang, Z., Hutchinson, J. & Kleckner, N. 2014. Crossover patterning by the beam-film model: analysis and implications. PLoS Genet, 10: e1004042.

Zhang, F., Tang, D., Shen, Y., Xue, Z., Shi, W., Ren, L., Du, G., Li, Y. and Cheng, Z. 2017. The F-Box Protein ZYGO1 Mediates Bouquet Formation to Promote Homologous Pairing, Synapsis, and Recombination in Rice Meiosis. Plant Cell, 29: 2597-2609.

Zhang, X., Scheuring, C. F., Zhang, M., Dong, J. J., Zhang, Y., Huang, J. J., Lee, M. K., Abbo, S., Sherman, A., Shtienberg, D., Chen, W., Muehlbauer, F. & Zhang, H. B. 2010. A BAC/BIBAC-based physical map of chickpea, Cicer arietinum L. BMC Genomics, 11: 501.

Zhang, J., Zhang, B., Su, H., Birchler, J. A. and Han, F. 2014. Molecular mechanisms of homologous chromosome pairing and segregation in plants. J Genet and Genomics, 41: 117-23.

Zhang, X., Scheuring, C.F., Zhang, M., Dong, J.J., Zhang, Y., Huang, J.J., Lee, M.K., Abbo, S., Sherman, A., Shtienberg, D. and Chen, W., 2010. A BAC/BIBAC-based physical map of chickpea, Cicer arietinum L. BMC Genomics, 11(1): 501.

Zhao M., 2012 BTriDB: a comprehensive database of triplicated blocks for Brassica lineage.

Zhao, M., 2017. [Online] Available at http://ocri-genomics.org/BTriDB/index.html [Accessed 2 June 2017]

Zickler, D. and Kleckner, N., 1998. The leptotene-zygotene transition of meiosis. Ann Rev of Genet, 32(1): 619-697.

Zickler, D. and Kleckner, N., 1999. Meiotic chromosomes: integrating structure and function. Ann Rev of Genet, 33(1): 603-754.

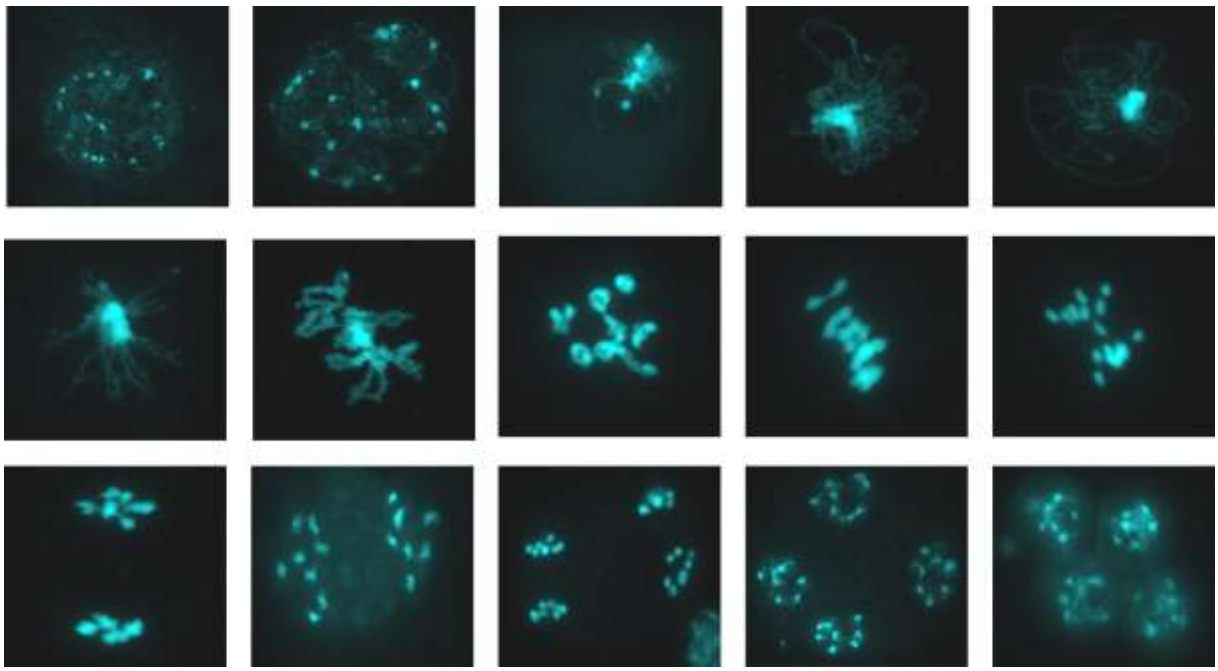
Zickler, D. and Kleckner, N., 2015. Recombination, pairing, and synapsis of homologs during meiosis. Cold Spring Harb Perspect Biol, 7(6): a016626.

Zoller JF, Yang Y., 2001. Herrmann RG, Hohmann U. Comparative genomic in situ hybridization (cGISH) analysis on plant chromosomes revealed by labeled Arabidopsis DNA. Chromosome Res, 9(5): 357−375.

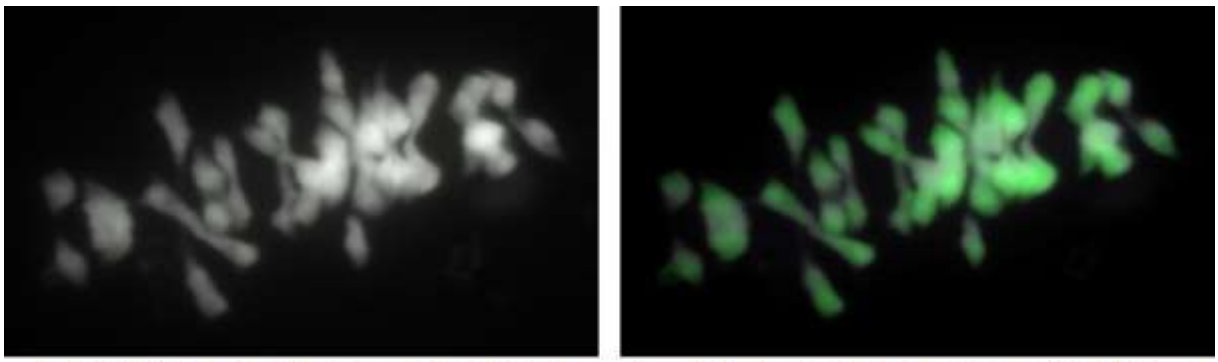
Zou, J., Hu, D., Liu, P., Raman, H., Liu, Z., Liu, X., Parkin, I. A., Chalhoub, B. and Meng, J. 2016. Co-linearity and divergence of the A subgenome of Brassica juncea compared with other Brassica species carrying different A subgenomes. BMC Genomics, 17: 18.

Appendix

Chromosomal behaviour under microscope: Meiotic atlas in Brassica (e.g. Brassica nigra)



Time course- Edu pulse *B. juncea*



M-I stage in *B. juncea after 40 hours*

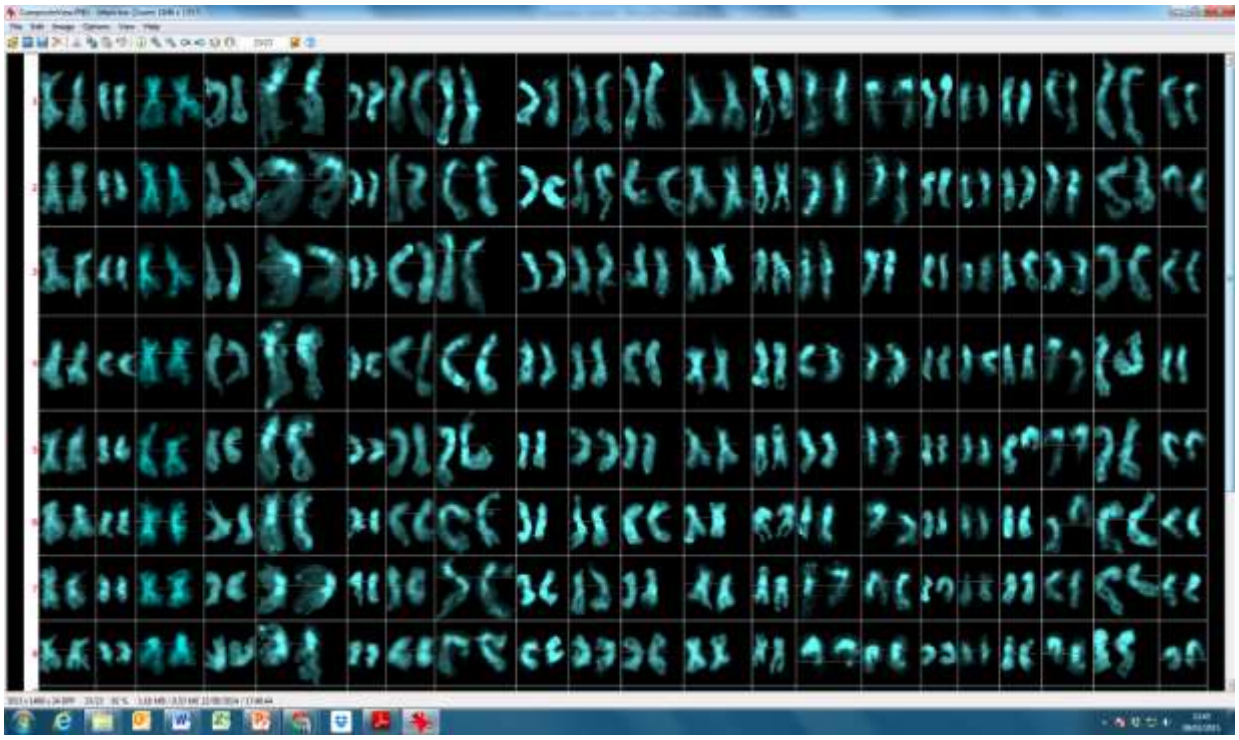
Edu detected as green after 40 hours of Edu pulse

Relative length of chromosomes for developing karyotype

	Relative length of chromosomes in ideotype								
	Chr1	Chr2	Chr3	Chr4	Chr5	Chr6	Chr7	Chr8	Total length
1	15.24	13.56	13.17	12.89	12.12	11.42	10.83	10.78	100
2	14.60	14.40	12.89	12.82	12.58	11.48	11.48	9.74	100
3	14.47	14.62	14.57	14.03	12.32	10.40	9.90	9.68	100
4	14.51	13.71	13.71	12.94	12.65	11.68	10.79	10.02	100
5	14.19	14.01	13.63	13.60	12.28	11.08	10.91	10.30	100
6	15.77	14.78	12.94	12.87	12.36	11.44	10.80	9.03	100
7	15.75	14.24	14.07	13.39	11.75	11.51	10.44	8.86	100
9	14.53	13.04	12.68	12.50	12.21	12.00	11.86	11.18	100
10	14.81	14.65	14.24	13.15	12.00	10.99	10.22	9.95	100
11	15.92	13.96	13.36	12.78	11.83	11.47	11.14	9.54	100
12	17.49	15.40	13.48	12.49	11.73	10.46	9.84	9.10	100
13	17.04	14.19	13.55	12.37	11.62	11.37	10.00	9.86	100
14	17.81	13.90	13.43	12.57	11.99	11.97	9.92	8.41	100
15	15.28	14.07	14.00	13.58	11.14	11.09	10.61	10.22	100
16	16.15	15.24	12.61	12.30	11.87	11.39	11.19	9.24	100
17	17.64	14.38	13.92	12.73	10.83	10.76	10.10	9.64	100
18	15.33	14.85	13.86	12.83	11.76	11.73	10.15	9.49	100
19	15.57	14.58	13.87	12.66	12.37	11.37	10.29	9.29	100
20	15.03	14.72	13.88	12.88	12.16	11.85	10.24	9.24	100
21	16.65	15.37	12.43	12.31	12.15	11.39	10.57	9.14	100
22	15.74	14.57	13.92	11.62	11.60	10.89	10.83	10.83	100
Average	15.69	14.39	13.53	12.82	11.97	11.32	10.58	9.69	100
St Dev	1.09	0.59	0.57	0.53	0.44	0.44	0.55	0.69	0
St Error	0.24	0.13	0.12	0.12	0.10	0.10	0.12	0.15	0

	Centromeric index							
Chr1	Chr2	Chr3	Chr4	Chr5	Chr6	Chr7	Chr8	
48.90	28.33	43.84	39.60	25.11	37.02	40.95	30.62	
27.23	49.76	37.77	38.77	40.60	40.00	22.09	42.25	
40.78	18.07	41.02	16.37	42.69	17.58	48.38	26.53	
19.36	38.06	46.29	50.09	41.78	47.73	19.26	23.62	
35.50	37.45	13.46	37.50	13.71	32.30	21.04	40.23	
47.95	21.89	27.37	50.00	43.25	30.36	46.69	47.92	
39.37	21.48	49.34	48.77	50.00	34.83	28.51	50.36	
16.02	48.74	31.03	47.75	22.63	46.35	44.53	24.72	
42.25	25.00	42.80	50.31	47.61	30.60	29.68	49.18	
39.53	42.68	21.63	49.39	24.81	28.29	49.90	49.42	
42.95	24.10	48.38	46.68	43.81	30.84	25.29	47.09	
49.84	48.82	29.48	41.08	24.28	24.82	42.18	43.63	
37.21	28.44	43.86	43.13	19.87	38.95	20.58	42.37	
44.94	42.27	23.83	22.60	20.61	35.08	42.60	47.75	
15.95	41.07	38.74	18.40	41.99	24.74	42.80	42.71	
41.55	17.56	42.82	49.59	36.57	29.64	50.00	49.09	
41.97	20.79	43.77	49.86	49.06	25.39	35.14	42.64	
43.42	36.92	27.94	42.78	25.65	47.32	49.53	34.48	
36.31	14.63	23.62	43.87	37.99	40.61	28.50	42.49	
36.74	29.75	47.41	38.98	47.78	33.28	28.78	44.87	
42.16	44.36	27.70	27.80	38.14	49.48	48.17	28.80	
Av	37.62	32.39	35.81	40.63	35.14	34.53	36.41	40.51
St Dev	9.94	11.41	10.42	10.67	11.28	8.58	11.10	8.73
St Error	2.17	2.49	2.27	2.33	2.46	1.87	2.42	1.91

Composite Karyotype



Genomic sizes of Brassica species

	Calculated				
	Genome size	9.78E+08	times	DNA content(pg)	
B rapa	1.05E+09	9.78E+08		1.078	
B nigra	1.26E+09	9.78E+08		1.294	
B juncea	2.13E+09	9.78E+08		2.184	
	Calculated				
	DNA content(pg)	Genome size	divided by	9.78E+08	
B rapa	1.08E+00	1058	1.06E+09	9.78E+08	978
B nigra	1.29E+00	1264	1.26E+09	9.78E+08	
B juncea	2.18E+00	2136	2.14E+09	9.78E+08	
C Value					
	Mbp	2X	1C	2C	
B rapa	529	1058	0.539	1.078	
B nigra	632	1264	0.647	1.294	
B juncea	1068	2136	1.092	2.184	

MLH1 foci counts

Nigra	
Image	Foci
1	14
7	11
12	13
1	11
5	13
6	13
7	14
11	12
12	14
13	13
14	14
15	15
16	13
17	13
18	14
19	14
21	15
22	13
23	14
25	13
26	15
27	14
28	11
30	14
31	12
33	14
34	12
35	12
36	13
37	13
39	13
40	13
41	12
42	12
43	13
44	13
45	12
46	16
47	13
48	11
49	11
50	14
51	11
52	14
53	10
54	13
55	14
56	13
57	15
58	13
59	12
60	10
61	13
62	14
63	13

B nigra	
Mean	13
Standard Error	0.17
Median	13
Mode	13
Standard Deviation	1.27
Minimum	10
Maximum	16
Sum	763
Count	59

Rapa	
Image	Foci
4	15
6	16
7	16
9	18
13	14
46	16
58	18
0	17
7	16
10	12
14	17
19	14
20	15
23	13
24	14
25	15
26	15
28	15
29	14
31	17
1	15
3	16
4	16
5	15
8	12
9	13
17	10
16	16
15	16
32	16

B rapa	
Mean	15
Standard Error	0.33
Median	15
Mode	16
Standard Deviation	1.80
Sample Variance	3.236782
Kurtosis	1.012714
Skewness	-0.82974
Range	8
Minimum	10
Maximum	18
Sum	452
Count	30

Juncea	
Image	Foci
2	26
3	25
7	24
8	29
13	24
20	23
21	28
22	27
23	25
24	27
25	25
26	27
27	29
28	26
30	28
31	23
32	24
34	28
5	22
6	25
8	23
11	23
12	27
13	27
14	28
15	22
16	25
18	31
19	23
20	29
21	23
22	23
23	21
25	27
26	23
27	25
28	21
29	30
30	26
35	21
37	22
38	26
39	28
40	27
42	25
43	22
44	25
45	26
46	24
47	26
48	29
49	26
50	27
51	30
53	25
5	30
7	32
8	30

B juncea	
Mean	26
Standard Error	0.37
Median	26
Mode	25
Standard Deviation	2.83
Sample Variance	8.009982
Kurtosis	-0.73694
Skewness	0.230358
Range	11
Minimum	21
Maximum	32
Sum	1513
Count	58

Chiasma counts

B napa	Cross Overs	CO	Ring	Rod											
	Mean	17.77	Mean	17.67	Mean	4.73	Mean	5.266667			8150				
	Standard Error	0.24	Standard Error	0.18	Standard Error	0.17	Standard Error	0.172429							
	Median	18	Median	18	Median	5	Median	5				CO			
	Mode	18	Mode	18	Mode	5	Mode	5							
	Standard Deviation	1.31	Standard Deviation	0.98	Standard Deviation	0.93	Standard Deviation	0.944433			Mean	8.20			
	Minimum	15	Minimum	15	Minimum	2	Minimum	4			Standard Error	0.26			
	Maximum	21	Maximum	19	Maximum	6	Maximu m	8			Median	8			
	Sum	550.77	Sum	547.67	Sum	146.73	Sum	158			Mode	9			
	Count	31	Count	31	Count	31	Count	30			Standard Deviation	1.31			
B juncea											Minimum	6			
	recovered 18 november 2016				Ring		Rod				Maximum	10			
											Sum	205			
	Mean	28.36	Mean	#DIV/0!	Mean	8.285714	Mean	9.71			Count	25			
	Standard Error	0.36	Standard Error	650.05.00	Standard Error	0.265789	Standard Error	0.27							
	Median	28	Median	#NUM!	Median	8	Median	10			Bivalent		Univalent	Multivalent	
	Mode	28	Mode	#N/A	Mode	8	Mode	10							
	Standard Deviation	1.34	Standard Deviation	#DIV/0!	Standard Deviation	0.994495	Standard Deviation	0.99			Mean	5.52	Mean	6.72	Mean 1.00
	Minimum	26	Minimum	0	Minimum	7	Minimum	8			Standard Error	0.18	Standard Error	0.36	Error 0.00
	Maximum	30	Maximum	0	Maximum	10	Maximu m	11			Median	6	Median	6	Median 1
	Sum	397	Sum	0	Sum	116	Sum	136			Mode	6	Mode	6	Mode #N/A
	Count	14	Count	0	Count	14	Count	14			Standard Deviation	0.92	Standard Deviation	1.43	Deviation #DIV/0!
											Minimum	4	Minimum	4	Minimum 1
											Maximum	7	Maximum	10	Maximum 1
B nigra											Sum	138	Sum	168	Sum 1
	Column1	CO		Ring		Rod					Count	25	Count	25	Count 1
	Mean	14.37	Mean	14.12	Mean	3.85	Mean	4.15							
	Standard Error	0.14	Standard Error	0.14	Standard Error	0.21	Standard Error	0.21							
	Median	14	Median	14	Median	4	Median	4							
	Mode	14	Mode	14	Mode	4	Mode	4							
	Standard Deviation	0.74	Standard Deviation	0.71	Standard Deviation	1.05	Standard Deviation	1.05							
	Minimum	13	Minimum	13	Minimum	2	Minimum	3							
	Maximum	16	Maximum	16	Maximum	5	Maximu m	6							
	Sum	388	Sum	367	Sum	100	Sum	108							
	Count	27	Count	26	Count	26	Count	26							

POSTER/ ORAL PRESENTATIONS

1. EMBO Meiosis meeting, Hvar Croatia	August 2017
2. Biosciences Graduate School Research Symposium, University of Birmingham	July 2017
3. 8th British Meiosis meeting, Imperial College London UK	April 2016
4. Biosciences Graduate School Research Symposium, University of Birmingham	April 2016
5. 7th British Meiosis meeting, International Centre for Life New Castle	Feb 2015
6. Percat Research Gala, University of Birmingham UK	Oct 2014
7. GARNet 2014- Arabidopsis: The ongoing Green Revolution, University of Bristol UK	Sept 2014