# Impact of Chromosomal Translocations (CTs) on reproductive isolation and fitness in natural yeast isolates

A thesis submitted to the University of Manchester for the Degree of Doctor of Philosophy (PhD) in Faculty of Biology, Medicine and Health

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## List of Abbreviations and Acronyms

| Abbreviations | Term |
|---|---|
| **°C** | Degrees Centigrade |
| **α** | Alpha |
| **M** | Micro |
| **Δ** | Deletant |
| **BIT** | Bridge-Induced Translocation |
| **Bp** | Base Pairs |
| **BSC** | Biological species concept |
| **Chr.** | Chromosome |
| **CHEF** | Clamped Homogenous Electric Field |
| **CHRs** | Cluster Homology Regions |
| **ChromPET** | Chromosomal Paired-End Tag |
| **C-limited** | Carbon limited |
| **CTs** | Chromosomal Translocations |
| **DMSO** | Dimethylsulphoxide |
| **DNA** | Deoxyribonucleic acid |
| **dNTPs** | Deoxynuclease |
| **DSB** | Double Strand Break |
| **EDTA** | Ethylene di-amine tetra-acetic acid |
| **ESC** | Ecological Species Concept |
| **Hr** | Hour |
| **HGT** | Horizontal Gene Transfer |
| **HML** | Hidden *MAT* Left |
| **HMR** | Hidden *MAT* Right |

| | |
|---|---|
| **HR** | Homologous Recombination |
| ***K. waltii*** | *Kluyveromyceswaltii* |
| **LB media** | Luria-Bertani media |
| **Li-Ac** | Lithium Acetate |
| **LTRs** | Long Term Repeats |
| **MAT** | mating-type |
| **MSC** | Morphological Species Concept |
| **NHEJ** | Non-Homologous End Joining |
| **N-limited** | Nitrogen limited |
| **ORFs** | Open Reading Frames |
| **OD** | optical density |
| **PCA** | Principal component analysis |
| **PCR** | polymerase chain reaction |
| **PEG** | Polyethylene Glycol |
| **PSC** | Phylogenetic Species Concept |
| **P-limited** | Phosphorus limited |
| **RNA** | Ribonucleic Acid |
| **SD** | Synthetic defined |
| **SGD** | Single Gene Duplication |
| **SGRP** | Saccharomyces Genome Resequencing Project |
| ***S. cariocanus*** | *Saccharomyces cariocanus* |
| ***S. cerevisiae*** | *Saccharomyces cerevisiae* |
| ***S. mikatae*** | *Saccharomyces mikatae* |
| ***S. paradoxus*** | *Saccharomyces paradoxus* |
| ***Sz. pombe*** | *Schizosaccharomyces pombe* |

| | |
|---|---|
| **Nacl** | Sodium chloride |
| **S-limited** | Sulphur limited |
| **TE** | Tris EDTA |
| **UCSC** | University of California Santa Cruz<br>Genome BrowserBioinformatics |
| **YPD** | yeast peptone dextrose |
| **WGD** | Whole-genome duplication |
| **WT** | Wild Type |

# Abstract

**The University of Manchester**

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**Doctor of Philosophy**

**Impact of Chromosomal Translocations on reproductive isolation and fitness in natural yeast isolates**

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Identifying the molecular mechanisms behind reproductive isolation between closely related yeast species provides avaluable understanding of their evolution. Sequence divergence and chromosomal rearrangements are the main post-zygotic barriers behind reproductive isolation within *Saccharomyces* '*sensu strico'* species, where hybrids are readily formed but sterile upon meiosis. *Saccharomyces paradoxus* and *Saccharomyces cariocanus* have an almost identical genome in terms of sequence, and therefore provide good model systems to explore the impact of karyotypic rearrangements on reproductive isolation. According to the biological species concept they are considered two different species despite having low sequence divergence. Since the karyotypic analysis revealed that the genomic differences are restricted to four chromosomal translocations, we hypothesized that such rearrangements may be the cause of low spore viability between them. To test this expectation, we engineered two chromosomal translocations in *S. paradoxus* YPS138, via Cre-*lox*P mediated recombination event, to render those parts of genome collinear to *S.cariocanus* UFRJ50816. Our analysis revealed that hybrids between *S. cariocanus* and engineered *S. paradoxus* harbouring two translocations showed a significant increase in spore viability (12.7%) compared to control hybrids harbouring five translocations (3.4%) ($P$=0.0031and $P$=0.0125, respectively, Two-sample t-test). Consequently, fitness in meiosis was improved four fold by undoing two

translocations. Given this result, the prediction for spore viability in complete collinear crossing would be around 50.8 %, which is still far from the value of ca. 100%, which would be expected for strains with very low sequence divergence and belonging to the same species. This indicates that other factors may contribute to meiotic fitness in these hybrids. Further investigation was carried to determine the genome structures by using the PacBio sequencing approach. Our DNA sequencing data revealed other, previously undetected, rearrangements in *S. cariocanus* strain: one new reciprocal translocation between chromosomes XIII and XIV and 11 inversions distributed in 6 chromosomes. The variations in meiotic viability observed in the engineered hybrids could be because of these 5 chromosomal translocations. Further experiments were also carried out to evaluate the impact of translocations on mitotic fitness and gene expression; we observed a significant drop in the mitotic fitness of engineered translocant strains under different nutritional and temperature stresses. These changes were also accompanied with alteration in genes expression throughout the genome. Our RNA- seq data revealed that many genes were up- or down- regulated because of the translocation. Several genes with altered expression in translocant strains are correlated with morphology changes when they are up- or down- regulated. Therefore, the cell morphology was evaluated under light microscopy and different abnormal cells were detected compared to the wild type. Irregular cell morphology included elongated and clumped cells. Overall, these data confirmed that chromosomal translocations were the cause of reproductive isolation between *S. paradoxus* and *S. cariocanus* and play an important role in altering the phenotype and gene expression.

## Declaration

No portion of this work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other University or other institute.

## Copyright Statement

## Dedication

I would like to express my gratitude to my parents **(Mum and Dad)** whom stood beside me the whole time of my project work; their blessing has always given me success throughout my life. My warm feelings must go to my sisters **(Bedour and Taiba)** and my brothers **(Husain andBader**) for their support and inspiration.

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# Chapter one

# Introduction

# 1. Introduction

## 1.1 Introduction of yeast genetics/genomics and physiology

### 1.1.1 Yeast genetics and genomics

Yeasts are eukaryotic microorganisms that belong to the kingdom of fungi, encompassing approximately 1,500 species (Kurtzman and Fell, 2006). Budding yeast is considered to be an attractive model for human research as their cellular and molecular systems can be easily controlled (Zeyl, 2006, Kuehne *et al*., 2007, Goujon *et al*., 2010). Outstanding features have been characterized in yeasts that make them promising candidates for their use in experimental studies; involving both biological (Sherman, 1998) and biomedical approaches (Wolfe, 2006). The features that make yeast an attractive experimental model include: efficient DNA transformation system, well-defined genetic system, small genome sizes (from 9 Mb to 21 Mb) (Dujon, 2010, Wolfe, 2006), short generation time, ease of genetic manipulation, and great similarity with higher eukaryotes (Goffeau *et al*., 2000, Landry *et al*., 2006). Because of its wide ecological, geographical, clinical and industrial distribution, yeast has become an efficient organism for understanding genome evolution and the diversity in many species (Landry *et al.,* 2006). Therefore, its genomic system gave us a better understanding of the adaptation process that resulted in response to different environmental changes and formation of new ecological niches.

In 1996, the first eukaryotic genome to be completely sequenced was the *Saccharomyces cerevisiae* (*S. cerevisiae*) genome. This sequencing was done with the collaboration of international scientists from different laboratories in North America, Europe and Japan (Goffeau *et al*., 1996). Therefore, this sequence is considered the reference for any newly-sequenced yeast strains and help in understanding the aligning and assembly parameters in order to develop sequence strategies for other eukaryotic genomes. Comparative analysis has identified that the genome of *S. cerevisiae* is comprised of 16 chromosomes with ca. 6,000

open reading frames (ORFs) (Goffeau *et al.*, 1996). Among these ORFs, 5,538 genes were represented by a minimum of 300 base pair (i.e. protein of 100 amino acids) (Kellis *et al*., 2003). Each of these ORFs has a unique systematic name that begins with the letter Y (indicating Yeast), followed by the chromosome number in which the A represents chromosome I and B represents chromosome II and so on. The next letter represents the chromosome arm that is either L for left or R for right and then a 3-digits code indicating the order of the genes starting from the centromere. The last letter is either W or C that represents the Watson strand or Crick strand (Mewes *et al*., 1997).

The analysis of yeast genome also identified 140 genes encoding ribosomal RNA (rRNA) located in chromosome XII, in addition to 40 and 275 genes representing small nuclear RNA (snRNA) and transfer RNA (tRNA), respectively. The total length of sequenced genome is 12,068 kb, starting from 230 kb (chromosome I) up to 1,532 kb (chromosome IV) (Goffeau *etal*., 1996). In 2002, the second yeast genome to be completely sequenced after *S. cerevisiae* genome was the *Schizosaccharomyces pombe* (*S. pombe*) yeast (Wood *et al*., 2002). The genomic structure of *S. pombe* was different from that of *S. cerevisiae* genome, thus comparing them did not offer many valuable information regarding the evolutionary genomics in yeast (Wood *et al*., 2002, Dujon, 2010). The differences was in the length of the genome in which the complete length of *S. pombe* sequenced genome is 13.8-Mb genome (Smith, 1987) with 4,940 protein coding genes (Wood *et al*., 2002) which was less than the predicted genes; 5,570±5,651 for *S. cerevisiae* (Blandin *et al.*, 2000, Wood *et al.*, 2001). 60.2% of *S. pombe* genome is comprised of protein-coding sequence (exons) incomparison to 71% in *S. cerevisiae* genome. The guanine and cytosine (GC) content in *S. pombe* genome is 36.0% as compared to 38.3% in *S. cerevisiae*. The number of transposable elements in *S. pombe* were found to be less than those in *S. cerevisiae*; 11 versus 59 (0.35% and 2.4% respectively) (Wood *et al*., 2002).

In 2010, about 40 different yeast species were completely sequenced including 10 yeast species from *Saccharomycetaceae* (Dujon, 2010). Later, thousands of *S. cerevisiae* strains have been sequenced with different degree of completion and around 100 of them have been assembled to the chromosomal level and deposited in International Nucleotide Sequence Database Collaboration databases (Engel and Cherry, 2013). Stropeet *et al*., (2016) carried of sequencing of 93 *S. cerevisiae* strains from different geographic and environmental origin susing Illumina HiSeq 2000, paired-end reads. These strains along with 7 other *S. cerevisiae* strains, were sequenced previously (Goffeau *et al*., 1996, Doniger *et al*., 2008, Wei *et al*., 2007, Dowell *et al*., 2010, Nishant *et al*., 2010), were added in novel genetic resource and named the "100-genomes" strains. These strains were assessed for different parameters including: chromosome colinearity with or without rearrangements and identification of novel genes relative to the reference genome S288C strain. In addition to that, they were analyzed for the population structure and the genotype-phenotype association among the 100-genome strains was also determined (Strope *et al*., 2015). The 100-genome strains are considered multiple purpose resource that may become useful for different aspect like, population genetics and quantitative genetics. Sequencing the genome of yeast is providing better understanding of the molecular mechanisms that causes reproductive isolation as well as the evolutionary history. *S. cerevisiae* has been used for several years for studying different processes in mammals, such as aging (Murakami and Kaeberlein, 2009), gene expression regulation (Biddick and Young, 2009), signal transduction (Hohmann *et al*., 2007), cell cycle (Nasheuer *et al*., 2002), apoptosis (Owsianowski *et al.*, 2008), and neurodegenerative disorders (Miller-Fleming *et al*., 2008). Therefore, it has become a valuable model system for studying the genetics and genomics, cell biology as well as biochemistry (Goffeau *et al.*, 1996, Spellman *et al*., 1998, Hartwell *et al*., 1974).

### 1.1.2 Life cycle

*S. cerevisiae* has a sexual as well as asexual reproductive cycle (Yeong, 2005) consequently, it can proliferate by mitotic and meiosis (Merlini *et al*., 2013) and can exist in both haploid (1*n*) and diploid (2*n*) states (Casamayor and Snyder, 2002). *Saccharomyces* yeast life cycle has been extensively studied in the laboratory, resulting in a firm understanding of the entire process. When grown in rich medium, yeast diploid cells are formed by the fusion of two haploid cells; *MAT**a*** and *MATα.* The diploid cell reproduces asexually (vegetative growth) dividing by mitosis and budding. When cells are grown in a medium lacking sufficient nitrogen to maintain mitosis, diploid cells can undergo meiosis, resulting in four haploid spores (Fabrizio and Longo, 2003), two for each mating type enclosed within an ascus called a tetrad (Figure 1.1) (Coluccio and Neiman, 2004). Nitrogen starvation might cause death for some diploids, while other diploids might produce less than four spores (Fabrizio and Longo, 2003). When tetrads are exposed to rich medium, the ascus wall breaks down with the help of degradative enzymes that digest the thick wall, causing a release of four haploid spores from the ascus.These spores germinate and become gametes that fuse with other gametes of opposite mating type to produce diploid cells. Diploid cells enter mitosis and reproduce asexually again until the starvation (Murphy and Zeyl, 2010, Nasmyth, 1985, Nasmyth, 1983).

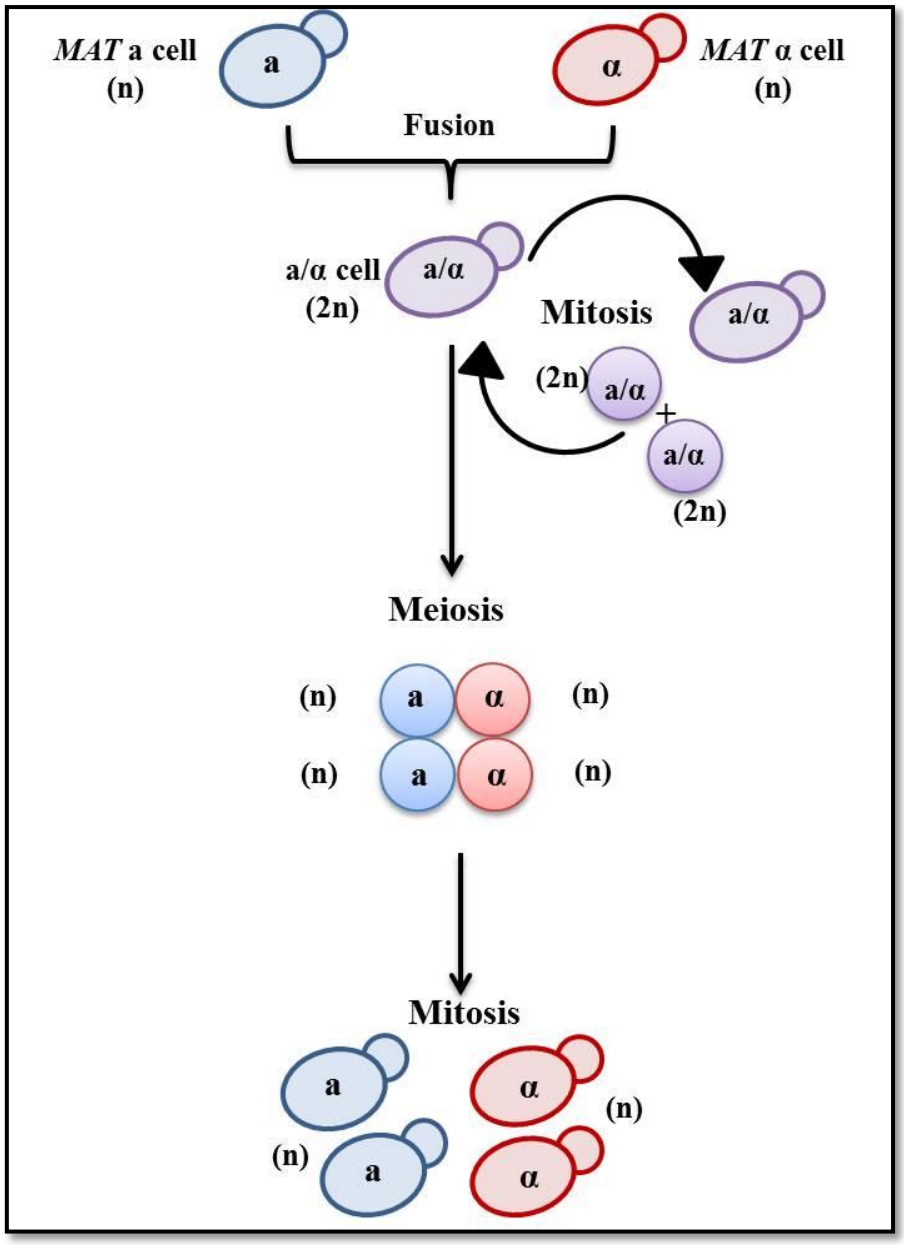


**Figure 1.1 The Life cycle of yeast consisting of sexual and asexual phases**

Two haploid cells of opposite matting type; **a** and α fuse together to form diploid cell (**a**/α). When grown in a rich medium, diploid cells undergo mitosis and continue to grow by budding to form two identical diploid cells. Upon starvation, the diploid cell undergoes meiosis forming a tetrad of haploid spores. Four spores germinate from the ascus and divide by mitosis. Figure modified from Griffiths *et al*., 2012.

The two life cycles in the *Saccharomyces* found in the nature can be classified as homothallism (self-fertile cells) and heterothallism (unable to self-fertilise) (Herskowitz, 1988). Most natural isolates are homothallic as they can switch mating type between **a** cell and α cell and therefore diplodization readily occurs. The mechanism of mating type switching is regulated by the *HO* gene which encodes for an endonuclease that cut the *MAT* locus. *HO* gene is expressed in haploid cells either in **a** or in α cells during the G1 phase of the life cycle and also in the mother cell (Harashima, 1994). Therefore, the homothallic haploid cell has the ability to switch mating genotype by the process called gene-conversion (Russell *etal*., 1986). The *HO* gene encodes a site-specific DNA endonuclease that cleaves the DNA at the recognition site in *MAT* locus, creating a double-stranded nick or gap in the DNA, thus initiating the mating-type switching from **a** to α and vice versa (Klar, 1987, Herskowitz, 1988). The resulting gap is repaired by two transcriptionally silence copies of *MAT* **a** and *MAT* α called Hidden *MAT* Left (*HML*) and Hidden *MAT* Right (*HMR*) (Herskowitz, 1988). These two loci are suppressed by silent information regulator genes (SIR) that produce proteins required for maintaining the silenced structure at the *HMR/L* loci (Nasmyth, 1982, Ravindra *et al*., 1999). As a result, **a** cell switches to α cell through replacing the silent locus *MAT***a** to *MAT*α by homologous recombination (Figure 1.2). On the other hand, heterothallic yeast cells maintain the same mating-type during the haploid growth cycle because they have defective and non-functional *HO* gene due to a substitution mutation (Meiron *et al*., 1995). Therefore, heterothallic yeast strains are unable to switch mating type, and are favourable for laboratory experiments as they facilitate the propagation of haploid cultures and genetics crosses between various strains (Landry *et al*., 2006).

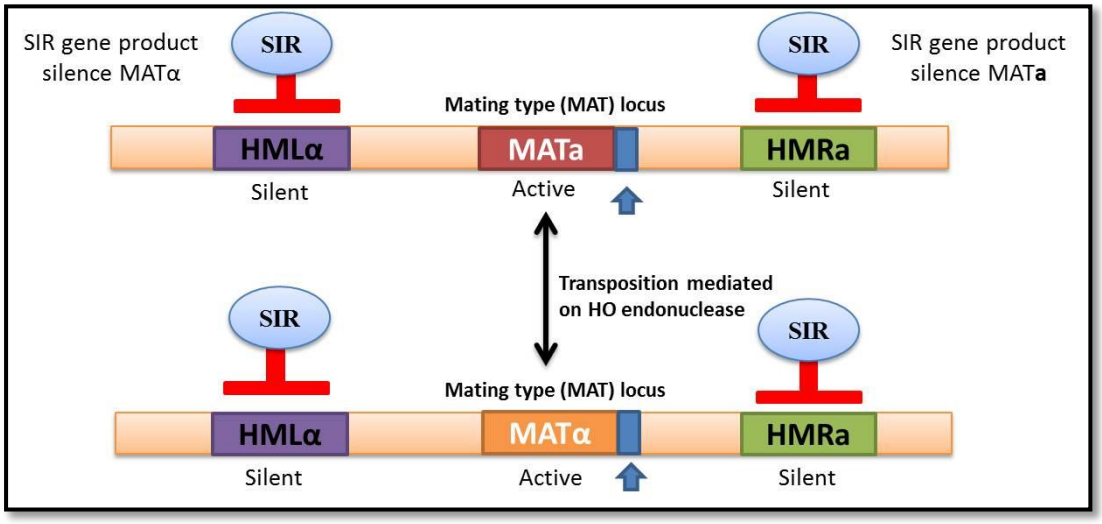


**Figure 1.2 Mating type switching in yeast**

*HO* gene encoded DNA endonuclease cleaves the DNA at *MAT* locus (blue arrows represent the *HO* cleavage sites). During cell switching, the conversion between *MAT* locus and the silent locus *HMR* or *HML* occurs. The genetic information in *MAT* is replaced by either of two silent loci (*HMR* and *HML*). DNA encoding the opposite mating type copies the genetic information from silent locus and the old mating type at *MAT* locus degredates. SIR gene encodes protein that is responsible for the suppression of silent locus.

There are three different ways through which a haploid cell returns to the diploid form after germination. They are: amphimixis (outcrossing), automixis (self-fertilization) and haplo-selfing (Auto-diploidization). Automixis might occur when some haploids fuse with other haploids from the same tetrad that were initially produced by the same meiosis. While, amphimixis can occurs between unrelated haploids from different tetrads (Knop, 2006). This type of mating occurs in less than 1% among mating types (Reuter *et al*., 2007). Auto-diploidization can also occur in haploids that have switched mating type that then fuse with their clone mates, producing homozygous diploid (Knop, 2006, Zakharov, 2005).

### 1.1.3 Physiology: The Crabtree effect

The yeast cellular energy metabolism produces an important energy source, namely Adenosine triphosphate (ATP) that is required for several processes like growth and transport. The majority of yeasts are capable of producing ATP from sugar through respiration and fermentation pathways. The glucose is degraded and converted into pyruvate through glycolysis pathway in both respiration and fermentation pathways. Briefly, each glucose produces 2 ATP and the resulting fructose is converted into 2 3-carbon sugars, followed by degradation of these sugars into pyruvate producing 2 ATP and 1 NADH per glucose. In fermentation, the pyruvate is further degraded into ethanol without producing ATP and does not required oxygen ($O_2$). Whereas, in respiration pathway, the pyruvate is oxidized completely to produce $CO_2$ and that yield more ATP (Verduyn *et al*., 1991, de Kok *et al*., 2012, van Gulik and Heijnen, 1995). In *S. cerevisiae*, the amount of ATP produced by the respiration pathway was found to be higher than that produced in fermentation pathway, 18 ATP versus 2 ATP per glucose, respectively (Pfeiffer and Morley, 2014). *S. cerevisiae* strains ferment glucose (sugar) to ethanol and carbon dioxide ($CO_2$) under anaerobic process (Pronk *et al*., 1996). However, when the concentration of the glucose and oxygen ($O_2$) are

high, the ethanol is produced aerobically. Therefore, the use of fermentation process in the presence of $O_2$ is referred to specific phenomenon, the Crabtree effect (Crabtree, 1929, De Deken, 1966, Postma *et al*., 1989, Van Urk *et al*., 1990), in which the Crabtree-positive yeasts such as *S. cerevisiae* and *S. pombe* degrade sugars to ethanol by fermentation while in the Crabtree-negative yeasts such as *Kluyveromyces lactis* and *Candida albicans* degrade the sugars to $CO_2$ by the respiration process (Pronk *et al*., 1996, Piskur *et al*., 2006). In Crabtree-positive yeasts, under high glucose concentration, the ethanol is produced by degradation of glucose (C6) to pyruvate (C3) via glycolysis and the energy (ATP) is released by substrate level phosphorylation and NADH (Piskur *et al*., 2006). This fermentation of sugar to ethanol in the presence of oxygen occurs through glucose repression mechanisms that inhibit respiratory protein coding genes and enzymes and thus ethanol is accumulated (De Deken, 1966, Johnston, 1999). As the glucose concentration is increased (above $6x10^{-3}$M) (De Deken, 1966), the aerobic fermentation rate also increased and eventually the production and accumulation of ethanol takes place.

The advantages of the Crabtree-positive yeasts including *sensu stricto* species, is their ability to adapt to the environmental stresses (Crabtree, 1929) including, low $O_2$ level and high ethanol concentration, the production and utilization of ethanol, and repression of glucose (Veiga *et al*., 2000). These properties prove to be useful for the selection of *Saccharomyces* yeasts strains for industrial fermentations including brewing (Hansen and Piskur, 2003). All Crabtree-positive *sensu stricto* species provide an important advantage in competing against the Crabtree-negative yeasts by accumulation of ethanol which inhibits their growth (Piskur *et al*., 2006, Sicard and Legras, 2011). That accumulated ethanol is then consumed by specific strategy called make-accumulate-consume that is another property of *sensu stricto* species (Piskur *et al*., 2006).

## 1.2 Saccharomyces *sensu stricto* complex

Yeast species belonging to the genus *Saccharomyces* have been divided into *'sensu stricto'* and '*sensu lato'* groups (Naumov, 1987, Fischer *et al*., 2000). The *Saccharomyces sensu stricto* group consists of nine species and many hybrid: *S. cerevisiae, Saccharomyces paradoxus* (*S. paradoxus*), *Saccharomyces cariocanus* (*S. cariocanus*), *Saccharomyces uvarum* (*S. uvarum*), *Saccharomyces mikatae* (S. *mikatae*), *Saccharomyces jurii* (S. *jurii*), *Saccharomyces kudriavzevii* (*S. kudriavzevii*), *Saccharomyces arboriculus* (*S. arboriculus*), *Saccharomyces eubayanus* (*S. eubayanus*) (Naumov *et al*., 2000, Kurtzman, 2003,Wang and Bai, 2008, Gayevskiy and Goddard, 2016, Baker *et al*., 2015) as well as *Saccharomyces pastorianus* resulting from hybrid crosses between *S. eubayanus* and *S. cerevisiae* (Tamai *et al*., 1998) and *S. bayanus* an hybrid between *S. cerevisiae* and *S. uvarum* (Gonzalez *et al*., 2006). *S. cerevisiae, S. uvarum, S. eubayanus, S. pastorianus* and hybrid between *S. cerevisiae* and *S. kudriavzevii* are associated with industrial fermentation processes whereas *S. paradoxus, S. cariocanus, S. arboriculus, S. kudriavzevii*and *S. mikatae*and *S. jurii* are isolated from natural habitats (Kurtzman, 2003, Wang and Bai, 2008). It is known that species within *sensu stricto* group, which are post-zygotically isolated (Naumov, 1987), can mate and generate viable hybrids, showing an absence of pre-zygotic barriers (Sniegowaski, 2002). In addition, yeasts within this group exhibit almost identical karyotypes with 16 chromosomes (Carle and Olsen, 1985, Cardinali and Martini, 1994, Naumov *et al*., 1992, Naumov, 1996). Alternatively, *Saccharomyces sensu lato* including species such as: *Saccharomyces servazzii* (*S. servazzii*), *Saccharomyces castellii* (*S. castellii*), *Saccharomyces dairenensis* (*S. dairenensis*), *Saccharomyces unisporus* (*S. unisporus*), *Saccharomyces exiguous* (*S. exiguus*) and *Saccharomyces kluyveri* (*S. kluyveri*) (Quesada and Cenis, 1995). The karyotypes of the yeast species within this group varies and is heterogeneous ranging from 8 to 16 (Petersen *et al*., 1999).

Comparing the size of mitochondrial DNA (mtDNAs) molecules between these two groups revealed that the *sensu stricto* species exhibit larger mtDNA molecules (64 to 85 kb) as compared with *Saccharomyces sensu lato* that exhibited smaller mtDNAs (23 to 48 kb). The intergenic guanosine-cytosine (G+C) clusters are represented in high amount in *Saccharomyces sensu stricto* that recognized are by *HaeIII* and *MspI* G+C-cutting restriction enzymes (Spirek *et al.*, 2003, Groth *et al*., 2000). The G+C content in *sensu lato* yeasts group is higher than those in *sensu stricto* group (≤ 24.5 mol% versus ≤ 18 mol%, respectively) that is recognized by *HaeIII* and *MspI* G +C- enzymes (Piskur *et al*., 1998). The gene order in *sensu stricto* group is slightly conserved because of which the chromosomes are thought to have high homology (Hunter 1996, Ryu 1996) with only 5% sequence divergence in the coding regions of the mitochondrial genes. Whereas, the *sensu lato* group represented 10 to 20 % diversity in the coding regions and the gene order was not conserved and showed some variation (Petersen *et al.*, 1999). *Saccharomyces sensu stricto* and *sensu lato* groups vary in their nuclear chromosomes number and of their mtDNA size and organization.

## 1.3 Evolution of the yeast genome

After the completion of *S. cerevisiae* genome sequence in 1996, yeast species have been widely used in different experimental studies (Goffeau *et al*., 1996, Mewes *et al*., 1997). This achievement has proved to be very valuable and beneficial as a reference for other eukaryotic as well as for human genome sequences. The genome of *Saccharomyces* has undergone several changes that contribute to its current shapes (Kellis *et al*., 2003). *Saccharomyces sensu strico* group underwent whole-genome duplication (WGD) and this is the main mechanism that contributed to its genome evolution (Ohno, 1970). Whole-genome duplication (WGD) refers to the doubling of the entire genome in a single event, causing one of the two results: auto-polyploidy events (duplication of its own genome), or allo-polyploidy events (genome duplication via hybridization of two closely related species) (Liti and Louis, 2005) followed by gene loss and genome reshaping (Wolfe and Shields, 1997, Gordon *et a*l., 2009). The *Saccharomyces sensu strico* ancestor has undergone a WGD event that occurred approximately 100 million years ago (Wolfe and Shields, 1997), where 90% of the duplicated genes were lost.

In 1997, Wolfe and Shields were the first scientists who analyzed the event of whole-genome duplication by using BLAST-P amino-acid sequence to search for all yeast proteins. Several genes were observed on one chromosome, had a series of paralogous (two copies of the same gene) on another chromosome with the same order. The duplicated regions were detected as diagonals and with conserved transcriptional orientation. These were 55 large duplicated chromosomal regions which contained 376 pairs of paralogous genes, likely to be formed by a single duplication event (tetraploidy) followed by reciprocal translocations. Only 63% was the average of sequence similarity of the amino acids between the gene pairs. Also only 25% of the duplicated genes were found within each block and the rest were single copy. These observations indicated that each complete block was duplicated followed by deletion of

several genes. In addition to that, the orientation of the duplicated blocks from centromere to telomere was also conserved in each two copies of genes. From these observations they confirmed that the duplicated blocks were formed by translocations which caused the orientation to be in one direction and not random, because if the blocks were formed by independent duplication, the orientation would be random. Their hypothesis proposed that the genome of *S. cerevisiae* originated from an ancient WGD and the 55 blocks were preserved without any rearrangement during yeast evolution (Wolfe and Shields, 1997). In 2004, *Kluyveromyceswaltii* (non-WGD species) that had emerged from *S. cerevisiae* lineage before WGD were sequenced and analyzed to prove the hypothesis of WGD. The non-WGD genomes were observed to had a ‘‘double conserved synteny’’ with the genome of *S. cerevisiae*, in which two regions of *S. cerevisiae* aligned into pairs with each region of *K. waltii*. Their analysis revealed that the WGD covered the whole *S. cerevisiae* genome, which is comprised of 16 centromeres distributed into 8 ancestral pairs that were found to be syntenic with non-WGD species centromeres. They proved that the WGD happened in yeast after the separation from *K. waltii* therefore, *S. cerevisiae* is degenerate tetraploid. As a conclusion, the WGD event may happen at the stage of haploid or diploid through auto-polyploidy or by the fusion of two closely related species forming hybrids through allo-polyploidy (Kellis *et al*., 2004). It has been shown that in *S. cerevisiae* genome, about 551 pairs of duplicated genes out of 5,774 protein coding genes, were formed by the WGD and were followed by 114 genome rearrangements (Byrne and Wolfe, 2005, Gordon *et al*., 2009). The remaining 500 paralogous gene pairs are divided into three types (Kellis *et al*., 2003). The first type includes large duplicated blocks with conserved gene orientation known as cluster homology regions (CHRs); this kind of duplication was detected in *S. cerevisiae* and showed some of the paralogous genes pairs in its genome (Lalo *et al*., 1993, Melnick and Sherman, 1993, Smith, 1987). The second type called segmental and single gene duplication

was represented by similar genes singly duplicated and distributed through the genome. Segmental duplication could occur either as intra-chromosomal or inter-chromosomal duplication of a DNA segment on the genome. Intra-chromosomal duplications have been detected more often than inter-chromosomal duplications (42 vs. 6 of 48) (Achaz *et al*., 2000, Koszul *et al*., 2004). Whereas the third duplication called sub-telomeric duplication, which is detected in *S. cerevisiae* (Louis and Borts, 1995), was found in the sub-telomeric regions that included middle repetitive elements (Liti and Louis, 2005). Therefore, the WGD species contributed massively to the changes that take place in the chromosomal number and structure during the evolution of yeast (Gordon *et al*., 2011).

Several other molecular mechanisms that were found to be responsible for the yeast genome evolution changes, include expansions of tandem gene arrays, segmental duplication, single gene duplication (SGD) (Dujon, 2010), and horizontal gene transfer (HGT) (Liti and Louis, 2005). HGT also played an important role in the evolution of yeast history (Piskur *et al*., 2006) These mechanisms are summarized in the Table 1.1.

HGT plays an important role in both prokaryotic and eukaryotic genomes evolution, in which the genetic material is exchanged between two unrelated species (Andersson, 2005). The transfer of genetic materials may occur between mitochondria and nuclear DNA within the same eukaryotic cell and also can occur between distant species (Timmis *et al*., 2004). Several researches detected genes in yeast genome that were originally transferred from bacteria as a result of HGT such as *URA1* (Gojkovic *et al*., 2004, Nara *et al*., 2000, Zameitat *et al*., 2004) and *BDS1* genes (Hall *et al*., 2005). *URA1* and *BDS1* genes were both found in the S288C *S. cerevisiae* genome and those were originally from bacterial genome, are best examples of HGT in yeast (Hall *et al*., 2005, Gojkovic *et al*., 2004, Nara *et al*., 2000, Zameitat *et al*., 2004). This mechanism has been found to be beneficial to the yeast genome

as *URA1* is required for uracil synthesis that allows the cell to grow under anaerobic conditions and *BDS1* helps catalysing the sulphate from different biological sources (Hall *et al*., 2005). Another *S. cerevisiae* strain EC118, acquired genes from non- *S. cerevisiae* donors regions encoding 34 genes that were found to be responsible for carbon and nitrogen metabolism that are important for yeast fermentation (Novo *et al*., 2009). Therefore, HGT provides useful information regarding the life style of the organisms.

**Table 1.1** Molecular mechanisms shaping the evolution of the yeast genome

| Mechanisms | Descriptions | References |
|---|---|---|
| **Whole-genome duplication** | An evolutionary event that doubles the genetics content in the genome, resulting in multiple copies of the gene. | Wolfe and Shields, 1997, Hittinger, 2013, Wolfe, 2015 |
| **Expansions of tandem gene arrays** | An evolutionary incident in which clusters of similar or identical genes were distributed in the genome between species. | Petes, 1980, Szostak and Wu, 1980, Dujon, 2010 |
| **Segmental duplication** | Duplication of large chromosomal DNA segments (10 to 1000 kb) that were not randomly distributed in the yeast genome. | Ohno *et al*., 1968, Marques-Bonet *et al*., 2009 |
| **Single gene duplication** | An event in which a single gene duplicated within the genome can either becomes fixed or rapidly lost. | Lynch and Conery, 2000 |
| **Horizontal gene transfer** | An evolutionary process describes the movement or incorporation of genetic material into a genome through transformation, conjugation, transduction or any other mechanisms. | Hittinger, 2013, Piskur and Langkjaer, 2004 |

## 1.4 *Saccharomyces paradoxus* and *Saccharomyces cariocanus* as models for studying impact of rearrangements on reproductive isolation and fitness

Over the years, *Saccharomyces paradoxus* has become an attractive model organism for studying population genetics and genomics of wild yeast and it is closely related to *S. cerevisiae* showing similarity in their genome organization and physiology (Liti and Schacherer, 2011, Sampaio and Goncalves, 2008, Sniegowski *et al*., 2002). There is about 14% DNA sequence divergence between these two species (Liti *et al*., 2006, Liti *et al*., 2009) and were found to be biochemically (Barnett *et al*., 1990) and phenotypically (Sweeney *et al*., 2004, Barnett *et al*., 2000) indistinguishable. However, unlike *S. cerevisiae*, *S. paradoxus* is not associated with human activity (Fischer *et al*., 2000, Kellis *et al*., 2003) and it is generally limited to natural environmental niches (Borneman and Pretorius, 2015). *S. paradoxus* has 16 linear chromosomes (Naumov *et al*., 1992) in its haploid form with approximately 12 million base pairs (bp) in its total genome length (Kellis *et al*., 2003). It is usually easily isolated from oak exudates, tree bark and in some oak-associated soil (Sniegowski *et al*., 2002, Naumov *et al*., 1998, Sampaio and Goncalves, 2008). Genomic sequencing data of *S. paradoxus* revealed three highly diverged geographic populations isolated from Europe, Far East Asia and North America (Liti *et al*., 2006, Liti and Louis, 2005), in addition to one isolate from Hawaii (Naumov *et al*., 2000). The level of sequence divergence is higher between European or Far Eastern and North American (4.6%) than between European and Far Eastern (1.5%). Several data demonstrated that crosses of all three subpopulation revealed partial reproductive isolations with spore viability ranging between 0.67 to ~80% (Liti *et al*., 2006, Sniegowski *et al*., 2002, Greig *et al*., 2003) while, crosses within the same subpopulation revealed more than 90% viable spore (Greig *et al*., 2003). The reason behind that partial reproductive isolation was the differences in the DNA sequences between the

strains (Liti *et al*., 2006). Not only the sequence divergence causes low spore viability but also the presence of translocations can affect the fertility (see section 1.5).

Three type of reproductions are observed in natural populations of *S. paradoxus* including; outcrossing (inter-ascus), clonal reproduction and auto-diploidization (inbreeding) (Johnson *et al*., 2004). *S. paradoxus* populations exhibited high levels of mating within the same tetrad and very rare instance of outcrossing as observed by Johnson *et al.,* 2004. In 2008, the sequence of two subpopulations; 12 from Europe and 8 from Far East Asia were analyzed by Tsai *et al.,* (2008) to evaluate the alternative mode of reproduction. DNA sequence variation in chromosome III was examined by calculating the mutational and recombinational diversity to evaluate the two estimated population sizes. If the type of reproduction was sexual, the values must be almost equal. However, they observed differences by three orders of magnitude among these two population sizes. These indicated that the two *S. paradoxus* populations undergo sexual cycle (including mating and sporulation) every 1,000 asexual (vegetative) cycles. In addition, they compared the recombination diversity as function of distance from *MAT* locus in chromosome III and estimated the mating frequency. As a result, 94% represent mating within the same tetrad (intra-tetrad), 5% with a clone mate after switching the mating type (auto-diploidization) and 1% for outcrossing (Tsai *et al*., 2008). As a result, the genetic variation in *S. paradoxus* species have been clearly determined from all these types of reproduction, thus illustrating that *S. paradoxus* populations reproduced asexually more often than sexually and the majority of mating occurs within same tetrad with no genetic variation.

*Saccharomyces cariocanus,* which is found in Brazil (Morais *et al*., 1992), shows high sequence identity to *S. paradoxus* with only 0.29% sequence divergence (Liti *et al*., 2006, Liti *et al*., 2009). It was isolated from South America and characterized by four reciprocal

translocations comprising eight chromosomes; IX/XV (9/15), XII/XIV (12/14), IV/XI (4/11) and XVI/II (16/2) (Fischer *et al*., 2000). Translocation between XVI/II chromosomes resulted from an ectopic recombination event, in which the cross over occurs at non-homologous region (Montgomery, 1987, Montgomery 1991), and found between two duplicated genes; *TEF1* (YPR080W) and *TEF2* (YBR118W). However, the translocations between IX/XV, XII/XIV and IV/XI are due to transposons of yeast (TY) elements, including both full length transposons and long terminal repeats (LTRs) (Fischer *et al*., 2000). Translocations mostly occur between these TY elements and the breakpoints occur near these transposons (Mathiopoulos *et al*., 1998, Fischer *et al*., 2000, Dunham *et al*., 2002). These TY elements can undergo homologous recombination with other TY element resulting in chromosomal rearrangements (Downs *et al*., 1985, Kupiec and Petes, 1988).

Crosses between *S. cariocanus* and *S. paradoxus* strains result in very low spore viability regardless of their low sequence divergence (0.29%) (Liti *et al.*, 2006, Liti *et al*., 2009). Because of this, scientists considered them as two different species based on the Biological Species Concept (BSC) (Mayr, 1942). However, due to the low sequence divergence, some researchers considered the *S. cariocanus* a South American sub-population of *S. paradoxus* (Liti *et al*., 2009, Liti *et al*., 2006, Naumov *et al*., 2000, Boynton and Greig, 2014).

Since their genomes differ by four reciprocal translocations, these two species can become model systems for studying and understanding the evolution of translocations as reproductive isolation (RI) barriers. Different RI barriers are mentioned in the following section and the impact of translocation on spore viability is mentioned in section 1.5.

## 1.5 Speciation and Reproductive isolation barriers

Defining species concepts is a critical subject that depends on different criteria on how one species can be distinguished from another one (Kwon-Chung and Varma, 2006). In 1955, Kottelat divided the species into several group in which each group shared similar sets of characters. These characters allow the species in one group to be distinguished from the species in the other group (Kottelat, 1955). The definition of species can vary according to different criteria: such as morphology, (morphological species concept, MSC) (Mayr, 2001, Giraud *et al*., 2008) evolutionary history (phylogenetic species concept, PSC) (Hittinger, 2013), ecological parameters (ecological species concept, ESC) or fertility (biological species concept, BSC) (Liti *et al*., 2006, Giraud *et al*., 2008, Mayr, 2001, Mayr & Ashlock, 1991, Mayden, 1997). Each of these species concepts has some strengths and weaknesses. In the MSC, the species are grouped according to their morphological characteristics (Mayr, 2001, Giraud *et al*., 2008). Therefore, the species can be easily recognized from the other species as they can be distinguished phenotypically and the similarity and differences in their morphology can be easily noticed (Cronquist, 1978). The other advantage of this species concept, it can be applied for both sexual and asexual species, however; it does not fit within population genetics. The BSC has defined species as group of organisms that can breed successfully together but are unable to breed with other such groups, belong to the same species (Liti *et al*., 2006, Giraud *et al*., 2008, Mayr, 2001). The BSC reflects the species genetics and can apply only to the sexual organisms. So, it cannot be applicable in case of two species that never had a chance to meet and interbreed as well as species that reproduce asexually. The PSC has defined the species as small group that classified as one group, share a common parental ancestor in their genes (Hittinger, 2013). The PSC depends mainly on the analysis of the DNA sequence of the genetic relationships (Cracraft, 1983, Kwon-Chung, 2006). The ESC has classified the species regarding their adaptation to specific resources in

the environment (Mayr, 2001, Giraud *et al*., 2008, Hittinger, 2013). It can be applied for both sexual and asexual species. Interbreeding in nature is required to define and identify the species concepts; however the presence of any barrier, such as sequence variation and chromosomal rearrangements may affect this interbreeding (Liti *et al*., 2006).

Reproductive isolation is a collection of mechanisms that prevent two species from mating successfully or generating fertile offspring as a result of geographical, behavioural, physiological and genetic barriers (Wu and Ting, 2004). Reproductive isolation can be divided into pre-zygotic that act before mating, and post-zygotic that act after mating (Giraud *et al*., 2008).

Pre-zygotic isolation (or pre-mating isolation) refers to the absence of mating or fertilization between species due to ecological, behavioural or mechanical factors (Wu and Ting, 2004). The expected causes of pre-zygotic isolation are the differences in the mating preference between species and/or difference in the timing of spores germination due to genetic differences between species (Maclean and Greig, 2008). Murphy *et al*., (2006), examined how pre-zygotic isolation occurs in yeast by performing mating trials with *S. cerevisiae* and *S. paradoxus* species. They performed cell-to-cell mate choice by placing one haploid cell with define mating type of one species with two mating possibilities of the opposite mating type cell. They observed that *S. cerevisiae* cells mated significantly more often with other *S. cerevisiae* cells than with *S. paradoxus*. The explanation of that was the presence of mating propensity differences in which the *S. cerevisiae* cells mated faster and higher than *S. paradoxus* cells, thus *S. cerevisiae* cells controlled the mating interactions (Murphy *et al*., 2006). Similar observations were detected by Maclean and Greig (2008), who observed that species significantly preferred to mate with the same species rather than different species. Their analysis suggested that the mate performance occurred because of the differences in the

spore germination timing between wild type species (Maclean and Greig, 2008). Further study proved that vegetative cells of *S. cerevisiae* and *S. paradoxus* significantly preferred mating with *S. cerevisiae* cells in both spore-to-spore and cell-to-cell mating because of the differences in the timing of mating and germination (Murphy and Zeyl, 2012). These above studies hypothesized different reasons behind the pre-mating isolation that exist between yeast strains.

Post-zygotic isolation (or post-mating isolation) is defined as the case where members of two species can mate but the hybrids generated are either non-viable or sterile (Wu and Ting, 2004). Species within the *sensu stricto* group can mate and generate viable hybrids, (Hunter *et al*., 1996, Sebastiani *et al*., 2002) however, spores that are produced by F1 hybrids are non-viable (Greig, 2009). Several studies highlighted different mechanisms that strongly affect the fertility of F1 hybrid specifically Dobzhansky-Muller incompatibility, sequence divergence and chromosomal rearrangements (CRs) (Muller, 1942, Chambers *et al*., 1996, Hunter et al., 1996, Naumov *et al*., 2000, Delneri *et al*., 2003). DNA sequence divergence of about 10 % to 30 % can prevent recombination and pairing of homologous chromosomes resulting in improper meiosis (Hunter *et al*., 1996). CRs, particularly translocation, may produce sterlitiy hybrids through the induction of quadrivalent configuration and that resulted from missegregation of chromosomes during meiosis (Stebbins, 1958, King, 1987). In the following sections each of these post-zygotic mechanisms are described individually.

### 1.5.1 Dobzhansky-Muller incompatibility

Genetic incompatibility is an important mechanism that complements DNA sequence divergence in yeasts. This model was first proposed by Dobzhansky-Muller (Muller, 1942) and occurs between epistatically interacting genes. According to this model, when the ancestral species divided to create two daughter lineages, incompatible changes occurred in alternative members of a pair of loci, though they got co-adapted (Liti *et al.,* 2006). Although these changes are neutral or sometimes beneficial, the diverged genes, when brought together in a hybrid will interact in such a way that it reduces viability (also called negative epistasis). These genes can invoke strong reproductive isolation and are called speciation genes. The advent of genome-wide sequencing technology has made it possible to precisely evaluate the onset of intraspecific reproductive isolation among various yeast species. Recently, studies carried out by two different research groups, on *S. cerevisiae* and *S. paradoxus* have reported that chromosomal rearrangements are the major mechanism that contributes to reduced offspring viability in yeast (Charron *et al.*, 2014; Hou *et al.*, 2014). Strikingly, these groups observed no evidence of Dobzhansky-Muller incompatibility in both the species. As a matter of fact, most of the studies that crossed yeast isolates and estimated the offspring viability were carried out under controlled laboratory conditions. Such conditions provide access to rich permissive media that optimizes yeast growth. However, if the diverse environmental stresses that a natural yeast population encounters are taken into consideration, Dobzhansky-Muller incompatibility appears to be a common phenomenon (Hou *et al.,* 2015). To evaluate this hypothesis, Hou *et al*., conducted a systematic survey by using 27 natural yeast isolates from various sources across different continents. All the strains selected in the study had high offspring viability when crossed with S288c reference strain. However, when these strains were exposed to 20 different culture conditions, the offspring viability decreased from 1 to 62%. The results of this study clearly indicate that Dobzhansky-Muller incompatibility

readily segregates within yeast natural populations and contributes to intraspecific reproductive isolation (Hou *et al.,* 2016).

### 1.5.2 Sequence divergence (SD)

Proper genetic recombination is obtained when the DNA sequences are homologous to each other and thus can align in meiosis and exchange DNA via recombination. Therefore, non-homologous genetic material reduces the recombination efficiency (Hunter *et al*., 1996). Several studies have investigated the causes of hybrid sterility that occurs between yeast species. The main mechanisms causing the meiotic sterility is the SD, in fact it was shown that SD directly correlated with the spore viability (Liti *et al*., 2006, Sniegowski *et al*., 2002, Greig *et al*., 2003). As sequence divergence increases the spore viability decreases and vice versa. Several experiments were carried out to test the impact of DNA variations on yeast fertility.

*S. paradoxus* populations (Far East, America, Europe and Hawaii) exhibit 4% genetic variations (Liti *et al*., 2009, Leducq *et al*., 2014) and that variation may occur at early stage of the speciation (Boynton and Greig, 2014). *S. paradoxus* haploid cells from these populations can mate and generate viable hybrids during mitosis, but upon meiosis about 86% of the spores were found to be unviable (Liti *et al*., 2006, Charron *et al*., 2014, Greig *et al*., 2003, Kuehne *et al*., 2007). The DNA sequence divergence between these populations led to improper meiotic segregation. Similar observations were detected by Bing *et al*., (2014), which confirmed that only 7% sequence differences between *S. eubayanus* populations caused the reduction of spore viability to 18% (Bing *et al*., 2014). These data suggested that sequence divergence as postzygotic barrier is sufficient to reduce the hybrids spore viability (Liti *et al*., 2006). Interestingly, when two closely related species, such as *S. paradoxus* and *S.*

*cariocanus* are crossed together, about 95% of their resulting spores are unviable despite their low sequence differences (0.29%) (Liti *et al*., 2006, Liti *et al*., 2009). Therefore, the presence of other barriers like CRs may reduce the spore viability between them.

### 1.5.3 Chromosomal rearrangements (CRs)

Chromosomal rearrangements, which cause changes in chromosomal structure, are caused by breakage of DNA double helices at two different locations in the genome, followed by re-joining of the broken ends. This process might cause new arrangements of the chromosome in the genome including deletions, duplications, inversions and translocations (Zuffardi *et al*., 2009, Lee *et al*., 2007). These rearrangements are found to be a contributing factor in the evolution and speciation of yeast species (Fischer *et al*., 2000, Ryu *et al*., 1996, Ryu *et al*., 1998) and plant speciation (Rieseberg and Willis, 2007). CRs can either reduce or prevent the chromosomal recombination and that result in preventing the gene flow and produce low viable spores (Rieseberg, 2001, Noor *et al*., 2001). Yeasts have underwent different rearrangements in their genome during their evolution including, changing in number of chromosomes (Keogh *et al*., 1998, Langkjaer *et al*., 2000), reciprocal translocations that have been detected in *S. cariocanus*, *S. uvarum* and *S. mikatae* related to *S. cerevisiae* (Fischer *et al*., 2000) and duplication of several genes (Wolfe and Shields, 1997, Hou *et al*., 2014). In human, DNA damage that cause chromosomal abnormality occurs in response to failure of DNA repair mechanisms, which function to prevent any error during replication, causing many human diseases (Kolodner *et al*., 2002, McKinnon and Caldecott, 2007).

Chromosomal rearrangements can cause sterility in many species as they can interrupt meiosis in hybrids; therefore they became significant factors that are responsible for reproductive isolation and speciation (Giraud *et al*., 2008, Greig *et al*., 2003). Chromosomal

translocation is considered as a common genetic abnormality that has contributed to genome instability and is linked to many diseases in yeast and higher organisms (Lemoine *et al*., 2005).

In 1914, Theodor Boveri proposed the first evidence of involving the chromosomal translocation in human cancers (Boveri, 2008) and many other diseases (Klemke *et al*., 2011, Teicher *et al*., 2011, Rowley, 1973, Nowell, 1985, Zech *et al*., 1976). Translocation arises as a result of failure in repairing the errors that has occurred during double strand break (DSB). The DSB happen throughout physiological processes; as a result of DNA replication errors (Meaburn *et al*., 2007) and pathological process, as well as the exposure to different types of radiation and oxidative stress (Lieber, 1998). Also it can happen if there is viral infection (Weitzman *et al*., 2010) and when meiotic recombination starts (Keeney and Neale, 2006). Two mechanisms that are required for repairing the DSB are: homologous recombination (HR) and non-homologous end joining (NHEJ) (Brugmans *et al*., 2007, Petes, 1991, Nickoloffand Hoekstra, 1998). In yeast, HR is the active repair mechanism that requires *RAD52* (YML032C) gene to start repairing the errors (Paques and Haber, 1999, Nickoloffand Hoekstra, 1998). However, mutation in the *RAD52* activates the NHEJ repair mechanism that involves two proteins; yKu70p and yKu80p (Critchlow and Jackson, 1998). HR requires the presence of homologous chromosome or sister chromatid that contain DNA sequence homologous to that of damaged chromosome. Consequently, HR retrieves the information that was lost in the damaged chromosome and repairs the errors (Lieber, 2010,van den Bosch *et al*., 2002). The NHEJ does not require the presence of sequence homology because the damaged DNA ends are ligated directly (van den Bosch *et al*., 2002). Failing in any of these two repair mechanisms would result in mutations and cell death (Jackson, 2001, Thacker, 1986, Kanaar *et al*., 1998).

Reciprocal translocation, which is one type of translocation, occurs when two non-homologous chromosomes break and exchange fragments. In a cell, heterozygote for one translocation during the first meiotic division, the two normal chromosomes and the two translocated chromosomes pair together forming a quadrivalent configuration. There are three ways that the quadrivalent can be resolved in anaphase, involving either alternate segregation or adjacent segregation of chromosomes. In the case of alternate segregation, the normal chromosomes are pulled to one pole of the cell and the translocated chromosomes are pulled to the other pole. This type of segregation produces four viable gametes; two normal chromosomes and two balanced translocations. In the case of adjacent I segregation, the homologous centromeres dis-join pulling one normal chromosome and one translocated chromosome from the other chromosome to one pole of the cell and the other normal chromosome and translocated chromosometo the other pole. While the adjacent II segregation, which is considered rare segregation, one normal chromosome and one translocated chromosome from the same chromosome are pulled to one pole of the cell and the other normal chromosome and translocated chromosome to the other pole. Both adjacent segregations yield unbalanced and unviable gametes, each containing a large deletion and a large duplication (Greilhuber *et al.,* 2013, Udall, *et al*., 2005, Nicolas, *et al*., 2007) (Figure 1.3).

Translocations have been found in *Saccharomyces sensu stricto* species; four in *Saccharomyces uvarum* (Fischer *et al*., 2000, Ryu *et al*., 1996), four in *Saccharomyces cariocanus*, one in each *Saccharomyces mikatae* strains (IFO1816 and IFO1815) (Fischer *etal*., 2000), two in *Saccharomyces eubayanus* (Baker *et al*., 2015) and one in *Saccharomyces arboricolus* (Liti *et al*., 2013). These naturally occurring translocations were studied extensively to estimate the reasons behind the reproductive isolation between yeast species. The impact of chromosomal translocations on meiotic fitness is mentioned in section 1.6.

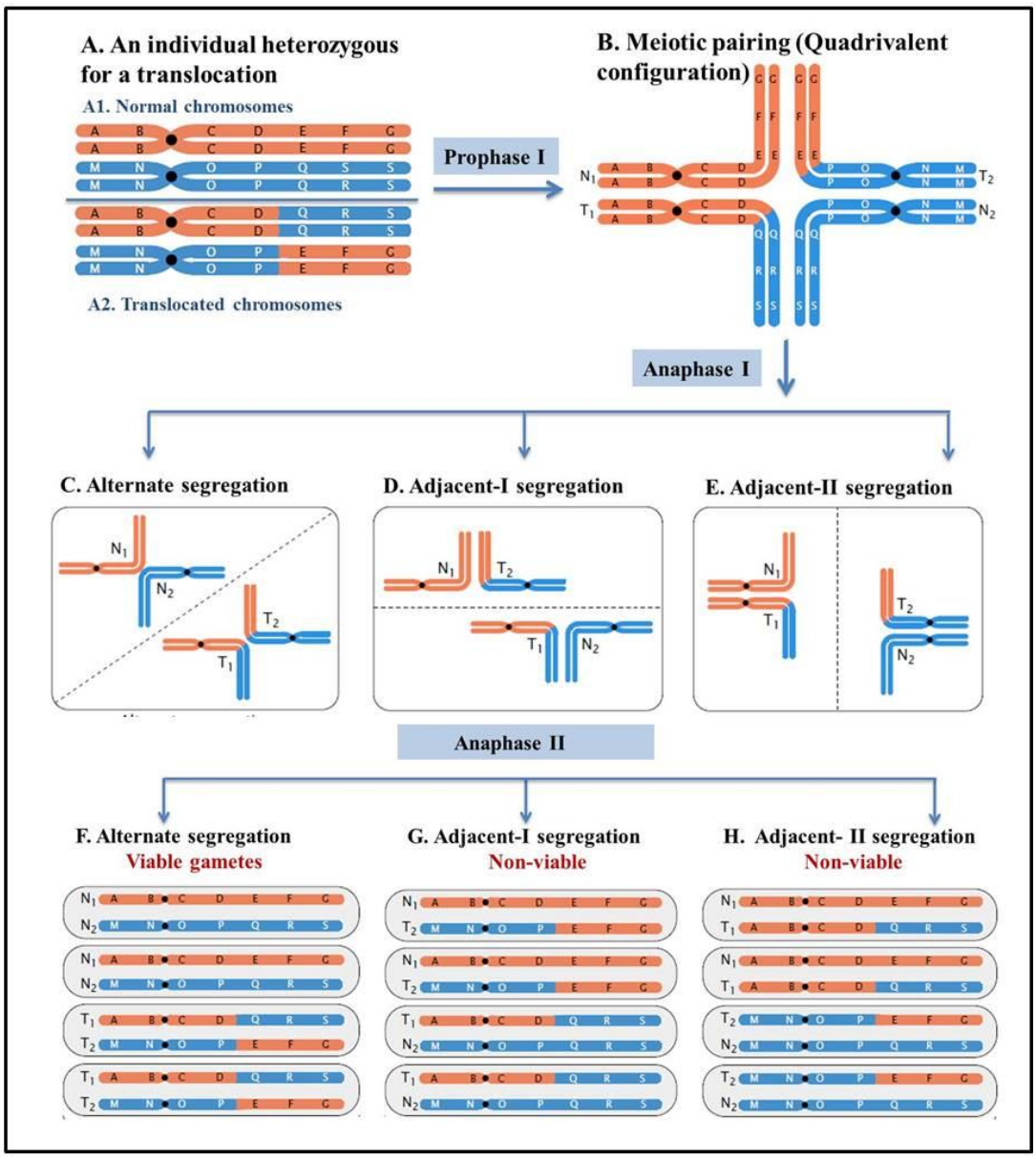


**Figure 1.3 Chromosomal segregation possibilities in reciprocal translocation heterozygote.**

During meiosis, two normal chromosomes **(Panel A.1)** and two translocated chromosomes **(Panel A.2)** pair together in prophase I resulting in quadrivalent configuration **(Panel B)**. After the meiotic paring, three ways that the quadrivalent can be resolved in anaphase I, either alternate segregation (**Panel C)** or adjacent segregation of chromosomes (**Panels D and E).** In alternate segregation, Normal chromosomes move toward one pole and the translocated chromosomes move to the other (**Panel C).** In adjacent I and II segregations, each pole contain one normal and one translocated chromosomes **(Panel D and E).** During anaphase II, alternate segregation yields balance gametes with normal and translocated chromosomes **(Panel F)** or adjacent segregations yield unbalanced gamete with large duplications and deletions **(Panels G and H).** (Figure modified from Benjamin A. Pierce, 2009).

## 1.6 Impact of chromosomal translocations on *Saccharomyces yeast* species

Several researches have highlighted the impact of translocation on gamete viability (Fischer *et al*., 2000, Naumov *et al*., 2000, Delneri *et al*., 2003, Liti *et al*., 2006). Four translocations make an important contribution to hybrid sterility between *S. paradoxus* and *S. cariocanus* as observed by Fischer *et al*, 2000. They showed that crosses between them produced very low spore viability, of 0.66% (Fischer *et al*., 2000). Similar observations were obtained after mating *S. cariocanus* UFRJ50971 and *S. cariocanus* UFRJ50816 strains with different geographical populations of *S. paradoxus* strains including European Far-East Asian, North American and Hawaiian populations. All these mating produced sterile hybrids (Naumov *et al*., 2000). Delneri *et al*., (2003) revealed that crossing *S. cerevisiae* strains that differed in the presence of translocations, producing low spore fertility, suggested that translocations strongly affect the yeast fertility and contributed to the reproductive isolation (Delneri *et al*., 2003). Further studies showed that CRs add an important contribution to hybrid sterility between *S. cariocanus* and *S. paradoxus* strains that produces approximately 5% viable spores following crossing and tetrads analysis. They concluded that reciprocal translocation decreases the spore viability to 50% and non-reciprocal translocation to 25% (Liti *et al*., 2006). Hybrid sterility and gamete viability between *Saccharomyces* species are strongly caused by several reproductive mechanisms that may drive the evolutionary changes between these species.

## 1.7 Strategy for creating chromosomal translocations

Several efficient molecular experiments can be performed in vivo to generate reciprocal translocation, using PCR-mediated gene disruption strategy combined with Cre-*lox*P recombination system (Delneri *et al*., 2003) and non-reciprocal translocation using bridge-induced translocation (BIT) (Tosato *et al*., 2005, Tosato *et al*., 2013).

PCR-mediated gene disruption strategy and Cre-*lox*P recombination are two efficient molecular tools for creating CRs including; CTs, inversions, deletions, and insertions. PCR-mediated gene disruption strategy is considered a powerful technique for studying gene function and only requires a minimum of 45bp DNA sequence on either side of marker gene for efficient integration by homologues recombination into the genome (Baudin *et al*., 1993, McElver and Weber, 1992). Cre-*lox*P recombination is a site-specific recombination technology applied both in eukaryotic and prokaryotic systems. This strategy was applied in yeast *S. cerevisiae*, describing the first application of Cre-*lox*P system (Sauer, 1987) and was used to study gene expression in mammalian cell lines and transgenic mice (Sauer and Henderson, 1988).

The Cre-*lox*P system was found to be useful for many applications, such as controlling gene expression, deleting undesired DNA sequence, creating chromosomal translocation (Delneri *et al*., 2003). In this system, Cre gene encodes Cre protein, which is 38-kDa site-specific recombinase derived from bacteriophage P1, catalyzes the site-specific recombination between the two DNA recognition sites, called *lox*P (Langer *et al*., 2002, Hamilton and Abremski, 1984). The *lox*P sequence is a 34bp consensus sequence composed of two flanking 13bp inverted repeated sequences, where the Cre-recombinase binds, separated by 8bp core sequence, where recombination occurs (Zhang and Lutz, 2002).

The molecular mechanism of recombination starts with Cre protein that recognizes and binds to the both *lox*P sites, performing a double strand break, followed by re-joining the ends and removing the marker cassette (Figure 1.4). The outcome of recombination depends on the orientation and location of the *lox*P sites, that determine whether Cre recombinase induces deletion, inversion, or chromosomal translocation. When the *lox*P sites are oriented in the same direction, Cre recombinase mediates a deletion. However, when they are oriented in opposite directions, Cre recombinase creates an inversion between the two sites. On the other hand, when the *lox*P sites are located on different chromosomes, it is possible for CT to be created by Cre recombinase (Nagy, 2000).

The dominant $kan^r$ marker gene is efficiently used in gene disruption experiments in a variety of yeast strains. This was also used in the form of *lox*P-*kanMX*-*lox*P cassette that combines the beneficial $kan^r$ marker with the Cre-*lox*P recombination system. The *lox*P-*kanMX*-*lox*P cassette can be integrated with high efficiency into genomic locus of interest by homologous recombination using PCR. Following the transformation of this cassette, the Cre recombinase, which is expressed by *GAL1* promoter, removed the *KanMX*, leaving a single *lox*P site in the chromosome (Guldener *et al.*, 1996). In 1999, Goldstein and Mccusker created new resistance marker cassettes; *hph* and *nat* cassettes that were found to be resistance to antibiotics hygromycin B and nourseothricin, respectively. Later, several researchers modified the previous cassettes and also constructed new selectable marker cassettes. For example, *KlURA3*, *LYS2* and *SpHIS5* flanked by two *lox*P sequences were replaced from *KanMX* marker cassette (Delneri *et al*., 2000). *hphNT1*and *natNT2* marker cassettes with different terminators make them suitable to be used at the same time in one yeast strain (Jank *et al*., 2004). Also, four new gene-disruption cassettes including *lox*P-*natNT2*-*lox*P, *lox2272–natNT2–lox-2272*, *lox*P-*hphNT1*-*lox*P,and *loxLE-hphNT1-loxRE* were generated by Carter and Delneri (Carter and Delneri, 2010). All these selectable marker

cassettes were constructed to facilitate the generation of multiple gene deletions and accelerate the whole deletion process. Thus, these markers were found to be very useful for creating several mutations in one strain. In addition, they do not have any sequence homology to the yeast genome hence, reducing the chance of integration to other location. Resistance marker cassettes; *kan*, *hph* and *nat* have ideal and widespread use in experimental studies due to their good features (Goldstein and McCusker, 1999).

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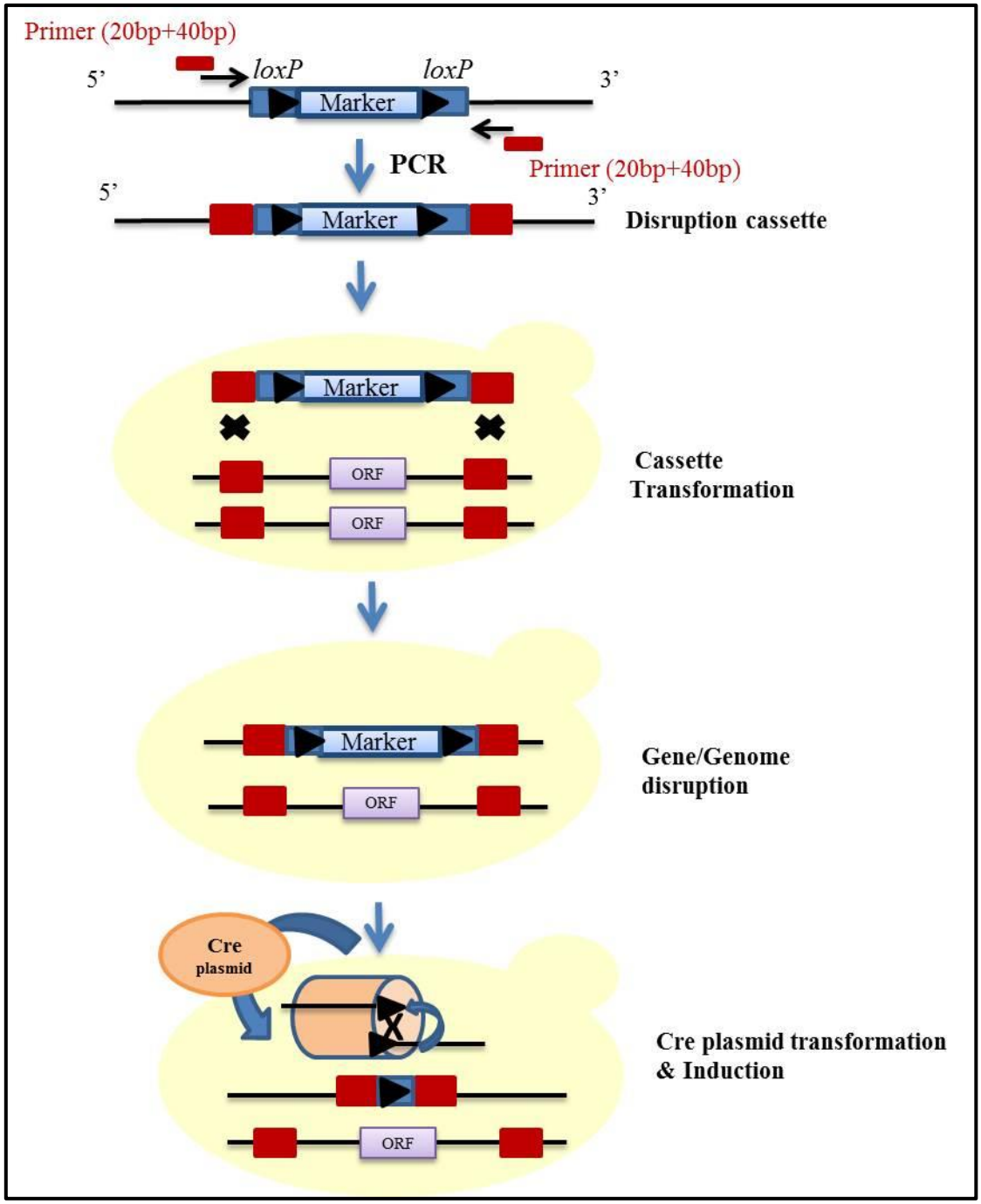


**Figure 1.4 The *lox*P/Cre gene disruption and marker rescue procedure**

The gene disruption cassette (marker) flanked by two 34 bp *lox*P sequences is amplified by PCR using oligonucleotide primers consisting of 20 bp specific to cassette and 40 bp specific to the gene/ genome. The cassette is transformed into the yeast cells and gene disruption occurs by homologous recombination. Expression of the Cre recombinase results in the removal of the marker gene, leaving behind a single *lox*P site at the chromosomal locus. Figure modified from Gueldener *et al.*, (2002).

## 1.8 Experimental Aims

*S. paradoxus* and *S. cariocanus* are reproductively isolated, and exhibit high sequence similarity except for four translocations presented in *S. cariocanus* genome. We hypothesized that it is possible that such rearrangements are the cause of low spore viability in *S. paradoxus* and *S. cariocanus* hybrids. Moreover, we hypothesized that translocations also contribute to the phenotypic fitness and gene expression. These data will inform yeast scientists and taxonomists on the appropriateness of using either BSC or PSC as species concept for these fungi.

**Approaches to examine our hypotheses:**

**1- Evaluating meiosis fitness:**

PCR-mediated gene replacement method combined with Cre-*lox*P mediated rearrangements technique were carried out to construct two chromosomal translocations in *S. paradoxus* YPS138 strains to render those parts of the genome collinear to that of *S. cariocanus*. Hybrids from these two species were tested for spore viability.

**2- Evaluating mitotic fitness and phenotypes :**

Microplate growth assay was carried out to evaluate the effect of translocation on yeast mitotic growth and to understand whether these translocations bring fitness advantages under different environmental conditions, including three different temperatures.

Light microscopy was carried out to test any morphological changes that may occur because of the translocation.

### 3- Evaluating gene expression:

Transcriptomic study was carried out by using RNAseq technology to examine the changes in gene expression as a response to translocation.

## 1.9 Flow diagram

**Four main questions were addressed in current research**

Q1. Is CT the sole responsible for the reproductive isolation between *S. paradoxus* and *S. cariocanus*?

**Method:**

- Two CTs in *S. paradoxus* YPS138 were created using Cre/*lox*P.
- Hybrids were created by crossing *S. paradoxus* with *S. cariocanus*.
- FACS analysis was performed for the validation of the ploidy.
- Sporulation of hybrids & tetrads dissection were performed.
- Genome sequence of *S. cariocanus* UFRJ50816 by PacBio DNA sequencing.

**Results and Discussion:**

- Hybrids of *S. cariocanus*/*S. paradoxus* (carrying 2 translocations) reversed showed a significant increase in spore viability (S.V) (12.7%) compared to control hybrids (3.4%) ($P$=0.0125).
- By reversing the genome rearrangements in *S. paradoxus* strain, the S.V increased by ca. 2 fold, so, the maximum S.V that we predict for the collinear cross is 50.8%.
- Two types of rearrangements were identified in *S. cariocanus* genome: one T and 11 inversions.
- Five translocations explained the variations observed in meiotic viability.

Q2. Does CT bring growth advantages or disadvantageous on yeast strains fitness under different environmental conditions?

**Method:**

- Fitness assays were carried out under different conditions. Including YPD, F1, C-limited, N-limited, P-limited and S- limited at 16 °C, 30 °C and 35 °C.

**Results and Discussion:**

- **At 30 °C and 35 °C:** significant drop in the mitotic fitness was observed in the translocant strains and the control non-translocant S. para-$NT_{12/14}T_{9/15}$, in comparison to the wild types and the non-translocant control S. para-$NT_{9/15}$.
- **At 16 °C:** significant drop in the mitotic fitness was observed regarding their maximum biomass and significant drop in C-limited media of all engineered strains only regarding their growth rate.
- The first CT (IX/XV) impaired the mitotic fitness of *S. paradoxus* strain.
- The second CT (XII/XIV) had no further effect on the phenotype (Neutral).

## Four main questions were addressed in current research

Q3. Does the gene expression change globally or only genes near the translocation breakpoints change as a response to translocations?

**Method:**

- Total RNA from wild types and all engineered strains including three biological replicates were extracted and purified using QIAGEN RNeasy Mini Kit.
- RNA sequencing was carried out using Illumina Hiseq 2500.

**Results and Discussion:**

- Significant changes in genes expression were observed in S. para $T_{9/15}$ vs S. para $NT_{9/15}$ involving 219 genes.
- 29 genes are located on the translocated chromosome IX/XV.
- Gene expression changed globally rather than being restricted to the breakpoints region.
- Many genes found to be co-expressed with other genes, so any change in the expression of one of these genes, may contribute to the changes of the others.
- Gene Ontology analysis revealed that most of the genes are involved in different aspects of metabolisms.

Q4. Does the CT affect the cell morphology?

**Method:**

- Light microscopy was carried out to visualize cell morphology of the translocant strains and wild type strains.

**Results and Discussion:**

- Abnormal cell morphology was detected in translocant strains; S. para-$T_{9/15}$ and S. para-$T_{12/14}T_{9/15}$ (66.2% and 36.4%, respectively).
- Gene Ontology analysis revealed that some of the genes are involved in general cellular structure.
- Morphological changes were observed in translocant strains due to the changes in the expression of many genes that are important for maintenance of cell morphology like; *LYS1*, *LYS12*, *SPO24*, *MET32* and *DLD2*.

# Chapter Two

# Materials and Methods

# 2. Materials and Methods

## 2.1 *Saccharomyces* strains

*Saccharomyces paradoxus* (*MAT***a**) YPS138 and *Saccharomyces cariocanus* (*MAT**a***) UFRJ50816 background strains were used for construction of translocant and non-translocant strains, as well as the hybrids. A List of strains used in the current study is provided in Table 2.1.

**Table 2.1** Complete list of strains used in current study

| Strains | Genotype | Source |
|---|---|---|
| ***S. cariocanus* UFRJ50816** | Prototrophic diploid strain | Provided by Daniela Delneri Lab |
| ***S. cariocanus* UFRJ50816** | *MAT***a**; HOΔ1::natNT2 | This study |
| ***S. paradoxus* YPS138** | *MAT***a**/α; Ura3Δ::KanMX | Liti Lab |
| ***S. paradoxus* YPS138** | *MAT***a**; *lox*P-*natNT2-lox*P and *lox*P-*hphNT1-lox*P cassettes inserted in intergenic regions upstream of MNT3 and THI20 genes | Provided by Daniela Delneri Lab |

## 2.2 Oligonucleotides

Saccharomyces Genome Re-sequencing Project (SGRP) software was used for downloading *S. paradoxus* YPS138 sequence (http://www.moseslab.csb.utoronto.ca/sgrp/). The *S. cariocanus* genome was sequenced by PacBio sequencing and uploaded to the University of California Santa Cruz (UCSC) Genome Browser Bioinformatics website (https://genome.ucsc.edu/). PCR primers were designed using the Primer3 programme and were blasted using the SGRP BLAST (Basic Local Alignment Search Tool) server. The Oligonucleotide sequences are provided in Appendix Tables A1 and A2.

## 2.3 Plasmids

Yeast transformation was carried out using the plasmid pSH-*ble*$^{r}$ Cre, which included the Cre recombinase gene. Plasmids PZC1, PZC2, PZC4 and pUG6, which encoded the hygromycin B, clonNAT (for both PZC2 and PZC4), and Geneticin (G418) resistance genes, respectively, were used as templates. These templates were used for production of marker cassettes *lox*P-*hphNT1-lox*P, *lox*P-*natNT2-lox*P, *lox2272-natNT2-lox2272* (Carter and Delneri, 2010), and *lox*P-*KanMX-lox*P (Guldener *et al*., 1996) for replacing the intergenic regions or target gene.

## 2.4 Yeast and Bacterial Media

### 2.4.1 Yeast media

- **YPD Liquid and solid media:** Yeast extract peptone dextrose **(**YPD) liquid media was prepared with 1% (w/v) Bacto$^{TM}$-yeast extract, 2% (w/v) yeast peptone and 2% (w/v) D-glucose. YPD solid media (YPD agar plate) was prepared in the same way as liquid media with the addition of 2% (w/v) agar.

- **YPD containing clonNAT:** YPD media containing the desired 100μg/ml clonNAT antibiotic (Werner BioAgents, Jena, Germany).
- **YPD containing hygromycin B**: YPD media containing the desired 300μg/ml hygromycin (Duchefa Biochemie).
- **YPD containing geneticin:** YPD media containing the desired 300μg/ml geneticin (G418) (GibcoBRL).
- **YPD containing phleomycin:** YPD media containing the desired 10-20μg/ml phleomycin (Invivo-Gen).
- **YPD containing galactose:** YPD media was prepared from 1% (w/v) Bacto™-yeast extract, 2% (w/v) yeast peptone and 2% (w/v) galactose.
- **YPD containing raffinose:** YPD media containing 10% (w/v) raffinose as a carbon source.
- **Synthetic dextrose SD media containing geneticin:** Synthetic dextrose media containing 0.67% (w/v) yeast nitrogen base without ammonium sulphate (YNB), 0.24% (w/v) Urea, 2% (w/v) agar, and 300 μg/ml geneticin.
- **Pre-sporulation media:** pre-sporulation medium containing 0.8% (w/v) Bacto™-yeast extract, 0.3% (w/v) Bacto™-peptone and 50 ml of 40% (w/v) glucose.
- **Sporulation solid media:** sporulation media containing 5 g potassium acetate, 0.5 g yeast extract, 0.313 ml 40% glucose and 10 g agar in 500 ml distilled water.
- **Limited media:** Nitrogen- (N), carbon- (C-), phosphorus- (P-) and sulphur- (S-) limited media were prepared by addition of mineral salts specific to each media, 0.5 ml of 10,000 x trace element solution I, 0.5 ml of 10,000 x trace element solution II ($FeCl_3$), and water up to 400 ml. The solution was autoclaved. A carbon source consisting of 25 ml of 40% (w/v) glucose was added to all limited media except for C-limited media, which received 3.125 ml. Filter-sterilised vitamins were added; 0.825

ml of 600 x stock was added to all media, amino acid Uracil 5 ml of 0.2 g/100 ml stock was added, and water was added up to 500 ml. F1 media was prepared in the same way as the C-limited preparation with 2% (w/v) glucose. The concentrations of mineral salts for each specific media, trace element media and vitamin stock solution are listed below (Tables 2.2, 2.3 and 2.4).

**Table 2.2** Mineral salt media

| **Mineral salt media** | **C-lim** | **N-lim** | **P-lim** | **S-lim** |
|---|---|---|---|---|
| $(NH4)_2SO_4$ | 3.13 g/l | 0.46 g/l | 3.13 g/l | 0.024g/l |
| $NH_4Cl$ | | | | 2.5 g/l |
| $KH_2PO_4$ | 2 g/l | 2 g/l | 0.054 g/l | 2 g/l |
| $KCl_2$ | | | 1.10 g/l | |
| $MgSO_4.7H_2O$ | 0.55 g/l | 0.55 g/l | 0.55 g/l | |
| $MgCl_2.2H_2O$ | | | | 0.45 g/l |
| NaCl | 0.10 g/l | 0.10 g/l | 0.10 g/l | 0.10 g/l |
| $CaCl_2.2H_2O$ | 0.09 g/l | 0.09 g/l | 0.09 g/l | 0.09 g/l |

**Table 2.3** Trace element media

| **Trace element media** | **10,000 x stock** | **Final concentration** |
|---|---|---|
| **Solution I** | | |
| ZnSO4.7H2O | 0.7 g/l | 0.07 mg/l |
| CuSO4.5H2O | 0.1 g/l | 0.01 mg/l |
| H3BO3 | 0.1 g/l | 0.01 mg/l |
| KI | 0.1 g/l | 0.01 mg/l |
| **Solution II** | | |
| FeCl3.6H2O | 0.5 g/l | 0.05 mg/l |

**Table 2.4** Vitamin stock solutions

| **Vitamin stock solution (Filter sterilize)** | **600 x stock** | **Final concentration** |
|---|---|---|
| Inositol | 37.2 g/l | 62 mg/l |
| Thiamine/HCL | 8.4 g/l | 14 mg/l |
| Pyridoxine | 2.4 g/l | 4 mg/l |
| Ca-panthothenate | 2.4 g/l | 4 mg/l |
| Biotin | 0.18 g/l | 0.3 mg/l |

### 2.4.2 Bacterial media

- **Lysogeny Broth: Lysogeny Broth** (Bertani G., 2004) (LB) media containing 10 mg/ml bacto-tryptone, 5mg/ml Bacto$^{TM}$-yeast extract, and 10 mg/ml sodium chloride (NaCl).

## 2.5 Colony PCR reagents and conditions

BIOTAQ DNA Polymerase (catalogue number BIO-21040) and MyTaq DNA Polymerase (catalogue number BIO-21105) were used for confirmation PCR. Preparation of the templates were carried out by suspending a small amount of colony in 50 µl of sterile MilliQ water and heating at 95 °C for 10 minutes followed by cooling the ruptured cells on ice for 5 minutes. PCR was performed in 50 µl reaction mixtures and the components are listed below (Table 2.5).

**Table 2.5** PCR Reaction Mixtures

| **PCR reaction mixtures** | |
|---|---|
| **BIOTAQ Master Mix :** | |
| 10mMdNTPs | 1 µl |
| Template DNA(10 ng/µl) | Suspend colony in $H_2O$, heat for 10 minutes |
| 10x Reaction Buffer | 5 µl |
| 50mM $MgCl_2$ | 1.5 µl |
| Oligonucleotide primer (each) | 100 µM (1 µl) |
| 5x BIOTAQ DNA Polymerase | 0.5 µl |
| Sterile water | Up to 50 µl |
| **MyTaq Master Mix:** | |
| 5 x MyTaq reaction buffer | 10 µl |
| Template DNA | Suspend colony in $H_2O$, heat for 10 minutes |
| Oligonucleotide primer (each) | 100 µM (1 µl) |
| 5 x MyTaq DNA Polymerase | 0.5 µl |
| Sterile water | Up to 50 µl |

The reaction conditions were carried out as the following; the mixtures were heated to 95°C for 3 minutes, followed by 35 cycles of: 45 seconds at 95°C, 45 seconds at 55°C and 3 minutes at 72°C with a final extension of 5 minutes at 72°C with a concluding hold at 4°C. Additional PCR reagents or conditions that used are mentioned in the following sections if required.

## 2.6 Genomic DNA extraction

*S. cariocanus* strain was grown overnight in YPD culture followed by DNA extraction using Qiagen Genomic-tip kit following manufacturer's recommended procedure. Genomic DNA extraction was performed at Johns Hopkins Deep Sequencing and Microarray Core Facility in the United States of America (USA). All DNA samples were used for PacBio sequencing.

## 2.7 Plasmid DNA extraction and restriction digestion

PsH-Cre *ble* plasmid was extracted using QIAprep spin miniprep kit (QIAgen catalogue no.27104) following the manufacture's protocol, which provided an expected yield of high-copy plasmid DNA of up to 20 µg. *E. coli* cultures were incubated overnight in 5 ml LB medium and centrifuged for 10 minutes at 6000 x g. Pelleted bacterial cells were re-suspended in 250 µl Buffer P1 and transferred to a microcentrifuge tube, followed by addition of 250 µl Buffer P2 and 350 µl of Buffer N3. Suspensions were mixed together by inverting the tube six times, and then the mixture was centrifuged for 10 minutes at 18000 x g. The supernatant was transferred to the QIAprep spin column, centrifuged for 1 minuteat 18000 x g, and washed twice with 500 µl PB Buffer and 750 µl PE Buffer. DNA was eluted by adding 50 µl water to the QIAprep spin column and centrifuging for 1 minute at 18000 x g.

- ***Hind*III restriction enzyme** was used to digest the extracted Cre plasmid. The reaction mixture was incubated for one hour at 37°C (Table 2.6). The digested and undigested DNA samples were analyzed and confirmed by 1% agarose gel electrophoresis.
- ***EcoRV* restriction enzyme** was used to digest the extracted Cre plasmid marker disruption cassettes; *lox*P-*natNT2*-*lox*P, *lox2272*-*natNT2*-*lox2272, lox*P-*hphNT1*-*lox*P and *lox*P-*KanMX*-*lox*P. The reaction mixture was incubated for 15-30 minutes at 37°C (Table 2.6). The digested and undigested DNA samples were confirmed by 1% agarose gel electrophoresis.

**Table 2.6** Restriction digestion mixtures

| **Reaction Mixture:** | |
|---|---|
| ***Hind*III** | |
| 10 units restriction enzyme | 2 µl |
| 100-200ng plasmid DNA | 2 µl |
| 10 x Reaction Buffer | 2 µl |
| Sterile water | Up to 20 µl |
| | |
| ***EcoRV*** | |
| 10 units restriction enzyme | 0.5 µl |
| 1µg plasmid DNA | 500 ng/Concentration of the plasmid |
| 10 x NEBuffer | 2.5 µl |
| Sterile water | Up to 25 µl |

## 2.8 Amplification of marker gene cassettes by PCR

All marker disruption cassettes were amplified prior to transformation into yeast genome. Specific primers were designed to correspond to the intergenic regions and genes of interest (Appendix Table A.1). Amplifications of *lox*P-*natNT2-lox*P and *lox2272-natNT2-lox2272* were carried out using GC-RICH PCR kit (Roche catalogue number 12 140 306 001). The reaction mixtures consisted of two master mixes, and the components are listed below (Table 2.7).

**Table 2.7** PCR reaction set-up

| **PCR reaction set-up** | |
|---|---|
| **Master Mix 1:** | |
| dNTPs | 0.2 mM |
| Template DNA | 0.2 – 0.5 µg |
| 5x GC-RICH Reaction Buffer containing DMSO | 5 µl |
| Oligonucleotide primer (each) | 0.2 µM |
| Sterile water | Up to 35 µl |
| **Master Mix 2:** | |
| 5x GC RICH PCR reaction buffer | 10 µl |
| 5x Taq Polymerase enzyme | 1 µl |
| Sterile water | Up to 15 µl |

The reaction conditions used were an initial start of 3 minutes at 97 °C and then 10 cycles of: 1 minutes at 95 °C for DNA denaturation to allow hybridization of oligonucleotide primers; 30 seconds at 55 °C for annealing of primers; and 2 minutes 40 seconds at 68 °C for DNA extension. This was followed by 20 cycles with the same conditions as previously except for the addition of 20 seconds per cycle to the extension step. A final extension step was carried out for 5 minutes at 72 °C (Janke *et al*., 2004).

Amplifications of *lox*P-*hphNT1-lox*P and *lox*P-*KanMX-lox*P were carried out using BIOTAQ DNA Polymerase kit (Bioline catalogue number 21040) with an additional buffer just used for *lox*P-*hphNT1-lox*P. The reaction mixture is listed below (Table 2.8).

**Table 2.8** PCR reaction conditions

| **PCR reaction conditions** | |
|---|---|
| **Master Mix:** | |
| 10mMdNTPs | 1µl |
| Template DNA | 0.2 – 0.5µg (1 µl) |
| 10x Reaction Buffer | 5µl |
| 50mM $MgCl_2$ | 1.5µl |
| Oligonucleotide primer (each) | 0.2µM |
| 5x BIOTAQ DNA Polymerase | 0.5 µl |
| Sterile water | Up to 50 µl |
| **Additional buffer for hph cassette** | |
| 10x buffer 1 (500mM Tris/HCL pH9.2, 22.5mM MgCl2, 160mM $(NH4)SO_4$) | |

The reaction conditions obtained for *lox*P-*hphNT1*-*lox*P amplification were similar to *lox*P-*natNT2*-*lox*P conditions. While the reaction conditions for *lox*P-*KanMX*-*lox*P were performed as described by Delneri *et al.,*(2003) in which the annealing temperature was 55 °C and with an extension time of 2 minutes (Delneri *et al*., 2003).

## 2.9 Transformation of marker cassettes and plasmid

### 2.9.1 Transformation by lithium acetate

A high-efficiency lithium acetate transformation method from Gietz and Schiestl (2007) was performed with some modifications. A colony of yeast was grown overnight in 10 ml YPD media at 30 °C in a shaker incubator. The culture was diluted to an optical density (OD) at 595 nm of 0.2 in 50 ml YPD media. This was incubated for 3 to 4 hours to reach $OD_{595nm}$ of 0.7. Then the cells were centrifuged for 3 minutes at 6000 x g and washed twice in 10 ml milliQ water (sterile water) and once in 10 ml lithium acetate with trice EDTA buffer (Li-Ac/TE). Cells were re-suspended in 300 µl of Li-Ac/TE buffer and incubated for 15 minutes at 30 °C. Cells (50 µl) were then added to the transformation mixture containing 425 µl of 50% (w/v) polyethylene glycol 3350 ($PEG_{3350}$), 1M Li-Ac and TE (10X), 25 µl of single-stranded carrier DNA (2 mg/ml), and 1µl of purified Cre plasmid. The mixture was incubated for 30 minutes at 30 °C followed by heat-shock at 42 °C for 20 minutes. 100 µl and 200 µl of the transformation mixtures were plated on appropriate selection plates and incubated for 3 days at 30 °C.

### 2.9.2 Transformation by electroporation

In order to transform yeast cells with the Cre plasmid, the electroporation methods of Becker and Guarente (1991) and Gannon *et al*., (1988) were used, with some modification. Preparation of electrocompetent cells was carried out as follows: a colony of yeast was grown overnight in 10 ml YPD media at 30 °C in a shaker incubator, followed by dilution of the culture to optical density (OD) at 595nm of 0.2 in 50-100 ml YPD media. The suspension was incubated from 3 to 4 hours to reach $OD_{595nm}$ of 0.7. The cells were chilled in an ice water bath for 15 minutes to stop growth of the yeast cells, then the cells were decanted into a

sterile 50 ml tube and were pelleted by centrifugation at 3000 x g for 5 minutes at 4°C. The Supernatant was discarded and the centrifuge tubes with the cell pellets were placed on ice followed by addition of 5 - 10 ml ice-cold sterile water to each tube and the cell pellets were re-suspend by vortexing. After that, the volume in each centrifuge tube was brought to 50 ml and cells were pelleted by centrifugation at 3000 x g for 5 minutes at 4 °C. The cells were washed again with 50 ml ice-cold sterile water. The cell pellets were re-suspended in 20 ml ice-cold 1 M sorbitol, transferred to a chilled microcentrifuge tube, centrifuged at 3000 x g for 5 minutes at 4 °C, and the supernatant was discarded. After that, the cell pellet was re-suspended in 0.5 ml of sterile, ice-cold 1 M sorbitol and was kept on ice for electroporation.

Electroporation was carried out as follows: DNA samples (5-100 ng in a volume of 5 µl) were pipetted into sterile 1.5 ml microcentrifuge tube and placed on ice. Using 0.2 cm cuvettes, 40 µl of competent cells were added to each 5 µl DNA sample, mixed gently, and incubated on ice for 5 minutes. The MicroPulser (BIO-RAD) is pre-programmed with different sittings according to the organism being used, therefor it was set to *S. cerevisiae* 0.2 cm cuvette (Sc2) program at a voltage (V) of 1.5 kV and at field strength (E) of 12.5 kV/cm. The DNA-cell samples were transferred to the electroporation cuvette. The cuvette was placed in the chamber slide, the slide was pushed into the chamber until the cuvette was seated between the contacts in the base of the chamber, and the cells were pulsed once followed by addition of 1 ml of ice-cold 1 M sorbitol to the cuvette. The suspension was then transferred into a sterile tube. The time constant was recorded and should be close to 5 milliseconds. Then the cells were plated on selective YPD media containing 1 M sorbitol and were incubated for 48-72 hours at 30 °C. The preparation of 1 M sorbitol for electroporation is listed in Table 2.9.

**Table 2.9** Sorbitol preparation for electroporation

| | |
|---|---|
| **1M sorbitol** | |
| Sorbitol | 182.2 g/L } dissolve |
| Water | 800ml } |
| Bring to volume with water | 1 L |

## 2.10 Generation of chromosomal translocations by Cre/*lox*P gene disruption and marker rescue procedure

PCR-mediated gene replacement method (Wach *et al*., 1994) was carried out for inserting all marker cassettes into the yeast genome at specific break points (Fischer *et al*., 2000). Two PCR checking primers were designed to correspond to the 3' and 5' regions of all marker cassettes and two primers corresponded to the intergenic regions of chromosomes of interest. The transformants were confirmed by diagnostic colony PCR using specific sets of primers (Appendix Table A.2). Strains with confirmed transformant cassettes were used for plasmid transformation and for induction to generate translocation.

### 2.10.1 Induction of pCre-*ble* plasmid

pSH-*ble*$^{r}$ Cre plasmids (0.25- 0.5µg) were transformed into engineered strains possessing resistance marker cassettes by electroporation (Becker and Guarente, 1991). Induction of Cre recombinase gene by *GAL1* promoter was carried out by growing the cells overnight in 10 ml YP-raffinose media at 30°C. The next day, cells were inoculated in YP-galactose media and incubated for 3 to 4 hours at 30 °C. The induced cells were serially diluted (10-fold dilutions), plated on YPD solid media and incubated for two days at 30 °C. Colonies grown on YPD plates were picked again on YPD plates and replica plated on YPD supplemented with phleomycin, hygromycin and clonNat (for the selection of *S. paradoxus*) and YPD, phleomycin, geneticin and hygromycin (for the selection of *S. cariocanus*). All colonies that lost the marker cassettes and Cre plasmids were verified by diagnostic colony PCR to confirm the translocants and non-translocants.

### 2.10.2 Colony PCR for Confirmation of translocant and non-translocant engineered strains

The engineered translocant and control non-translocant strains were verified by colony PCR using BIOTAQ DNA Polymerase and MyTaq DNA Polymerase with different sets of primers corresponding to each chromosome (Appendix Table A.2). The reaction mixture and conditions were carried out as described in Section 2.5. 40 µl of the PCR product was loaded on 1% or 1.5% (w/v) agarose gel electrophoresis in 1xTAE buffer, run for one hour at 100 volts and visualized by ethidium bromide staining (30 µL + 300 ml 1 x TAE) on a transilluminator.

### 2.10.3 Translocation in *S. paradoxus* strain

Two chromosomal translocations of the five existing in *S. cariocanus* UFRJ50816 strain were engineered in *S. paradoxus* YPS138 (*MAT* **a**) strain via the cre-*lox*P recombination method (Carter and Delneri, 2010, Colson *et al*., 2004). Engineered YPS138 strain carried two marker cassettes: *lox*P-*hphNT1*-*lox*P and *lox*P-*natNT2*-*lox*P was used to create the first CT between IX and XV chromosomes. The same YPS138 strain possessing the first translocation was used to create the second translocation, and the process was carried out by transformation of marker disruption cassettes *lox2272-natNT2-lox2272* between chromosomes XII and XIV.

### 2.10.4 Translocation in *S. cariocanus* strains

One chromosomal translocation of the five existing in *S. cariocanus* UFRJ50816 strain was reversed between chromosomes IX and XV. The first marker cassette, *lox*P-*hphNT1*-*lox*P, was inserted between translocation breakpoints YIL014w and YOL055c, and the second cassette, *lox*P-*kanMX*-*lox*P, between YIL015w and YOL054w.

## 2.11 Diploid sporulation

*S. cariocanus* wild type strain and *Saccharomyces* hybrid strains (*S. paradoxus* x *S. cariocanus*) were grown overnight at 30°C in rich pre-sporulation medium. Cells were washed twice with sterile MilliQ water and plated on minimal sporulation medium. The plates were incubated for yeast sporulation at 20°C for 5 to 7 days. Light microscopy was used to visualize the haploid spores.

## 2.12 Generation of hybrids and Tetrads dissection

Engineered *S. paradoxus* translocants and non-translocants were put in physical contact with each of four spores of *S. cariocanus* to create hybrids with 3 translocations (3T), 4 translocations (4T) and 5 translocations (5T) using a Singer Instruments MSM micromanipulator as described previously (Delneri *et al*., 2003). The *S. cariocanus* tetrads were first dissolved in 1.2 M sorbitol with 5 mg/ml zymolase to digest the ascus wall, and incubated at 37 °C for 8 minutes. 10 µl of cells from digested asci were streaked on YPD plates on one side and *S. paradoxus* cells on the opposite side. The mating plates were incubated at 30 °C for 2 to 3 days and hybrids were selected on synthetic dextrose media containing 300 µg/ml geneticin (G418) and 0.24 % urea. All hybrids with confirmed ploidy were sporulated on sporulation medium and dissected with a Singer MSM-300

micromanipulator. Two-sample t-tests were conducted to detect the significant differences between the spore viability of the hybrids, values of $P<0.05$ were considered significant.

### 2.13 Fluorescence Activated Cell Sorting (FACS) analysis

FACS analysis was performed as previously described (Haase and Reed, 2002, Johnson and Kolodner, 1995) with some modifications for confirming the ploidy of all the hybrids. Cells were grown overnight in 5 ml liquid YPD to reach $1x10^6$ cells/m, fixed in 3.5 ml of 95% (v/v) ethanol, and incubated for 2 hours at room temperature. Fixed cells were collected by centrifugation for 5 minutes at 8000 x g, the ethanol fixative was poured off, and cells were washed once in 1ml sterile water and once with 0.5 ml of 2 mg/ml RNase solution (Sigma, Aldrich UK). Cells were incubated for two hours at 37 °C followed by treatment with 0.2 ml of 5 mg/ml protease solution for 30 minutes at 37 °C. Cells were then stained with 1ml of 1μM SYTOX® Green (Molecular Probes, Eugene, OR). The stained cells were sonicated for 20 seconds on low power using a Diagenode Biorupter (Diagenode Corporation, Belgium) and DNA content was determined using a Beckman Coulter CyNn™ ADP flow cytometer (Beckman, Coulter, USA) with excitation wavelength of 588 nm. The DNA contents were analyzed by comparing the fluorescence intensities with those of two reference strains: *S. paradoxus* YPS138 (haploid) and *S. cariocanus* UFRJ50816 (diploid). Measurements were performed on three independent biological replicates.

### 2.14 PacBio sequencing

Around 10 μg genomic DNA of *S. cariocanus* strain UFRJ50816 was first DNA damage-repaired, sheared with Covaris G-tube, end-repaired and exonuclease-treated. A 10-20 kb size SMRTbell library was prepared by ligation of hairpin adaptors at both ends according to manufacturer instructions (Pacific Bioscience, No: 100-259-100). The resulting library was then size-selected using Blue Pippin with a 7-10 kb cutoff. A sequencing run was performed

for 4 hours on a PacBio RS II using P6/C4 n chemistry. The genome was assembled using SMRT analysis HGAP3 pipeline using default setting with genome size of 12 Mb. The PacBio sequencing was carried out at Johns Hopkins Deep Sequencing and Microarray Core Facility.

### 2.15 Annotation of *S. cariocanus* genome

Protein coding gene models were predicted using Augustus (Stanke and Morgenstern, 2005) and the Yeast Genome Annotation Pipeline (Byrne and Wolfe, 2005). Annotated proteins from the *S. cerevisiae* S288C and *S. paradoxus* YPS138 genomes (Scannell *et al*., 2011) (www.yeastgenome.org/download-data/sequence, genome version R64-2-1) were aligned to the assembly using BLAST (Altschul *et al*., 1990) to provide support for the *de novo* predictions. A final set of 5,794 gene models was produced using the Apollo annotation tool (Lewis *et al*., 2002). The protein sequences were functionally annotated using InterproScan (Goujon *et al*., 2010). Orthologous relationships with *S. cerevisiae* S288C sequences were calculated using InParanoid (Berglund *et al*., 2008). Non-coding RNAs were annotated by searching the RFAM database (Nawrocki *et al*., 2015) using Infernal (Nawrocki and Eddy, 2013). Further tRNA predictions were produced using tRNAscan (Lowe and Eddy, 1997). Repeat sequences were identified by searching Repbase (Jurka *et al*., 2005) using Repeat Masker (http://www.repeatmasker.org). The dotplot was constructed by aligning *S. cariocanus* genome to the *S. cerevisiae* S288C genome using NUCmer and plotted using MUMmerplot (Kurtz *et al*., 2004).

## 2.16 Growth fitness assays

Fitness assays under different nutritional and thermal stresses were carried out as previously described with some modifications (Naseeb and Delneri, 2012) using an optima microplate reader (FLUOstar Omega, BMG LABTEACH). The fitness of S. *cariocanus*, *S. paradoxus*, S. para-$T_{9/15}$, S. para-$NT_{9/15}$, S. para-$T_{12/14}T_{9/15}$ and S. para-$NT_{12/14}T_{9/15}$ (See Table 3.2) was measured in different media: YPD, F1 media, C- limited, N- limited, P- limited and S-limited media at three different temperatures (16 °C, 30 °C and 35 °C) for 24-48 hours in YPD and for 72 hours in all limited media. The growth rates of *S. cariocanus*, S. car-4T and S. car-$NT_{(5T)}$ (See Table 3.2) was measured in YPD, N- limited, P- limited and S-limited media at 35 °C for 48 hours. Overnight culture was prepared in 5 ml YPD media, washed twice in distilled water and re-suspended in 5 ml YPD or limited media to an $OD_{595}$ of 0.05. 200 µl of diluted culture and blank control media were aliquoted into each well of a 96-well plate (Costar, 3596). Each engineered strain control was run in three replicates (each with three to six technical replicates). The $OD_{595}$ of the growth was measured every 5 minutes with shaking for 1 minute. Data was analyzed using polynomial regression using the package grofit (R version 1.1.1-1) to estimate both maximum growth rates and maximum biomass (Kahm *et al*., 2010). The P value was calculated by Mann-Whitney test and corrected by normal approximation (Bauer, 1972).

## 2.17 Generation of haploid strains (*HO* deletion)

Haploid strains were generated by deleting the *HO* gene by transformation of *lox2272-natNT2-lox2272* marker cassette using lithium acetate protocol (section 2.9). Primers for cassette insertion and confirmation were designed to target the *HO* gene and their sequences are listed in Appendix Tables A1 and A2. These confirmation primers were designed approximately 400 bp upstream and downstream of the *HO* deletion. The deletion strains were confirmed by colony PCR, confirmed *HO* deletion strains were sporulated (Section 2.10), and tetrads were dissected (Section 2.11). All dissected plates were incubated at 30 °C for two days and replica-plated on YPD containing clonNAT. Only two spores from each tetrad should survive on clonNAT plates. Ten spores were selected to be tested for the mating type.

## 2.18 Identification of mating types

Confirmed deletion haploid strains were crossed with two tester strains: *S. paradoxus- MAT **a*** and *S. paradoxus-MAT α* (Ura3Δ::KanMX). All strains were grown overnight in 3 ml YPD media at 30 °C. Using a 96-well plate, 10 µl of each tester strain was added to each well, followed by the addition of unknown mating type strains to each well that already containing either *MAT **a*** or *MATα* tester strain. The 96-well plate was incubated overnight at 30 °C, then the mixed cultures were replica-plated onto YP containing G418 and clonNAT using a 96-well pen tool, and incubated at 30 °C for 48 hours. Strains with opposite mating types were only grown on the plates containing the markers; therefore **a** and α mating types were identified by the formation of prototrophs with α and **a** strains, respectively. Engineered strains with defined mating types were used for undoing one translocation in *S. cariocanus* and fitness testing using bioscreen.

## 2.19 RNA extraction

Prior to RNA sequencing, total RNA from 6 engineered strains (including three biological replicates) was extracted and purified using QIAGEN RNeasy Mini Kit (Qiagen, catalogue number 74104) to yield 25 μg of total RNA. RNA was extracted from three replicates of wild type strains: *S. paradoxus* YPS138 (*MAT* **a**), *S. cariocanus* UFRJ50816 and each translocant and non-translocant strains. Following the manufacturer's instructions, yeast cells were grown overnight in 5 ml YPD media, diluted to $OD_{595}$ of 0.1 in 15 ml media, and incubated for 4 hours at 30 °Cto reach $OD_{595}$ of 0.5. Pellets of cells were harvested by centrifugation at 1000 x g for 5 minutes at 4 °C, re-suspended in 2 ml freshly prepared Y1 Buffer (1 M sorbitol, 0.1 M EDTA, pH 7.4 0.1% β-ME and lyticase) and incubated for 30 minutes at 30 °C. Spheroplasts were pelleted by centrifugation for 5 minutes at 300 x g and the supernatant was discarded. 350 μl RLT Buffer was added to the homogenized lysate, which was then centrifuged for 3 minutes at 1000 x g followed by addition 350 μl of 70% (v/v) ethanol. Samples were transferred to RNeasy spin column, centrifuged for 15 seconds at ≥ 8000 x g and flow-through was discarded. 700 μl of Buffer RW1 and 500 μl Buffer RPE were added separately, and the samples were centrifuged for 15 seconds at ≥ 8000 x g. 500 μl of Buffer RPE was added then the suspension was centrifuged for 2 minutes at ≥ 8000 x g, and placed in a 1.5 ml collection tube. 50 μl RNase-free water was added to each tube, which was centrifuged for 1 minute at ≥ 8000 x g.

## 2.20 Quantification and Qualification of DNA and RNA

NanoDropLite Ultraviolet spectrophotometer (Thermo Scientific, USA) was used for the assessment of DNA and RNA concentrations. Measurement of RNA was taken at $OD_{260nm}$ and the concentration was 44 μg/ml. The quality of DNA and RNA were assessed by 1% and 1.5% agarose gels, respectively, stained with ethidium bromide and visualized in a Gel Doc™ XR+ with Image Lab™ software (BIO RAD).

## 2.21 RNA sequencing (Illumina sequencing)

Total RNA from wild type strains; *S. paradoxus* YPS138 (*MAT* **a**) and *S. cariocanus* UFRJ50816, and all engineered strains; S. para-$T_{9/15}$, S. para-$NT_{9/15}$, S. para- $T_{9/15}T_{12/14}$ and S.para-$NT_{12/14}T_{9/15}$ (including three biological replicates each) were extracted and purified using QIAGEN RNeasy Mini Kit as described in Section 2.19. Strains were grown on rich YPD media and the sequencing of purified total RNA was carried out using Illumina Hiseq 2500 (Illumina, USA) platform. Whole steps of preparation of the RNA library and the sequencing process were performed at Manchester University in the Genomic Technologies Core Facility. TruSeq Stranded mRNA low throughput LT Sample Prep Kit was using with low sample protocol (LS) (Catalogue number RS-122-9004DOC) according to the manufacturer's protocol.

The analysis of RNA data was carried out in the Bioinformatics department at Manchester University. Quality control (QC) tool was performed with the FastQC tool for high throughput sequencing data (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). FastQ screen was obtained to screen a library of sequencing against a set of sequence databases (http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/)(Anders and Huber, 2010). Mapping and aligning RNA-set datasets were done using Spliced Transcript Alignment to Reference (Jauniaux *et al*. 1978, Dobin *et al*., 2013). Counts were normalized

to adjust the total number of reads using HTSeq-count (Anders and Huber, 2010). Principal component analysis (PCA) was performed from the normalization of the data and differential expression (DE) using DESeq2 (Love *et al.*, 2014). Gene Ontology (GO) analysis was obtainedby using the GO Term Finder (Version 0.83) against the entire *Saccharomyces* Genome Database at default settings on all significantly up- and down-regulated genes (http://www.yeastgenome.org/cgi-bin/GO/goTermFinder.pl).

### 2.22 Light microscopy for determine Cell morphology

Yeast cells were grown in 5 ml YPD media at 30 °C to stationary phase, then diluted to reach an $OD_{595nm}$ of 0.5 – 1 ($1x10^6$ cells/ml). The cells were first fixed with 3.7% formaldehyde and incubated for one hour at 30 °C, followed by washing the cells twice with 1 ml 1x (v/v) $Ca^{2+}$/$Mg^{2+}$-free phosphate buffered saline (PBS) and re-suspended in 1x PBS Buffer containing 0.5 Mm EDTA to prevent clump formation (Giaever *et al*., 2002). The cells were sonicated briefly, placed on slides, and visualized by Nikon DS-QilMc camera and NIS-Elements BR 3.2 software.

The analysis of abnormal cell morphology was conducted by classifying the cells into five classes: elongated, round, small, large, and clumped cells from a total of 25 fields from each biological replicate.

### 2.23 Storage and maintenance of engineered strains

All the confirmed engineered strains and hybrids were stored at -80 °C for further use in the future. Strains were grown in 5 ml YPD media overnight at 30 °C shaker incubator, then 1 ml of the culture was mixed with 500 µl of 50% (w/v) glycerol.

# Chapter Three

# Results

# 3. Results

## 3.1 Construction of *S. paradoxus* strains with chromosomal translocations

*S. paradoxus* YPS138 engineered strains with one and two chromosomal translocations were constructed successfully using Cre-*lox*P recombination system (Colson *et al*., 2004, Delneri *et al*., 2003, Delneri *et al*., 2000) to render these parts of the genome collinear with that in *S. cariocanus* UFRJ50816 strain. The locations of the breakpoints of these two chromosomal translocations were previously defined by Fischer *et al.*, 2000, therefore the marker cassettes were inserted in the intergenic regions between these translocation breakpoints (Table 3.1). Two marker cassettes, *lox*P-*natNT2*-*lox*P and *lox*P-*hphNT1*-*lox*P, were used to create the first chromosomal translocation and inserted between two ORFs: YIL014w/YIL015w, on chromosome IX; and YOL054w/YOL055c, on chromosome XV, respectively. The second translocation was created by inserting the *lox2272*-*natNT2*-*lox2272* cassette first between ORFs YLL023c and YLL026w on chromosome XII then was removed by Cre/*loxP* system. Then the same cassette was inserted between YNL284c and YNL286w on chromosome XIV in the same strain that possessed the first translocation. Transformant colonies that grew on YPD media containing antibiotic selectable markers (neoceothricin and hygromycin) were selected to undergo Cre plasmid transformation to induce recombination between *lox* sites. This plasmid carries genes specific to the Cre recombinase enzyme, which was induced by growing cells in YP-raffinose then in YP-galactose containing media.

Following the induction, all colonies that lost the selectable marker cassettes were confirmed by diagnostic colony PCR using two primers pairs that are specific to the marker cassette and to the genome of interest. *S. paradoxus* YPS138 strains possessing chromosomal translocations and their controls will be referred as S. para-$T_{9/15}$ (carrying one CT), S. para-$NT_{9/15}$ (carrying *lox*P scar without translocation), S. para-$T_{12/14}$-$T_{9/15}$ (carrying first and

second translocations) and S. para-$NT_{12/14}$-$T_{9/15}$ (carrying *lox*P or *lox*2272 scars with first translocation) (Table 3.2). Engineered controls underwent the whole process of the CTs generation containing *lox*P or *lox2272* scars but without translocations. The following steps represent the results of whole processes of the construction of strains carrying CTs.

**Table 3.1** Chromosomal translocation breakpoints in *S. cariocanus* strain representing ORFs that mapped in translocation involving chromosomes IX, XV, XII and XIV

| Species | Translocation | Breakpoint mapping interval |
|---|---|---|
| ***S. cariocanus*** | XV t $IX_L$ | YIL014w-YIL015w |
| | $IX_L$ t $XV_L$ | YOL054w- YOL055c |
| | XIV t $XII_L$ | YLL023c- YLL026w |
| | XII t $XIV_L$ | YNL284c- YNL286w |

**Table 3.2** Complete list of engineered strains and hybrids created in current study

| **Engineered strains (Given name)** | **Strain description** |
|---|---|
| *S. paradoxus* (S. para-$T_{9/15}$) | *S. paradoxus* translocant strain possessing one translocation between chromosomes IX/XV. |
| *S. paradoxus* (S. para-$NT_{9/15}$) | *S. paradoxus* control (non-translocant) strain possessing *lox*P scar without translocation. |
| *S. paradoxus* (S. para- $T_{9/15}T_{12/14}$) | *S. paradoxus* translocant strain possessing two translocations: first translocation between chromosomes IX and XV; and second translocation between chromosomes XII and XIV. |
| *S. paradoxus* (S.para-$NT_{12/14}T_{9/15}$) | *S. paradoxus* control (non-translocant) strain possessing *lox2272* scar and carrying first translocation. |
| *S. cariocanus* (S. car-4T) | *S. cariocanus* translocant strain possessing four translocations; translocation between chromosomes IX/XV was reverse to the normal chromosomal position. |
| *S. cariocanus* (S. car-$NT_{(5T)}$) | *S. cariocanus* control non-translocant strain possessing five translocations and *lox*P scar between chromosomes IX/XV. |
| **Engineered hybrids (Given name)** | **Strain descriptions** |
| *S. paradoxus* 1T crossed with *S. cariocanus* (S. para-$T_{9/15}$ × *S. cariocanus*). 4T system | Hybrid constructed by crossing engineered *S. paradoxus* strain possessing one translocation and *S. cariocanus* strain carrying five translocations (4 Ts were detected by Fischer *et al.*, 2000 and one new T detected in current study) to render one translocation and generate hybrid possessing four translocations. |
| *S. paradoxus* 2T crossed with *S. cariocanus* (S. para- $T_{9/15}$ $T_{12/14}$ × *S. cariocanus*). 3T system | Hybrid constructed by crossing engineered *S. paradoxus* strain possessing two translocations and *S. cariocanus* strain carrying five translocations to render two translocations and generate hybrid possessing two translocations. |
| *S. paradoxus* $NT_{9/15}$ crossed with *S. cariocanus* (S. para-$NT_{9/15}$×*S. cariocanus*). 5T system | Control hybrid constructed by crossing control *S. paradoxus* strain for the first translocation (S. para-$NT_{9/15}$) and *S. cariocanus* to generate hybrid possessing five translocations. |

### 3.1.1 Validation of plasmid DNA via restriction digestion

Plasmids pSH-*ble*$^{r}$ Cre, pFA6a-*lox*P-*hphNT1*-*lox*P (PZC1), pFA6a-*lox*P-*natNT2*-*lox*P (PZC2), pFA6a-*lox2272*-*natNT2*-*lox2272* (PZC4) and pUG6-*lox*P-*KanMX*-*lox*P were extracted and digested with *HindIII* (for pSH-*ble*$^{r}$ Cre plasmid) and *EcoRV* (for PZC1, PZC2, PZC4 and pUG6) and confirmed on 1% (w/v) agarose gel electrophoresis. The predicted length sizes of all digested plasmids are 7218bp for Cre-ble, 4235bp for PZC1, 3852bp for PZC2, 3827bp for PZC4 and 4009bp for pUG6 (Figure 3.1).

### 3.1.2 Amplification of marker gene cassettes by PCR

All marker gene cassettes (*lox*P-*hphNT1*-*lox*P, *lox*P-*natNT2*-*lox*P, *lox2272*-*natNT2*-*lox2272* and *lox*P-*KanMX*-*lox*P*)* were amplified by PCR using specific oligonucleotide primers (Appendix, Table A.1). These PCR primers contained 20bp universal annealing sequences and 60bp to 80bp flanking sequencing that are homologous to the intergenic regions between chromosomal breakpoints. The resulting amplified cassettes were confirmed and visualised on 1% (w/v) agarose gel electrophoresis (Figure 3.2).

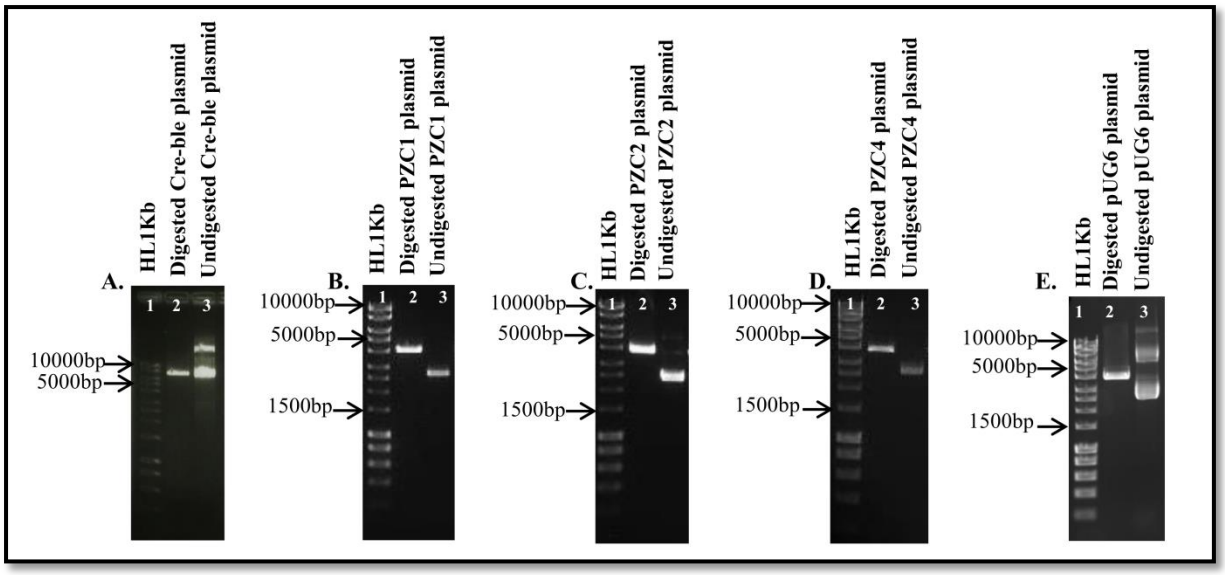


**Figure 3.1 Digestion of plasmids with restriction enzymes**

DNA profile of undigested and digested plasmids with *HindIII* **(Panel A)** and *EcoRV* **(Panels B, C, D and E)** on 1% agarose gel. **Panel A**: digestion of Cre-ble; **Panel B**: digestion of PZC1; **Panel C**: digestion of PZC2, **Panel D**: digestion of PZC4; Panel E: digestion of pUG6. Lanes 1: Hyperladder 1Kb **(all Panels)**. Lanes 2 represent bands for linearized Cre-ble (7218bp), PZC1 (4235bp), PZC2 (3852bp), PZC4 (3827bp) and pUG6 (4009bp) plasmids. Lanes 3 represent bands of undigested Cre-ble, PZC1, PZC2, PZC4 and pUG6 plasmids.

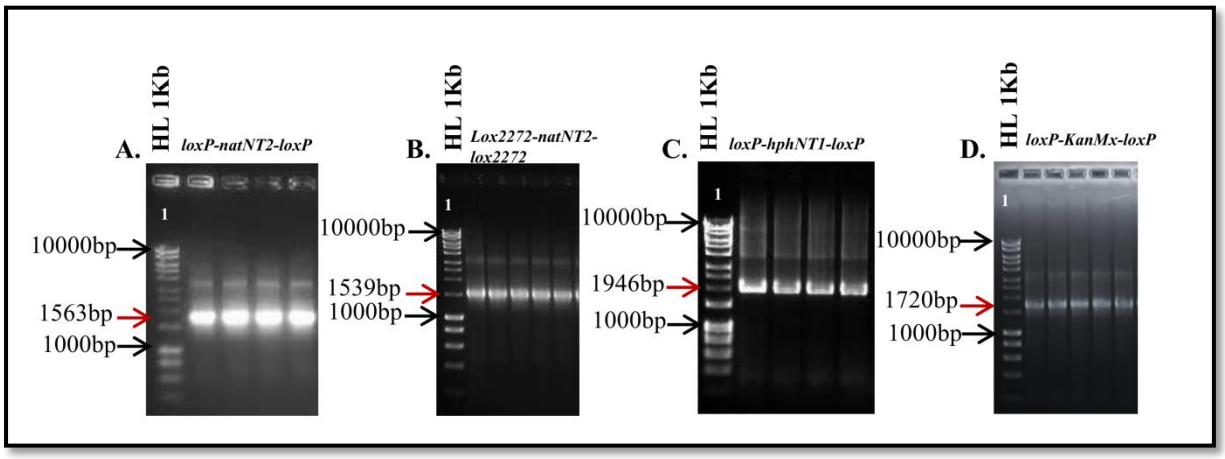


**Figure 3.2 Amplification of: *lox*P-natNT2-*lox*P (A), *lox2272*-natNT2-*lox2272* (B), *lox*P-hphNT1-*lox*P (C) and *lox*P-KanMX-*lox*P (D) marker gene cassettes**

Electrophoresis of amplified PCR products on 1% agarose gels. Lane 1 represents Hyperladder 1Kb **(All Panels)**. All bands represent the amplified cassettes which give **Panel A**: 1563bp PCR product of *lox*P-*natNT2*-*lox*P, **Panel B**: 1539bp PCR product of *lox2272*-*natNT2*-*lox2272*, **Panel C**: 1946bp PCR product of *lox*P-*hphNT1*-*lox*P; and **Panel D**: 1720bp PCR product of *lox*P-*KanMX*-*lox*P.

### 3.1.3 Transformation and selection of the marker cassettes and plasmid

Transformation of the marker cassettes in the haploid *S. paradoxus* YPS138 strains was carried out to create chromosomal translocations. Successful transformation was indicated by growth on YPD containing different selectable antibiotic markers. These antibiotic markers were added to the YPD media depending on the marker cassette used for each transformation. Three independent colonies were selected from each transformation. Plasmid was transformed successfully to the same strain and the colonies were selected from YP containing phleomycin antibiotic marker.

All the selected colonies were confirmed to have the relevant antibiotic resistance by re-streaking onto new antibiotic plates, and also by diagnostic PCR (See section 3.1.4). All confirmed colonies were stored at - 80 °C.

### 3.1.4 Diagnostic colony PCR

- **Confirmation of marker cassettes insertion by PCR**

To confirm the successful insertion of all marker cassettes into the *S. paradoxus* YPS138 genome, diagnostic colony PCR was performed using four specific checking primers (Table A.2). All expected band sizes were confirmed via 1% (w/v) agarose gel electrophoresis and were obtained after using a combination of primer pairs specific to the cassette and to the genome (Table 3.2) (Figure 3.3).

- **Confirmation of translocant and non-translocant colonies**

In order to confirm the creation of translocation in *S. paradoxus* YPS138 strains, all colonies that lost both marker cassettes and Cre plasmid were checked by diagnostic colony PCR. All the expected band sizes were confirmed with 1% (w/v), 1.5% (w/v) and 3.5% (w/v) agarose gel electrophoresis (Table 3.3). Two sets of PCR confirmation primers were designed to

correspond to both chromosomes that were selected for translocation (see Section 2.10.2). Translocant colonies were confirmed by PCR primers (Table A.2) specific to chromosomes IX/XV and XII/XIV, while non-translocant colonies were confirmed by primers corresponding to each chromosome individually (Figures 3.4 and 3.5). Three colonies that confirmed the translocants and non-translocants were selected as three biological replicates and were used for crossing with *S. cariocanus* to create hybrids.

**Table 3.3** Expected band sizes of PCR products for primer sets used to confirm the correct integration of the *lox*P and *lox2272* sites and to verify the rearrangements in *S. paradoxus* strains.

| **Position of *lox*P and *lox2272* sites insertion in *S. paradoxus*** | **Primer Names** | **Fragment sizes (bp)** | |
|---|---|---|---|
| **Chromosome XII** | Conf.12.F+nat up | 1185 | |
| | Conf.12.R+nat down | 1223 | |
| **Chromosome XIV** | Conf.14.F+nat up | 1049 | |
| | Conf.14.R+nat down | 1702 | |
| **Rearrangements in *S. paradoxus*** | **Primer Names** | **Fragment sizes (bp)** | |
| | | **Translocation** | **Non-translocation** |
| **CT: IX/XV*** | Ch9F+Ch9R | - | 322 |
| | Ch15F+Ch15R | - | 477 |
| | Ch9F+Ch15R | 400 | - |
| | Ch15F+Ch9R | 399 | - |
| **CT: XII/XIV **** | Chk.12F+ Chk.12R | - | 1222 |
| | Chk.14F+ Chk.14R | - | 1000 |
| | Chk.12F+Chk.14 R | 1190 | - |
| | Chk.14F+ Chk.12R | 635 | - |

* Chromosomal translocation between chromosomes IX and XV

** Chromosomal translocation between chromosomes XII and XIV

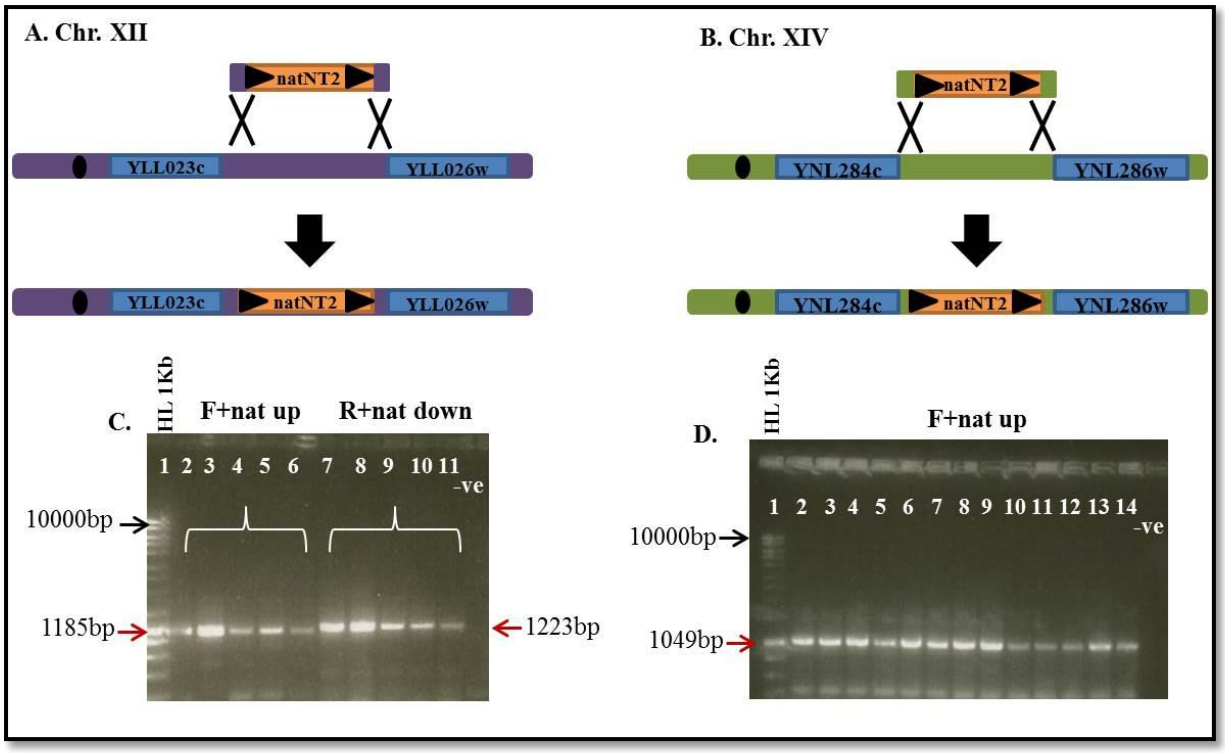


**Figure 3.3 Colony PCR for confirmation of cassette insertion**

**Panels A and B** represent a cartoon of the insertion of amplified *lox2272-natNT2-lox2272* marker cassettes by homologous recombination into intergenic regions between specific translocation breakpoints at chromosomes XII and XIV. **Panels C and D** represent 1% (w/v) agarose gels of cassettes inserted into S. paradoxus-$T_{9/15}$ strain. Lanes1 show Hyperladder 1Kb. **Panel C**: lanes 2 to 6 and lanes 7 to 11 represent transformant colonies confirmed by Conf.12.F+nat up (1185bp) and Conf.12.R+nat down primers (1223bp), respectively. **Panel D**: Lanes 2 to 14 represent transformant colonies confirmed by Conf.14.F+nat up primers (1049bp). Negative controls are presented as –ve.

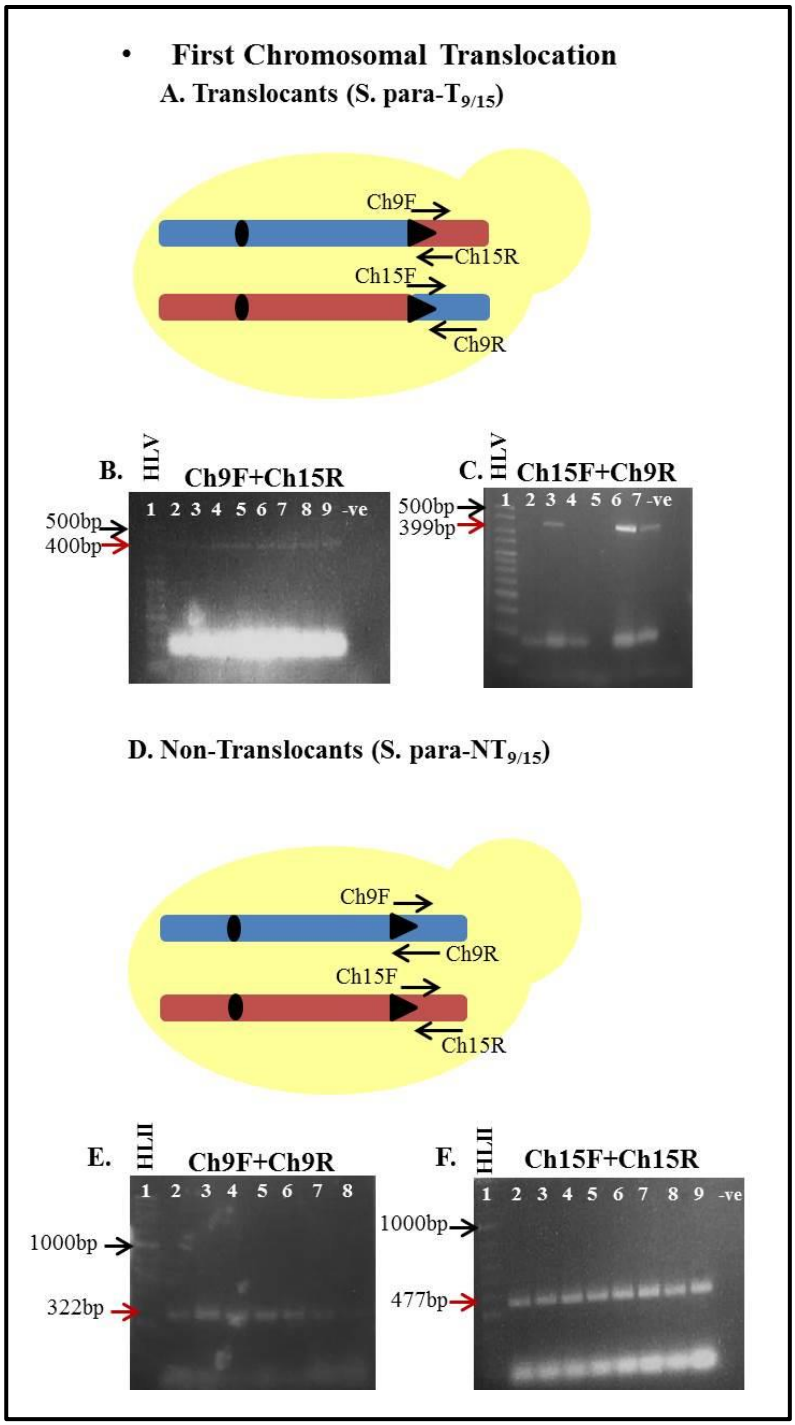


**Figure 3.4 Colony PCR for confirmation of translocations and non-translocations of first chromosomal translocation**

First chromosomal translocationwas created between chromosomes IX/XV. Translocants **(Panel A)** and non-translocants chromosomes **(Panel D)** confirmed by using combination of checking primers. Confirmation of translocation (**Panels B and C)**; Lanes1 show Hyperladder V (Bioline), lanes 2 to 9 and lanes 3, 6 and 7 represent translocants confirmed by Ch9F+Ch15R primers (400bp) and Ch15F+Ch9R primers (399bp), (**Panels B** and **C**, **respectively)**. Confirmation of non-translocations **(Panels E and F)**; Lanes 1 show Hyperladders II (Bioline), lanes 2 to 9 and lanes 2 to 8 represent non-translocants confirmed by Ch9F+Ch9R primers (322bp) and Ch15F+Ch15R primers (477bp) **(Panels E and F**, respectively). Negative controls are presented as –ve.

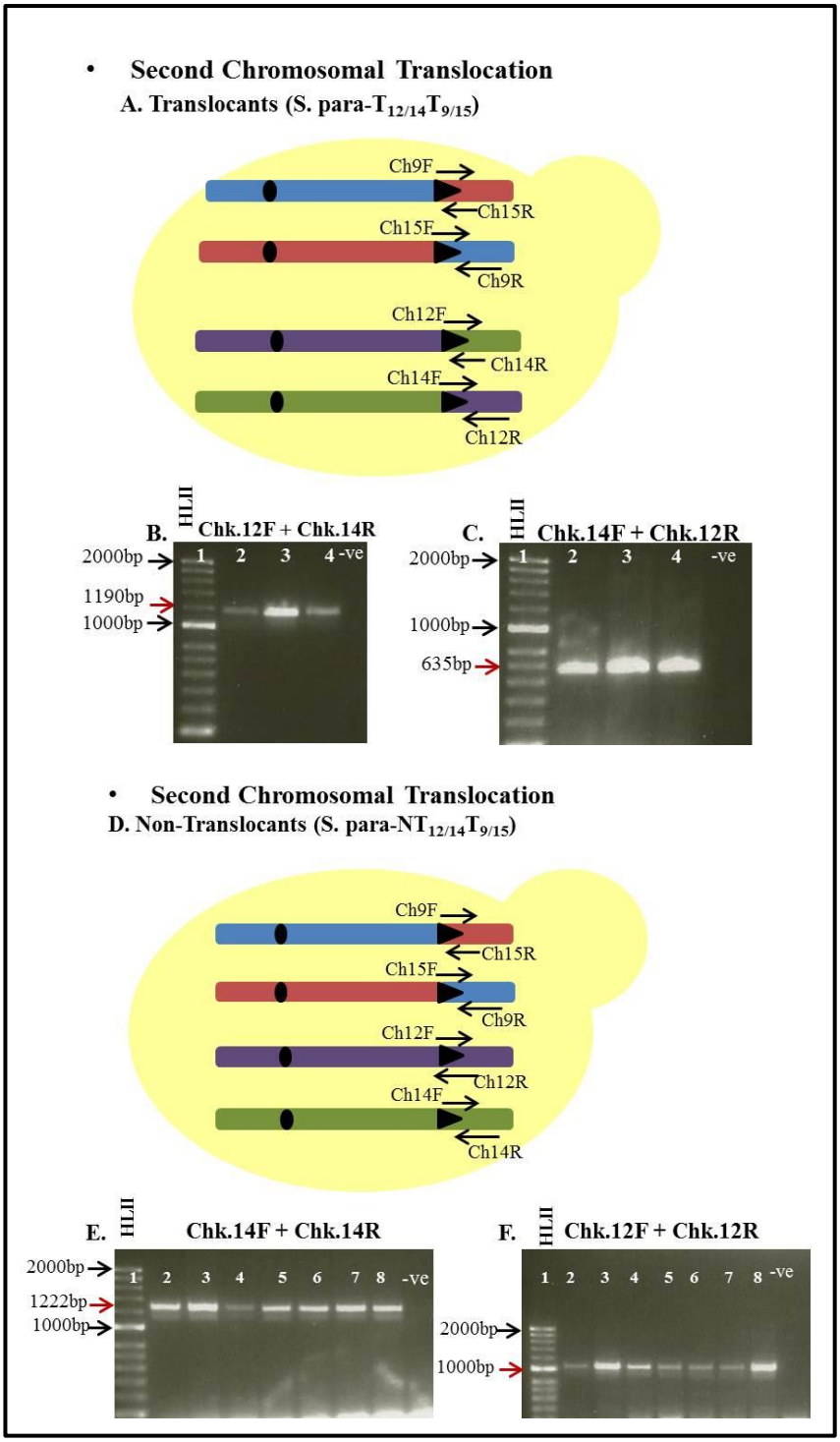


**Figure 3.5 Colony PCR for confirmation of translocations and non-translocations of second chromosomal translocation**

Second chromosomal translocationwas created between chromosomes XII/XIV. Translocants **(Panel A)** and non-translocants chromosomes **(Panel D)** are shown in diagrams and confirmed by using combination of checking primers.Confirmation of translocations **(Panels B and C)**; Lanes1 show Hyperladder II (Bioline), and lanes 2 to 4 represent translocants confirmed by Ch12F+Ch14R primers (1190bp) and Ch14F+Ch12R primers (399bp). Confirmation of non-translocations **(Panels E and F)**; Lanes 1 show Hyperladder II (Bioline), lanes 2 to 8 and 2 to 7 represent non-translocants confirmed by Ch12F+Ch12R primers (1222bp) and Ch14F+Ch14R primers (1000bp) **(Panels E and F, respectively)**. Negative controls are presented as –ve.

## 3.2 Generation of *S. cariocanus* and *S. paradoxus* hybrids by micromanipulation and tetrads analysis

*Saccharomyces cariocanus* cells were induced to undergo meiosis during the 7 days of sporulation, resulting in four haploid spores enclosed in ascus. Each of these spores was crossed with translocated and non-translocated haploid cells of *S. paradoxus* (*MAT* ***a***) using a micromanipulation needle. *S. cariocanus* is a homothallic strain and therefore the spore needs to be mated with a *S. paradoxus* haploid cell before it divides and undergoes mating type switching. Three biological replicates from each engineered strain were used to create the hybrids. The total number of mating created by crossing *S. cariocanus* spores with *S. paradoxus* translocated cells (S. para-$T_{9/15}$ and S. para-$T_{12/14}T_{9/15}$), including all biological replicates, was 531. In addition, 100 mating were carried out with *S. paradoxus* non-translocated cells (S. para-$NT_{9/15}$) (Figure 3.6). Moreover, 25 cell-cell mating were conducted as controls: S. para- $T_{9/15}$ cells (*MAT* **a**) with S. para-$NT_{9/15}$ (*MAT* α), and S. para-$NT_{9/15}$ cells (*MAT* **a**) with S. para-$NT_{9/15}$ (*MAT* α). All hybrids were selected on SD medium (for *S. cariocanus* selection) containing G418 (for *S. paradoxus* selection) (Figure 3.7).

Hybrid systems carrying five CTs (5T system) (4 Ts were detected by Fischer *et al.*, 2000 and one new T detected in current study), four CTs (4T system) and three CTs (3T system) are referred as S. car X S. para-$NT_{9/15}$, S. car X S. para-$T_{9/15}$ (undoing one CT) and S. car X S. para- $T_{12/14}T_{9/15}$ (undoing two CTs). Control systems including intraspecific crossing between *S. paradoxus* strains with different rearrangements and mating types are referred as S. para-$T_{9/15}$ X S. para-$NT_{9/15}$ and S. para-$NT_{9/15}$ (*MAT* **a**) X S. para-$NT_{9/15}$ (*MAT* α).

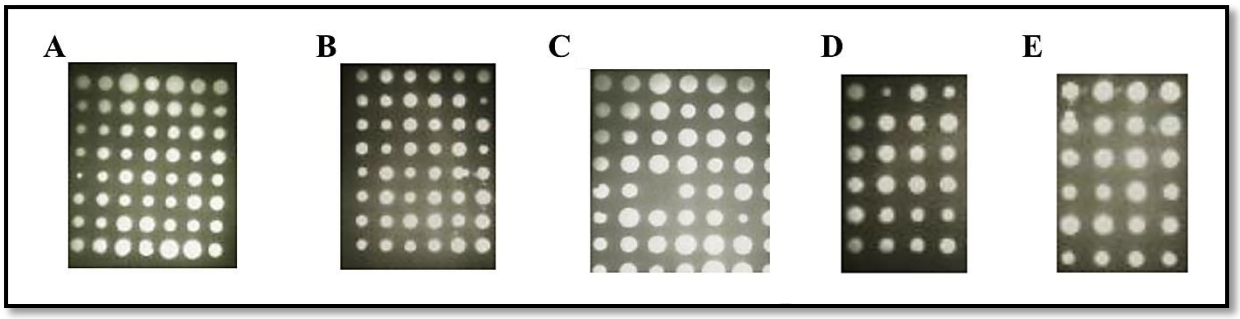


**Figure 3.6 Hybrids obtained from crossing *S. cariocanus* with *S. paradoxus* strains and *S. paradoxus* with *S. paradoxus* strains.**

Each image represents growth of colonies obtained from crossing each of the four spores of*S. cariocanus* tetrad with *S. paradoxus* haploid cells. Example of engineered hybrids between S. para-$T_{9/15}$ and *S. cariocanus* **(Panel A)**, S. para-$NT_{9/15}$ and *S. cariocanus* **(Panel B)** andS. para- $T_{12/14}T_{9/15}$ and *S. cariocanus* **(Panel C)**. Example images of control hybrids between S. para-$NT_{9/15}$ and S. para-$NT_{9/15}$ **(Panel D)** and S. para-$NT_{9/15}$ and S. para-$T_{9/15}$ **(Panel E).**

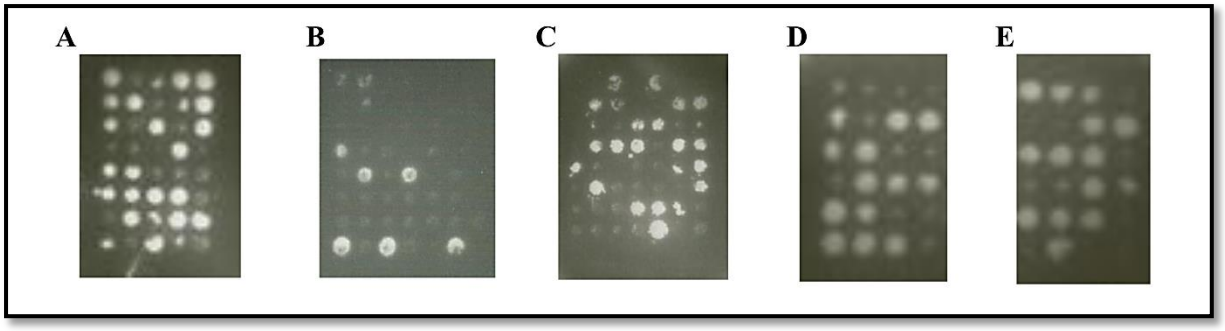


**Figure 3.7 Selection of hybrids on SD medium containing resistant antibiotic geneticin (G148) and urea**

Each image represents growth of only viable hybrids after replica plated on SD+G418+Urea (for interspecific crossing) and on YPD+G418+Uracil (for intraspecific crossing). Example images of viable hybrids of S. para-$T_{9/15}$ and *S. cariocanus* **(Panel A)**, S. para-$NT_{9/15}$ and *S. cariocanus* **(Panel B)** and S. para- $T_{12/14}T_{9/15}$ and *S. cariocanus* **(Panel C).** Example images of control viable hybrids between S. para-$NT_{9/15}$ and S. para-$NT_{9/15}$ **(Panel D)** and S. para-$NT_{9/15}$ and S. para-$T_{9/15}$ **(Panel E).**

### 3.2.1 Fluorescence Activated Cell Sorting analysis (FACS analysis)

FACS analysis was performed to check the ploidy of the *S. cariocanus* strain, the haploid *S. paradoxus* YPA138 strain, and the translocated and non-translocated engineered strains. This was carried out to detect any aneuploidy that may have occurred during the strain construction. A fluorescence histogram was createdfor each yeast strain representing the yeast cell number (count) versus fluorescence (CYTOX green) intensity. The DNA content of all engineered hybrids was compared to a haploid *S. paradoxus* (*MAT* ***a***) control strain and a diploid *S. cariocanus* control strain. The FACS analysis confirmed that all engineered hybrids were diploid, which represented a similar DNA content of that of *S. cariocanus* control strain (Figure 3.8). Therefore, these hybrids were sporulated and quantified by tetrad analysis.

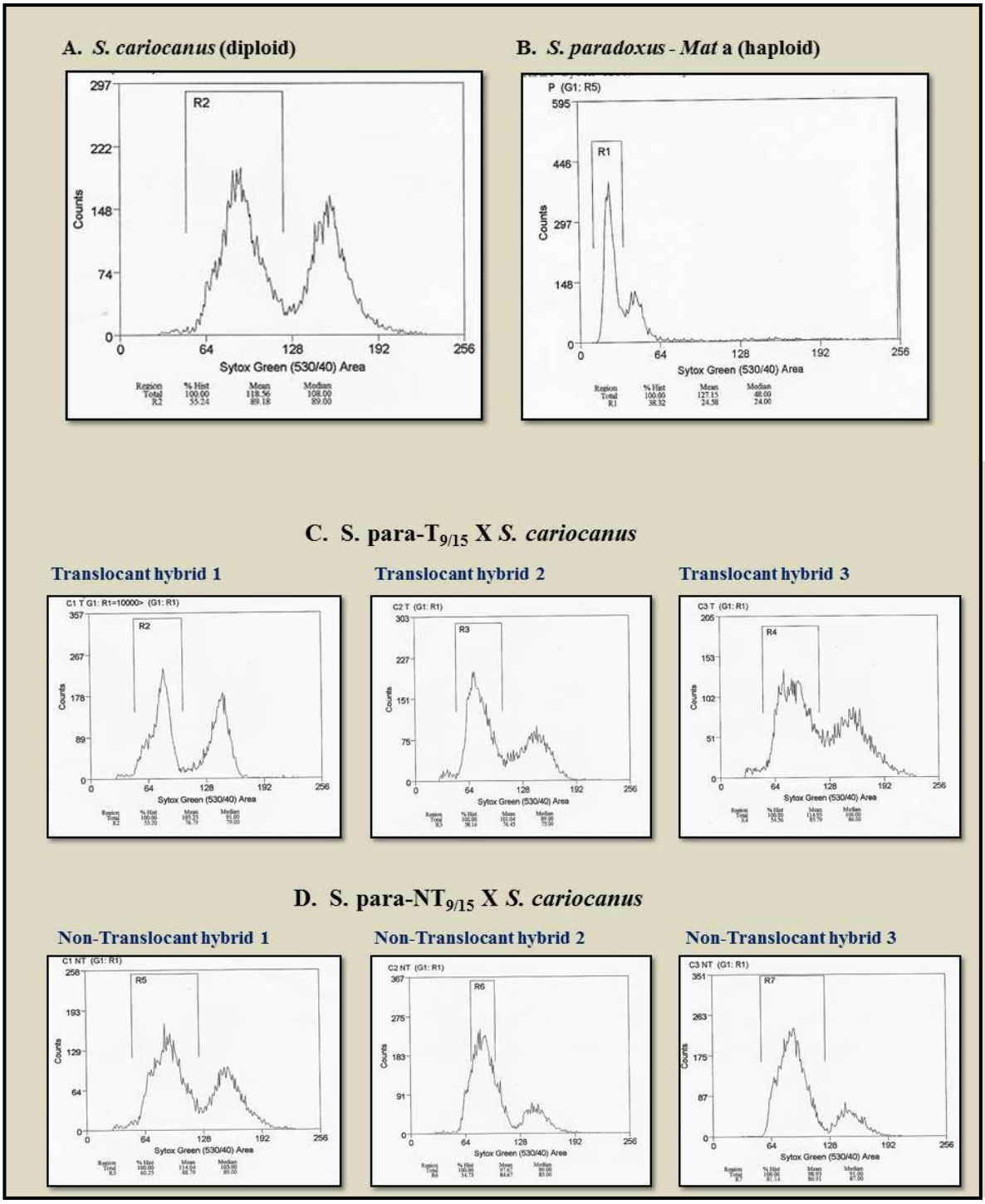


**Figure 3.8 Flow cytometry histograms of control strains and engineered hybrids**

The ploidy of engineered hybrids was evaluated by comparison of DNA to *S. cariocanus* diploid strain **(Panel A)** and *S. paradoxus* haploid strain **(Panel B)** by FACS analysis. Data are represented as cell number (*counts*) versus fluorescence intensity. Histograms show viable hybrids obtained from crosses of S. para-$T_{9/15}$ with *S. cariocanus* **(Panel C)** and S. para- $NT_{9/15}$ with *S. cariocanus* **(Panel D).**

## 3.3 The impact of reciprocal chromosomal translocations on reproductive isolation between *S. cariocanus* and *S. paradoxus* species (meiotic fitness)

In order to evaluate the effect of chromosomal translocations on meiotic fitness of yeast hybrids, both interspecific (between *S. paradoxus* and *S. cariocanus*) and intraspecific (between *S. paradoxus* strains) crossings were tested. Three replicates that were tested in each cross produced similar spore viability results, and these results were averaged. Fischer *et al*., 2000 detected 4 translocations in *S. cariocanus* and following PacBio sequencing (the result is addressed later in section 3.4), we found a 5$^{th}$ translocation in *S. cariocanus*, therefore, the nomenclature of *S. cariocanus* will be S.car-5T. Overall, 1536 spores were dissected for hybrids with 4T and 5T systems, and 3072 total spores for hybrids carrying 3T system. Comparing the hybrids fertility between the translocants and non-translocants, very low spore viability was observed in the control hybrid carrying five heterozygous reciprocal translocations (5T system), in which only 3.4% viable spores were obtained. On the other hand, hybrids carrying 3 and 4 heterozygous reciprocal translocations (3T and 4T systems) showed significant increase in spore viability compared to the 5T system (Table 3.3) ($P$=0.0031 and $P$=0.0125, respectively, Two-sample t-test, GraphPad software, GraphPad PRISM 7). As expected from our hypothesis, by reversing the genome rearrangements in *S. paradoxus* strain, the spore viability increased by ca. two fold. Our data revealed that by reducing the number of CTs in the engineered hybrids from five to four, these results in doubling the spore viability from 3.4% to 6.9%. Reducing further the CTs from four to three, the spore viability doubles again from 6.9% to 12.7% (Table 3.4 and Figure 3.9). The tetrad dissection of the control intraspecific cross between two collinear strains, namely S. para-$NT_{9/15}$ × S. para-$NT_{9/15}$, showed very high spore viability (93%). The control cross between two non-collinear strains of *S. paradoxus*, namely *S.* para-$NT_{9/15}$ × S. para-$T_{9/15}$, produced only 51 % viable spores (Table 3.4 and Fig 3.9). These controls were performed to ensure

that our engineered strains do not carry other mutations or unwanted rearrangements that can affect the spore viability (Table 3.4 and Figure 3.9).

**Table 3.4** Spore viability results and total number of spores analyzed of engineered hybrids

| **Systems** | **Hybrid strains** | **No. of spores dissected** | **No. of viable spores** | **Mean spore viability** (±SE∗) |
|---|---|---|---|---|
| Engineered systems | S. para-$NT_{9/15}$ X *S. cariocanus* (5T system) | 1536 | 52 | **3.4%** (0.287) |
| | S. para-$T_{9/15}$ X *S. cariocanus* (4T system) | 1536 | 106 | **6.9%** (0.652) |
| | S. para- $T_{12/14}T_{9/15}$- X *S. cariocanus* (3T system) | 3072 | 389 | **12.7%** (2.643) |
| Control systems | S. para-$NT_{9/15}$ X S. para-$NT_{9/15}$ | 500 | 464 | **93%** (1.08) |
| | S. para-$NT_{9/15}$ X S. para-$T_{9/15}$ | 300 | 153 | **51%** (0.707) |

∗Standard error

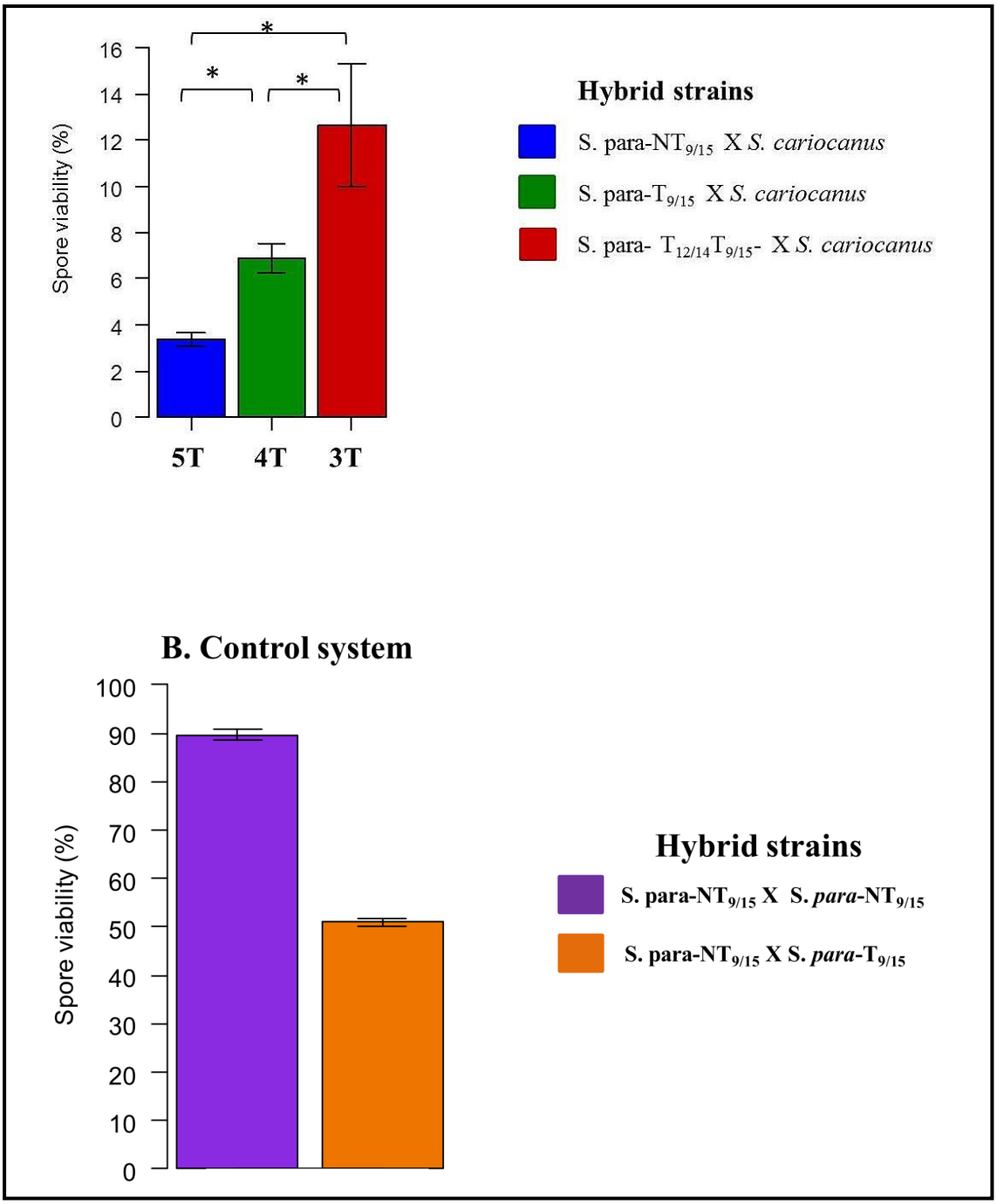


**Figure 3.9 Spore viabilities of engineered and control hybrids**

Bar charts represent the percentages of the average spore viability of each hybrid (including three biological replicates). Significant changes in spore viability were observed in hybrids carrying 2 and 3 heterozygote reciprocal translocations in comparison to hybrids carrying 4T system ( * = $P$<0.05) **(Panel A)**. Control system shows the expected spore viability **(Panel B)**.

## 3.4 Genome sequence and assembly of *S. cariocanus* UFRJ50816 strain by PacBio DNA sequencing.

From spore viability data, we observed that the percentage of fertile spores was doubled for each CT undone. Because of this trend, we expected that by removing the other translocations, resulting in collinear genome between *S. cariocanus* and *S. paradoxus*, the percentage of fertility of the hybrids will quadruplicate. Accordingly, given a starting point of ca. 12.7% spore viability, the maximum spore viability that we predict for the interspecific collinear cross is 50.8%. This percentage would be quite low for two collinear strains with their given genome similarity. Sequence divergence as another postzygotic barrier has been suggested, and tested for the 49.2% missing fertility. Our results were compared with the findings of Liti *et al*., (2006) and we observed that more than 70% to 80% spore viability is predicted from crossing of two collinear strains having the same sequence divergence as *S. paradoxus* and *S. cariocanus* (our strains) (Table 3.5 and Figure 3.10) . Thus, our spore viability from collinear crossing is expected to be more than 50%, and this suggested the presence of different barriers, such as other rearrangements (Figure 3.10).

**Table 3.5** Summary of the percentages of spore viability and sequence divergence of the collinear genomes extracted from Liti *et al*., (2006) and current study

| Crosses | Viable spores (%) | SD |
|---|---|---|
| Sp N44 x Sp NBRC 1804 | **86.79*** | **0.084** |
| Sp N17 x Sp YPS138 | **34.11*** | **4.631** |
| Sp N44 x Sp CBS432 | **77.13*** | **1.156** |
| Sp N44 x Sp YPS125 | **36.32*** | **4.567** |
| S. para YPS138 X S. car (complete collinear crossing) | **~50.8**** | **0.29** |

*Results from (Liti *et al*., 2006)
**Our expected result

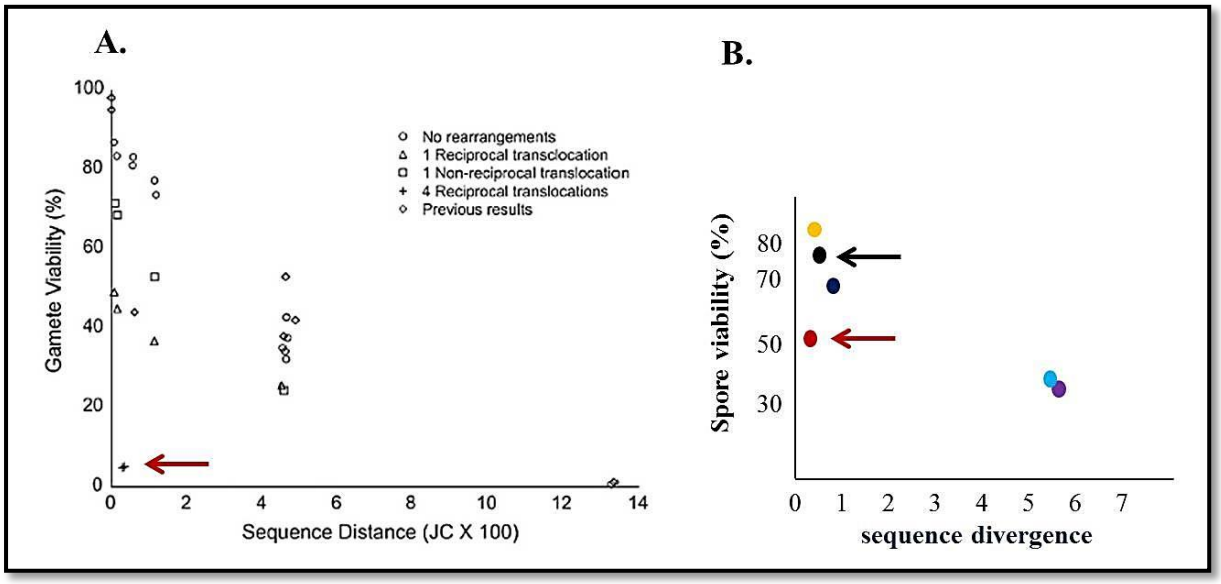


**Figure 3.10 Correlations between sequence divergence and spore viability (SV)**

Sequence divergence and spore viability were plotted against each other on a linear scale **(Panel A)** and representation of all SV outcomes from collinear crossing **(Panel B)**. Spore viability decreased with high sequence variation between species, and in the presence of translocation (red arrow) (**Panel A**). Following collinear crosses, more than 80% of SV was produced bySp N44 x Sp NBRC 1804 (yellow dot) which have similar sequence divergence with our species, and 50% SV is expected from our strains (red dot). The expected SV of our strains should be more than 70% (black dot) and this is higher than our result **(Panel B)**.

For that reason, we hypothesized that there could be other type of rearrangements, such as one translocation or some inversions that have not been identified previously in *S. cariocanus* genome and may contribute to the drop in meiotic fitness. The *S. cariocanus* genome was re-sequenced by PacBio DNA sequencing and the genome structures were aligned between *S. cariocanus* and *S. cerevisiae* S288C genome using NUCmer software, and the chromosomes' structures were visualized using MUMmerplot (Kurtz *et al.*, 2004) (Figures 3.11 and 3.12). Two types of rearrangements were identified in *S. cariocanus* genome: one new chromosomal translocation and 11 new inversions that had not been observed or described in previous studies (Table 3.6). The 4 chromosomal translocations previously identified were also clearly detected (Figures 3.13, 3.14, 3.15 and 3.16) (Fischer *et al*., 2000).

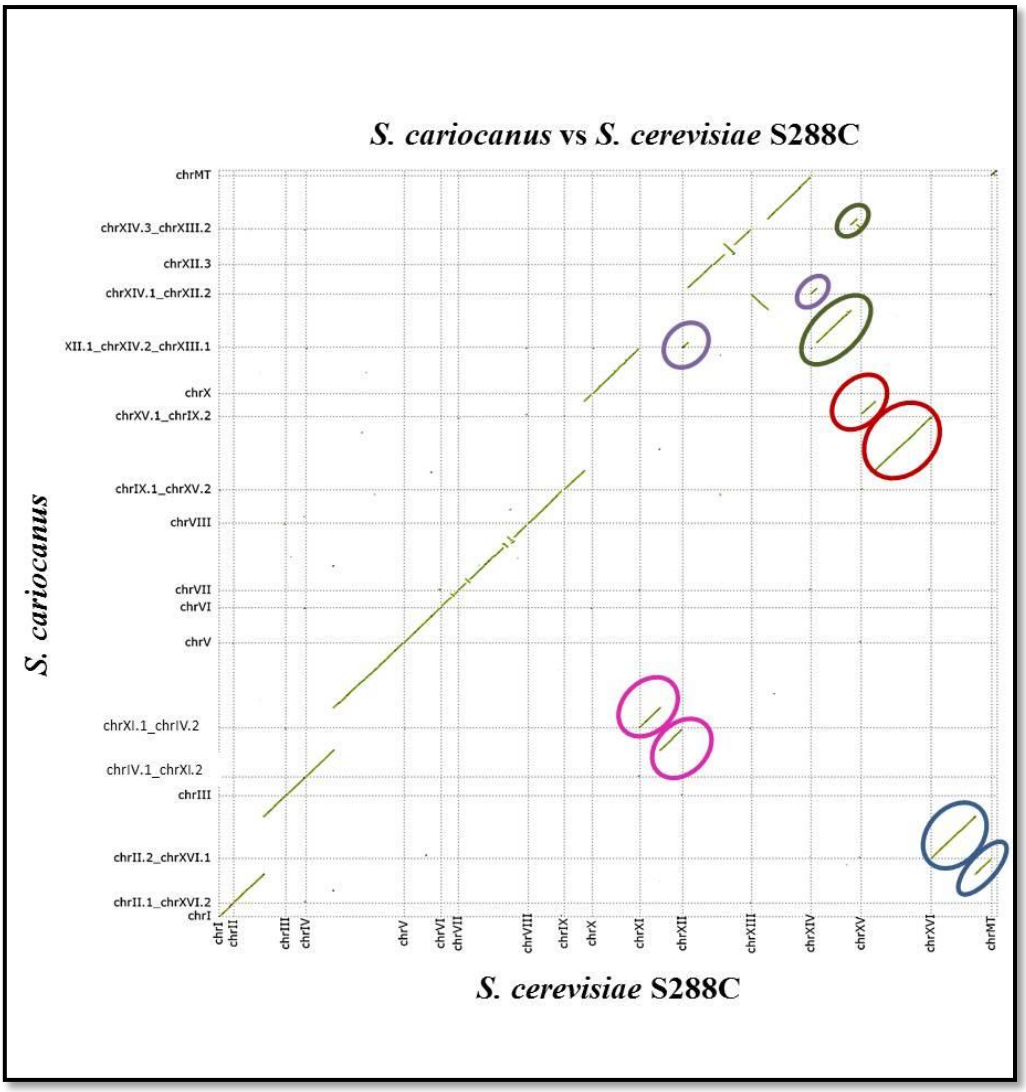


**Figure 3.11 Dotplot representation of *S. cariocanus* genome versus *S. cerevisiae* genome**

On the x-axis are plotted the *S. cerevisiae* chromosomes and on the y-axis the *S. cariocanus* chromosomes. If a *S. cariocanus* chromosome is completely collinear with the *S. cerevisiae* homologues, this is shown as an uninterrupted diagonal line in each dotted box. The five chromosomal translocations existing between the two strains are presented as interrupted and split lines in the dotted box, while the inversions are re-presented by lines oriented at approximately 90° angle in the dotted box. Circles of the same colour show chromosomal translocation between IX/XV (red circles), XIV/XII (purple circles), XI/IV (pink circles), II/XVI (blue circles) and the newly-defined translocation XIV/XIII (green circles).

**Each translocation was defined/circled as in the following example: ChrIV of *S. cerevisiae***: on the x-axis, the column of the plot that contains chrIV of *S. cerevisiae* is split over two sequences in *S. cariocanus* when look up in the diagonal line. The right side from these two *S. cariocanus* sequences have lines in the column of *S. cerevisiae* chrXI. This is the known translocation of chromosomes IV/XI.

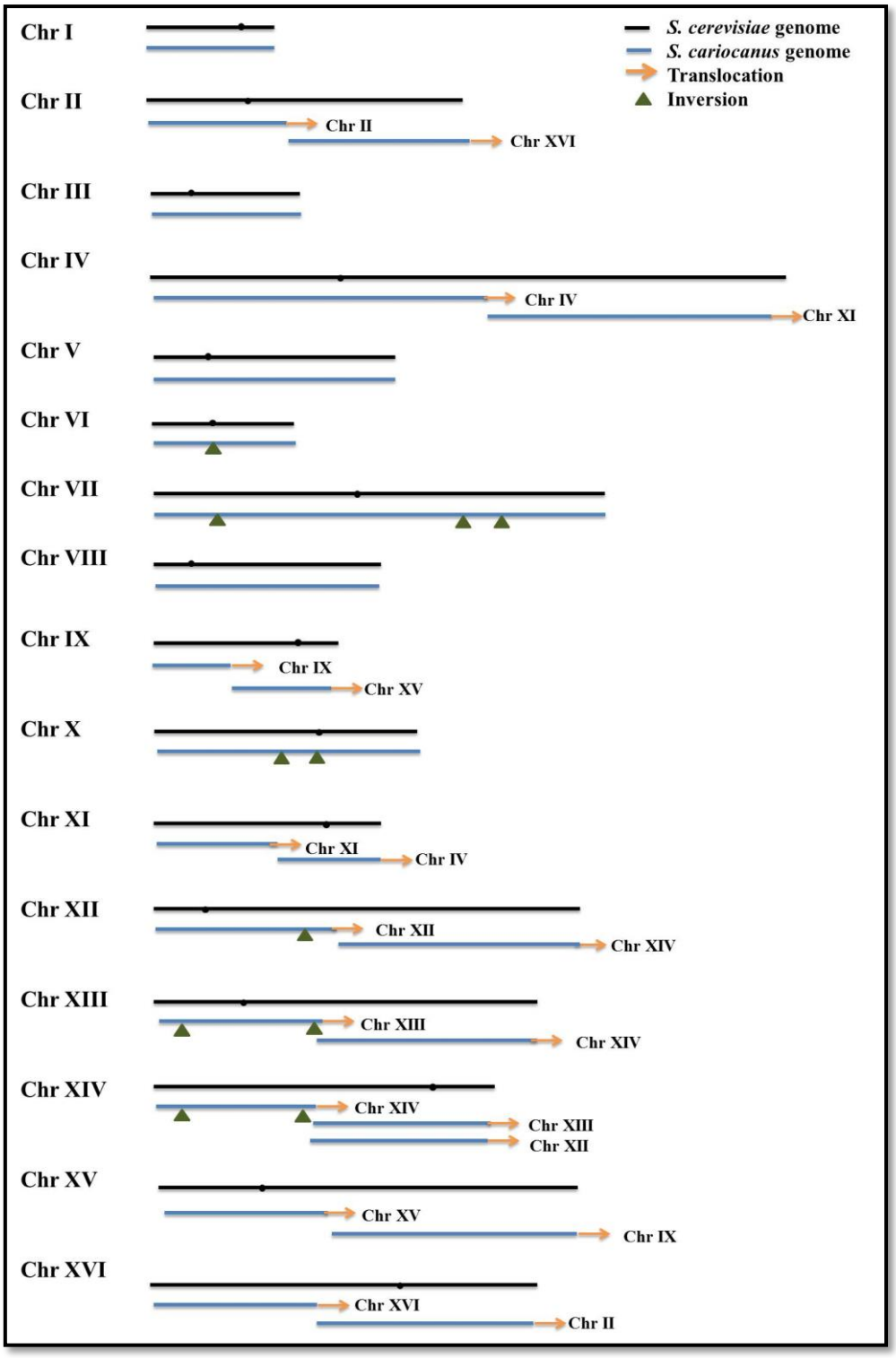


**Figure 3.12 Chromosomal structure of of *S. cariocanus* genome relative to *S. cerevisiae* S288C genome.**

The black horizontal bars indicate the *S. cerevisiae* genome. Chromosomal translocations between chromosomes IX/XV, XII/XIV, XI/IV, II/XVI and XIII/XIV at different chromosomes are indicated by orange arrows. The approximate locations of the centre of 11 inversions (not the size of the inversions) relative to the *S. cerevisiae* genome are indicated by green triangles.

**Table 3.6** New rearrangements including chromosomal translocation and inversions observed in *S. cariocanus* strain

| Chromosomal location | Type of rearrangement | Size of rearrangement (±3 ) | Coordination | Recombining sequences |
|---|---|---|---|---|
| **XIV t $XIII_R$**<br>**XIII t $XIV_R$** | translocation | 164 kb<br>695 kb | 1- 165 kb<br>155-850 kb | (AC)n , A-rich and TY2_LTR<br>TY1_LTR and (TGGTA)n |
| **VI** | One Inversion<br>(Pericentric) | 42 kb | 177- 219 kb | (ATATAC)n and (CATA)n |
| **VII** | Three inversions<br>(Paracentric) | i) 63 kb<br>ii) 45 kb<br>iii) 31 kb | 127- 190 kb<br>710- 755 kb<br>846- 877 kb | TY4_LTR and (ATTTTTA)n<br>TY4 and TY4<br>TY1_LTR and TY4 |
| **X** | Two inversions<br>(Paracentric) | i) 20 kb<br>ii) 16 kb | 352-372 kb<br>534-550 kb | TY3-LTR<br>TY3-LTR and TY |
| **XII** | One Inversion<br>(Paracentric) | 140 kb | 200-340 kb | TY1_LTR and TY4 |
| **XIII** | Two Inversions<br>(Paracentric) | i) 7 kb<br>ii) 7 kb | 198-205 kb<br>861-868 kb | (CTCTTT)n and TY<br>(TGGTA)n and (TGAT)n |
| **XIV** | Two Inversions<br>(Paracentric) | i) 55 kb<br>ii) 7 kb | 10-65 kb<br>862-868kb | (AC)n<br>(TGGTA)n and (TGAT)n |

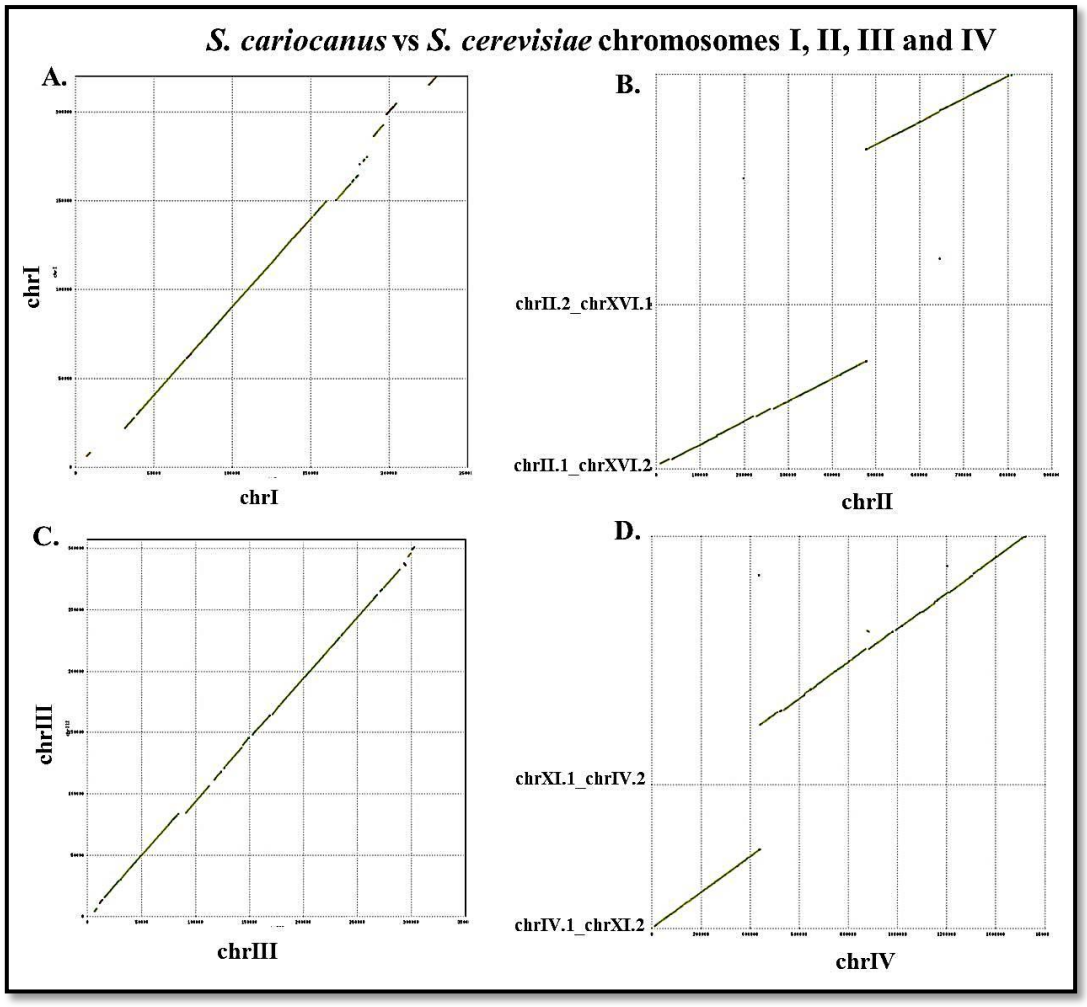


**Figure 3.13 Dotplot representation of *S. cariocanus* genome versus *S. cerevisiae* chromosomes I, II, III and IV**

On the x-axis are plotted the *S. cerevisiae* chromosomes and on the y-axis the *S. cariocanus* chromosomes. If a *S. cariocanus* chromosome is completely collinear with the *S. cerevisiae* homologues, this is shown as an uninterrupted diagonal line in each dotted box **(Panels A and C)**. Chromosomal translocations (XII/XVI and XI/IV) existing between the two strains are presented as interrupted and split lines in the dotted box **(Panels B and D).**

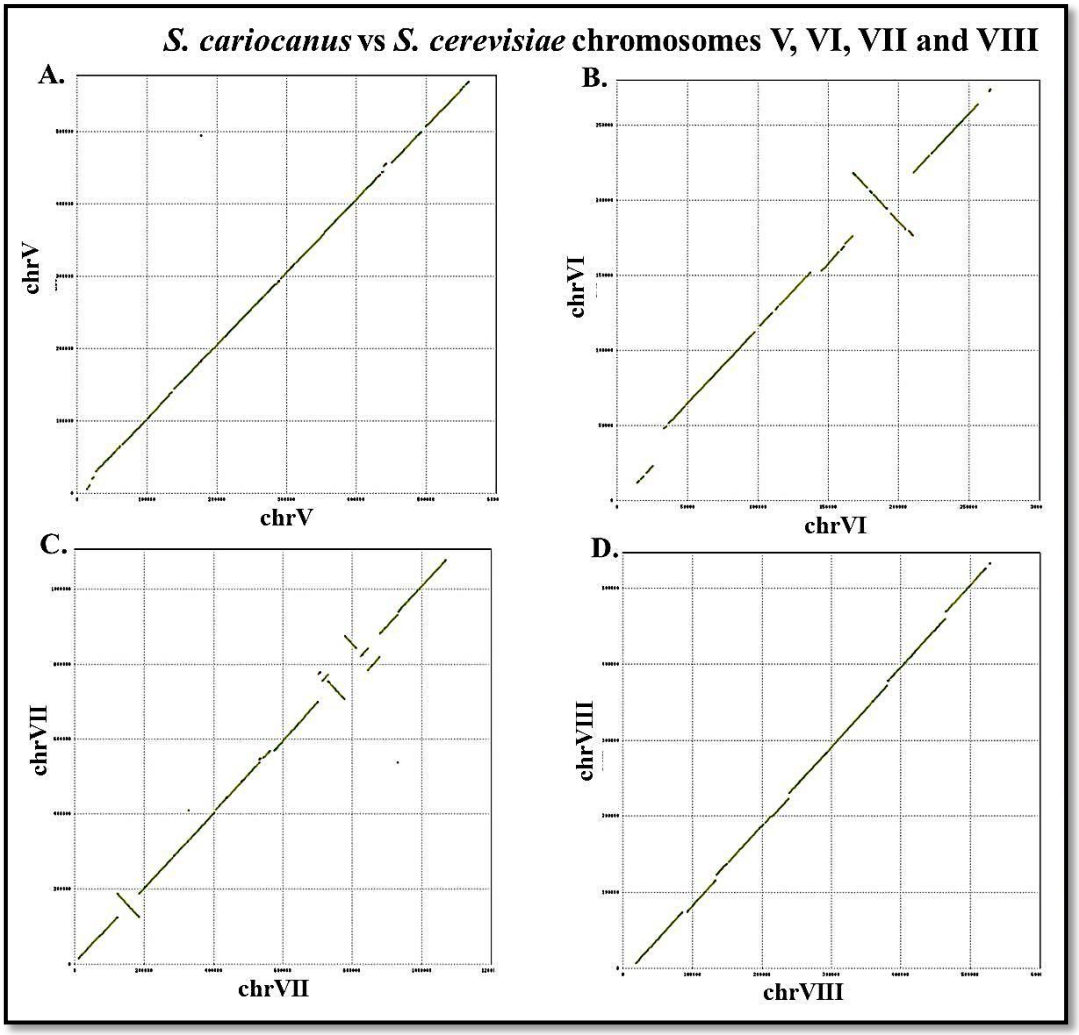


**Figure 3.14 Dotplot representation of *S. cariocanus* genome versus *S. cerevisiae* chromosomes V, VI, VII and VIII**

On the x-axis are plotted the *S. cerevisiae* chromosomes and on the y-axis the *S. cariocanus* chromosomes. If a *S. cariocanus* chromosome is completely collinear with the *S. cerevisiae* homologues, this is shown as an uninterrupted diagonal line in each dotted box **(Panels A and D)**. Chromosomal inversions at chromosomes VI and VII are re-presented by lines oriented at approximately 90° angle in the dotted box **(Panels B and C)**.

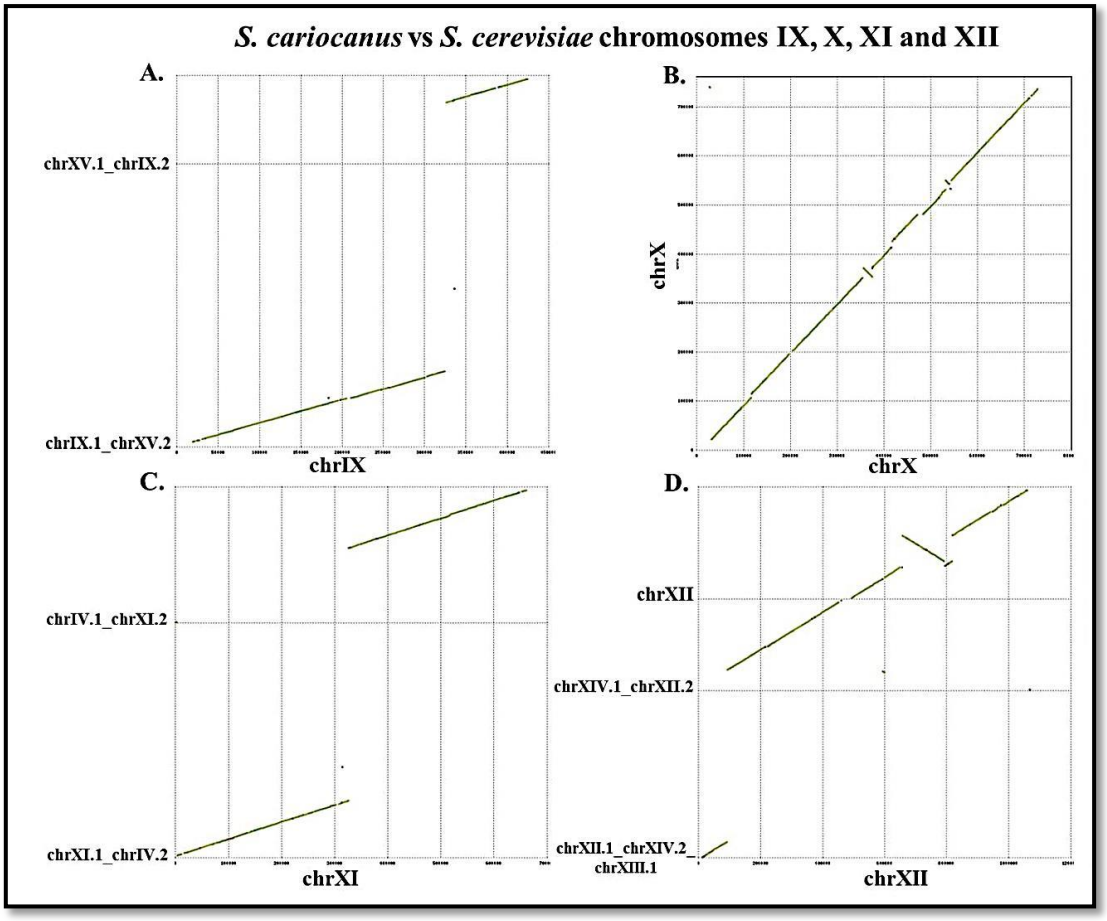


**Figure 3.15 Dotplot representation of *S. cariocanus* genome versus *S. cerevisiae* chromosomes IX X, XI and XII**

On the x-axis are plotted the *S. cerevisiae* chromosomes and on the y-axis the *S. cariocanus* chromosomes. If a *S. cariocanus* chromosome is completely collinear with the *S. cerevisiae* homologues, this is shown as an uninterrupted diagonal line in each dotted box **(Panels A and C)**. Chromosomal translocations (XII/XVI and XI/IV) existing between the two strains are presented as interrupted and split lines in the dotted box (**Panels B and D).**

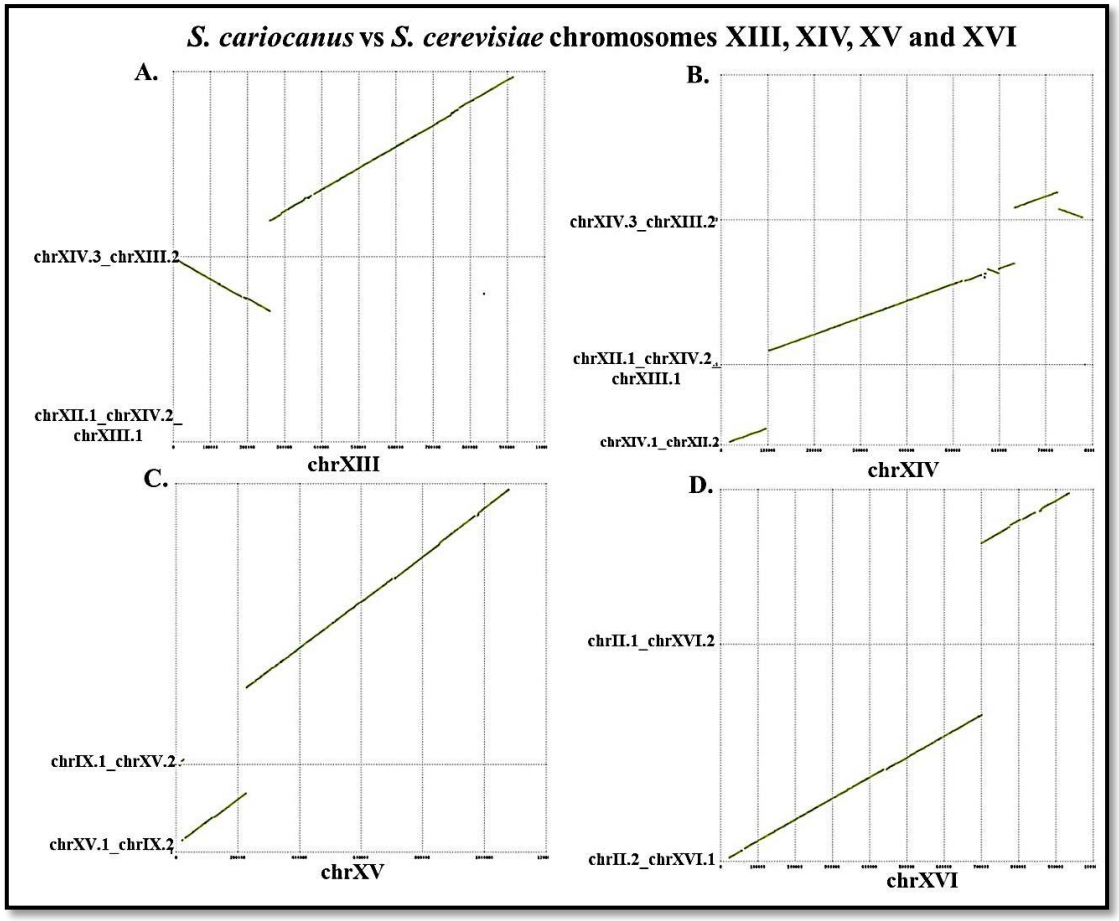


**Figure 3.16 Dotplot representation of *S. cariocanus* genome versus *S. cerevisiae* chromosomes XIII XIV, XV and XVI**

On the x-axis are plotted the *S. cerevisiae* chromosomes and on the y-axis the *S. cariocanus* chromosomes. Chromosomal translocations (XIV/XIII, IX/XV and II/XVI) existing between the two strains are presented as interrupted and split lines in the dotted box **(Panels B, C and D).** Chromosomal inversions at chromosomes XIII and XIV are re-presented by lines oriented at approximately 90° angle in the dotted box **(Panels A and B)**.

The newly-defined chromosomal translocation is occurred between chromosomes XIII and XIV (Figure 3.17) and located near two specific breakpoints: *PRE5* (YMR314W) gene, at chromosome XIII; and *ESF2* (YNR054C) gene, at chromosome XIV. These breakpoints were identified using the UCSC genome browser (https://genome.ucsc.edu/). Eleven inversions took place at six different chromosomes (VI, VII, X, XII, XIII and XIV), involving one pericentric and tenparacentric inversions, with sizes ranging from ~ 7 kb up to 140 kb (Figures 3.18, 3.19 and 3.20). All these newly-defined rearrangements were flanked by transposons elements (TY); either long terminal repeats (LTRs) or full length transposons at both boundary regions. In addition to those rearrangements, a duplication of *YOP1* (YPR028W) gene, from a membrane-integrated protein family, was detected on chromosome I. As a result, meiotic fitness of our engineered hybrids must be affected by the presence of the newly-defined chromosomal rearrangements that results in reduced the mitotic fitness (Section 3.5). All these data were uploaded to the UCSC Genome Browser (https://genome.ucsc.edu/). In the UCSC Genome Browser images, the chromosomes are represented by green reads, the inversions are represented by red reads, and the translocations are represented by white spaces between the red and green reads (Figures 3.17, 3.18, 3.19 and 3.20).

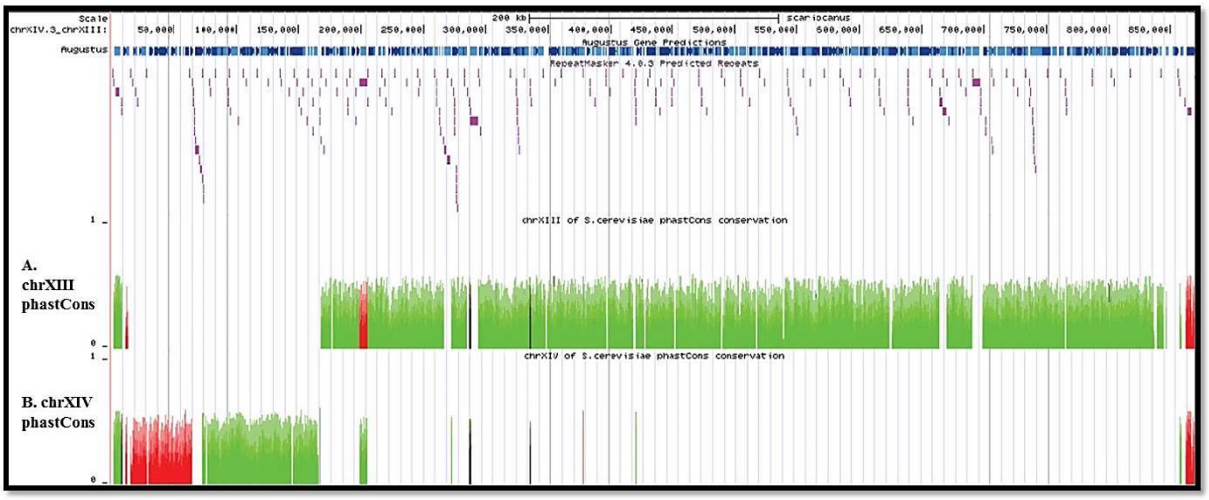


**Figure 3.17 Representation of newly-defined chromosomal translocation between chromosomes XIII and XIV of *S. cariocanus* strain in the UCSC Genome Browser**

Chromosomes XIII and XIV of *S. cariocanus* strain were mapped to *S. cerevisiae* S288C strainand revealed one extra chromosomal translocation between chromosomes XIII and XIV. The green reads represent the chromosome, the red reads show chromosomal inversions, and the white spaces between the green and red reads represent the chromosomal translocations.

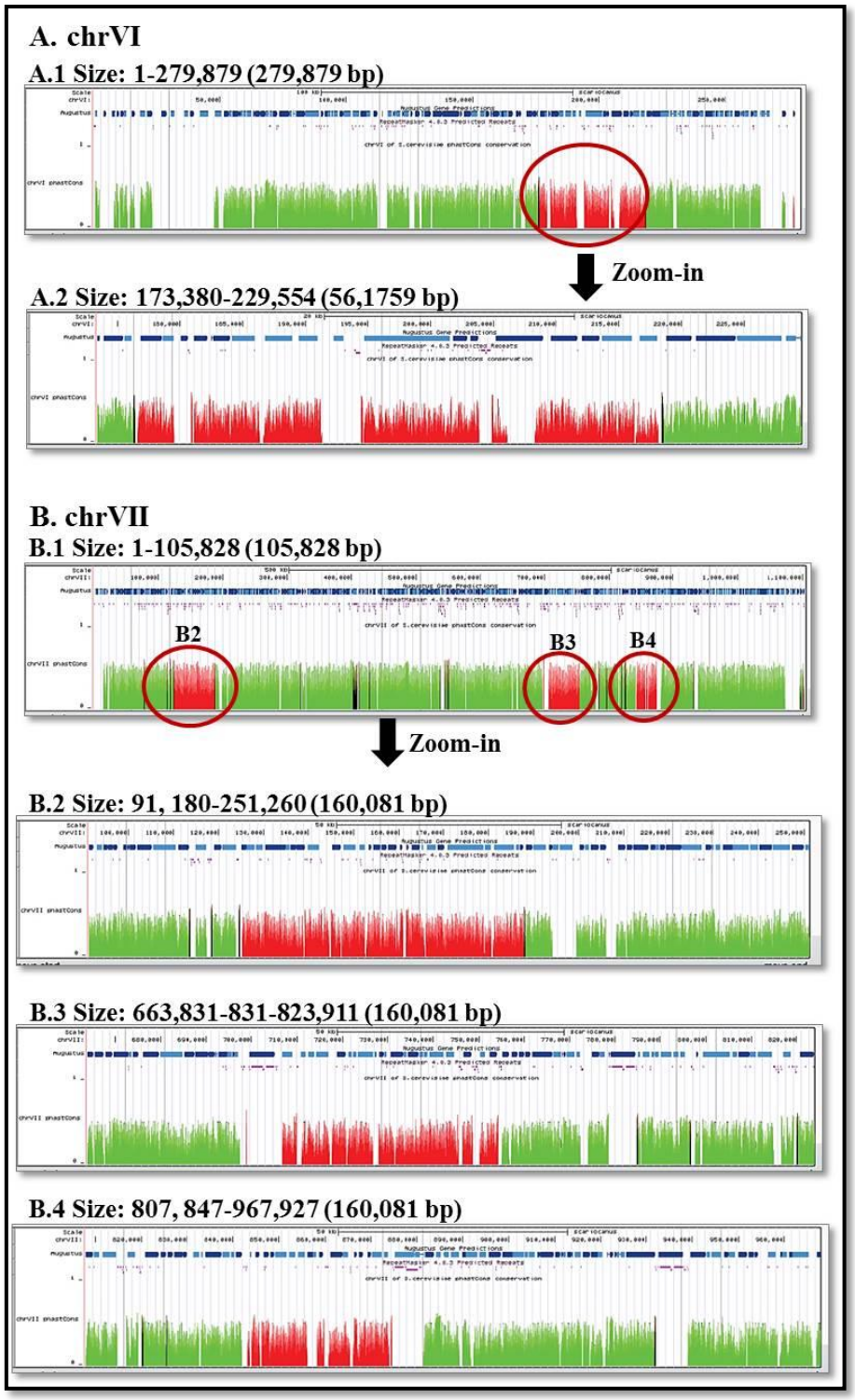


**Figure 3.18 Representation of newly-defined inversions at chromosomes VI and VII of *S. cariocanus* strain in the UCSC Genome Browser**

Chromosomes VI and VII of *S. cariocanus* strain were mapped to *S. cerevisiae* S288C strainand revealed one inversion at chromosome VI **(Panel A.1)** and three inversions at chromosome VIII **(Panel B.1)**. Panels **A.2, B.2, B.3** and **B.4** represent magnification of each inversion. The green reads represent the chromosome and the red reads show chromosomal inversions.

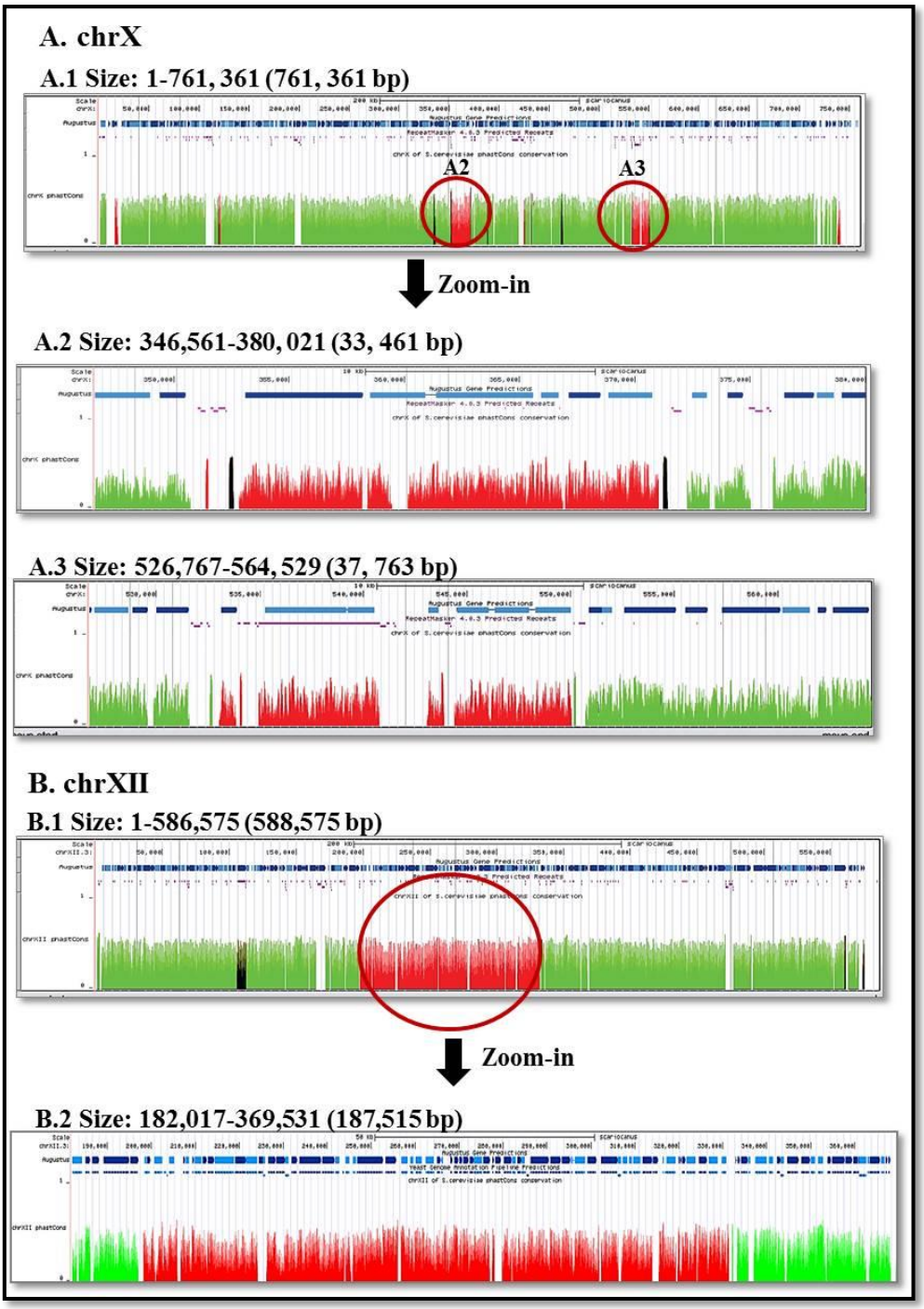


**Figure 3.19 Representation of newly-defined inversions at chromosomes XI and XII of *S. cariocanus* strain in the UCSC Genome Browser**

Chromosomes XI and XII of *S. cariocanus* strain were mapped to *S. cerevisiae* S288C strainand revealed two inversions at chromosome XI **(Panel A.1)** and one inversion at chromosome XIII **(Panel B.1)**. **Panels A.2, A.2, A.3** and **B.2** represent a magnification of each inversion. The green reads represent the chromosome and the red reads show chromosomal inversions.

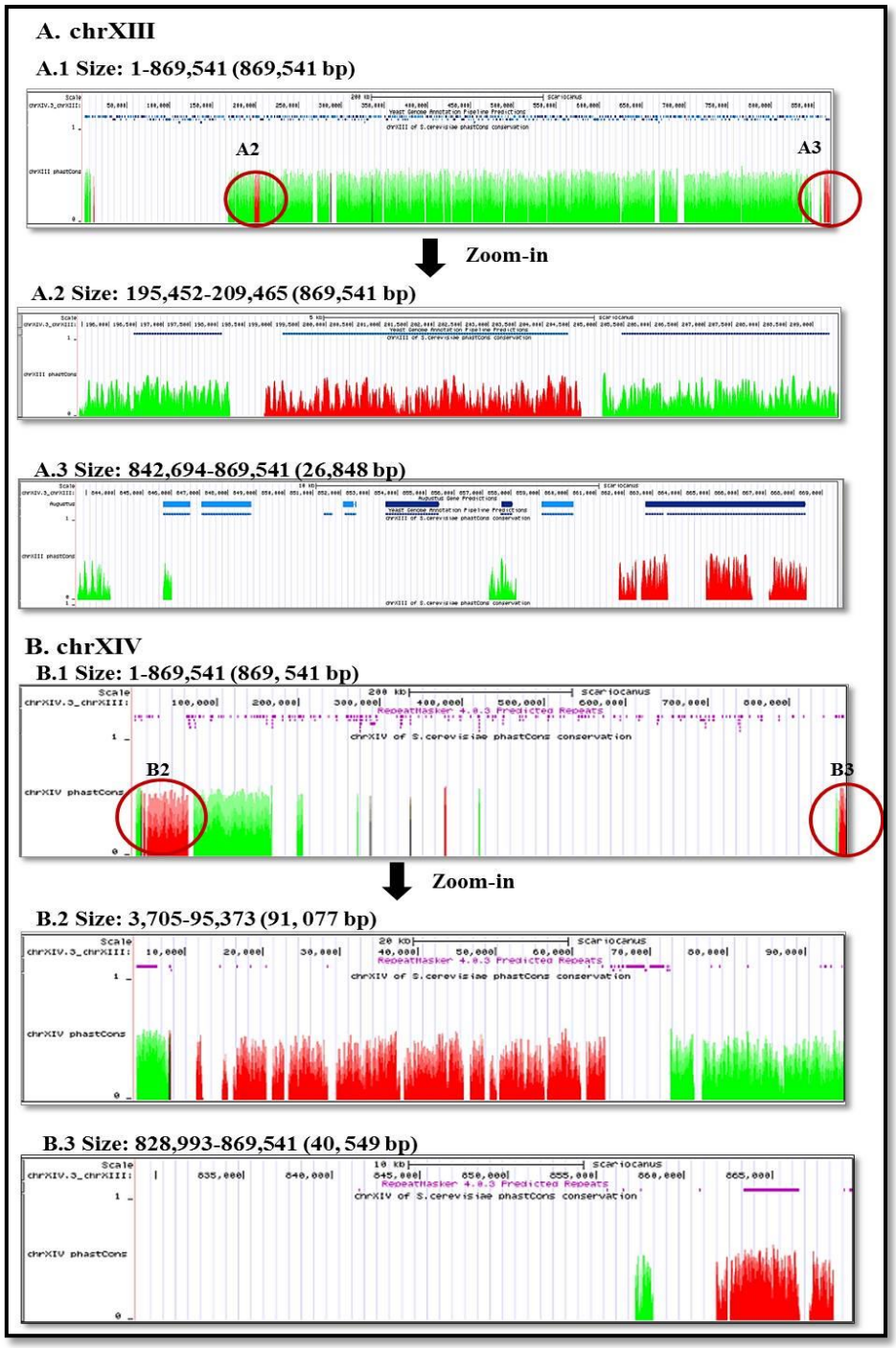


**Figure 3.20 Representation of newly-defined inversions at chromosomes XIII and XIV of *S. cariocanus* strain in the UCSC Genome Browser**

Chromosomes XIII and XIV of *S. cariocanus* strain were mapped to *S. cerevisiae* S288C strainand revealed two inversions at chromosome XIII **(Panel A.1)** and two inversions at chromosome XIV **(Panel B.1)**. **Panels A.2, A.2, A.3** and **B.2** represent magnification of each inversion. The green reads represent the chromosome, the red reads show chromosomal inversions, and the white spaces between the green and red reads represent the chromosomal translocations.

## 3.5 Effect of chromosomal translocations (CT) on the mitotic fitness of *S. paradoxus* and *S. cariocanus* strains under different experimental conditions

To evaluate the mitotic fitness effect of chromosomal translocation in the engineered strains carrying CTs and their controls, fitness assays were carried out under different nutritional and temperatures conditions. Six media including YPD, F1, C-limited, N-limited, P-limited and S- limited and three temperatures (16 °C, 30 °C and 35 °C) were tested on three replicates of each wild type, *S. cariocanus* and *S. paradoxus*, and on each engineered strain, S. para-$T_{9/15}$, S. para-$NT_{9/15}$, S. para- $T_{12/14}T_{9/15}$, and S. para- $NT_{12/14}T_{9/15}$. The mitotic fitness of all three replicates for each strain was consistent and therefore their results were averaged.

**At 30 °C**, after comparing all engineered strains regarding their maximum growth rate and maximum biomass in all media, a significant drop in the mitotic fitness was observed. This drop in fitness were detected in all translocant strains (S. para-$T_{9/15}$ and S. para-$T_{9/15}T_{12/14}$) and the non-translocant control for the second translocation which possessed the first translocation (S. para-$NT_{12/14}T_{9/15}$) in comparisons to all wild type strains (*S. cariocanus* and *S. paradoxus*) and the collinear non-translocant control S. para-$NT_{9/15}$ ($P$<0.05, Mann-Whitney test) (Figures 3.21, 3.22, 3.23, 3.24, 3.25 and 3.26).

**At 35 °C**, similar observations were obtained as at 30 °C. Comparing the strains regarding their maximum growth rate and maximum biomass in all media, a significant drop in the mitotic fitness was detected all translocant strains (S. para-$T_{9/15}$ and S. para-$T_{9/15}T_{12/14}$) and the non-translocants control (S. para-$NT_{12/14}T_{9/15}$) in comparisons to all wild type strains (*S. cariocanus* and *S. paradoxus*) and the non-translocant control S. para-$NT_{9/15}$ ($P$<0.05, Mann-Whitney test) (Figures 3.21, 3.22, 3.23, 3.24, 3.25 and 3.26).

**At 16 °C**, comparing all engineered strains regarding their maximum biomass, significant drop in the fitness was observed in the engineered translocant strains S. para-$T_{9/15}$ and S. para-$T_{9/15}T_{12/14}$ and the control non-translocant S. para-$NT_{12/14}T_{9/15}$, in comparison to the wild types and the non-translocant control S. para-$NT_{9/15}$ ($P$<0.05, Mann-Whitney test). Interestingly, when comparing them according to their maximum growth rate, a significant drop in the fitness was observed only on C-limited media of all engineered strains (S. para-$T_{9/15}$, S. para-$T_{9/15}T_{12/14}$ and S. para-$NT_{12/14}T_{9/15}$) in comparison with wild types and control non-translocant (S. para-$NT_{9/15}$) ($P$>0.05, Mann-Whitney test). However, no significant differences in the fitness were observed in all the other media (Figures 3.21, 3.22, 3.23, 3.24, 3.25 and 3.26).

Our observations indicated that, regardless of the media and temperature conditions, all translocant strains exhibited a drop in their fitness compared to the collinear control according to their maximum biomass.

Furthermore, we recognized that the fitness of the strain carrying two translocations, S. para-$T_{9/15}T_{12/14}$ was comparable to the strain with one translocation, S. para-$T_{9/15}$, indicating that the addition of the second translocation did not add any further reduction in the fitness of the strain.

Taking together all the fitness results, we observed significant decreases in the fitness in the engineered strains carrying one translocation (S. para-$T_{9/15}$), two translocations (S. para-$T_{12/14}T_{9/15}$) and the control for two translocations (S. para-$NT_{12/14}T_{9/15}$) in all limited media at all temperature according to their maximum biomass ($P$<0.05, Mann-Whitney test) (Figure 3.27). Accordingly, our observations conferred that CTs affect *S. paradoxus* fitness strains under different environmental conditions.

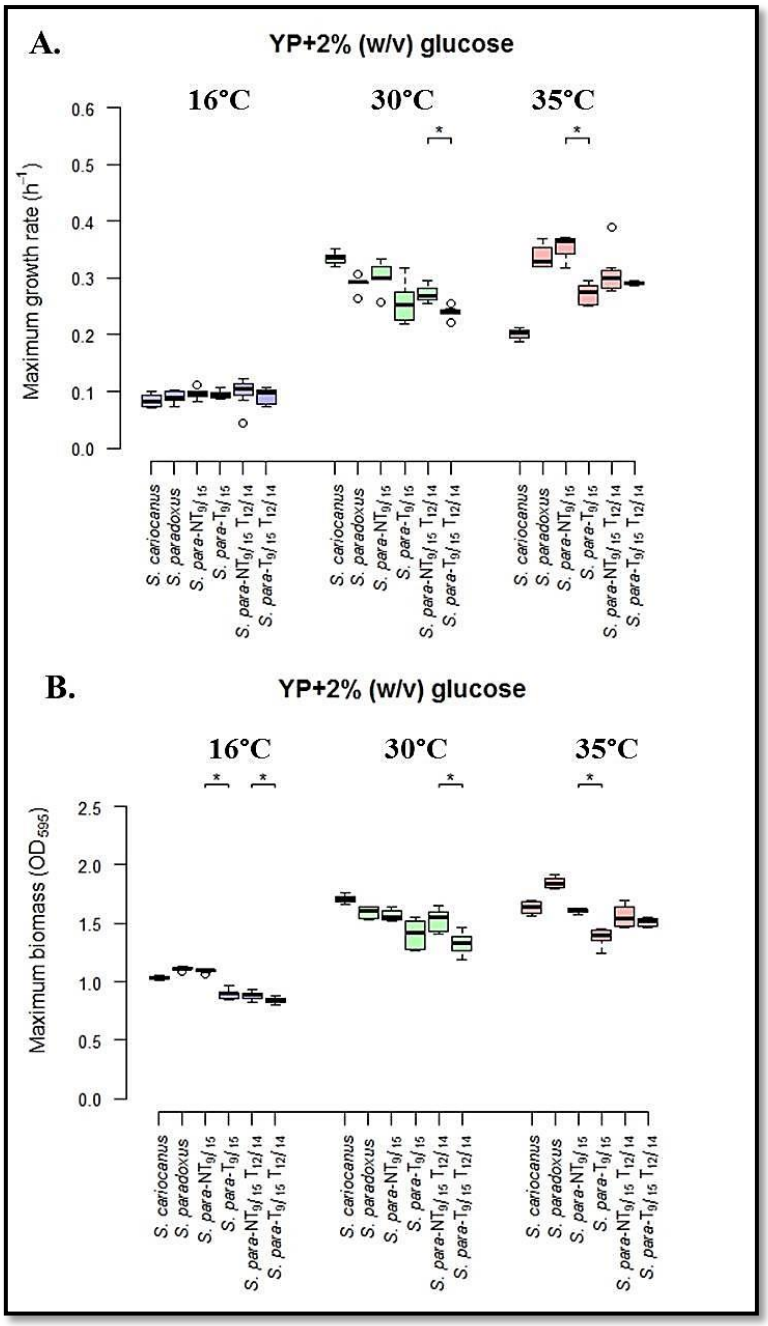


**Figure 3.21 Mitotic fitness of *S. cariocanus, S. paradoxus* and engineered translocant and non-translocant strains in YPD media**

Fitness boxplots represent both maximum growth rate **(Panel A**) and maximum biomass **(Panel B)** of all strains in YPD at 16°C, 30°C and 35°C. Purple, green and pink boxplots represent all strains at 16°C, 30°C and 35°C respectively. Each boxplot shows the distribution of data based on the minimum, median and maximum number. Each strain was tested with three biological replicates (with three technical each) (**P*<0.05, Mann-Whitney test). Significant drops in growth fitness were observed in translocant strains and non-translocant S. para-$NT_{12/14}T_{9/15}$ incomparisons to wild type strains and non-translocant S. para-$NT_{9/15}$ at all temperatures. No significant differences were observed at 16°C according to their maximum growth rate.

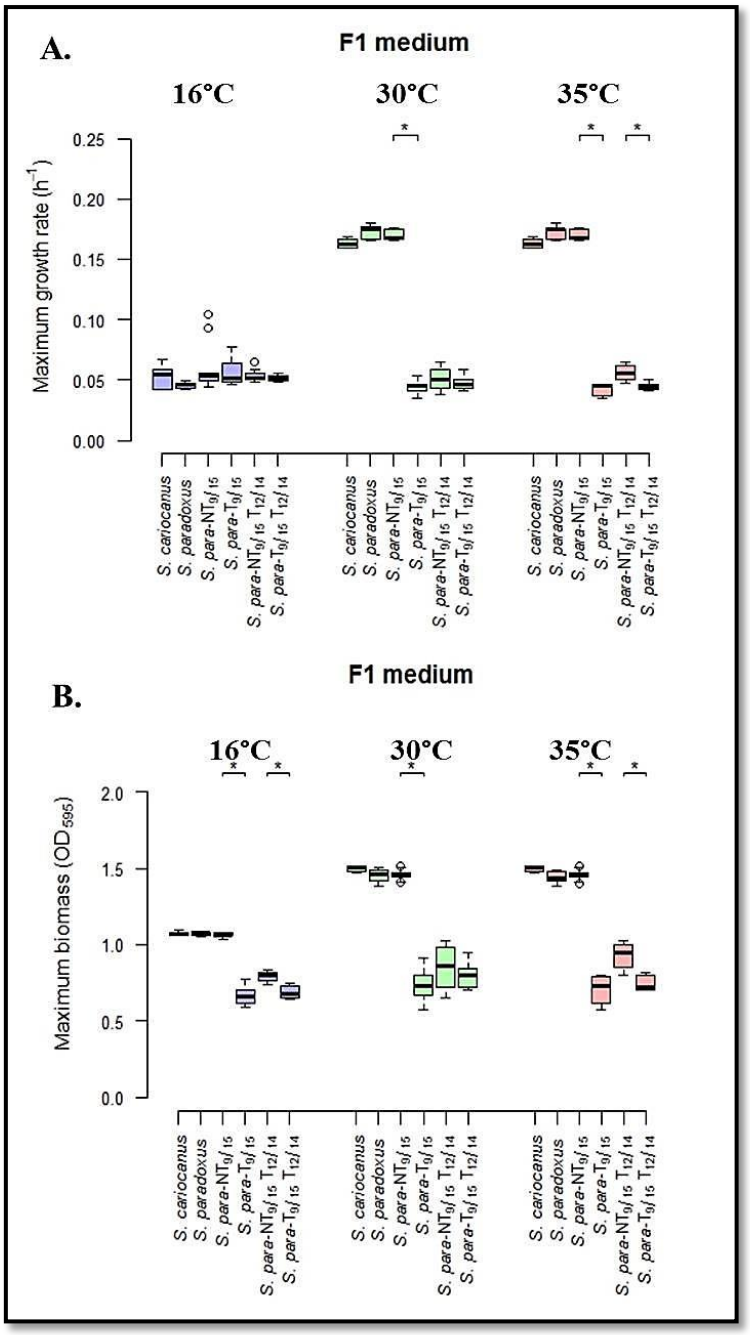


**Figure 3.22 Mitotic fitness of *S. cariocanus, S. paradoxus* and engineered translocant and non-translocant strains in F1 media**

Fitness boxplots represent both maximum growth rate **(Panel A)** and maximum biomass **(Panel B)** of all strains in F1 media at 16°C, 30°C and 35°C. Purple, green and pink boxplots represent all strains at 16°C, 30°C and 35°C respectively. Each boxplot shows the distribution of data based on the minimum, median and maximum number. Each strain was tested with three biological replicates (with three technical each) (**P*<0.05, Mann-Whitney test). Significant drops in growth fitness were observed in translocant strains and non-translocant S. para-$NT_{12/14}T_{9/15}$ incomparisons to wild type strains and non-translocant S. para-$NT_{9/15}$ at all temperatures. No significant differences were observed at 16°C according to their maximum growth rate.

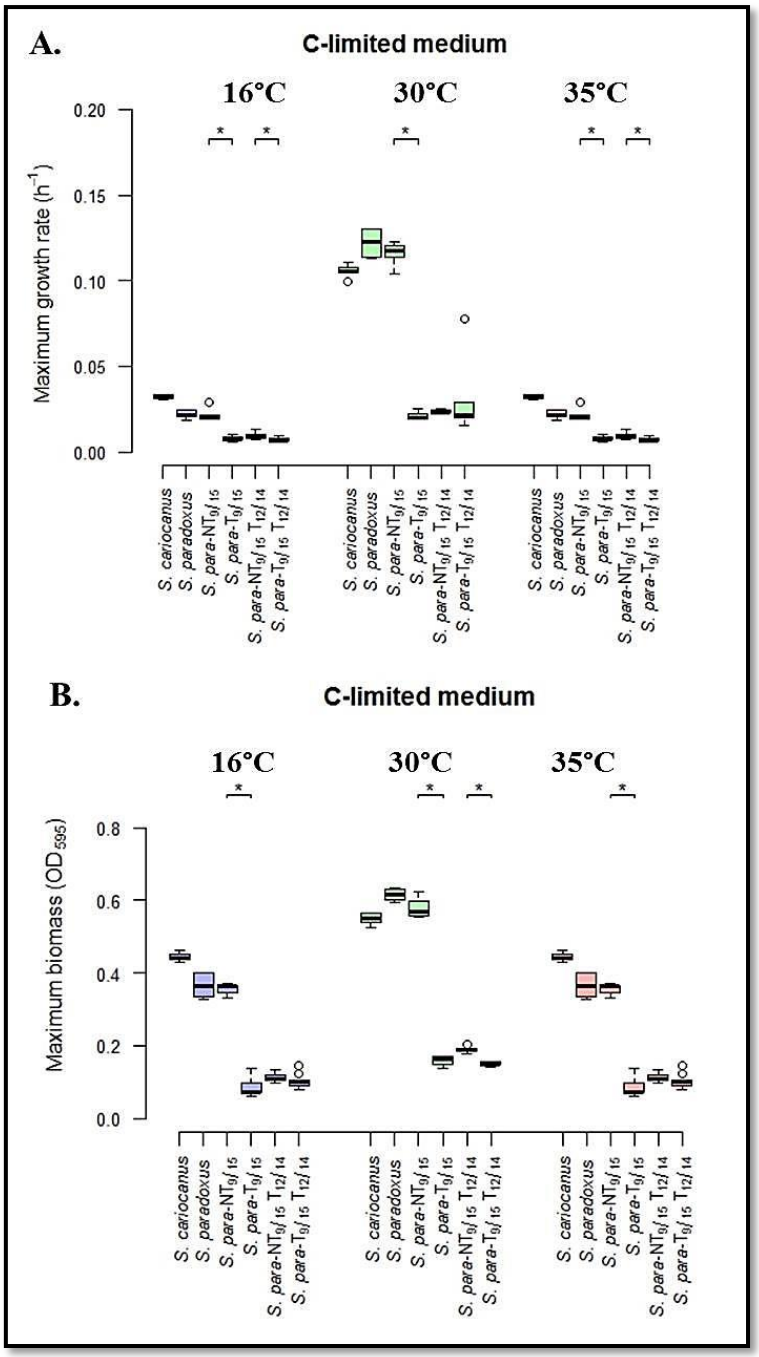


**Figure 3.23 Mitotic fitness of *S. cariocanus*, *S. paradoxus* and engineered translocant and non-translocant strains in C-limited media**

Fitness boxplots represent both maximum growth rate **(Panel A)** and maximum biomass **(Panel B)** of all strains in C-limited media at 16°C, 30°C and 35°C. Purple, green and pink boxplots represent all strains at 16°C, 30°C and 35°C respectively. Each boxplot shows the distribution of data based on the minimum, median and maximum number. Each strain was tested with three biological replicates (with three technical each) (*$P$<0.05, Mann-Whitney test). Significant drops in growth fitness were observed in translocant strains and non-translocant S. para-$NT_{12/14}T_{9/15}$ incomparisons to wild type strains and non-translocant S. para-$NT_{9/15}$ at all temperatures.

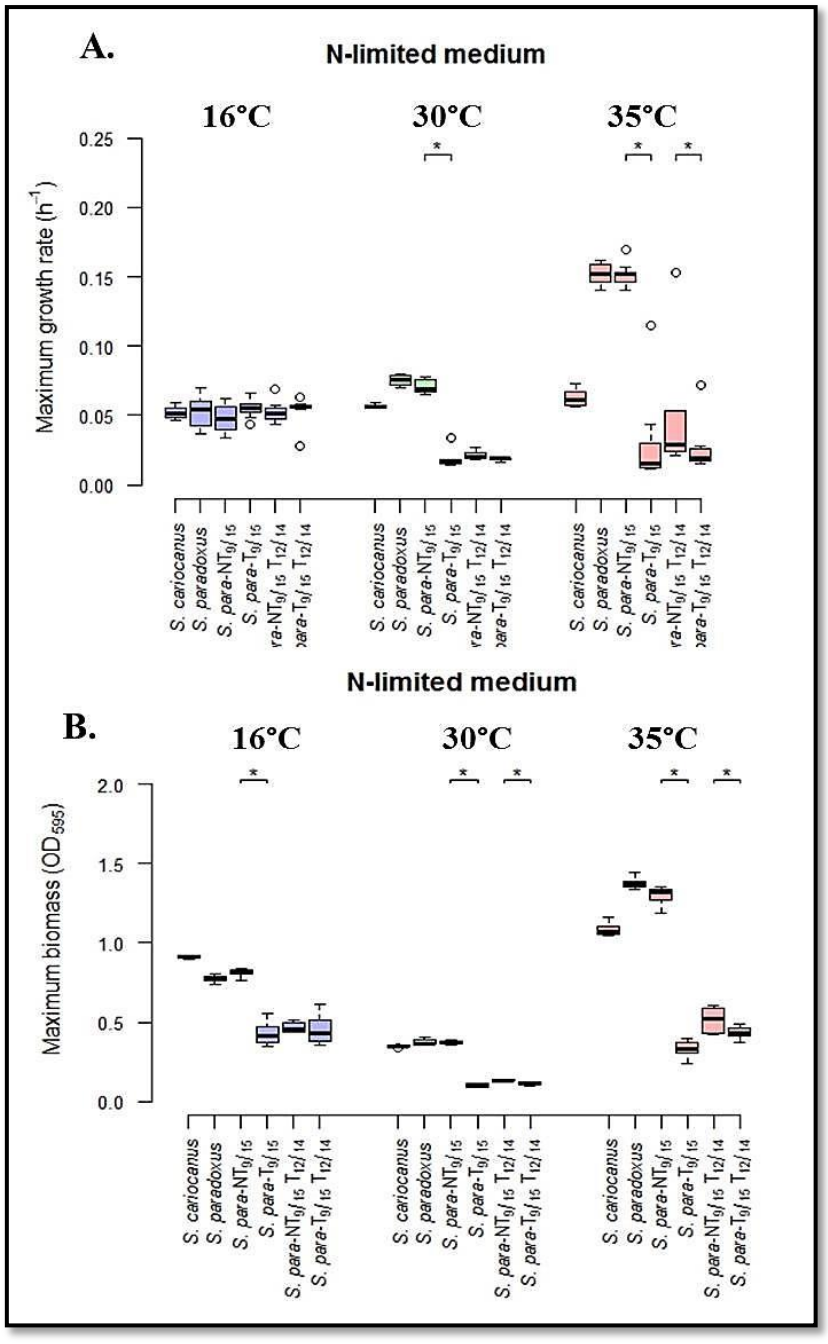


**Figure 3.24 Mitotic fitness of *S. cariocanus, S. paradoxus* and engineered translocant and non-translocant strains in N-limited media**

Fitness boxplots represent both maximum growth rate **(Panel A)** and maximum biomass **(Panel B)** of all strains in N-limited media at 16 °C, 30 °C and 35 °C. Purple, green and pink boxplots represent all strains at 16 °C, 30 °C and 35 °C respectively. Each boxplot shows the distribution of data based on the minimum, median and maximum number. Each strain was tested with three biological replicates (with three technical each) (*$P$<0.05, Mann-Whitney test). Significant drops in growth fitness were observed in translocant strains and non-translocant S. para-$NT_{12/14}T_{9/15}$ incomparisons to wild type strains and non-translocant S. para-$NT_{9/15}$ at all temperatures. No significant differences were observed at 16°C according to their maximum growth rate.

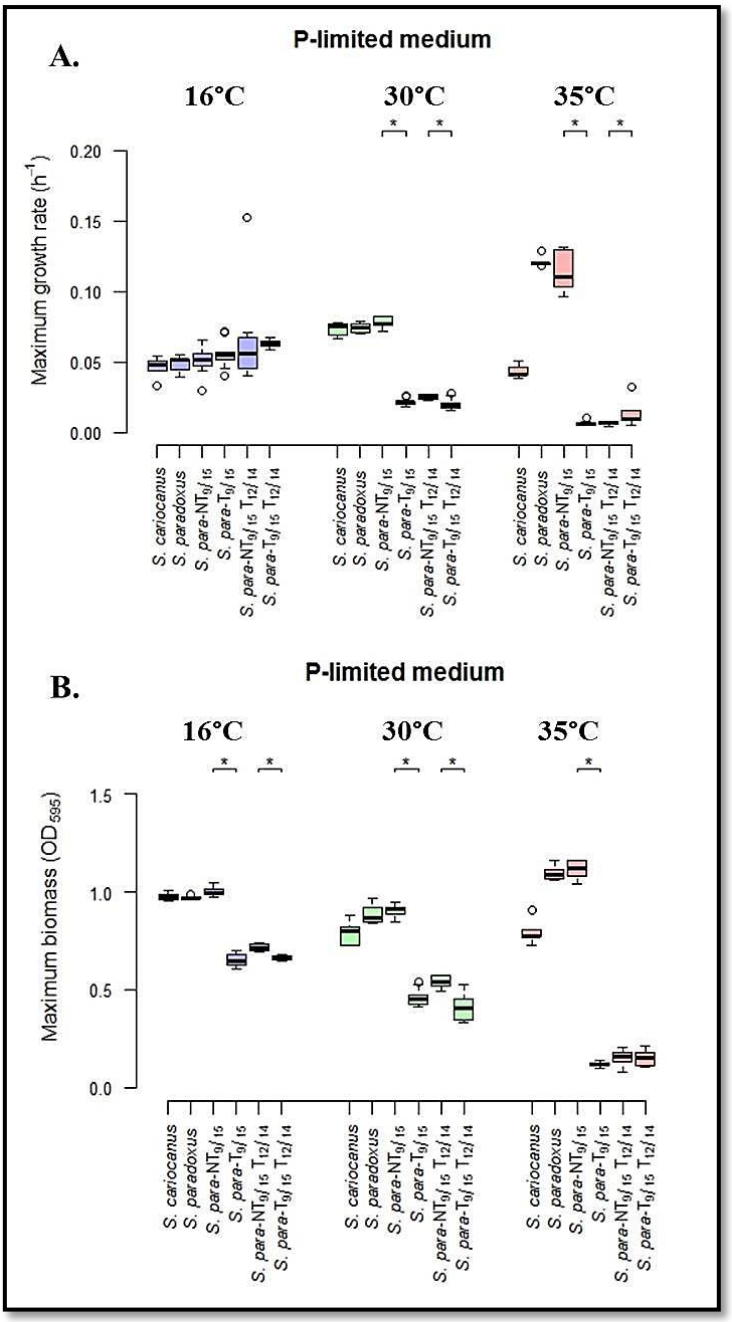


**Figure 3.25 Mitotic fitness of *S. cariocanus, S. paradoxus* strains and engineered translocant and non-translocant strains in P-limited media**

Fitness boxplots represent both maximum growth rate **(Panel A)** and maximum biomass **(Panel B)** of all strains in P-limited media at 16 °C, 30 °C and 35 °C. Purple, green and pink boxplots represent all strains at 16 °C, 30 °C and 35 °C respectively. Each boxplot shows the distribution of data based on the minimum, median and maximum number. Each strain was tested with three biological replicates (with three technical each) (**P* <0.05, Mann-Whitney test). Significant drops in growth fitness were observed in translocant strains and non-translocant S. para-$NT_{12/14}T_{9/15}$ incomparisons to wild type strains and non-translocant S. para-$NT_{9/15}$ at all temperatures. No significant differences were observed at 16°C according to their maximum growth rate.

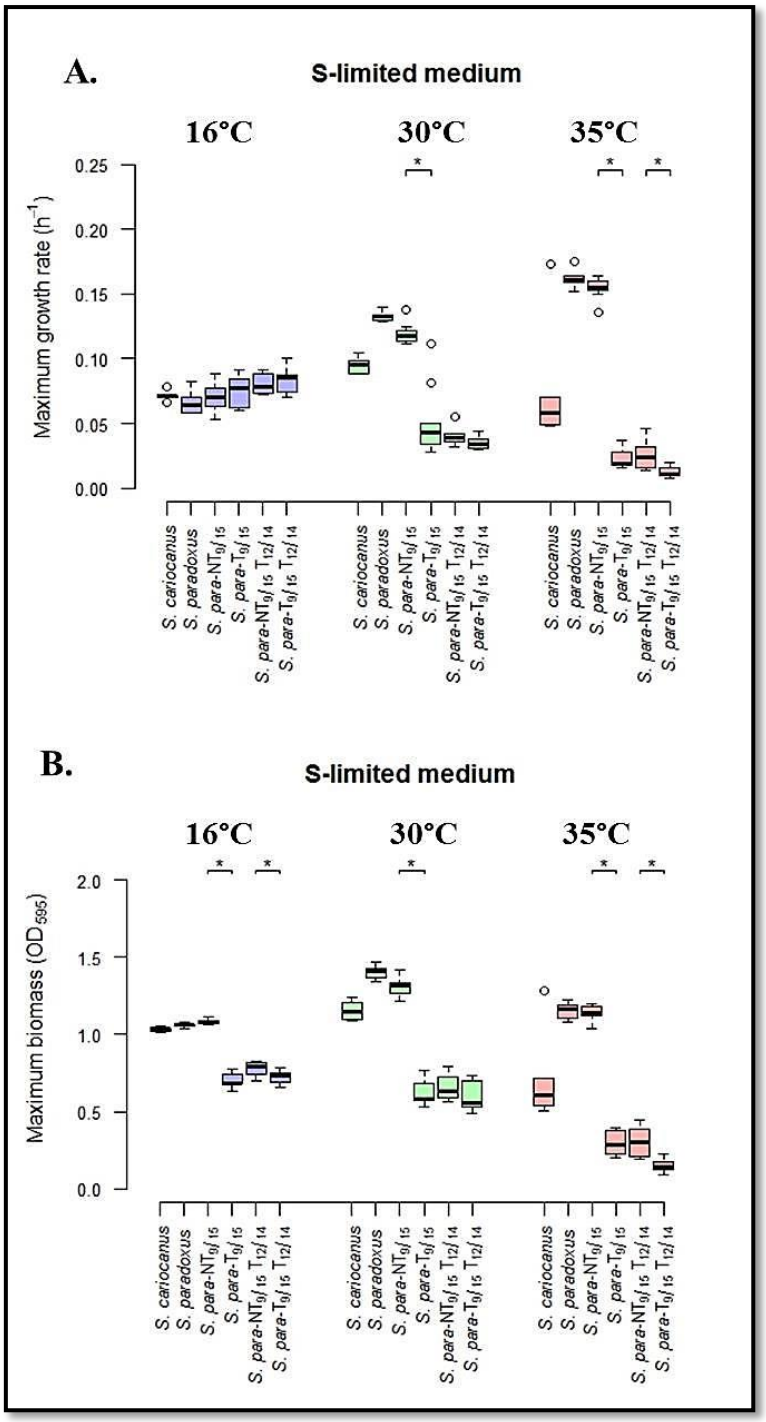


**Figure 3.26 Mitotic fitness of *S. cariocanus*, *S. paradoxus* strains and engineered translocant and non-translocant strains in S-limited media**

Fitness boxplots represent both maximum growth rate **(Panel A)** and maximum biomass **(Panel B)** of all strains in S-limited media at 16 °C, 30 °C and 35 °C. Purple, green and pink boxplots represent all strains at 16 °C, 30 °C and 35 °C respectively. Each boxplot shows the distribution of data based on the minimum, median and maximum number. Each strain was tested with three biological replicates (with three technical each) (*$P$ <0.05, Mann-Whitney test).

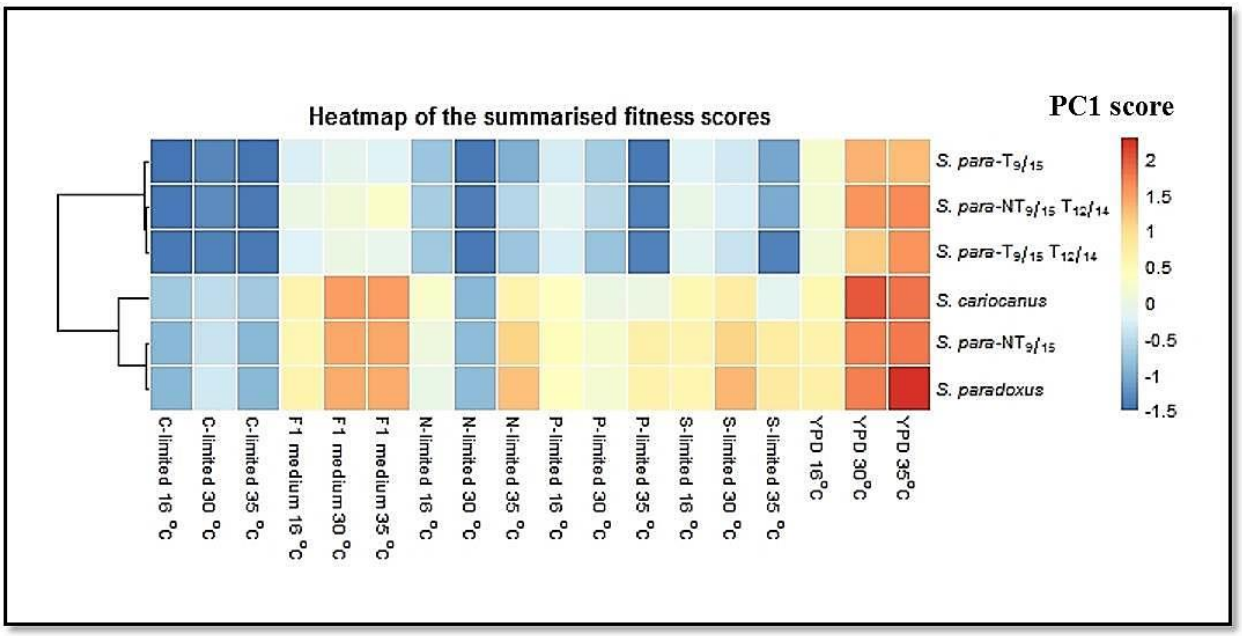


**Figure 3.27 Heatmap representing the mitotic fitness of translocant, non-translocant, and wild type strains under different nutritional and temperature conditions**

The colour scale represents the fitness of all strains in which the blue scale shows very low fitness (from -1.5) up to the red scale shows the normal fitness (2). Taking together all growth parameters, there is a drop in the fitness of translocant strains in comparison to the wild types *S. cariocanus* and *S. paradoxus* and non-translocant S. para-$NT_{9/15}$ in all limited media. Heatmap of PC1 scores derived from the three growth parameters (max. growth rate, lag phase and max. biomass) estimated by local polynomial regression implemented in the R package *grofit*.

When analyzing the fitness of the parents, remarkably, we observed a low fitness of *S. cariocanus* strain specifically in three limited media, N-limited, P-limited and S-limited, at 35 °C compared with the *S. paradoxus* wild type and other conditions. This fitness pattern of *S. cariocanus* is consistent with the drop in fitness that we see in *S. paradoxus* translocated strains mimicking part of *S. cariocanus* genome. We therefore suggest that the presence of chromosomal rearrangements, including translocations (five CTs) may contribute to this fitness reduction in *S. cariocanus* in these specific media and may be severely affecting its growth at 35 °C. To test our hypothesis, we selected the CT between chromosomes IX/XV that caused the most severe phenotype in our *S. paradoxus* engineered strain and reversed it in *S. cariocanus* strain to analyze the resulting fitness.

### 3.6 Effect of removing/ reversing one chromosomal translocation on the mitotic fitness of *S. cariocanus* strain

As we observed reduction in the fitness of *S. cariocanus* at 35 °C in three limited media (N-limited, P-limited and S-limited), we reversed one chromosomal translocation between chromosomes IX and XV to see whether this causes an improvement of the mitotic fitness. The analysis of PacBio sequences revealed that the translocation between chromosomes IX and XV are found between YIL014w (in chromosome IX) and YOL055c (in chromosome XV) on unitig 10 and between YIL015w (in chromosome IX) and YOL054w (in chromosome XV) on unitig 1. The PacBio sequence was used for designing the oligonucleotide primers for cassettes insertion and confirmation of translocant and non-translocant strains. In the following sections, the construction of a strain of reversing one translocation between IX/XV and a fitness assay analysis will be described.

### 3.6.1 Creation of stable haploid strain of *S. cariocanus*

In order to create a heterothallic stable *S. cariocanus* haploid strain from *S. cariocanus* diploid strain that was only available in our lab, the *HO* gene was deleted using the *lox2272-natNT2-lox2272* marker cassette. This deletion was created to prevent mating type-switching and to ensure that the strain existed in astable haploid state.

Oligonucleotide primers were designed for *HO* gene deletion cassettes which were homologous to the first and last 60 nucleotides of the *HO* gene, and to 20 nucleotides of universal primer annealing sequences (Tables A.1 and A.2). The *HO* mutant *S. cariocanus* strains were confirmed by colony PCR (Figure 3.28) and subsequently sporulated. Tetrad dissection was carried out and followed by replica plating on YPD-containing ClonNAT resistance antibiotic to detect the haploid strain carrying the *HO* deletion. Only two spores those that contain the marker cassettes would grow on selectable marker plates and thus would be identified as carrying the deletion of *HO* (Figure 3.29). Spores that were confirmed to have the *HO* deletion were selected for mating type identification.

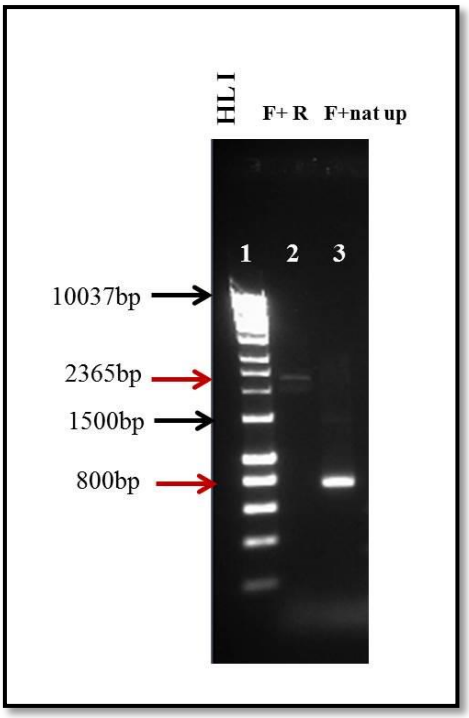


**Figure 3.28 Colony PCR for confirmation of *lox2272-natNT2-lox2272* cassette insertion into *HO* gene position**

1% (w/v) agarose gels of cassettes inserted into *S. cariocanus* haploid strain. Lanes1 show Hyperladder 1Kb, lanes 2 and 3 represent transformant colonies confirmed by Conf. F+R (2365bp) and Conf. F + nat up primers (800bp), respectively.

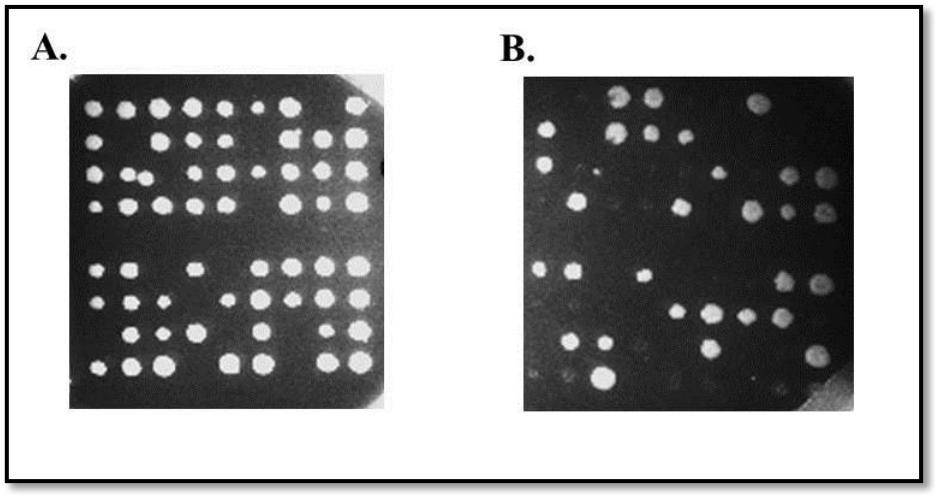


**Figure 3.29 *S. cariocanus* tetrads dissection and replica plating**

Growth of *S. cariocanus* spores after dissection of tetrads (dissected vertically on the plate **(Panel A)** and only two spores were viable when replicated on YPD- containig ClonNAT **(Panel B**).

### 3.6.2 Mating-type identification of *S. cariocanus* haploid *HO* deletion strain

Identification of *MAT* ***a*** and *MAT* α types were obtained by crossing two spores from one tetrad with a known mating-type *S. paradoxus-MAT* **a** and *S. paradoxus-MAT* α tester strains. Since the tester strain contain the auxotrophic marker Ura3 deleted with *KanMX* resistance marker cassette (-Ura3), a prototrophic diploid strains (heterozygous for Ura3) were generated with *S. cariocanus* spores (Figure 3.30). Diploids were selected on YPD-containing ClonNat and geneticin resistance markers (G418).

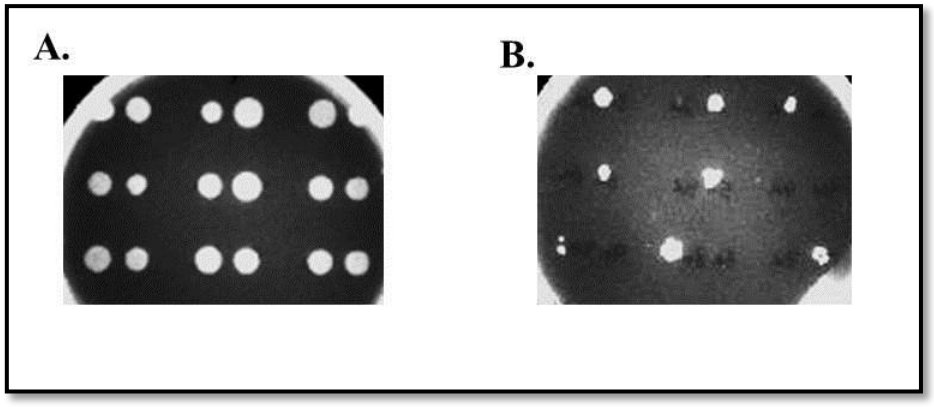


**Figure 3.30 *S. cariocanus* mating type identification**

Colonies of *S. cariocanus* spores mated with two tester strain *S. paradoxus MAT* **a** and *MAT* α strains (G418 resistant) **(Panel A)**. Selective plate for diploid where only diploids containig CloNAT + G418 markers can grow **(Panel B)**.

### 3.6.3 Reversing one chromosomal translocation between chromosomes IX and XV

In order to evaluate whether the translocation naturally present between IX/XV in *S. caricanus* has an impact on fitness, the CT between chromosomes IX/XV was reversed. Two marker cassettes, *lox*P-*hphNT1*-*lox*P and *lox*P-*kanMX*-*lox*P, were inserted between specific breakpoints in *S. cariocanus MAT* **a** strain followed by successful removal of marker cassettes by Cre recombinase methods. The correct insertion of these cassettes was verified by diagnostic PCR and the expected band sizes were confirmed by agarose gel electrophoresis (Table 3.7) (Figures 3.31 and 3.32). Primers used for confirmation of insertion and removing of marker cassettes were designed to be specific to the cassettes and chromosomal intergenic regions.

*S. cariocanus* engineered strains possessing the reversed CTs at chromosomes IX and XV are therefore referred to as S. car-4T. The controls that underwent the same process of reversing the translocations but still carrying all translocations is referred to as S. car-$NT_{(5T)}$. These strains were used to assess the effect of CTs on the fitness growth after undoing the translocation.

**Table 3.7** Expected band sizes of PCR products for primer sets used to confirm the correct integration of the *lox*P sites and to verify the rearrangements in *S. cariocanus* strains

| **Position of *lox*P site insertions in *S. cariocanus*** | **Primer Names** | **Fragment sizes (bp)** | |
|---|---|---|---|
| **ChromosomesIX/XV** | Conf.kan.F+nat up | 840 | |
| | Conf.kan.R+nat down | 1400 | |
| **ChromosomesIX/XV** | Conf.hph.F+hph up | 932 | |
| | Conf.hph.R+nat down | 1035 | |
| **Rearrangements in *S.cariocanus*** | **Primer Names** | **Fragment sizes (bp)** | |
| | | **Translocation** | **Non-translocation** |
| **IX/IX∗** | Ch9.hph.F+Ch9.kanR | - | 400 |
| **IX/XV∗∗ between YIL014w/YOL055c** | Ch9.hph.F+Ch15hph.R | 359 | - |
| **XV/XV∗** | Ch15.kanF+ Ch15.hphR | - | 491 |
| **IX/XV∗∗ between YIL015w/YOL054w** | Ch15.hph.F+Ch9hph.R | 1202 | - |

∗ Reversing chromosomal translocation to the normal chromosomal positions

∗∗ Chromosomal translocation between chromosomes IX and XV were found between YIL014w and YOL055c and between YIL014w/YOL055c

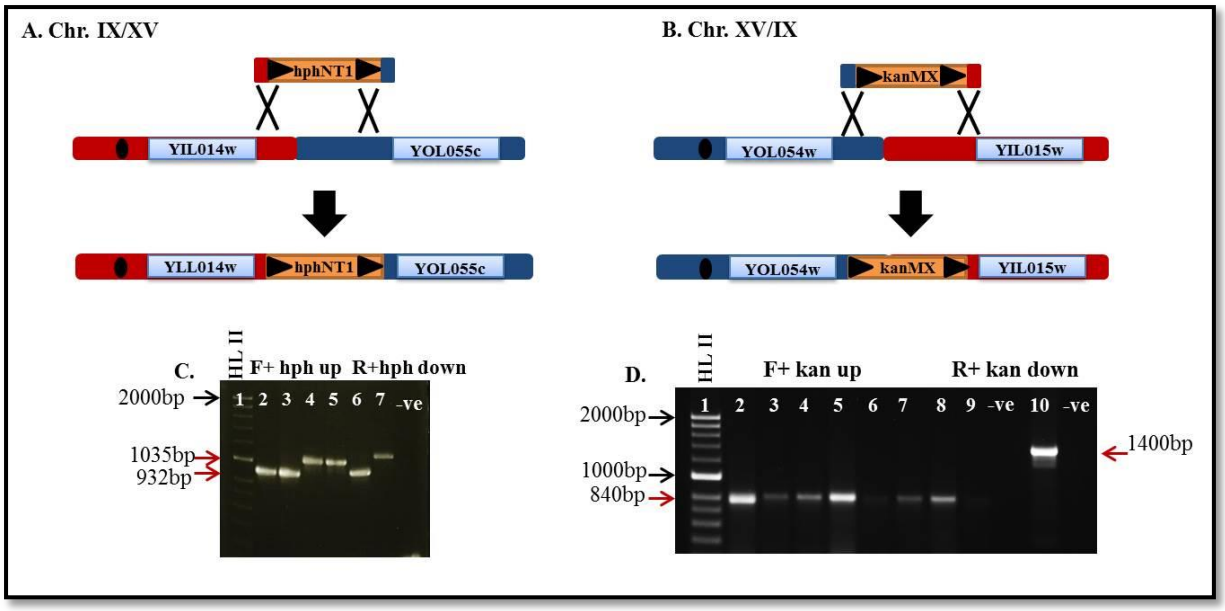


**Figure 3.31 Colony PCR for confirmation of cassettes insertion into *S. cariocanus* haploid strains**

**Panels A and B** represent the insertion of amplified *lox*P-*hphNT1*-*lox*P and *lox*P-*kanMX*-*lox*P marker cassettes by homologous recombination into intergenic regions between specific translocation breakpoints at chromosomes IX and XV. **Panels C and D** represent 1.5% (w/v) agarose gels of cassettes inserted into *S. cariocanus* haploid strains. Lanes1 show Hyperladder 1I (Bioline), lanes 2, 3 and 6 represent transformant colonies confirmed by Conf. F+ hph up primers (932bp), and lanes 4 and 5 confirmed by Conf. R+ hph down primers (1035bp) (**Panel C**). Lanes 2 to 9 represent transformant colonies confirmed by Conf.F+nat up (840bp) and lane 10 confirmed by Conf.R+nat down primers (1400bp) **(Panel D).** Negative controls are presented as –ve.

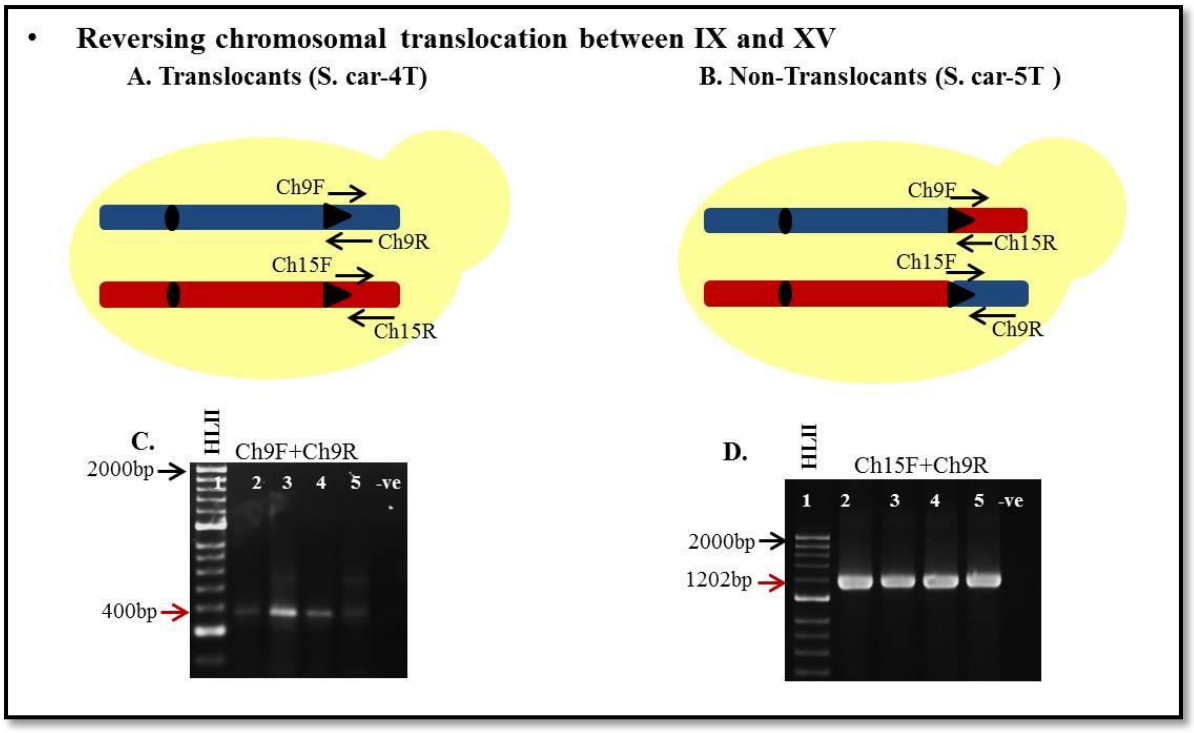


**Figure 3.32 Colony PCR for confirmation of translocations and non-translocations *S. cariocanus* strains**

Following Cre recombinase induction, chromosomal translocation was reversed between chromosomes IX/XV. Translocants **(Panel A.1)** and non-translocants chromosomes **(Panel B)** are shown in diagrams and confirmed by using combinations of checking primers. **Panels C and D** represent 1.5% (w/v) agarose gels for confirmation of translocant and non-translocant colonies. Lanes1 represent Hyperladder II (Bioline) **(Panels C and D)**, lanes 2 to 5 represent translocant colonies that possessed 4T confirmed by Ch9F+Ch9R primers (400bp) **(Panel C)**. Lanes 2 to 5 represent non-translocants that possessed 5T confirmed by Ch15F+Ch9R primers (1202bp) **(Panel D)**. Negative controls are presented as –ve.

### 3.6.4 Fitness assay analysis of *S. cariocanus* engineered strains at 35 °C

It was hypothesized that the chromosomal translocation that was located between chromosomes IX/XV is responsible for the low fitness in *S. cariocanus* strains. By reversing this translocation, we would see whether the fitness improves. A fitness assay was performed for three biological replicates of engineered translocants S. car-4T, non-translocant S. car-$NT_{(5T)}$ strains, and wild type *S. cariocanus* at 35 °C in selected media including YPD, N-limited, P-limited and S-limited media. These media were selected because we observed a drop in the fitness of the wild type *S. cariocanus* strain in comparison to its fitness in other media and conditions.

Comparing the fitness of the engineered strain with its control in YPD media, S. car-4T translocant strains exhibited a significant improvement of the maximum biomass ($P$ <0.05, Wilcoxon rank sum test), while the improved growth rate did not appear large enough to be significant. In all the limited media, significant differences of maximum biomass and maximum growth rate were observed in translocant S. car-4T strains in comparison to non-translocant S. car-$NT_{(5T)}$ strains ($P$ <0.01, Wilcoxon rank sum test) (Figure 3.33). The difference in fitness was not always significant between S.car-4T and *S. cariocanus* wild-type probably due to the fact *S. cariocanus* did not undergo any genetic manipulation, while both S. car-$NT_{(5T)}$ and S. car-4T underwent three rounds of transformation procedures (two rounds of *lox*P insertion and one Cre plasmid transformation; see figs 3.4 and 3.5). Therefore, the correct comparison to understand the effect of the translocation on fitness must be done between S. car-$NT_{(5T)}$ and S. car-4T. Our findings were exciting and proved that the translocation between chromosomes IX and XV was indeed responsible for the lower fitness of *S. cariocanus* strains compared to the *S. paradoxus*.

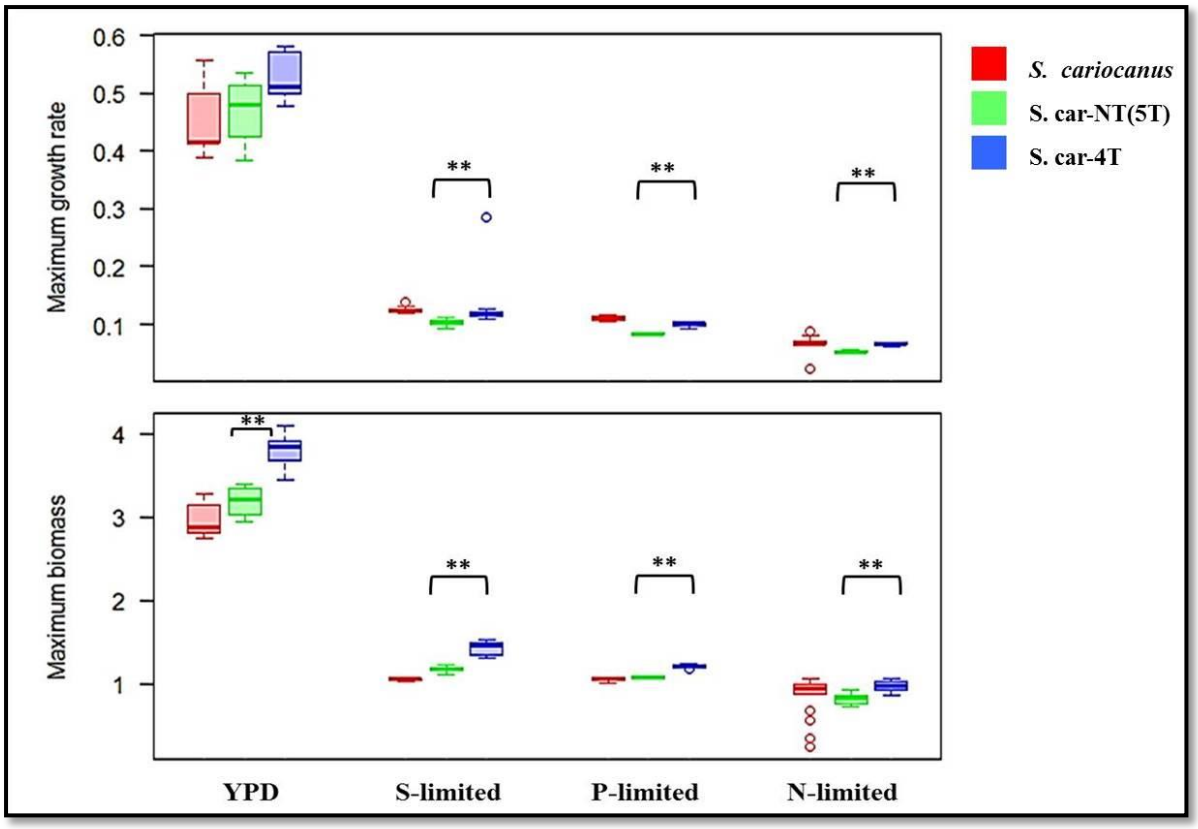


**Figure 3.33 The fitness of engineered *S. cariocanus* translocant, non-translocant and wild type strain in YPD, N-limited, P-limited and S-limited at 35 °C**

Three biological replicates were tested from each strain and error bars represented the standard error of the mean. Significant improvement in the fitness of *S. cariocanus* strain possessing 4 translocations (S. car-4T) in comparisons to the control *S. cariocanus* non-translocant strain (S. car-$NT_{(5T)}$) can be seen in all nutrient limited media. Significant improvement of maximum biomass for S. car-4T can be also seen in rich YPD media.

## 3.7 Impact of chromosomal translocation on Global gene expression using illumina sequencing (RNA-seq)

In order to test whether the changes in mitotic and meiotic fitness are combined with alteration in gene expression, global transcriptomic profile via RNAseq was performed. Total RNA from six strains; *S. paradoxus* YPS138 (*MAT* **a**), *S. cariocanus* UFRJ50816, S. para-$T_{9/15}$, S. para-$NT_{9/15}$, S. para- $T_{9/15}T_{12/14}$ and S.para-$NT_{12/14}T_{9/15}$ including three biological replicates each were extracted and purified using QIAGEN RNeasy Mini Kit (Qiagen, catalogue number 74104). All these strains were grown on rich YPD media and the RNA sequencing was carried out using Illumina Hiseq 2500 (Illumina, USA) platform. Principal component analysis (PCA) and Gene Ontology were performed to analyze and utilize the information obtained from gene expression data for all tested strains.

### 3.7.1 Principal component analysis (PCA)

To identify the similarity and dissimilarity in gene expression data, principal component analysis (PCA) was performed for all engineered strains, their controls, and the wild type strains including all the biological replicates. We observed that engineered translocant strains, namely S. para-$T_{9/15}$ (strain with one translocation) and S. para-$T_{12/14}T_{9/15}$ (strain with two translocations), and the second control strain, S. para-$NT_{12/14}T_{9/15}$ (carrying one translocation) were clustered separately from the parental strain *S. paradoxus* and the first non-translocant control strain, S. para-$NT_{9/15}$ (Figure 3.34). Accordingly, these results confirmed that the chromosomal translocation that was constructed in the *S. paradoxus* strains affects gene expression. Next, we analyzed which genes were up-regulated or down-regulated due to the presence of the CTs.

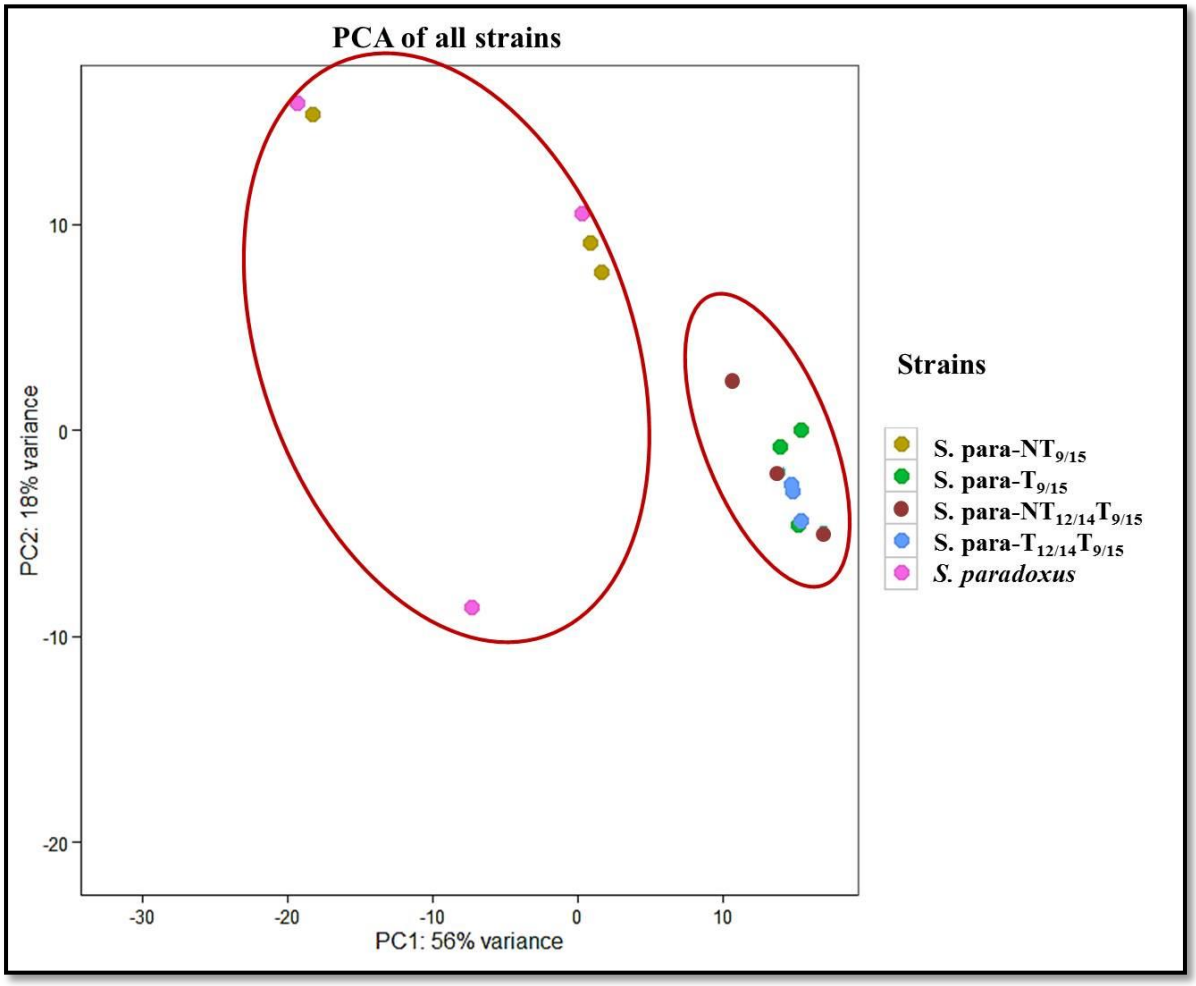


**Figure 3.34 Principle component analysis (PCA) of RNA sequencing data of all engineered strains**

PCA represents overall variation of three biological replicates (with three technical replicates each) for each strain including wild type *S. paradoxus* YPS138 haploid stain, the translocant, and the control engineered strains. Non-translocant S. para-$NT_{9/15}$ control stains are clustered with the *S. paradoxus* wild type strain while, all engineered translocant strains and the second control S. para-$NT_{12/14}T_{9/15}$, carrying one translocation,are clustering in one group in term of genes expression.

### 3.7.2 Identification of total number of genes with differential changing in expression

Differential gene expression (either up- or down- regulated) was assessed in each engineered translocant strain in comparison to its control non-translocant strain, and the differences were considered to be significant if the q-value was ≤ 0.05 and the fold change was between ≥ 2. A total of 219 genes were significantly differentially expressed in the translocant S. para-$T_{9/15}$ strain in comparisons to its control S. para-$NT_{9/15}$ strain (q-value ≤ 0.05) (Figure 3.35). However, when compared the differences in gene expression in translocant S. para-$T_{12/14}T_{9/15}$ strains and its control S. para- $NT_{12/14}T_{9/15}$ strains, no significant difference was observed (data not shown). Together, our results indicated that the translocation $T_{12/14}$ did not have a significant effect on the expression of the genes.

The analysis of the RNAseq data for S. para-$T_{9/15}$ revealed that 146 (66%) and 73 (33%) genes were down- and up -regulated, respectively, in this strain carrying the translocation $T_{9/15}$ (Figures 3.36 and 3.37). Twenty-nine (13.24%) genes out of 219 genes were located on the translocated chromosomes IX/XV. Out of these 29 genes: 15 genes were found to be down-regulated, of which six genes were located on chromosome IX and nine genes were located on chromosome XV and 14 genes were found to be up-regulated, of which three genes on chromosome IX and 11 genes at chromosome XV (Figure 3.38). We also realized that chromosome I does not include any alteration in gene expression, and this is likely due to its size (~230.218 kb) which is considered very small. Chromosomes III and VI have only three and five altered genes, respectively, and that also is likely due to their sizes (316.620 kb and 270.161 kb). Also number of genes may be few that are presented in chromosome I that doesn't has much genes to be changes.

Our gene expression data indicates that the CTs presented in *S. paradoxus* strains not only contributes to the changes in genes expression in translocated chromosomes, but also in the other parts of the genome. In fact, in the top 20 differentially expressed genes, there are only six genes (two up-regulated and four down-regualted genes) that are present on the translocated chromosomes (Tables 3. 8 and 3.9).

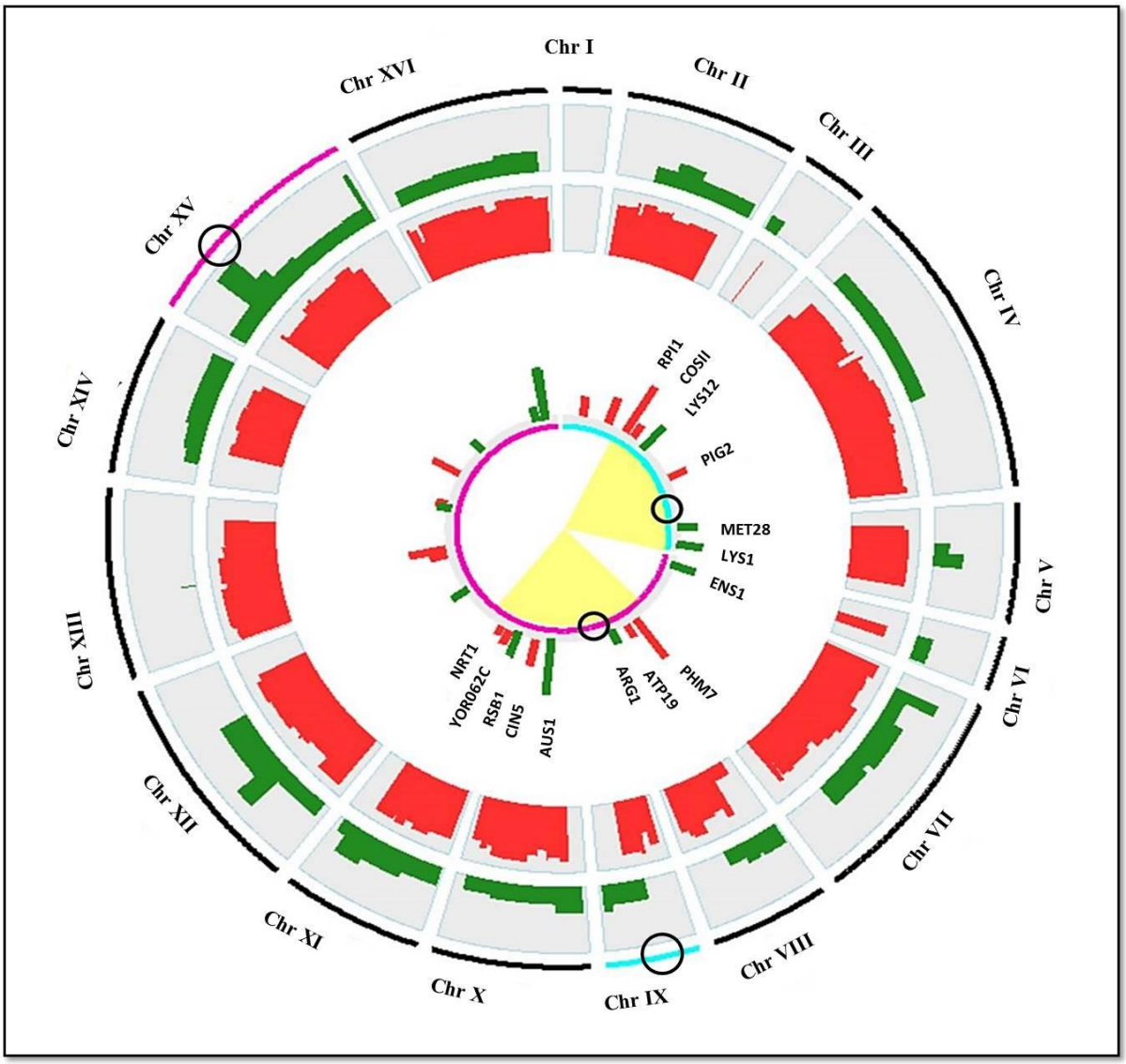


**Figure 3.35 Gene expression map of the engineered translocant S. para-$T_{9/15}$ relative to its control strain**

The chromosomes are presented as black lines, except for chromosomes IX and XV which possessed the chromosomal translocation which are blue and pink, respectively. The distribution of up- and down-regulated genes is presented in green and red histograms, respectively. Genes that are up- and down-regulated on chromosomes IX and XV are shown in the central circus plot. The translocation breakpoints are circled by black ovals.

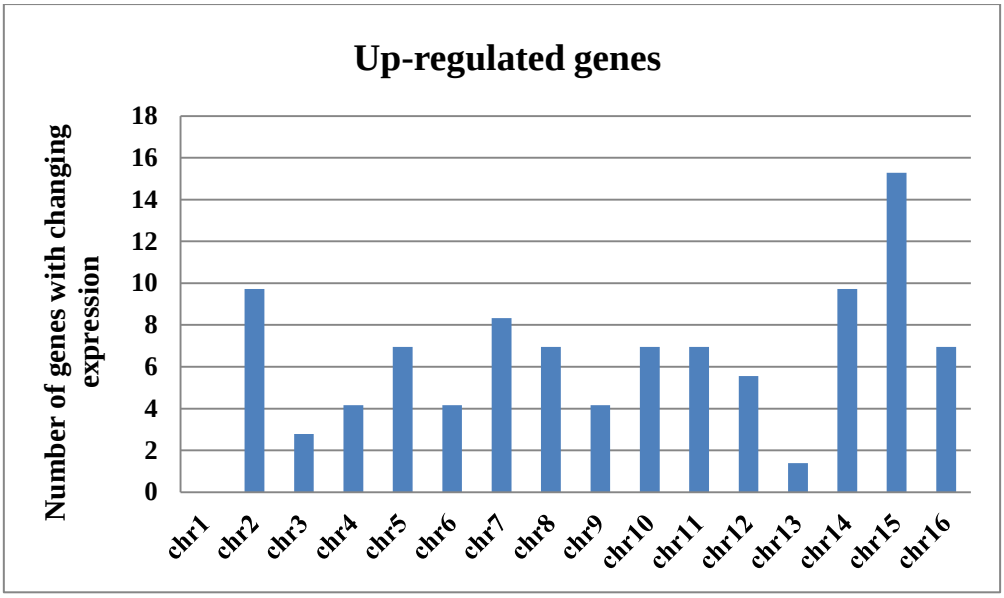


**Figure 3.36 Total number of up-regulated genes in S. para-$T_{9/15}$ strains**

Number of genes that are up-regulated in each chromosome in the strain S. para-$T_{9/15}$ relative to its control strain. Chromosome 15 has the highst number of up-regulated genes and chromosome 13 has the lowest number of genes. No significant changes in genes expression in chromosome 1.

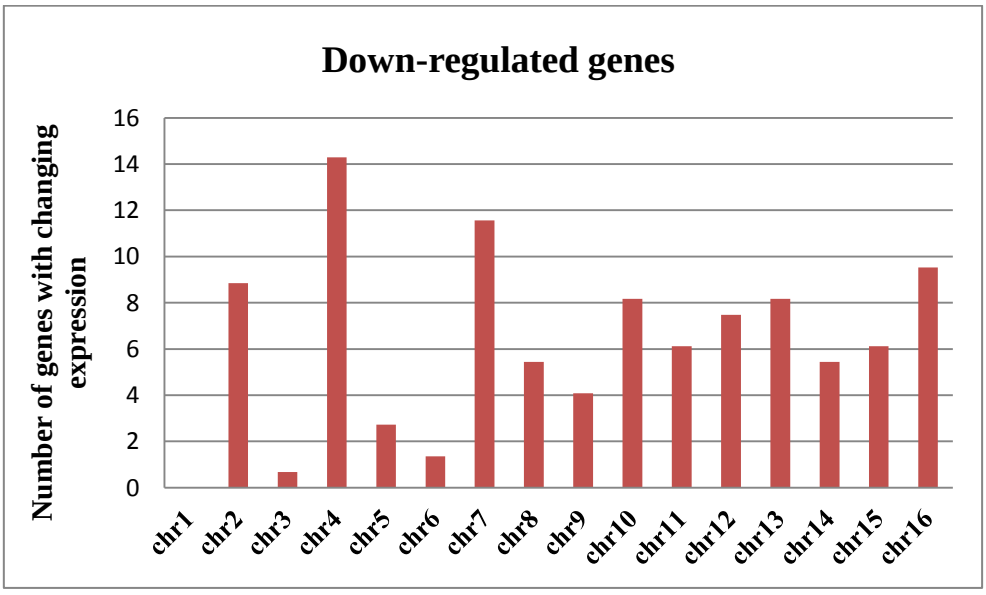


**Figure 3.37 Total number of down-regulated genes in S. para-$T_{9/15}$ strains**

Number of genes that are down-regulated in each chromosome in the strain S. para-$T_{9/15}$ relative to its control strain. Chromosome 4 has the highst number of down-regulated genes and chromosome 3 has the lowest number of genes. No significant changes in genes expression in chromosome 1.

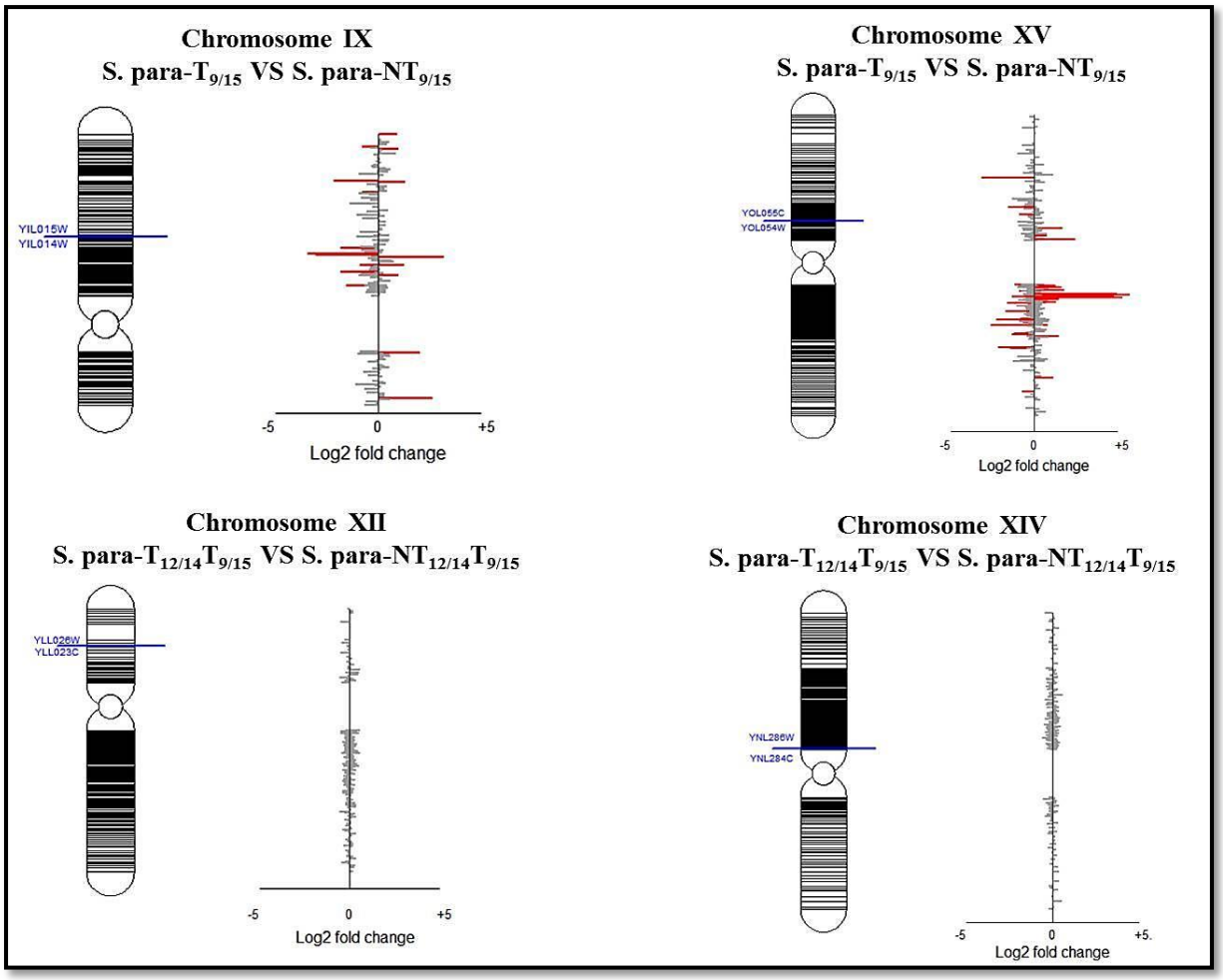


**Figure 3.38 Representation of chromsomes IX, XV, XII and XIV with the locations of breakpoints and significantly expressed genes**

Red lines represent the distribution of the genes that are significantly up- and down-regulated on chromosomes IX and XV of translocant S.para-$T_{9/15}$strains in comparison to non-translocant S. para-$NT_{9/15}$. Comparing the expression of genes in translocant S. para-$T_{12/14}1T_{9/15}$ strains with its control, S.para-$NT_{12/14}$-$T_{9/15}$, revealed no significant differences between them (fold change $\geq 2$, q-value $< 0.05$).

**Table 3.8** Top 20 genes highly up-regulated in S. para-$T_{9/15}$ strain in comparison to its control S. para-$NT_{9/15}$ strain

Yellow highlighted genes are presented in translocated chromosome XV

| Standard Name | Systematic Name | Chromosomal position | log2FoldChange | q-value |
|---|---|---|---|---|
| UBP11 | YKR098C | ChrXI | 1.0039 | 0.000584 |
| ZWF1 | YNL241C | ChrXIV | 1.0097 | 6.40000E-05 |
| PXP2 | YJR111C | ChrX | 1.0109 | 6.60000E-05 |
| HOM3 | YER052C | ChrV | 1.0243 | 1.00000E-06 |
| HIS1 | YER055C | ChrV | 1.0483 | 0.000365 |
| MET5 | YJR137C | ChrX | 1.0497 | 0.041463 |
| RAD59 | YDL059C | ChrIV | 1.0715 | 0.000831 |
| COT1 | YOR316C | ChrXV | 1.0788 | 0.035309 |
| SUL1 | YBR294W | ChrII | 1.0795 | 0.007751 |
| - | YHR112C | ChrVIII | 1.0813 | 0.00000E+00 |
| HXK1 | YFR053C | ChrVI | 1.0818 | 0.00001 |
| HIS7 | YBR248C | ChrII | 1.0841 | 0.00000E+00 |
| ARG7 | YMR062C | ChrXIII | 1.0866 | 0.000034 |
| CTP1 | YBR291C | ChrII | 1.0877 | 0.000418 |
| MET32 | YDR253C | ChrIV | 1.0938 | 0.030984 |
| ISU1 | YPL135W | ChrXVI | 1.0974 | 0.003373 |
| - | YGL117W | ChrVII | 1.0991 | 0.000753 |
| ODC2 | YOR222W | ChrXV | 1.0993 | 0.00009 |
| LEU2 | YCL018W | ChrIII | 1.1012 | 0.000011 |
| MET10 | YFR030W | ChrVI | 1.1117 | 0.00115 |

**Table 3.9** Top 20 genes that are highly down-regulated in S. para-$T_{9/15}$ strain in comparisons to its control S. para-$NT_{9/15}$ strain

Yellow highlighted genes are presented in translocated chromosomes IX/XV

| Standard Name | Systematic Name | Chromosomal position | log2FoldChange | q-value |
|---|---|---|---|---|
| - | YHR033W | Chr VIII | -4.1096 | 0.0000E+00 |
| INH1 | YDL181W | Chr IV | -3.0994 | 0.0000E+00 |
| FMP16 | YDR070C | Chr IV | -2.6849 | 0.0000E+00 |
| RPI1 | YIL119C | Chr IX | -2.5904 | 0.0000E+00 |
| RTN2 | YDL204W | Chr IV | -2.3863 | 0.0000E+00 |
| PHM7 | YOL084W | Chr XV | -2.3567 | 1.7700E-07 |
| USV1 | YPL230W | Chr XVI | -2.3465 | 0.0000E+00 |
| RPI1 | YIL119C | Chr IX | -2.2977 | 1.6100E-07 |
| RTC3 | YHR087W | Chr VIII | -2.189 | 3.0000E-09 |
| CLD1 | YGR110W | Chr VII | -2.15 | 1.3000E-08 |
| TAT1 | YBR069C | Chr II | -2.0939 | 2.6000E-08 |
| YHB1 | YGR234W | Chr VII | -2.0939 | 0.0000E+00 |
| JEN1 | YKL217W | Chr XI | -2.0874 | 1.1300E-06 |
| COX4 | YGL187C | Chr VII | -2.0067 | 0.0000E+00 |
| MCR1 | YKL150W | Chr XI | -1.9927 | 2.0000E-09 |
| - | YJR149W | Chr X | -1.9925 | 3.5300E-06 |
| PRY3 | YJL078C | Chr X | -1.9841 | 0.0000E+00 |
| ARO9 | YHR137W | Chr VIII | -1.9809 | 3.6000E-05 |
| GAC1 | YOR178C | Chr XV | -1.9674 | 1.2800E-06 |
| ACH1 | YBL015W | Chr II | -1.9174 | 0.0000E+00 |

### 3.7.3 Gene Ontology analysis (GO)

To define which particular genes cluster in one group in terms of their biological process and molecular function, Gene Ontology analysis (GO) was performed using the Gene Ontology GO Term Finder (Version 0.83) (http://www.yeastgenome.org/cgi-bin/GO/goTermFinder.pl). In term of biological process, the analysis of significantly differentially up-regulated and down-regulated genes revealed that the CTs mostly affected genes involved in amino acid metabolic process and ATP metabolic process, respectively (Tables 3.10 and 3.11). When classified according to their molecular function, most of the up-regulated genes are involved in oxidoreductase activity and down-regulated genes are involved in hydrogen ion transmembrane transporter activity (Tables 3.12 and 3.13).

**Table 3.10** Gene Ontology analysis for the biological process of the **up-regulated** genes in translocant S. para-$T_{9/15}$ strain VS non-translocant S. para- $NT_{9/15}$ control strain

| GO ID | GO_term | Cluster frequency | P-value | FDR* |
|---|---|---|---|---|
| GO:0006520 | cellular amino acid metabolic process | 24 out of 72 genes, 33.3% | 3.48E-15 | <0.001 |
| GO:0008652 | cellular amino acid biosynthetic process | 18 out of 72 genes, 25.0% | 1.55E-13 | |
| GO:1901605 | alpha-amino acid metabolic process | 19 out of 72 genes, 26.4% | 2.69E-13 | |
| GO:1901607 | alpha-amino acid biosynthetic process | 17 out of 72 genes, 23.6% | 3.82E-13 | |
| GO:0019752 | carboxylic acid metabolic process | 27 out of 72 genes, 37.5% | 3.89E-13 | |
| GO:0043436 | oxoacid metabolic process | 27 out of 72 genes, 37.5% | 1.03E-12 | |
| GO:0006082 | organic acid metabolic process | 27 out of 72 genes, 37.5% | 1.03E-12 | |
| GO:00016053 | organic acid biosynthetic process | 19 out of 72 genes, 26.4% | 3.04E-12 | |
| GO:0046394 | carboxylic acid biosynthetic process | 19 out of 72 genes, 26.4% | 3.04E-12 | |
| GO:0000096 | sulfur amino acid metabolic process | 10 out of 72 genes, 13.9% | 3.23E-09 | |

∗ False Discovery Rate

**Table 3.11** Gene Ontology analysis for the biological process of the **down-regulated** genes in translocant S. para-$T_{9/15}$ strain VS non-translocant S. para- $NT_{9/15}$ control strain

| GO ID | GO_Term | Cluster frequency | P-value | FDR* |
|---|---|---|---|---|
| GO:0046034 | ATP metabolic process | 31 out of 146 genes, 21.2% | 2.72E-40 | <0.001 |
| GO:0009205 | purine ribonucleoside triphosphate metabolic process | 31 out of 146 genes, 21.2% | 2.19E-39 | |
| GO:0009144 | purine nucleoside triphosphate metabolic process | 31 out of 146 genes, 21.2% | 1.52E-38 | |
| GO:0009199 | ribonucleoside triphosphate metabolic process | 31 out of 146 genes, 21.2% | 1.52E-38 | |
| GO:0009141 | nucleoside triphosphate metabolic process | 31 out of 146 genes, 21.2% | 1.15E-35 | |
| GO:0009126 | purine nucleoside monophosphate metabolic process | 32 out of 146 genes, 21.9% | 6.89E-30 | |
| GO:0009167 | purine ribonucleoside monophosphate metabolic process | 32 out of 146 genes, 21.9% | 6.89E-30 | |
| GO:0009161 | ribonucleoside monophosphate metabolic process | 32 out of 146 genes, 21.9% | 1.59E-29 | |
| GO:0009123 | nucleoside monophosphate metabolic process | 32 out of 146 genes, 21.9% | 3.62E-29 | |
| GO:0009150 | purine ribonucleotide metabolic process | 32 out of 146 genes, 21.9% | 2.24E-27 | |

∗ False Discovery Rate

**Table 3.12** Gene Ontology analysis for the molecular function of the **up-regulated** genes in translocant S. para-$T_{9/15}$ strain VS non-translocant S. para- $NT_{9/15}$ control strain

| GO ID | GO_term | Cluster frequency | P-value | FDR* |
|---|---|---|---|---|
| GO:0016491 | oxidoreductase activity | 17 out of 72 genes, 23.6% | 1.82E-07 | <0.001 |
| GO:0016723 | oxidoreductase activity, oxidizing metal ions, NAD or NADP as acceptor | 4 out of 72 genes, 5.6% | 3.36E-05 | |
| GO:0000293 | ferric-chelate reductase activity | 4 out of 72 genes, 5.6% | 3.36E-05 | |
| GO:0016722 | oxidoreductase activity, oxidizing metal ions | 4 out of 72 genes, 5.6% | 0.00046 | |
| GO:0022891 | substrate-specific transmembrane transporter activity | 13 out of 72 genes, 18.1% | 0.00068 | |
| GO:0022892 | substrate-specific transporter activity | 14 out of 72 genes, 19.4% | 0.00092 | |
| GO:0008509 | anion transmembrane transporter activity | 7 out of 72 genes, 9.7% | 0.00156 | |
| GO:0022857 | transmembrane transporter activity | 13 out of 72 genes, 18.1% | 0.00162 | |
| GO:0005215 | transporter activity | 14 out of 72 genes, 19.4% | 0.00479 | |
| GO:0008514 | organic anion transmembrane transporter activity | 6 out of 72 genes, 8.3% | 0.00495 | |

∗ False Discovery Rate

**Table 3.13** Gene Ontology analysis for the molecular function of the **down-regulated** genes in translocant S. para-$T_{9/15}$ strain VS non-translocant S. para- $NT_{9/15}$ control strain

| GO ID | GO_term | Cluster frequency | P-value | FDR* |
|---|---|---|---|---|
| GO:0015078 | hydrogen ion transmembrane transporter activity | 27 out of 146 genes, 18.5% | 1.17E-28 | <0.001 |
| GO:0015077 | monovalent inorganic cation transmembrane transporter activity | 29 out of 146 genes, 19.9% | 7.71E-27 | |
| GO:0015075 | ion transmembrane transporter activity | 37 out of 146 genes, 25.3% | 1.57E-22 | |
| GO:0008324 | cation transmembrane transporter activity | 32 out of 146 genes, 21.9% | 4.73E-22 | |
| GO:0022890 | inorganic cation transmembrane transporter activity | 29 out of 146 genes, 19.9% | 2.89E-21 | |
| GO:0022891 | substrate-specific transmembrane transporter activity | 40 out of 146 genes, 27.4% | 1.25E-20 | |
| GO:0022857 | transmembrane transporter activity | 41 out of 146 genes, 28.1% | 2.94E-20 | |
| GO:0046933 | proton-transporting ATP synthase activity, rotational mechanism | 14 out of 146 genes, 9.6% | 9.30E-20 | |
| GO:0022892 | substrate-specific transporter activity | 41 out of 146 genes, 28.1% | 1.13E-18 | |
| GO:0005215 | transporter activity | 42 out of 146 genes, 28.8% | 3.88E-17 | |

∗ False Discovery Rate

### 3.7.3 Analyzing the expression of genes around the translocation breakpoints

To directly evaluate the effect of CTs on gene expression in the immediate surrounding regions of the translocation breakpoints, 20 genes on either side of the breakpoints YIL014w, YIL015w, YOL054w and YOL055c were analyzed. The only gene that is found within the breakpoint vicinity and showed changes in its expression is *ARG1* (YOL058W). *ARG1* is located downstream of the translocation breakpoint YOL055C gene at chromosome XV and was found to be up-regulated by 1.3-fold in the translocant S. para- $T_{9/15}$ strain in comparison to the non-translocant S. para- $NT_{9/15}$ control strain. This gene is involved in the arginine biosynthesis pathway and codes for an enzyme called argininosuccinate synthase (Crabeel *et al*., 1988) which catalyzes the eighth step in the arginine biosynthesis pathway (Jauniaux *et al.*, 1978).

The STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database (http://string-db.org, version 10.0) was used to extract information of how groups of genes are co-regulated or co-expressed together (Szklarczyk *et al.*, 2015). *ARG1* was tested with all the 218 genes to evaluate if their any interaction between them by using http://string-db.or website. *ARG1* is co-expressed with *YHI9* (YHR029C), *ORT1* (YOR130C), *LYS1* (YIR034C), *LYS9* (YNR050C), *ARG4* (YHR018C), *ICY2* (YPL250C), *PCL5* (YHR071W) and YGL117W (Figure 3.39). All these genes were up-regulated by 1.3, 1.4, 1.9, 1.6, 1.6, 1.3, 1.5 and 1.1 fold, respectively. *ORT1*, *LYS1*, *LYS9* and *ALD4* are located at the translocated chromosomes XV, IX, XIV and XV, respectively, whereas, *YHI9*, *ARG4*, *ICY2*, *PCL5* and YGL117W are located at chromosome VIII, VIII, XVI, VIII and VII, respectively. Translocation caused alteration in *ARG1* expression near the breakpoints and that may cause differences in the expression of those co-expressed genes distributed throughout the genome.

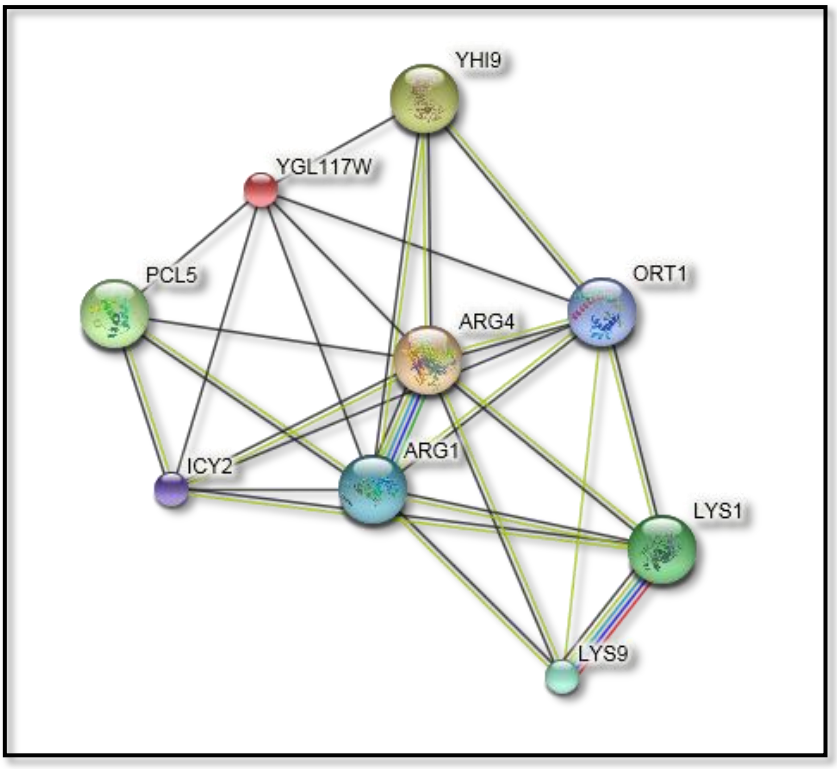


**Figure 3.39: Graphical representation of STRING network view of *ARG1* interactions.** *ARG1* gene was compared with the 218 gene with altered expression. Several interactions were detected between *ARG1* gene and 8 different genes. Lines between the genes are colored regarding the types of interaction. Gray colour indicates that two genes are co-expressed together.

Several genes are located in the translocated chromosomes IX/XV but far away from breakpoints, considered important transcription factors for the regulation of the transcription. One of these genes is *RPI1* (YIL119C) was found to be down-regulated by - 2.5 fold in translocated S. para- $T_{9/15}$ strain relative to non-translocant S. para- $NT_{9/15}$ control strain and is located in translocated chromosome IX. *RPI1* is a transcription factor that involves in the cell wall integrity signalling pathway during the stationary phase (Sobering *et al.*, 2002) and encodes a negative regulator of the Ras/cAMP pathway (Kim and Powers 1991). Sobering *et al*., 2002 showed that the overexpression of *RPI1* gene resulting in alteration of the transcriptional regulation of many genes that involved in cell wall metabolism by elevating their mRNA level. Since, changing in the expression of *RPI1* gene observed in our translocant strains, it could affect the expression of other genes distributed throughout the genome.

*CIN5* (YOR028C) was found to be down-regulated by - 1.6 fold in translocated S. para- $T_{9/15}$ strain relative to non-translocant S. para- $NT_{9/15}$ control strain and is located in translocated chromosome XV. *CIN5* is a transcription factor that physically interacts with *Tup1* and consideres co-repressor with Tup1-Ssn6 complex. The Tup1-Ssn6 complex is essential for the suppression of many genes that are activated during cellular stresses and in response to alterations in growth conditions (Boorsma *et al*., 2008, Hanlon *et al*., 2011). *CIN5* is co-expressed with other genes like *TPO4* (YOR273C) and *YAK1* (YJL141C) and are located at translocated chromosome XV and chromosome X, respectively (gene interaction is obtained from RTRING website, http://string-db.or). Interestingly, both these genes are down-regulated by -1.7 and - 1.3 fold in translocated S. para- $T_{9/15}$ strain. *TPO4* is a membrane transporter protein that involved in polyamines exportation (Tomitori, *et al*., 2001) and it is member of the Drug: H+ Antiporter-1 (DHA1) family (Gbelska, *et al.*, 2006).

Genes interactions were tested using RTRING-db database and several interactions were observed amonge 219 genes. Since the expression of *TPO4* was changed in our translocated strain, it could be one reason for observing the changes in other genes distributed throughout the genome like *TOS8* (YGL096W), *YAK1*, *CIN5* and *GSY1* (YFR015C) (Figure 3.40). All of these genes are co-expressed together and they interact together, so any change in the expression of one of them may contribute to the changes of the rest.

*TOS8* (YGL096W) was found to be down-regulated by 1.6 fold in translocated S. para- $T_{9/15}$ strain and it is specific DNA binding that associated with chromatin (Horak *et al.* 2002, Byrne and Wolfe 2005). *TOS8* binds to two important transcription factors; SBF (Swi4-swi6 cell cycle box binding factor) and MBF (*Mlu*I binding factor) that are regulating the START of the cell cycle with several other transcription factors (Andrews and Herskowitz 1989, Breeden and Mikesell 1991, Koch *et al*., 1993, Dirick *et al.*, 1995) and they bind to 235 genes promoters (Iyer *et al*., 2001). This complex regulates the G1 to S phase progression, initiates DNA replication, transcription, formation of spindle pole complex and protein synthesis (Horak *et al.* 2002). Therefore, when the expression of *TOS8* is down-regulated that may contribute to the function of the other genes.

*GSY1* was found to be down-regulated by -1.6 fold and is involved in 10-15% of glycogen synthase activity (Farkas, *et al.*, 1991). *GSY1* co-expressed with *GAD1* (YMR250W), *CTT1* (YGR088W), *SOL4* (YGR248W), *OM45* (YIL136W), *TFS1* (YLR178C), and *MSC1* (YML128C) (Figure 3.41) and ll of them were foun to be down-regulated by -1.6, -1.6, -1.5, -1.6, -1.7 and -1.6 fold, respectively. Translocation affects the expression of *OM45* and *TFS1* since they are located in the translocaated chromosomes IX and XII respectively and hence they caused the reduction of the expression of the other genes that distributed in different chromosome.

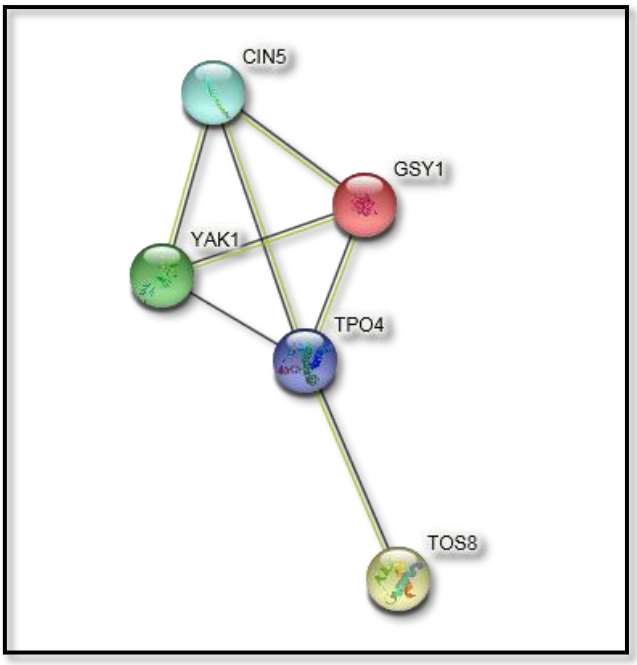


**Figure 3.40: Graphical representation of STRING network view of *TOP4* interactions.** *TPO4* gene was compared with the 218 gene with altered expression. Several interactions were detected between *TPO4* gene and 8 different genes. Lines between the genes are colored regarding the types of interaction. Gray colour indicates that two genes are co-expressed together.

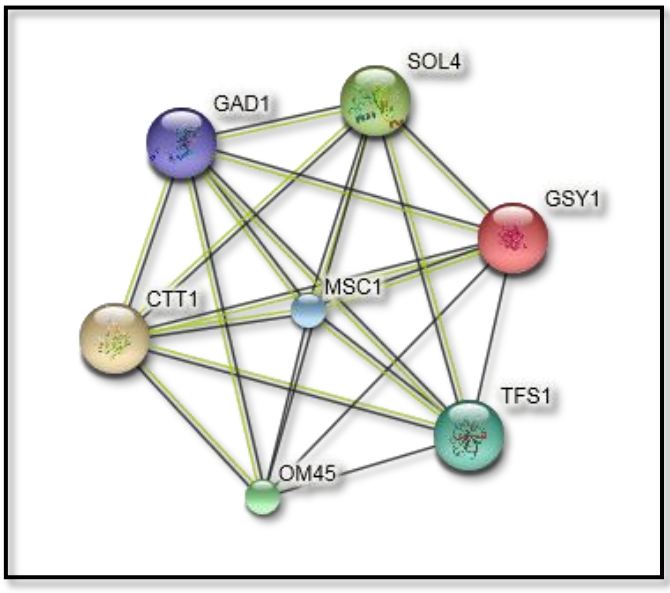


**Figure 3.41: Graphical representation of STRING network view of *GSY1* interactions.** *GSY1* gene was compared with the 218 gene with altered expression. Several interactions were detected between *GSY1* gene and 8 different genes. Lines between the genes are colored regarding the types of interaction. Gray colour indicates that two genes are co-expressed together.

Other interactions were found between genes involve in methionine biosynthesis and surprisingly all of them were up-regulated in in translocated S. para- $T_{9/15}$ strain. *MET32* (YDR253C) is transcription factor that involved in transcriptional regulation of several genes (Blaiseau, *et al.*, 1997). It acts as positive regulator at the promoter region of the *MET14* (YKL001C) and negative regulator at *MET17* (YLR303W) promoter regions (Blaiseau, *et al*., 1997, Thomas and Surdin-Kerjan, 1997). Both *MET14* and *MET17* were also up-regulated by 1.8 and 2.0 fold, respectively. *MET32* is co-expressed with *MET2* (YNL277W), *MET10* (YFR030W), *MET16* (YPR167C) and *MET28* (YIR017C) (Figure 3.42) and all of them were up-regulated by 2.8, 1.1, 1.5 and1.5 fold, respectively. *MET28* and *MET2* are located in the translocated chromosomes IX and XIV, respectively. Therefore, several genes distributed throughout the genome were found to be affected by the translocations or by genes located in the translocated chromosomes, therefore these genes are co-expressed with other genes located in different chromosomes and that explain the global transcriptional changes.

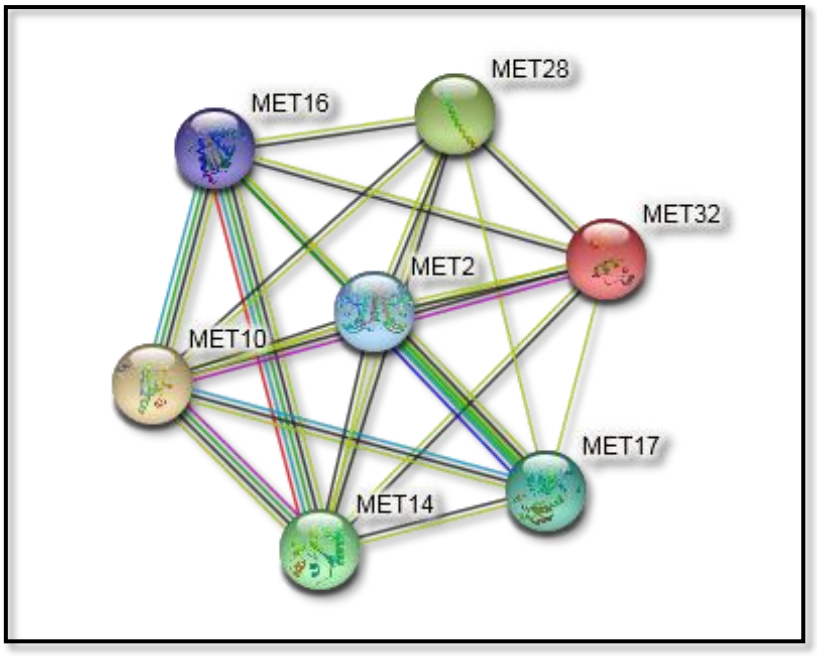


**Figure 3.42: Graphical representation of STRING network view of *MET32* interactions.** *MET32* gene was compared with the 218 gene with altered expression. Several interactions were detected between *MET32* gene and 8 different genes. Lines between the genes are colored regarding the types of interaction. Gray colour indicates that two genes are co-expressed together. Pink and light blue colours indicate known interaction that experimentally determined and from curated database, respectively. Green colour indicates gene neighbourhood.

## 3.8 Evaluating the effect of chromosomal translocation on cell morphology in strains carrying translocation relative to the wild type controls

There were differences both in meiotic and mitotic fitness in the strain carrying translocation, and we recognized that several genes in our data are correlated with morphology changes when they are overexpressed or mutated. Therefore as next step, we decided to check whether the cell morphology is also affected by CT.

Three biological replicates for each engineered strains; S. para-$T_{9/15}$, S. para-$T_{12/14}T_{9/15}$ and parental strains; *S. paradoxus* and *S. cariocanus* were examined by light microscopy to observe any morphological changes. For each biological replicate, the total number of normal cell morphology and the total number of abnormal cell morphology were calculated. Then, for all biological replicates the average of normal and abnormal cell morphology were calculated separately. Morphological phenotypes were classified as abnormal phenotypes when they were elongated, round, small, large or clumped cells (see Section 2.22 and Figure 3.43). Approximately 66.2 % of abnormal cell population were found in the translocant S. para-$T_{9/15}$ strains and 36.4 % in translocant S. para-$T_{12/14}T_{9/15}$ strains in comparison to parental strains *S. paradoxus* and *S. cariocanus* that were showed 6.2 % and 18 % abnormal cell shape, respectively (Table 3.14) (Figures 3.44 and 3.45). Wherase, approximately 33.8% of normal cell population were found in the translocant S. para-$T_{9/15}$ and 63.6% in translocant S. para-$T_{12/14}T_{9/15}$ strains in comparison to parental strains *S. paradoxus* and *S. cariocanus* that showed 93.8% and 82% (Table 3.14) (Figures 3.44 and 3.45).

Strain caarrying second translocation (S. para-$T_{12/14}T_{9/15}$) showed less variation in the cell morphology and no further improvement in mitotic fitness either compared with strain carrying first translocation (S. para-$T_{9/15}$). From these data, we can confirm that first CT not only significantly contributed to the meiotic and mitotic fitness levels, but also affect the cell morphology.

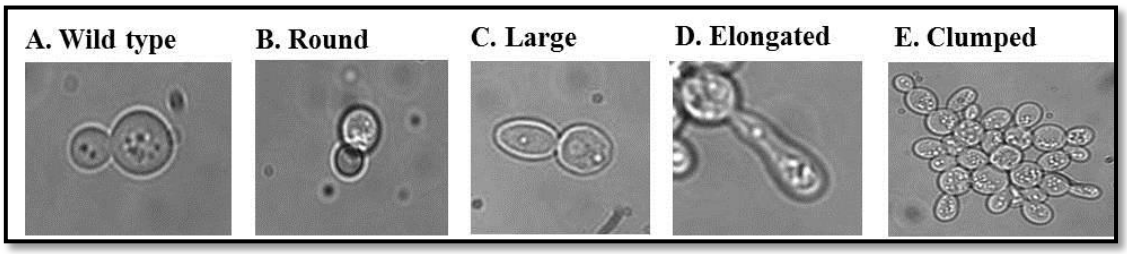


**Figure 3.43 Microphotography of five phenotypic classifications of abnormal morphology**
Abnormal cell morphology was grouped into several categories as normal cell shape **(Panel A)**, round shape **(Panel B)**, large oval bud **(Panel C)**, elongated bud **(Panel D)** and clumped cell **(Panel E)**.

**Table 3.14** Normal (N) and abnormal (Ab) cell morphology in translocant strains, and wild-type parents

| Strain name | Number of N morphological phenotype (±SE∗) | Number of Ab morphological phenotype (±SE∗) | % of N | % of Ab |
|---|---|---|---|---|
| S. para-$T_{9/15}$ | 165 (1.21) | 342 (4.08) | 33.8% | 66.2% |
| S. para-$T_{12/14}$ $T_{9/15}$ | 238 (3.63) | 126 (2.03) | 63.6% | 36.4% |
| *S. paradoxus* | 211 (3.95) | 14 (0.38) | 93.8% | 6.2% |
| *S. cariocanus* | 238 (5.27) | 52 (0.94) | 82% | 18% |

∗Standard error

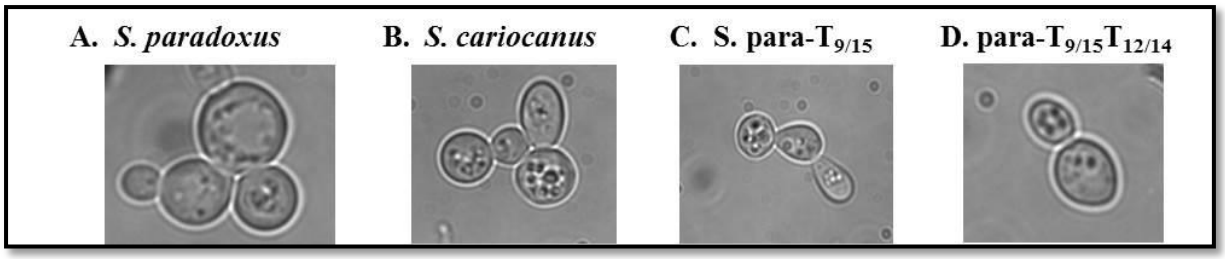


**Figure 3.44 Microphotography of single cells of engineered translocants, and parental strains**

Normal morphological phenotypes were observed in the majority of the cell population of *S. paradoxus* and *S. cariocanus* strains **(Panels A and B, respectively)**. Abnormal cell morphologies were detected in all engineered translocant strains (S.para-$T_{9/15}$ and S. para-$T_{12/14}T_{9/15}$) **(Panels C and D, respectively)**. Magnification 100 x.

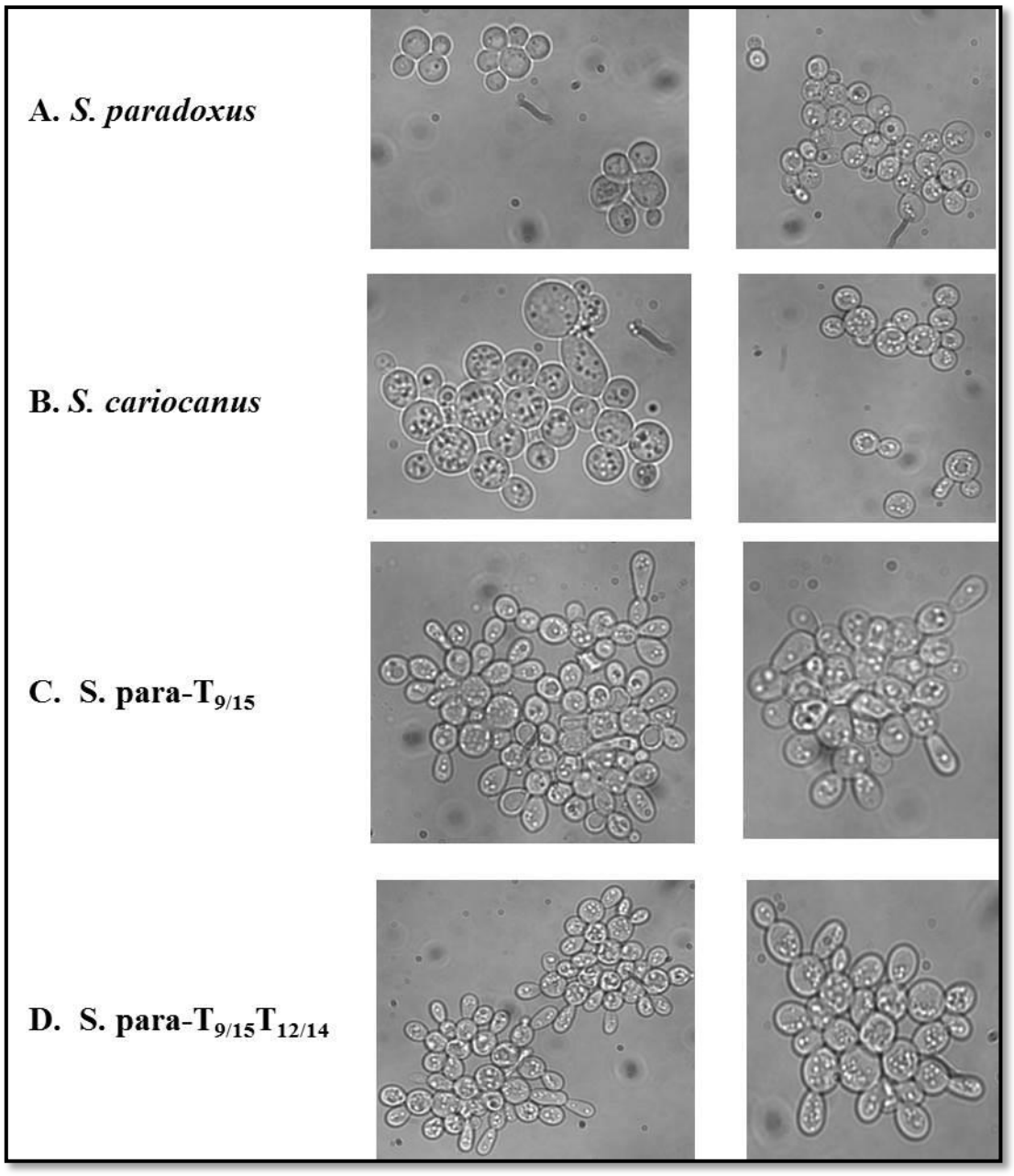


**Figure 3.45 Microphotography of clumping cell morphology in all engineered strains and wild types**

Normal phenotype was observed in the majority of *S. paradoxus*, and *S. cariocanus* cell populations **(Panels A and B)**. Elongated, large, and round cells were observed in S.para-$T_{9/15}$ **(Panel C)** and S. para-$T_{12/14}T_{9/15}$ **(Panel D)**. Magnification 100 x.

Morphological changes were observed in our engineered strains due to the changes in the expression of many genes that are important for maintenance of cell morphology. We conclude that translocations altered gene expression and hence cell morphology was altered.

In our expression data, several genes which are important for maintenance of cell morphology had their expressions altered. These genes were caused abnormal cell wall morphology, abnormal bud morphology, abnormal cell shape, and decreased or increased cell size when they are overexpressed, mutated or deleted (Hurtado S., *et al*., 2014, Zhang J., *et al*., 2002; Watanabe M, *et al*., 2009; Ragni E., *et al*., 2011, Ephrussi and Slonimski, 1955, Herrmann and Funes, 2005, Briza P., *et al*., 2002, Ren P., *et al*., 2016, Ni L. and Snyder M., 2001, Dungrawala H., *et al.,* 2012). These genes; *LYS1* (YIR034C), *LYS12* (YIL094C), *SPO24* (YPR036W-A), *MET32* (YDR253C), *DLD2* (YDL178W), *CCW12* (YLR110C), *COX5A* (YNL052W), *COX5B* (YIL111W), *AMA1* (YGR225W), *CIP1* (YPL014W), *ROM2* (YLR371W), *MIR1* (YJR077C), *ATP4* (YPL078C) and *PRY3* (YJL078C), were differentially changing in their expression in strain S. para-$T_{9/15}$ compared with its control (Table 3.15). Interestingly, these genes are located in translocated chromosomes IX, XII and XIV and they exhibited altered gene expression. *LYS1*, *LYS12*, and *COX5B* are located in translocated chromosome IX, *CCW12*, and *ROM2* are located in translocated chromosome XII and *COX5A* is located in chromosome XIV. *DLD2* and *PRY3* are located in chromosome IV and X respectively (Table 3.15). The analysis of all these genes is summarized further in Table 3.15. Gene interactions were tested using RTRING-db database and two interactions were observed. *COX5A* is co-expressed with *COX5B*, *MIR1* and *ATP4* together and *LYS1* is co-expressed with *LYS12*. All of these genes when they are mutated or overexpressed, they caused abnormal morphology. Changes in the expression of genes important for preserve the morphological phenotype may be the reason for detecting cell abnormality in the engineered *S. paradoxus* strains with translocated chromosomes.

**Table 3.15** List of genes with altered expressions in strain carrying first translocation S. para-$T_{9/15}$ compared with its control were caused abnormal cell morphology

| Standard Name (Systematic Name) | Chr. position | Our expression data | log2 Fold Change | Literature data | References |
|---|---|---|---|---|---|
| *LYS1* (YIR034C) | Chr IX | Up-regulated | 1.969215695 | Overexpression: Increased filamentous growth | Jin *et al*., 2008, Shively *etal*., 2013 |
| *LYS12* (YIL094C) | Chr IX | Up-regulated | 2.384475358 | Overexpression: Increased invasive growth | Jin *et al.*, 2008, Shively *et al*., 2013 |
| *SPO24* (YPR036W-A) | Chr XVI | Up-regulated | 1.215354773 | Mutation: Abnormal cell shape | Hurtado S., *et al*., 2014 |
| *MET32* (YDR253C) | Chr IV | Up-regulated | 1.093826177 | Mutation: Increased cell size | Zhang J., *et al*., 2002 |
| *DLD2* (YDL178W) | Chr IV | Up-regulated | 1.857011699 | Overexpression: Abnormal cellular morphology | Hachiya *et al*., 2004 |
| *CCW12* (YLR110C) | Chr XII | Down- regulated | -1.907630688 | Mutation:<br>- Abnormal bud shape<br>- Abnormal cell wall morphology | Watanabe M, *et al*., 2009 Ragni E., *et al*., 2011 |
| *COX5A* (YNL052W) | Chr XIV | Down- regulated | -1.582020293 | Mutation: Abnormal yeast growth and colony size | Ephrussi and Slonimski, 1955, Herrmann and Funes, 2005) |
| *COX5B* (YIL111W) | Chr IX | Down- regulated | -1.153699176 | Mutation: Abnormal cell shape | Zhang J., *et al*., 2002 |
| *AMA1* (YGR225W) | Chr VII | Down- regulated | -1.582020293 | Mutation: Absent spore wall formation | Briza P., *et al*., 2002 |

| | | | | | |
|---|---|---|---|---|---|
| *CIP1* (YPL014W) | Chr XVI | Down- regulated | -1.189697997 | Mutation:<br>Decreased cell size | Ren P., *et al.*, 2016 |
| *ROM2* (YLR371W) | Chr XII | Down- regulated | -1.16063882 | Mutation:<br>- Abnormal cell shape<br>- Abnormal bud morphology<br>- Abnormal cell size | Ni L. and Snyder M., 2001<br>Dungrawala H., *et al.*, 2012 |
| *MIR1* (YJR077C) | Chr X | Down- regulated | -1.152871901 | Mutation:<br>- Abnormal bud morphology | Watanabe M., *et al.*, 2009 |
| *ATP4* (YPL078C) | Chr XVI | Down- regulated | -1.144248037 | Mutation:<br>Decreased cell size | Jorgensen P., *et al.*, 2002 |
| *PRY3* (YJL078C) | Chr X | Down- regulated | -1.984131338 | Deletion:<br>Abnormal morphology | Michaillat, L., and Mayer, A. 2013. |

# Chapter Four

# Discussion

# 4. Discussion

The *Saccharomyces* species have been considerd an excellent model system for comparative studies in population genetics, genome evolution and speciation (Kellis *et al*., 2003, Liti and Louis, 2005, Borneman and Pretorius, 2015). The ancestral yeast genome underwent a whole genome duplication event ca. 100 million year ago followed by extensive genome reshaping including chromosomal rearrangements (Fischer *et al*, 2001), segmental duplications (Dujon *et al*., 2004) and gene loss (Llorente *et al*, 2000, Fischer *et al*, 2001). These events significantly influenced the phylogenetic division and burst of speciation in *Saccharomyces* complex (Scannel *et al*., 2007). Therefore, the analysis of the genome of extant yeast species and strains provide a better understanding and knowledge on the evolutionary process (Liti and Louis, 2005).

Alternatively, reproductive barriers exist among various yeast species, and this has been widely investigated in the past few years. The reproductive isolation prevents the free flow of genes between diverging populations and is an integral part of the speciation process. Two types of reproductive isolation, premating (prezygotic) and postmating (postzygotic), occurs in yeast. Until recently, prezygotic reproductive isolation or unsuccessful gamete recognition in yeast was mostly overlooked. In mating trial experiments, no evidence for active species recognition in mate choice was observed in two closely related yeast species, *S. cerevisiae* and *S. paradoxus* (Murphy *et al*., 2006). However, in wild-type spores of *S. cerevisiae* and *S. paradoxus*, a clear discrimination was noticed when a choice of species was given, and hybridization occurred only when no mate choice was available (Maclean and Greig, 2008). It is thought that prezygotic isolation might have evolved due to natural selection to prevent the formation of sterile hybrids, though this is considered to be a relatively weak reproductive barrier.

Postzygotic reproductive isolation caused by hybrid sterility occurs at several stages in yeasts. Factors affecting F1 meiosis, spore germination or gamete mitosis in hybrids could potentially lead to postzygotic sterility. Chromosomal rearrangements such as deletions, translocations, and inversions play a role in yeast hybrid sterility. Chromosomal rearrangements induce multivalents at meiosis that are prone to missegregation and can result in the development of the spores that are aneuploid. Four large-scale translocations that rearrange the genome have been reported in *S. sensu stricto* species (Fischer *et al.,* 2000) whereas *S. cerevisiae* and *S. paradoxus* are collinear and have very small inversions (Kellis *et al.,* 2003) that rarely reduce fertility. Chromosomal rearrangements cannot be considered as a major contributing factor for speciation (Delneri *et al.*, 2003) because several yeast species have few or no chromosomal rearrangements, but have hybrids that are sterile.

The second and the most crucial contributing factor for postzygotic reproductive isolation is the DNA sequence divergence (Chambers *et al.*, 1996; Hunter *et al.,* 1996). Homologous recombination that takes place during meiosis requires the formation of an intermediary heteroduplex DNA structure (Holliday junctions) that contains complementary strands from the two recombining chromosomes. If the DNA sequences diverge, mismatches occur which gets repaired by the mismatch repair system. This could potentially lead to gene conversions and termination of recombination, a process known as anti-recombination. It is quite evident now that sequence divergence in yeasts can cause improper meiotic recombination, segregation, and can induce F1 hybrid sterility (Greig *et al.*, 2003).

Another mechanism that complements sequence divergence is the Dobzhansky-Muller incompatibility that occurs between epistatic genes (Muller, 1942). According to this model, the ancestral species divided to create two daughter lineages that carry diverged genes from its ancestral sequence. Although these changes are neutral or sometimes beneficial, the diverged genes, when brought together in a hybrid can reduce viability. These genes can

invoke strong reproductive isolation and are called speciation genes. Dobzhansky-Muller incompatibility can either be dominant (expressed in F1 hybrid diploids) or recessive (expressed in F1 haploid gametes or homozygous diploid F2 progeny), and their contribution to reproductive isolation in yeasts is equivocal. Theoretically, F1 hybrid sterility in yeast can be caused by an abnormal meiosis (a dominant incompatibility) or due to spore germination defects (this could be either dominant or recessive).

To determine the role of dominant incompatibility in hybrid sterility in yeasts, Greig *et al*., created pseudo-haploids from non-hybrid diploids by deleting a single copy of *MAT* locus. They observed that spore viability of the hybrids of the pseudo-haploids was not significantly different from those of normal haploid intraspecific crosses. These results excluded the possibility that dominant incompatibility could contribute to hybrid sterility (Greig *et al.*, 2002a). On the other hand, to determine recessive incompatibility, individual chromosomes of *S. cerevisiae* were replaced with homologous counterparts from *S. paradoxus* (Greig *et al.*, 2007). The results showed that recessive speciation genes do not play a major role yeast speciation. Nevertheless, indirect evidence demonstrated the association between recessive incompatibility and decreased hybrid fertility in yeasts (Greig *et al.*, 2002b). Despite the popularity of Dobzhansky-Muller model, only a very few speciation genes have been identified. Moreover, the elusiveness of speciation genes could be due to the modified Dobzhansky-Muller mechanism that behaves in a similar fashion (but with significant differences) as the Dobzhansky-Muller incompatibility (Werth and Windham, 1991). The classic Dobzhansky-Muller model involves co-adapted alleles segregating at pairs of loci and arises due to adaptive substitutions, whereas the modified model suggests that the loss of hybrid fitness occurs due to the gaining of a null copy of the previously duplicated genes from each of the parental genotypes. Modified Dobzhansky-Muller mechanism is important because a large number of genes were duplicated and lost following the ancestral whole-

genome duplication event, and could provide a molecular explanation for the speciation process.

Genomic and karyotypic data of *S. paradoxus* and *S. cariocanus* genomes revealed a very low level of sequence diversity and the presence of four translocations which are likely to contribute to the reproductive isolation between them (Naumov *et al*., 2000, Fischer *et al*., 2000). According to the biological species concept (BSC), *S. paradoxus* and *S. cariocanus* are in fact considered two separate species because they are reproductively isolated from each other (percentage of viable spore for heterozygote crosses is $\leq 1$ %) (Naumov 1987, Naumov *et al*.1992, Hunter *et al*. 1996, Liti *et al*., 2006). The current study aims to understand whether chromosomal rearrangements are, in this case, the sole responsible for reproductive isolation since the sequence divergence between the two species is very low (comparable to what it can be seen in two strains of the same species). Chromosomal translocations induce multivalents formation at meiosis stage and the resulting gamete viability drop to 50% for each reciprocal translocation (Fischer *et al*., 2000). We hypothesized that by undoing one translocation this should lead to a doubling in spore viability and eventually, if colinearity is restored, restore fertility fully. Two CTs were created in *S. paradoxus* genome (without disrupting any coding sequence) using *lox*P/Cre recombinase methodology (Delneri *et al*., 2000, Carter and Delneri, 2010). The engineered strains were then crossed with *S. cariocanus* to see whether a higher amount of viable spores were produced. Our data showed, as expected, that the fertility quadruplicated (from 3.4% to 12.7%) in heterozygote crosses where two out of five translocations were undone (*P*-value <0.05, two sample t-test). Based on this result, we predicted that our engineered hybrids will only ever be able to reach 50.8 % spore viability in collinear crossings: if we undo another translocation the fertility would double reaching 25.4 %, and double again to 50.8 % for collinear genomes. Sequence divergence could perhaps account for the remaining 50.8 % infertility, and to test this we

compared our results with those of Liti *et al*., (2006) on the impact of sequence divergence on reproductive isolation in different yeast strains. The expected amount of spore viability in collinear crossings with a sequence divergence similar to that one between *S. paradoxus* and *S. cariocanus* should be ca. 80% (Table 3.5). Consequently, there are still ca. 30% on infertility that cannot be accounted by the translocations or sequence divergence. This led us to hypothesise either the presence of other CRs (inversions/translocations previously undetected) or the presence of allelic incompatibilities that may contribute to this reduction in meiotic fitness.

PacBio DNA sequencing of our *S. cariocanus* strain confirmed our expectations and revealed one new chromosomal translocation between chromosomes XIII/XIV and 11 inversions ranging from ~7 to 140 kbthat have not been characterized previously. The newly defined translocation with the others 4 translocations (total 5 translocations) may affect the meiotic results of our engineered hybrids and were caused the variation in meiotic viability, since CRs contributes to the F1 gametes producing unbalance segregation of several genes and preventing these gametes from receiving a complete genome composition (Orr *et al*., 2004).

Previous researches clarified the contribution of CRs to the hybrid viability and proved that translocations can drop the hybrids meiotic fertility of ≤ 40 % (Avelar *et al*., 2013), 44 % to 86 % (Hou *et al*., 2014), ≤ 44% (Zanders *et al*., 2014) and inversions can be lethal (Naseeb *et al*., 2016). Therefore, these experimental results are a strong evidence of the contribution of CRs to reproductive isolation in yeast. Nevertheless, compared to sequence divergence, the role of chromosomal rearrangements in driving speciation is of secondary importance in yeast species since they spend large part of their life cycle by reproduce asexually. However, chromosomal rearrangements re-inforce the isolation barriers by reducing the fertility (Sites and Moritz, 1987, Dobzhansky, 1933, Coyne *et al*., 1993, Delneri *et al*., 2003). Our data

provide strong evidence that *S. paradoxus* and *S. cariocanus* are reproductively isolated solely because of the CRs.

*S. paradoxus* and *S. cariocanus* are two biological species due to chromosomal rearrangements; however they are not two different phylogenetic species because they do not have enough sequence divergence (Liti, 2015). So, in term of taxonomical classification, two questions arise from this example 1. Should we choose one of the two species concepts and apply it consistently to the *Saccharomycetes* 2. Which one of these two species concepts would encapsulate better the evolutionary trajectory of *Saccharomycetes* species? The concept of the biological species (BSC) depends upon the concept of breeding and fertility and is linked to meiosis. So, if two yeasts cannot produce fertile offsprings they are considered to belong to different species that are reproductively isolated. The phylogentic species concept (PSC) is based on sequence divergence and it is largely applied to organisms which reproduce asexually via mitosis. Yeasts reproduce both sexually (meiosis, outcrossing) and asexually (mitosis) and therefore it is easy to see how both BSC and PSC can be applied to this case. One way for the yeast taxonomists to make a decision is to see whether outcrossing happens often enough in yeast to support the use of BSC. The scientific communities present different views on this matter. The outcrossing between *S. cerevisiae* and *S. paradoxus* in the wild has been measured and found to be a fairly rare event, with only 315 outcrossing events out of 16 million divisions (Ruderfer *et al*., 2006). Other studies estimated that the majority of mating events occurs within spores of the same tetrad with only 1% of real outcrossing (Knop, 2006, Tsai *et al*., 2008). On the other hand, there are other studies that are claiming that outcrossing between yeasts occurs regularly and at high frequency both in insects guts (Reuter *et al*., 2007, Stefanini *et al.*, 2016) and in the lab (Murphy and Zeyl, 2010).

Since it is very easy and inexpensive to test reproductive isolation between yeast strains and outcrossing occurs at least at some appreciable frequency, perhaps the BSC would still be the best way to classify these yeasts.

Another important question addressed in the current study was whether the CTs are affecting the phenotype of *S. paradoxus* and *S. cariocanus* strains. The fitness of our engineered strains was tested under different conditions and the analysis revealed that the CTs were either disadvantageous or had a neutral effect. The phenotypic outcome depends both on the position of the translocation (either between IX/XV and XII/XIV chromosomes) and the environments in which the strain are exposed. Low mitotic fitness was detected in engineered *S. paradoxus* translocant strains compared with wild type strains and non-translocant strains in all conditions tested at 30 °C, 35 °C (when classified them according to maximum growth rate and biomass) and at 16 °C (when classified them according to maximum biomass). Remarkably, when comparing them according to maximum growth rate at 16 °C, significant drop in the fitness was observed only in C-limited media. These observations confirmed that CTs are strongly affecting the strains mitotic fitness under different conditions. Interestingly, the fitness growth of *S. paradoxus* strain possessing two CTs was almost comparable to the strain possessing one translocation. These results indicated that while the first CT (IX/XV) was impairing the mitotic fitness of *S. paradoxus* strain and the second translocation (XII/XIV) had no further effect on the phenotype. Our data are in agreement with Colson *et al*., (2004) and Avelar *et al*., (2013), who observed that CRs affected the mitotic growth of yeast strains which were vary according to the environmental conditions used in the experiment. Specifically Avelar *et al*., (2013) showed that CRs can have either beneficial or deleterious effect to the strains in rich media and under different stressor and abiotic factors (Avelar *et al*., 2013); while Colson *et al*., (2004), showed that two CTs presented in yeast strain were provided growth advantages in C-limited condition (Colson *et al*., 2004).

Chromosomal inversions, which change gene orders and overall chromosomal structure, can also contribute to the *S. cerevisae* strains fitness as observed by Naseeb and Delneri, 2012. They constructed several *DAL2* inverions in *S. cerevisae* strains mimicking *N. castellii DAL* cluster and showed that inversion of *DAL2* reduces the mitotic fitness in nitrogen-limited media compared to control (Naseeb and Delneri, 2012). In 2016, Naseeb *et al.*, evaluated the impact of gene order on growth fitness in rich and minimal media by constructing 16 *S. cerevisae* strains possessing paracentric and pericentric inversions between Ty1 element. They observed that only 4 *S. cerevisae* strains possessed inversions that were lethal, while the others 12 strains did not show any significant fitness changes in the most tested media. They realized that the inversion (428 kb) in strain (XIV.inv) caused drop in meiotic fitness of 48% and lowered the growth fitness in 7 media conditions. While the inversion (288 kb) in strain (VII-B.inv) was reduced the meiotic fitness of 52% but did not change the mitotic fitness in all tested media. Overall, their results indicated that the size of the inversion does not correlate positively with the meiotic viability, and that mitotic fitness can vary from lethality to unchanged (i.e. wild-type phenotype).

The data presented in this thesis add to the bulk of evidence that CRs can be beneficial, disadvantageous, deleterious or neutral according to the position of translocation and the different environmental conditions. One reason for such differential phenotypes caused by CTs is that the synteny and the localization of genes is affected and this may induce changes in gene expression (Kleinjan and van Heyningen, 2005), which can cause variation in the fitness (Avelar *et al*., 2013).

Interestingly, the wild type *S. cariocanus* strain has a lower mitotic fitness at 35 °C in N-limited, P-limited and S-limited media compared to other conditions and to *S. paradoxus*. Furthermore, creating the translocation IX/XV in the *S. paradoxus* strain brought a disadvantage to the strain fitness; so, undoing the same translocation IX/XV in *S. cariocanus*

may confer a fitness benefit by placing the genes in different promoter regions. Since the *S. cariocanus* genome is highly similar to the *S. paradoxus* genome, we suggest that there may be a link between the alteration in gene expression and mitotic fitness between the two species. The translocation contributing to the fitness loss in *S. paradoxus* background was reversed in *S. cariocanus* using *lox*P/Cre recombinase method. Interestingly, by undoing the translocation between IX/XV chromosomes, the growth fitness of *S. cariocanus* improved in some conditions. This indicates that the translocation between chromosomes IX/XV brought a disadvantage to the fitness of *S. cariocanus* strain. We can hypothesise that the other four translocations may have compensated the disadvantage brought about by the IX/XV translocation and overall contributed to the current fitness of *S. cariocanus*.

Mitotic fitness variations observed in current study are likely caused by the changes happened in genes expression in strains possessing translocation. This expectation led us to analyze gene expression level by RNAseq in our engineered strains and we observed that several genes with altered expression were involved in cellular amino acid biosynthesis pathway. Thus, these genes could contribute to the reduction in the mitotic fitness under different environmental conditions.

The analysis of RNAseq data revealed that 219 gene in *S. paradoxus* translocant (S. para-$T_{9/15}$) strain were significantly differentially expressed in comparisons to the control non-translocant strain (S. para-$NT_{9/15}$). However, no significant changes were detected in the translocant strain (S. para-$T_{12/14}T_{9/15}$) in comparison to its control and this result was in agreement with the phenotypic data which showed that the translocation in S. para-$T_{9/15}$ was disadvantageous.

Gene Ontology analysis revealed that most down-regulated genes were enriched for Adenosine triphosphate (ATP) synthesis pathway. These are subunits of the respiratory complex which take part in electron transport chain in mitochondria. For example, *COX5A* (YNL052W) and its isoform *COX5B* (YIL111W) are part of subunit V of the cytochrom C oxidase, which is localized to the inner membrane, responsible for proton transport to create transmembrane potential and generate ATP. It has been shown that these genes are expressesd under different oxygen concetrations (Cumsky *et al*., 1985, Hodge *et al*., 1989, Trueblood and Poyton, 1987), *COX5A* is expressed aerobicly (>1µM $O_2$ concentration) and the *COX5B* anaerobicly (<1µM $O_2$ concentration) (Trueblood and Poyton, 1987, Burke *et al*., 1997). We observed that both *COX5A* and *COX5B* were significantly differentially expressed in translocant strain (S. para-$T_{9/15}$). It has been shown that mutations in the genes required for the expression or assembly of cytochrome c oxidase subunits can affect the yeast growth and colony size (Ephrussi and Slonimski, 1955, Herrmann and Funes, 2005). Therefore, changing in the expression of these genes may contribute to the mitotic fitness of engineered translocant strains. Interestingly, both *COX5A* and *COX5B* are located on the translocated chromosomes XIV and IX respectively.

Several other genes like *CCW12* (YLR110C) (Watanabe M, *et al*., 2009, Ragni E, *et al*. 2011), *AMA1* (YGR225W) (Briza P, *et al*., 2002), *CIP1* (YPL014W) (Ren P., *et al.*, 2016), *ROM2* (YLR371W) (Ni L. and Snyder M. 2001, Dungrawala H., *et al*., 2012), *MIR1* (YJR077C) (Watanabe M., *et al*., 2009), *ATP4* (YPL078C) (Jorgensen P., *et al*., 2002), and *PRY3* (YJL078C) (Michaillat, L., and Mayer, A. 2013) are important for maintenance of cell morphology when they are expressed normally. However these genes were down-regulated in our translocant strain (S. para-$T_{9/15}$) and that strong indication of impairment of cell morphology. All these genes will be discussed further in the cell morphology part.

Cellular amino acid metabolic process was a highly enriched GO category for the up-regulated genes. For example, the lysine biosynthesis pathway was clearly affected in the engineered translocated (S. par-$T_{9/15}$ strain) strains. *LYS12* (YIL094C), *LYS1* (YIR034C), *LYS9* (YNR050C) and *LYS2* (YBR115C) involved in lysine biosynthesis (Strassman and Ceci, 1965) were up-regulated by 2 folds. Interestingly three of these genes (*LYS12*, *LYS1* and *LYS9*) are located on the translocated chromosomes IX and XIV, whereas *LYS2* is located on chromosome II. Therefore, the CTs not only affected genes in the translocated chromosomes but also genes located in different chromosomes.

Analyzing genes surrounding the breakpoints, we found that only *ARG1* (YOL058W), involved in arginine biosynthesis pathway and located downstream of YIL055C (one of the breakpoints) on chromosome XV, is significantly overexpressed. This result indicated that CTs does not always affect the expression of all the genes around the breakpoints. In fact there were many genes with altered expression which were located far from the breakpoints. Several interactions were observed between *ARG1* and many genes; *YHI9*, *ORT1*, *LYS1*, *LYS9*, *ARG4*, *ICY2*, *PCL5* and YGL117W are located at different chromosomes that may explain the global transcription changes. All these genes were up-regulated by 1.3, 1.4, 1.9, 1.6, 1.6, 1.3, 1.5 and 1.1 fold, respectively. Therefore, gene expression changed globally rather than being restricted to the breakpoints region. Several genes are located in the translocated chromosomes contributed to the changes in the expressions of many genes distributed throughout the genome. Since there was alteration in the expression of many genes like *RPI1*, *TPO4*, *YAK1*, *OM45*, *TFS1*, *MET28* and *MET2* that are located in the translocated chromosomes and they found to be co-expressed with other genes distributed in non-translocated chromosomes. Any changes in the expression of these genes may contribute to the changes of the others since they are interacted and co-expressed together (genes interactions were perfurmed using http://string-db.or website). Some of these genes are

transcription factors like *RPI1*, *CIN5*, *MET28* and *MET32* that regulate the expression of many genes either near of them or located far away (Latchman, 1997). Therefore, genes that are presented in the translocation chromosomes are the origins of the global transcriptional changes. Other studies found a greater amount of genes located near the breakpoints whose gene expression was altered by CTs (Avelar *et al.*, 2013, Kleinjan and van Heyningen, 2005), sometimes over-expressed up to five times (Nikitin *et al*., 2008).

Cell morphology was also analyzed in our engineered and control strains, since alteration of the expression of many genes that are important for maintaining the cell morphology were observed in our RNAseq datas described above. The analysis of cell morphology of *S. paradoxus* strains carrying one and two translocations (S. para-$T_{9/15}$ and S. para-$T_{12/14}T_{9/15}$) revealed abnormal phenotype in 66.2 % and 36.4 % of the population. Strain with two translocations showed less alteration in the cell morphology compared with the strain carrying first translocation. This observation may confirm that the second translocation may become fixed in the population because improved some phenotypic trait from the first translocation. Interestingly, second translocation caused improve in meiotic fitness only without adding any mitotic fitness improvement compared with the strain carrying first translocation and does not make the cell morphology worse. We expected that changes in cell morphology are associated with the changes in genes expression and the presence of CTs. Our translocant strains exhibited an increased or decreased in the level of many genes that are important to maintain the cell morphology and that could explain the abnormality in cell morphology. *LYS1* (YIR034C) and *LYS12* (YIL094C) genes are responsible for abnormal cell morphology when they are only overexpressed and the overexpression of these two genes enhanced filamentous and pseudohyphal growth formations hence play important roles in changing the cell morphology (Shively *et al*., 2013, Jin *et al.*, 2008). In our expression data,

both *LYS1* and *LYS12* are overexpressed in our engineered S. para-$T_{9/15}$ strain compared to the non-translocant S. para-$NT_{9/15}$ control strains. Another gene; *SPO24* (YPR036W-A) (Hurtado S., *et al*. 2014) was up-regulated in S. para-$T_{9/15}$ translocant strain was analyzed for it potential association with alter cell shape (Hurtado S., *et al*., 2014**)**. Hurtado S., *et al*., (2014) created *SPO24*Δ homozygous diploid cells by replacing the gene with nat selectable marker and observed that cells of *SPO24*Δ strain was exhibit sporulation defective compared with wild type control strain. So, *SPO24* is an important protein for sporulation and mutation of this gene was affecting the cell shape. *DLD2* gene is a D-lactate dehydrogenase protein that plays an important role in maintaining the cell morphology (Chelstowska A, *et al*., 1999). Hachiya *et al*., 2004 observed that overexpression of *DLD2* cells formed multi-buds and elongated bud. Interestingly, this gene was found to be overexpreesed by 1.8 fold in translocant strain S. para-$T_{9/15}$ compared with it control and that may cause the bud to be elongated.

*CCW12* gene was down-regulated by -1.9 fold in translocant strain S. para-$T_{9/15}$ in comparison to it non-translocant control S. para-$NT_{9/15}$. *CCW12* is a cell wall protein that located on chromosome XII, found to be responsible for maintenance and organization of newly-synthesized cell wall (Mrsa V., *et al*. 1999, Ragni E., *et al*. 2011). Mutation of this gene is caused abnormal bud shape and abnormal cell wall morphology (Watanabe M., *et al*. 2009, Ragni E, *et al*., 2011). *CIP1* gene was down-regulated by -1.2 fold in translocant strain S. para-$T_{9/15}$ in comparison to it non-translocant control S. para-$NT_{9/15}$. *CIP1* gene is newly identified as negative regulator of cyclin-dependent kinases; interact with *Cdk1* during cell cycle phases (G/S-phase) (Ren *et al*., 2016). Cell division cycle is an essential process that is derived and controlled by cycline-dependent kinases especially *Cdk1*. Overexpression of *CIP1* was causing delayed of the yeast budding and blocked the DNA replication and that triggered filamentous growth. Mutation of *CIP1* caused faster progression of G1/S-phase as

indicated from the accumulation of G2/M population in the cell culture. Also, the size of the cells was significantly smaller in the mutant strains than the wild type strains using synchronization methods (Ren *et al*., 2016). Similar observations were detected in strain possessing mutation in *ATP4* gene, which is an important subunit of mitochondrial ATP synthase, exhibited small cell size as a result of accumulation of the cells in G1 phase (Jorgensen P., *et al*. 2002**)**. Given that some genes contributed to the cell size when they are mutated and caused them to become smaller. *ATP4* gene was also down-regulated by -1.1 fold in translocant strain S. para-$T_{9/15}$ in comparison to it non-translocant control S. para-$NT_{9/15}$. *ROM2* gene, which is important for bud morphology and cell growth, was mutated to evaluate the cell morphology under different temperatures using differential-interference contrast microscopy (Manning B.D., *et al*. 1997). They observed that *ROM2*Δ strain exhibited morphological defect including elongated bud (7%) and large bud at 30°C. When shifted the cells to 37°C, cells often formed small bud compared with wild type strains (49% vs 26%) and cells with elongated bud were detected only in the mutant strains (Manning B.D, *et al*. 1997). *ROM2* gene, which is down-regulated in our translocant strain, interestingly is located in translocated chromosome XII and could contribute to alter cell morphology. *AMA1* (YGR225W) is an important component in anaphase promoting complex that needed for meiosis I (Cooper *et al.,* 2000). Briza *et al*., (2002) showed that cells with deleted *AMA1* gene were arrested after S-phase forming only one nucleus, while few cells were formed without spore walls (Briza *et al*., 2002). This gene is down-regulated by -1.7 fold in translocant strain S. para-$T_{9/15}$ in comparison to it non-translocant control S. para-$NT_{9/15}$. *MIR1* (YJR077C) gene is a mitochondrial phosphate carrier (Murakami, *et al*., 1990) that is found to be down-regulated by -1.15 fold in strain carrying first translocation. Elongated buds were observed in cells with deleted *MIR1* gene which had a longer period of apical growth (one of the bud growth phase) than the wild type and that resulted in annormal bud (Watanabe *et al.,* 2009).

All the mentioned above genes were down-regulated in S. para-$T_{9/15}$ strain thus that may be strong indication for contributing to alter morphology. Therefore, translocation altered the genes expression that is important for maintenance of cell morphology and hence cell morphology was impaired.

Previous studies confirmed that CRs were causing variations in colony morphology (Suzuki *et al*., 1989, Rustchenko-Bulgac *et al.*, 1990) such as pseudohyphal cells and filamentous true hyphae (Suzuki *et al*., 1991). Also abnormal cells with elongated buds and pseudohyphal growth were observed in translocant strains (Rossi, 2010, Nikitin *et al*., 2008). These cells abnormality ranging from 10% to 20% of the cell population as observed by Nikitin *et al*., (2008) and from 2% to 20% of the cell population as detected by Rossi *et al*., (2010). These observations may explain the alteration observed in the cell morphology. Therefore, we anticipate that cell morphology is perturbed in our engineered strains carrying translocations.

# 5. Conclusions and Future work recommendations

Our finding highlighted the contribution of CTs to hybrids fertility, mitotic growth, differential gene expression and cell morphology and we proved that CTs has a major role in the organism fitness. Creating two translocations in *S. paradoxus* strains mimicking those translocations in *S. cariocanus* provide us better understanding of how translocation may contribute to the meiosis fitness. Hybrids carrying three translocations showed an enhancement in the spore viability compared with hybrids carrying five translocations and that confirmed the translocation has a major role in reproductive isolation. *S. paradoxus* engineered strains that differ by the presence of chromosomal translocations are a valuable resources for evaluation how the changes in the genome organization will affect the evolution and function of the genome.

We have presented novel chromosomal rearrangements in *S. cariocanus* strain including one translocation between XIII/XIV chromosomes and eleven inversions. These newly-defined rearrangements can further explain the reason behind very low fertility between them. The updated genome sequence of *S. cariocanus* strain will add valuable information to the previously *S. cariocanus* genome sequence regarding genome structure.

At phenotypic level; we observed that the fitness of *S. paradoxus* strain carrying one translocation was significantly dropped compared with it control strain in different environmental media. The fitness of the second translocation does not add any improvement in the fitness of the strain in all tested media. These data showed that the translocations are considered to be disadvantageous or neutral in mitotic fitness. Different environmental parameters should be further tested in future studies to discover which specific media can bring an advantageous or disadvantageous to the strain carrying translocation.

We also found that the expression of several genes was significantly altered in strain carrying first translocation. Those changes in the expression are combined with low mitotic fitness and that happened because of the translocation. Several genes are functions to maintain the cell morphology and the changes that observed in the morphology may be the result of altered those expressions. Microarray or real-time PCR can be performed in the future studies to evaluate the function of each gene that altered the morphology.

The cell morphology of our strains was evaluated under light microscopy and we showed that the translocation IX/ XV changes the phenotype of the cell. Further quantification can be done to evaluate the differences in cell size using synchronization methods. Overall, our findings are important in showing that the chromosomal translocation contributes significantly to both fitness and reproductive isolation in yeast species.

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# Appendix

**Table A.1 Nucleotide sequences of primers used for cassettes amplification.**

| **Primer Name** | **Purpose of using in current study** | **5'-3' nucleotide sequence** |
|---|---|---|
| Amp 12 F | Amplification of ***lox2272-natNT2-lox2272*** cassette and to be inserted between YLL023c and YLL026w at chromosome 12 | CCTGTTAGTTACCAAAAAAGAAAACTCAGCGTACTCGGCAATCAGTTC GTCTATCCTCATTCATATTCTACCTCTCGTCCCAGCTGAAGCTTCGTACG |
| Amp 12 R | | CAGACATGTGGTAAAGAAAACCTACAAAGAATTACACTGCAGGGAAA CCAAAAAGTCCCAGTTTGTGCACTTTGCAATTCGCCTTTGAGTGAGCTG ATAC |
| Amp 14 F | Amplification of ***lox2272-natNT2-lox2272*** cassette and to be inserted between YNL284c and YNL286w at chromosome 14 | ACACCCACATGACAGGAGATGCGTTGTTAAACTAGATAAAGCGTTATA TGGTCTCAAGCAGAGTCCTAAAGAATGGAACGCAGCTGAAGCTTCGTA CG |
| Amp 14 R | | CACTTGCTGCGATCACACAGTCATCAACATAAACTGCGATCATGAGTTT TTTATCCTTGGATTGGTATAGTCCTGGAGTAGCCTTTGAGTGAGCTGAT AC |
| Amp-F-hph | Amplification of ***loxP-hphNT1-loxP*** cassette and to be inserted between YIL014w and YOL055C at chromosomes 9 and 15 | CCTATCCTTCATCGGGAAGCATTAATCTATCAGAAAATAGGAACTATAC ATGTACATGTCGCTGAAGAAGTACGGAGGGCCTGTGTAGGACGTACGC TGCAGGTCGAC |
| Amp-R-hph | | GACGTACTACCTTTACTCACATATATGAACAAAGTAAAACTGTGCCGTT TAACCAAATAATACCACCTGTAAGAAGAATTTAATCTTGGCCACTATA GGGAGACCGGCAG |
| Amp-F-Kan | Amplification **of *loxP-KanMX-loxP***cassette and to be inserted between YIL015W and YOL054W at chromosomes 9 and 15 | GCACCGCATGTGATAATATACTACTAAATAGATGATATTAGAATCCAA TTCCAACAAATAGCCAACTACATCTGACATGACGTACGCTGCAGGTCG AC |
| Amp-R-Kan | | TTGCAGGAAAATGACACGCAGCGAACTTCAGACCTAAACTTAAATTTG TTCTTCCATTTCTATCTTGGATTGAAATTTGCCACTATAGGGAGACCGG CAG |

| **Primer Name** | **Purpose of using in current study** | **5'-3' nucleotide sequence** |
|---|---|---|
| Amp-4F-HO | Amplification of ***lox2272-natNT2-lox2272*** cassette and used for *HO* gene deletion | ATGCTTTCTGAGAACACAACTATTTTGATGGCCAGTGGTGAAATTAAAGA CATCGCAAAC CGTACGCTGCAGGTCGAC |
| Amp-4R-HO | Amplification of ***lox2272-natNT2-lox2272*** cassette and used for *HO* gene deletion | TTAGCATATACGCGCACCAACATTAGTACCACAGCTCTTATGGGGCCCAC GAACAGCATC CACTATAGGGAGACCGGCAG |

**Table A.2 Nucleotide sequences of primers used for confirmation.**

| Primer Name | Purpose of using in current study | 5'-3' nucleotide sequence |
|---|---|---|
| Nat-up | Confirmation of cassette insertion- primer specific to ***lox2272-natNT2-lox2272*** | ATGTCCTCGACGGTCAGC |
| Nat-down | Confirmation of cassette insertion- primer specific to ***lox2272-natNT2-lox2272*** | GCTGACCGTCGAGGACAT |
| hph-up | Confirmation of cassette insertion- primer specific to ***loxP-hphNT1-loxP*** | ACCTGCCTGAAACCGAACT |
| Hph-down | Confirmation of cassette insertion- primer specific to ***loxP-hphNT1-loxP*** | CTCTCGATGAGCTGA |
| Hph-F | Confirmation of cassette insertion- primer specific to ***loxP-hphNT1-loxP*** | GAGGGCAAAGGAATAATCT |
| Kan-up | Confirmation of cassette insertion- primer specific to ***loxP-KanMX-loxP*** | AGGAAAAGACTCACGTT |
| P2K-down | Confirmation of cassette insertion- primer specific to ***loxP-KanMX-loxP*** | TCGTCACTCATGGTGATTTC |
| Conf.12.F | Confirmation *of* ***lox2272-natNT2-lox2272***cassette insertion into chromosome 12 | GAGGACAAAGCATGCGAGAG |
| Conf.12.R | Confirmation of ***lox2272-natNT2-lox2272***cassette insertion into chromosome 12 | GCTGGTGTAGACGGGAGTTC |
| Conf.14.F | Confirmation of ***lox2272-natNT2-lox2272***cassette insertion into chromosome 14 | GAGGTGATATCCAGACGCCA |
| Conf.14.R | Confirmation of ***lox2272-natNT2-lox2272***cassette insertion into chromosome 14 | TTGGGTTGCAGATAGCTCCG |
| Conf.kan.FA | Confirmation of ***loxP-KanMX-loxP*** cassette insertion into translocated chromosomes 9/15 | GTGGTTGTTGGGATTCCATT |
| Conf.kan.RA | Confirmation of ***loxP-KanMX-loxP*** cassette insertion into translocated chromosomes 9/15 | GCTGGAGCATAATCCCTTCA |
| Conf.kan.RB | Confirmation of ***loxP-KanMX-loxP*** cassette insertion into translocated chromosomes 9/15 | TGCACCTATTGCAGAAGGAA |
| Conf.hph.FA | Confirmation of ***loxP-hphNT1-loxP*** cassette insertion into translocated chromosomes 9/15 | CCTATCCTTCATCGGGAAGC |
| Conf.hph.RA | Confirmation of ***loxP-hphNT1-loxP*** cassette insertion into translocated chromosomes 9/15 | AGCAACAATGGAATCCCAAC |
| Chk.9F | Confirmation of Translocants and Non-translocants of chromosome 9 | CAGGTTTGCTGAGTCATTGC |
| Chk.9R | Confirmation of Translocants and Non-translocants of chromosome 9 | CCTTTTGCAACCAACCCTTA |
| Chk.15F | Confirmation of Translocants and Non-translocants of chromosome 15 | GCTGCCAAGATTGTATTCAT |
| Chk.15R | Confirmation of Translocants and Non-translocants of chromosome 15 | GCGATTAACAGTCGCACGCC |
| Chk.12 F | Confirmation of Translocants and Non-translocants of chromosome 12 | GCTACAAACAGGCACCTGTG |

| | | |
|---|---|---|
| Chk.12 R | Confirmation of Translocants and Non-translocants of chromosome 12 | AGCGGCGGCATGTAATAAAT |
| Chk.14 F | Confirmation of Translocants and Non-translocants of chromosome 14 | GAGGTGATATCCAGACGCCA |
| Chk.14 R | Confirmation of Translocants and Non-translocants of chromosome 14 | GAAGTTCCGCTTCGGTTGAG |
| HO-conf. F | Confirmation of HO deletion | GCCGGCATTACTGACGTATC |
| HO-conf. R | Confirmation of HO deletion | TGCTCCTACCATTGATACCG |