Doctoral Dissertation

Development of a novel technology for genome engineering and genome-wide construction of a series of segmental aneuploids in *Saccharomyces cerevisiae*

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Chapter 1

General introduction

1.1 Genome engineering in chromosome level

Genome engineering is a recently developed technology that enables the large-scale manipulation of a genome and the simultaneous modification of many genes. It is expected to be a potential tool not only for generating strains with desired traits but also for understanding genome functions. Several genome engineering technologies have been established in the budding yeast *Saccharomyces cerevisiae* for the manipulation of a chromosome or genome on a large scale. Genome engineering is strategy or technology that redesigns or modifies targeted genetic information or genome of interest. Chromosome engineering is subset of genome engineering. It enables us to introduce defined chromosomal rearrangements such as small deletion, insertion, duplication, inversion or translocation into genome of interest. Here is the example of technology or strategy that was utilized to reconstruct, redesign or synthesize genetic information on the chromosome. Yeast artificial chromosomes (YACs) have been developed as an artificial chromosome that has a capability to carry large DNA fragments (Burke *et al*., 1987). YACs were applied to the creation of genomic libraries of the entire genomes of higher organisms such as mammalian genome in addition to genome manipulation in *S. cerevisiae*. Bridge-induced translocation (BIT) allows us to generate the translocation event at desired chromosomal regions by transformation with a DNA cassette containing a selectable marker flanked by two homologous sequences corresponding to two different chromosome locations (Tosato *et al*., 2005). PCR-mediated chromosome splitting (PCS) method enables us to split a chromosome into two smaller chromosomes at any desired site using PCR followed by a single transformation (Sugiyama *et al*., 2005). Application of PCS (Sugiyama *et al*., 2009) has provided valuable tools to manipulate and study the genome, including the chromosome shuffling method which allows

to swap selected chromosomal regions with the corresponding region of other strains (Sugiyama *et al*., 2006), PCR-mediated chromosome deletion (PCD) method, which can be exploited for deleting chromosomal region at any desired site in a single transformation per deletion event (Sugiyama *et al*., 2008), and genome reorganization technology which allows creating a huge variety of genome composition in yeast cells (Ueda *et al*., 2012). In genome reorganization technology, various chromosome regions were split to generate mini-chromosomes by PCS method. After introduction by mini-chromosome loss, cells with a variety of genome composition were created. This technology was exploited for strain improvement such as creation of yeast strains with ethanol tolerant phenotype (Park *et al*., 2012). Recently a technology to completely synthesize entire chromosome from oligo-nucleotides have also been developed (Dymond *et al*., 2011, Annaluru *et al*., 2014). In this study, the newly synthesized chromosome was designed with following principles. First, the change should confer near wild-type phenotype and fitness. Second, the destabilizing elements such as tRNA genes or transposons should be removed. Third, synthetic chromosome should incorporate genetic flexibility to facilitate future studies. Outcome of this work was the first artificial synthesis of the partial chromosome VI, a right arm of chromosome IX and entire chromosome III. Moreover, the entire synthesis of other chromosomes covering the whole genome of *S.cerevisiae* is in progress. This research group also developed a technology named Synthetic chromosome rearrangement and modification by LoxPsym-mediated Evolution technology (SCRaMble) (Dymond *et al*., 2011) to generate genome rearrangements including deletion and inversion in the synthetic chromosome. In SCRaMble technology, the insertion of loxP site after stop codons of each non-essential gene and at major genetic landmarks followed by the induction of expression of Cre recombinase allows the creation of cell with enormous genome diversity. Taken all together, those genome engineering techniques could be applied to the study on a large scale of genome

rearrangement and the subsequent investigation of the relationship between changed phenotype caused by altered genotype.

1.2 Origin of segmental aneuploidy

Segmental aneuploidy is recently noted type of chromosome rearrangements. It is the aberrant structure of chromosomes in which segments of chromosomes are gained or lost and is found to be involved in both growth defect and advantageous phenotypes on a broad range of organisms such as antifungal drug resistance in pathogenic yeasts, copper tolerance in natural yeasts living in area with high copper contents, morphological abnormality in maize, and human diseases exemplified by Down syndrome and tumors. (Bigner *et al*., 1988, Warburton, 1991, Crolla, 1998, Viersbach *et al*., 1998, Infante *et al*., 2003, Fuster *et al*., 2004, Selmecki *et al*., 2006, 2008, 2009, Makarevitch *et al*., 2008, Gresham *et al*., 2008, Lyle *et al*., 2009, Lucas *et al*., 2010, Jung *et al*., 2011, Borneman *et al*., 2011, Dunn *et al*., 2012, Brion *et al*., 2013, Chang *et al*., 2013, Weischenfeldt *et al*., 2013, Chen *et al*., 2013, Akalin *et al*., 2014)**.** In the scope of this thesis, duplication of chromosomal segments was focused. In yeast, segmental duplication of a large chromosomal region occurs spontaneously at a frequency of 10^{-9} to 10^{-10} per mitosis in the haploid genome (Koszul *et al*., 2004). The spontaneously segmental duplication was classified into four types (Dujon, 2006, 2010). The first type is intra-chromosomal duplication, in which internal chromosomal region is duplicated in tandem on the same chromosome arm (Koszul *et al*., 2004). The second type is inter-chromosomal duplication, in which chromosomal region is duplicated followed by moving to other chromosome, while original chromosome remains unaffected (Koszul *et al*., 2004). The third type is supernumerary chromosome, in which chromosomal region is duplicated, fused with other duplicated region of another chromosome, generating structurally abnormal extra chromosomes (Koszul *et al*., 2004). The forth type is episomal

chromosomes, in which duplicated region is converted into a new chromosome with a circular structure (Libuda and Winston, 2006).

Segmental duplication is generated as the consequence of DNA breakage. Mechanism of segmental duplication is classified into 2 large groups (Koszul and Fischer, 2009). The first groups are called conservative mechanisms, including inherited segmental mechanism, unequal crossing-over amplification (Smith, 1976, Ohta, 1976), Break-fusion-bridge (BFB) amplification (Murnane, 2006) and non-homologous end joining (NHEJ)-mediated formation of segmental duplication (Koszul *et al*., 2004). Inherited segmental mechanism occurs by a translocation of the large chromosome region from one chromosome to another, then segmental duplication appears in the offspring. Unequal crossing-over occurs between homologous sequences located either on the same sister chromatids, on the identical sister chromatids or on homologous chromosomes, and segmental duplications are subsequently generated. BFB mechanism occurs by the fusion between two sister-chromatids due to the loss of telomere, forming dicentric chromosome. During chromosome segregation in anaphase, each centromere is pulled toward opposite poles, then one daughter cell will carry chromosome with deletion, another daughter cell will carry chromosome with duplication. Since those two chromosomes lack telomeres, the BFB cycles will repeat and continue until those chromosomes obtain telomere. After multiple rounds of this process occur, it leads to duplication of chromosomal regions. NHEJ-mediated segmental duplication happens when two sister chromatids or homologous chromosome experience DSB at different sites, then improper repair mechanism occurs by NHEJ and results in segmental duplication. Second groups are called as replication-dependent mechanisms, including break induced replication (BIR) (Morrow *et al*., 1997, Payen *et al*., 2008) and microhomology/microsatellite-induced replication (MMIR) (Payen *et al*., 2008). BIR is a *RAD52* (homologous recombination protein) dependent mechanism and requires long homology for strand invasion. DSB end

sometimes invades homologous sequence either at non-allelic position, on sister chromatid, at upstream site of the DNA break point or on a different chromosome, then lead to direct tandem segmental duplication or non-reciprocal translocation. MMIR is mediated by microhomology or low-complexity DNA sequences and occurs in a *RAD52*-independent manner. However, formation of segmental duplication by the BIR and MMIR mechanism is dependent on Pol32, subunit of DNA polymerase Polδ for DNA synthesis step. In this study, the term "segmental duplication" is used to refer to amplification of a particular chromosomal region and "segmental aneuploidy" is used to refer to a duplication in which the chromosomal region is present as an independent chromosome.

1.3 Segmental aneuploidy and their consequences

Segmental duplications are generally associated with detrimental effects in multicellular organisms. For example, in maize, segmental duplication causes morphological abnormalities (Makarevitch *et al*., 2008). While in humans, segmental duplication resulting from supernumerary chromosomes is associated with tumor development and many diseases such as human breast cancer and cat eye syndrome (Bigner *et al*., 1988, Warburton, 1991, Crolla, 1998, Viersbach *et al*., 1998, Fuster *et al*., 2004, Lucas *et al*., 2010, Chen *et al*., 2013, Akalin *et al*., 2014). Similarly, although Down syndrome in humans is usually due to trisomy for chromosome 21, it can also occur as a result from partial (segmental) aneuploidy of chromosome 21 (Lyle *et al*., 2009). There are at least two possibilities that might explain the reason why aneuploidy lead to the detrimental effect. The first possibility is that the increase in dosage of a specific gene that is involved in the critical pathway of cell survival hampers the growth (Torres *et al*., 2007). The second possibility is that the presence in extra protein that is translated by duplicated genes located on an additional chromosome causes the

imbalances in protein homeostasis and lead to the defects in cell proliferation (Oromendia *et al*, 2012).

In yeast, partial chromosomal duplications may offer an evolutionary advantage through enabling adaptation to particular stresses in the environment (Infante *et al*., 2003, Gresham *et al*., 2008, Brion *et al*., 2013, Chang *et al*., 2013). For example, segmental aneuploids are occasionally found in industrial yeast strains such as those used for fermentation of wine and beer (Borneman *et al*., 2011, Dunn *et al*., 2012). Segmental duplication of chromosome VII and VIII that confer copper resistance have been found in natural yeast living around areas with high copper contents (Chang *et al*., 2013). Laboratory yeast strains were found to have segmental duplication of a specific region of chromosome II containing high affinity sulfate transporter (*SUL1*) after cultivation in sulfate limited condition (Gresham *et al*., 2008). In *Candida albicans*, a pathogenic yeast, fluconazole resistance is the result of duplication of the left arm of chromosome V that contains *ERG11* encoding a target of fluconazole and *TAC1* encoding a transcription regulator of the ABC transporter (Selmecki *et al*., 2006, 2008, 2009). These various examples illustrate the impact of segmental duplication on phenotype in unicellular and multicellular organisms.

1.4 The influences of chromosome segmental duplication on gene expression

It has been reported that gene expression are correlated proportionally to gene copy number on a duplicated region in yeast and mammals (Torres *et al*., 2007, Pavelka *et al*., 2010b). However, in Drosophila and plants, there is compensation of gene dosage changes at the transcription level that normalizes the expression level of genes on an additional chromosome to euploid level (Makarevitch and Harris, 2010, Zhang *et al*., 2010). Nevertheless phenotypic alterations are occasionally conferred by increased dosages of a single gene or the consequences of the combination of two or more genes on the duplicated

region (Selmecki *et al*., 2006, 2008, 2009, Gresham *et al*., 2008, Pavelka *et al*., 2010b, Chen *et al*., 2012, Chang *et al*., 2013). For example, in case of a dosage change of a single gene on duplicated region, segmental gain of a regions of chromosome II that contain high affinity sulfate transporter (*SUL1*) were found in evolved *S. cerevisiae* strain under sulfate limited condition (Gresham *et al*., 2008), aneuploidy of chromosome XIII confers 4-NQO drug resistance due to increased dosages of *ATR1* gene on duplicated region (Pavelka *et al*., 2010b). In case of the effect of multiple genes, aneuploidy of chromosome XV confers radicicol resistance because of the synergistic effect of *STI1* and *PDG5* and possibly other genes that are located in chromosome XV (Chen *et al*., 2012). Amplification of isochromosome 5 also confers fluconazole resistance in *C. albicans* as a result of increased dosages of *ERG11* and *TAC1* (Selmecki *et al*., 2006, 2008, 2009). Natural yeast strains that tolerate copper have segmental duplication of chromosome VII and VIII. This copper resistance was conferred by duplication of both of *CUP1* on chromosome VIII and *CUP2* gene on chromosome VII (Chang *et al*., 2013). Moreover, there are two effects that may occur by aneuploidy. First is *cis*-effect that is the effect by which dosage and expression of gene located on duplicated chromosome are changed (Pavelka *et al*., 2010b). Second is *trans*-effect that is the effect by which expression of genes on other chromosomes are changed. This change might result in phenotypic change if genes on the duplicated region are regulatory gene(s) for other multiple genes in its network (Rancati *et al*., 2008).

1.5 Detection of segmental aneuploidy

As emphasized in the previous section, segmental aneuploid play an important role in phenotypic alterations of various organisms. Many researchers have attempted to discover karyotypic variations that are the cause of specific phenotypic changes. To date, several technologies have been developed to analyze numeral and structural variation in the genome.

Here, the mainly used approaches that enable us to identify segmental aneuploidy are described. Those include electrophoresis-based karyotyping, fluorescent in situ hybridization (FISH) or based microarray approaches, and next-generation sequencing (NGS) technology. An electrophoresis-based technology for detecting the alteration of chromosome number and chromosome rearrangement is Pulse field gel electrophoresis (PFGE) coupled with Southern blot analysis (Infante *et al*., 2003, Koszul *et al*., 2004, Chang *et al*., 2013). Gross chromosome rearrangement and the changes of approximate chromosome size (100 bp to 10 Mb) could be detected, but the data on exact sequences could not be obtained by this method. Fluorescent in situ hybridization (FISH) is an approach that enables identification of the presence and localization of specific DNA on chromosome. It was usually exploited to detect chromosome rearrangement (at resolution of approximately 5 Mb for analysis of metaphase chromosome) in multicellular organisms (Liu *et al*., 1998, Kolialexi *et al*., 2006). Hybridization-based microarray approaches, including array comparative genomic hybridization (array CGH) and SNP microarrays are exploited for the detection of copy number variation (CNV) (Gresham *et al*., 2008, Dunn *et al*., 2012, Brion *et al*., 2013). SNP microarrays could also be used to detect single nucleotide polymorphism. However, hybridization-based microarray approaches could not specify the location of duplication or structure of chromosome rearrangement. Next-generation sequencing (NSG) technology has been developed in the past few years (Alkan *et al*., 2011). NGS technology allows us to identify the exact sequences, type, break point and copy number of structural variations. By using these approaches, especially microarray and DNA sequencing technologies, segmental duplication in genome of various organisms are being discovered rapidly. The characteristics of karyotype by CGH and/or whole genome sequencing were often analysed from strains grown in natural environments (Infante *et al*., 2003, Dunn *et al*., 2012, Chang *et al*., 2013) or laboratory evolved strains (Dunham *et al*., 2002). The strains harbouring segmental

aneuploidy and other mutations were frequently identified in those studies. To understand the biological roles of segmental aneuploidy clearly, cell that harbours only the segmental aneuploidy of interested region but no other mutation are required. Therefore, methodology to generate segmental aneuploidy at any genomic locus is needed.

1.6 Methodologies to construct whole chromosome duplication

In yeast, there are several techniques to generate duplication of whole chromosome, including treatment with antibiotics that cause chromosome segregation errors (Chen *et al*., 2012); a chromosome transfer strategy based on drug selection (Torres *et al*., 2007); disruption of genes involved in chromosome segregation fidelity (Rancati *et al*., 2008); induced nondisjunction of specific chromosomes using a conditional centromere (Anders *et al*., 2009); and meiotic progenies from polyploidy (Pavelka *et al*., 2010b). However, it should be again noted that all of these techniques are to cause duplication of the whole chromosome but not segmental duplication of chromosome. Methodologies to construct precise segmental aneuploidy are much more restricted. It has been reported that growth defect of mutants harboring single-gene deletion subsequently generated spontaneous large segmental duplications with random sizes to suppress the defect (Koszul *et al*., 2004). To date, however, methodology to construct an extra-chromosome with segmental duplication at a desired chromosomal region has never been developed.

1.7 Objective (of this study)

Since the available methods are unsuitable for constructing segmental duplications of specific chromosomal regions, I initiated the present study to develop a methodology with properties mentioned in previous sections. Here, I describe the development of a simple new technology, which I termed PCR-mediated chromosome duplication (PCDup) that can be

used in budding yeast to duplicate any desired chromosomal region as an independent chromosome.

In Chapter 1, I have already summarized the origin of segmental aneuploidy, the effect of segmental aneuploidy in different organisms, the influences of chromosome duplication on gene expression, method for identification and construction of chromosome rearrangement and genome engineering in chromosome level. In Chapter 2, I have demonstrated the principle and the performance of PCDup. The size limitation of segmental duplication constructed by PCDup technology was also determined. In Chapter 3, I have applied PCDup technology to construct the series of approximately 100-200 kb segmental duplications that covered the whole genome of *S. cerevisiae.* Interestingly, some chromosomal regions could not be duplicated; the implications of these interesting observations are considered later**.** Subsequently, the phenotypic alterations of those segmental aneuploid strains were investigated under environmental stresses. Moreover, the correlation between the presence of duplicated chromosome and observed phenotype were also verified. In Chapter 4, I discussed the importance of development and the utility of this novel genome engineering technology for generating an additional chromosome consisting of a defined genomic region. Finally, I emphasized that this new technology will not only be valuable for deciphering genome function, but also for breeding yeast strains with desirable stress resistance characteristics.

Chapter 2

Development of PCR-mediated chromosome duplication technology

2.1 Introduction

The development and application of high-throughput genome analysis methods, such as comparative genomic hybridization and next-generation sequencing (Alkan *et al*., 2011), have made it relatively easy to identify and analyze most types of novel genetic change not only at the chromosomal but also at the sub-chromosomal level. However, not all chromosomal changes are amenable to analysis by these new approaches. Although high-throughput genome analysis can detect chromosome copy number variation including segmental aneuploidy, it cannot distinguish among types of segmental duplication, such as tandem duplications, duplications inserted into an independent chromosome or generation of independent chromosome. As described in Chapter I, segmental duplication involving large chromosomal regions has great impact on phenotypic alterations in unicellular and multicellular organisms (Bigner *et al*., 1988, Warburton, 1991, Crolla, 1998, Viersbach *et al*., 1998, Dunham *et al*., 2002, Infante *et al*., 2003, Fuster *et al*., 2004, Selmecki *et al*., 2006, 2008, 2009, Makarevitch *et al*., 2008, Gresham *et al*., 2008, Lyle *et al*., 2009, Lucas *et al*., 2010, Borneman *et al*., 2011, Brion *et al*., 2013, Chang *et al*., 2013, Weischenfeldt *et al*., 2013, Chen *et al*., 2013, Akalin *et al*., 2014).

To date, very few organisms have been exploited for segmental aneuploidy research, although such studies have been performed in *S. cerevisiae* (Jung *et al*., 2011), Drosophila (Zhang *et al*., 2010), maize (Makarevitch *et al*., 2008) and mouse (Tybulewicz and Fisher, 2006). In contrast to multicellular organisms, a wide range of genetic tools is available to manipulate the *S. cerevisiae* genome and, therefore, *S. cerevisiae* may be the best available model organism for studying segmental aneuploidies. Several methods can be used to

duplicate whole chromosomes in yeast as described in Chapter I. However, methods for studying segmental aneuploids are much more restricted. Most of the information obtained from yeast regarding the relationship of segmental aneuploidy and the phenotype is derived from high-throughput analysis of karyotypic changes in natural populations (Infante *et al*., 2003, Dunn *et al*., 2012, Chang *et al*., 2013) or laboratory-generated strains (Dunham *et al*., 2002). In these populations and strains, it is unclear whether the observed phenotypic changes are a direct consequence of segmental aneuploidy and, additionally, it is difficult to delimit the region potentially responsible for any phenotypic changes. Since the methods for constructing segmental duplications of specific chromosomal regions in a targeted manner are lacking, my study was initiated to develop a methodology satisfying this demand. In this Chapter, I demonstrated the performance of the new technology by constructing segmental duplications of various lengths of several chromosomal regions and by testing the efficiency of the construction of segmental aneuploidy.

2.2 Materials and Methods

2.2.1 Yeast strains and plasmids

Saccharomyces cerevisiae strain BY4742 [*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*] was used as the parental strain for the construction of segmental aneuploidy. The plasmids used in this chapter are listed in Table 1. Yeast strains were grown at 30 °C in YPAD medium containing 5% (wt vol^{-1}) Difco^{TM} YPD broth (1% (wt vol^{-1}) yeast extract, 2% (wt vol^{-1}) Bacto-peptone and 2% (wt vol^{-1}) dextrose) supplemented with 0.04% (wt vol^{-1}) adenine (Wako), or selective medium (Amberg *et al*, 2005) containing 0.67% (wt vol^{-1}) yeast nitrogen base without amino acids (Difco) and 2% (wt vol^{-1}) glucose (Wako). If necessary, selective media were supplemented with appropriate amino acids (0.02 mg ml^{-1} l-typtophan, 0.02 mg ml^{-1} l-lysine, 0.03 mg ml^{-1} l-leucine, 0.02 mg ml^{-1} l-histidine, 0.02 mg ml^{-1} uracil and/or 0.02

mg ml^{-1} adenine). *Escherichia coli* strains were grown at 37 °C in LB medium (2% (wt vol^{-1}) LB broth; Sigma) with or without 75 µg ml^{-1} ampicillin (Wako). For solid media, 2% (wt vol^{-1}) agar (Wako) was added.

Table 1. Plasmids used in this study.

Plasmid	Description	Duplicating module	Remarks
p3008	The *loxP-CgLEU2-loxP* module containing plasmid constructed by modifying pUG6	A fragment containing the 5'-$(C_4A_2)_6$-3' telomere seed sequence and the *CgLEU2* cassette	Sugiyama *et al*., (2005)
p3009	The *loxP-CgHIS3-loxP* module containing plasmid constructed by modifying pUG6	A fragment containing the 5'-$(C_4A_2)_6$-3' telomere seed sequence and the *CgHIS3* cassette	Sugiyama *et al*., (2005)
p3122	The *loxP-CgLEU2-CEN4-loxP* module containing plasmid constructed by modifying pUG6	A fragment containing the 5'-$(C_4A_2)_6$-3' telomere seed sequence	Sugiyama *et al*., (2008)
p3276	*URA3* containing plasmid constructed by modifying pUG6	A fragment containing the 5'-$(C_4A_2)_6$-3' telomere seed sequence and the *URA3* cassette	Sugiyama *et al*., (2008)
p3279	The *loxP-CgHIS3-H4ARS-loxP* module containing plasmid constructed by modifying pUG6	A fragment containing 5'-$(C_4A_2)_6$-3' telomere seed sequence, *CgHIS3* and the *H4ARS* cassette	NBRP, YGRC, Japan
YCp50	*URA3* centromeric plasmid whose length is 7.8 kb	-	Rose *et al*., (1987)

2.2.2 Yeast genomic DNA and plasmid DNA extraction

Yeast cells were inoculated into YPAD medium and cultivated at 30°C overnight. Cells were collected and resuspended in DNA lysis buffer (containing 2% (wt vol^{-1}) TritonX-100, 1% (wt vol^{-1}) SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM Na_2EDTA), and glass beads were then added. Phenol chloroform was added and the solution was mixed vigorously at 4°C for 30 min. Next, TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)) was added and the solution was subjected to centrifugation. The aqueous phase was recovered, and DNA was precipitated with ethanol. The DNA pellets were air-dried and dissolved in TE buffer. The DNA concentration was measured using NanoDrop spectrophotometer.

Plasmid DNA was isolated from *E. coli* strains according to the alkaline lysis method (Sambrook *et al*., 1989). *E.coli* strains were cultured in LB plate supplemented with 75 µg ml^{-1} of ampicillin at 37°C overnight. The following day, cells were picked up and suspended in 100 µl of cold solution I (50 mM glucose, 10 mM EDTA (pH8.0), 20 mM Tris-HCl (pH8.0)). Then cells were lysed with 200 µl of freshly prepared solution II (0.2 N NaOH, 1% (wt vol^{-1}) sodium dodecyl sulfate (SDS)) and allowed to stand on ice for 5 min. Cell lysate was neutralized and precipitated by adding 150 µl of cold solution III (60 ml of 5 M sodium acetate, 11.5 ml of glacial acetic acid in 100 ml of total solution). Eppendorf tube was inverted gently and allowed to stand on ice for 20 min. Phenol:chloroform treatment and ethanol precipitation were performed. DNA pellets were air-dried and dissolved in 50 µl of TE buffer (pH8.0) containing RNaseA (Sigma).

2.2.3 PCR procedure for preparation of DNA duplicating modules

The primers used in this study are listed in Table 2. The *Saccharomyces* Genome Database (http://www.yeastgenome.org) was used to select the target region for duplication and to design primers. The two DNA modules required for PCDup were prepared by two rounds of PCR. In the first round of PCR, loxP-cas and CA primers were used to amplif**y** a DNA fragment from plasmid template **(**Table 1**)**.

Two DNA cassettes were amplified from the plasmids: one contained the telomere seed sequences, selectable marker and *CEN4* (fragment 1); the other contained the telomere seed sequences and a second selectable marker (fragment 2). In parallel, two DNA fragments (400 bp; fragments 3 and 4) with nucleotide sequences corresponding to the left and right ends of the target region were amplified from genomic DNA of strain BY4742. One pair of primers designated Cx-y-L-f and Cx-y-L-r and a second pair designated Cx-y-R-f and Cx-y-

R-r was used to amplify DNA fragments at the left and right ends of the target region, respectively (Table 2; x represents the chromosome number, y represents the size of duplicated chromosomal region, L represents the left end of target region, R represents the right end of target region, f represents a forward primer, and r represents a reverse primer). The Cx-y-L-f and Cx-y-L-r primers contained 20 bp sequences that respectively corresponded to the 5' and 3' ends of the fragment at the left end of the target region; the Cx-y-R-f and Cx-y-R-r primers likewise contained 20 bp sequences corresponding to the 5' and 3' ends of the fragment at the right end. In addition, the Cx-y-L-r and Cx-y-R-f primers also contained 30 bp annealing sequences complementary to the DNA fragment amplified from the plasmid to further amplify the duplicating module in the next step of PCR. After the first round of PCR, the 4 PCR products (fragments 1-4) were gel-purified using a Wizard SV Gel and PCR Clean-up System (Promega).

Next, overlap extension PCR was performed to amplify two duplicating DNA module: one target fragment (fragment 3 or 4) was combined with a marker cassette (fragment 1 or 2) by overlap extension PCR using primers Cx-y-L-r and CA, or primers Cx-y-R-f and CA. After amplification, the two PCR products were ethanol-precipitated.

The first round of PCR was performed using 1.0 U *Ex Taq* DNA Polymerase (Takara), approximately 50 ng of DNA template and 0.1 μM of each primer in a final volume of 50 μl. The following PCR cycle was used: the amplification of plasmid DNA (fragment 1 or 2); 94°C for 5 min; 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 3 min; and 72°C for 7 min. The amplification of genomic DNA (fragment 3 or 4); 94°C for 5 min; 30 cycles of 94°C for 30 seconds, for 30 seconds, and 72°C for 30 seconds; and 72°C for 7 min. The overlap extension PCR was performed using a final volume of 100 μl containing an equal amount of PCR product from the plasmid and genomic DNA, 2.0 U *Ex Taq* DNA Polymerase (Takara) and 1 μM of each primer. The following cycling profile was performed:

94°C for 5 min; 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 7 min; and 72°C for 7 min. All PCR amplifications were carried out on a Gene Amp PCR System 9700 (Applied Biosystems).

Table 2. Primers used for estimation of the maximum length of segmental chromosome duplication

Chromosomal region	Primer name	Nucleotide sequence (5'-3')
-	CA	CCCCAACCCCAACCCCAACCCCAACCCCAACCCCAAAGGCCACTAGTGGATCTGAT
-	loxP-cas	GGCCGCCAGCTGAAGCTTCG
Chr. I	C1-50k-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTAGCGTTGGTGAAAGGCACT
37,504 -87,735	C1-50k-L-r	GGTGCATAGTGTTTTAATGC
	C1-50k-R-f	AGAACGACCCCAGAATGTAC
	C1-50k-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCAGCAATGGGGACGATGATT
Chr. II	C2-150k-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTCTAAGCATCGACCTTAGAG
360,775-505,293	C2-150k-L-r	CAGACAAATCGCCATAGTCG
	C2-150k-R-f	CTGACCAAGAAAGAGCACGC
	C2-150k-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGTGGAACTTGCATATCGTT
Chr. IV	C4-250k-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAACCCACAAAACGAGATGGA
148,203-401,638	C4-250k-L-r	TCCTTGTAGCGCTGATACGA
	C4-250k-R-f	TCTTTTCATTATTGCTAGTA
	C4-250k-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAAAGTAGTTCATGATGCGGG
Chr. IV	C4-300k-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCATTCGATTTCCACTGCTTAT
97,475-401,638	C4-300k-L-r	CCTCGCATAAATTGGGAAAT
	C4-300k-R-f	TCTTTTCATTATTGCTAGTA
	C4-300k-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAAAGTAGTTCATGATGCGGG
Chr. IV	C4-350k-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCAAACAACATTTGTCCAAAA
50,000 - 401,638	C4-350k-L-r	TTCTGCAAACCAAAGAAAGA
	C4-350k-R-f	TCTTTTCATTATTGCTAGTA
	C4-350k-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAAAGTAGTTCATGATGCGGG
Chr. IV	C4-400k-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGTGCTCTTCTTGTTAACCCC
198,996-600,688	C4-400k-L-r	GGCCGCAATTGACGACACAC
	C4-400k-R-f	TCGAGGACAAAAAGGCATAT
	C4-400k-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGAGAATAAAATAGGTCAGGT
Chr. VIII	C8-50k-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCTCCTAGATGGTGGGATCCA
294,748- 346,028	C8-50k-L-r	GGCCAAACGGTCAAGATCAA
	C8-50k-R-f	GACTGGTTTTAATGGTATTG
	C8-50k-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGACCTCTTATAAAGATTCAA
Chr. VIII	C8-100k-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTTTGCGCAACTGTTGCCGTG
247,693-346,028	C8-100k-L-r	TTAACTTTGGGGACCATTGA
	C8-100k-R-f	GACTGGTTTTAATGGTATTG
	C8-100k-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGACCTCTTATAAAGATTCAA
Chr. VIII	C8-150k-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAGCGTGTCGCGTTCCTCGAA
192,203-346,028	C8-150k-L-r	TGGTATCTACCTGAAGTCTT
	C8-150k-R-f	GACTGGTTTTAATGGTATTG
	C8-150k-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGACCTCTTATAAAGATTCAA

Chromosomal region	Primer name	Nucleotide sequence (5'-3')
Chr. VIII	C8-200k-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTTCGTAGAAATGACTCCAAG
145,656-346,028	C8-200k-L-r	GAACGACCGAACATACAGTA
	C8-200k-R-f	GACTGGTTTTAATGGTATTG
	C8-200k-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGACCTCTTATAAAGATTCAA
Chr. X	C10-100k-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCACAGACAAGGTCATATCGCG
225,115-326,063	C10-100k-L-r	CTCTCATGGAGGGTGTAATT
	C10-100k-R-f	TTCCATTGACCACCGTCTAC
	C10-100k-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGCGAACTCTGTTTCATCAGG

2.2.4 Yeast transformation

Yeast cells were transformed according to the method of Gietz and Schiestl (Gietz and Schiestl, 2005). Yeast cells were cultured in YPAD at 30°C overnight. Cultures were inoculated in fresh YPAD media. After incubateing at 30°C for 3-4 hours until the O.D.600 reached at 0.8-1.0, cells were collected and washed with sterile water. Cell pellets were suspended with 0.1 M lithium acetate and centrifuged. The following reagents were added into cell pellets in order listed; 240 µl of 50% polyethylene glycol8000 (Wako), 36 µl of 1 M lithium acetate, 25 µl of 2 mg ml^{-1} salmon carrier DNA (Wako) (heat in boiling water for 5 min and chilled on ice for 5 min before using) and PCR product, then vortexed vigorously. After incubating at 37°C for 30 min followed by heat shock at 42°C for 20-25 min, cells were centrifuged and resuspended in sterile water. About 100 µl of cell suspension were spread on an appropriate selective media plate. For a selection of yeast transformants, cells were cultured on SC medium without leucine, or without leucine and histidine, or without leucine and uracil at 30°C for 4 days.

2.2.5 Karyotype analysis by PFGE and Southern blot analysis

Chromosome DNA plugs were prepared according to the method of Sheehan and Weiss (Sheehan and Weiss, 1990). Chromosomes were separated on 1% (wt vol^{-1}) pulsed-field gel electrophoresis gels in 0.5× TBE (Tris-borate-EDTA) buffer at 14°C using the

CHEF DRIII® System (Bio-Rad Laboratories), with a 60-second pulse for 15 hours, followed by a 90-second pulse for 9 hours, at 6 V cm^{-1}. The chromosomes were visualized and photographed under a UV transilluminator (UVP Bio Do-It Imaging System). Separated chromosomes were transferred onto a Hybond-N+ membrane using capillary blotting, and then cross-linked to the membrane by exposure to UV light (120 mJ cm^{-2}) using a UV cross-linker (Spectrolinker™ UV CROSSLINKER XL-1500) to fix DNA onto membrane. The membrane was hybridized with specific probes that were amplified by primers listed in Table 3. Probe labeling, hybridization, and hybridization signal detection were carried out using an ECL direct™ nucleic acid labeling and detection system (Amersham Biosciences). The film was exposed to membrane for 45 min and then developed in an X-ray film processor (FPM100; Fuji Film).

Table 3. Primers used to amplify probes for estimation of the maximum length of segmental chromosome duplication

Chromosomal region	Primer name	Nucleotide sequence (5'-3')
Chr. I	C1-50k-R-f	AGAACGACCCCAGAATGTAC
87,336-87,735	C1-50k-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCAGCAATGGGGACGATGATT
Chr. II	C2-150k-R-f	CTGACCAAGAAAGAGCACGC
504,894-505,293	C2-150k-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGTGGAACTTGCATATCGTT
Chr. VIII	Chr.8-probe3-f	CAAGTCCGTGCTGTCAAGGA
325,648-326,147	Chr.8-probe3-r	CAATAACGGCCAATGGCTTG
Chr. IV	C4-250k-R-f	TCTTTTCATTATTGCTAGTA
401,239-401,638	C4-250k-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAAAGTAGTTCATGATGCGGG
Chr. X	C10-check-f	CTGATGAATGGACAATGCAT
247,685-248,184	C10-check-r	GCTCGATGATGAGCCTCTTA

2.2.6 Mitotic stability of segmentally duplicated chromosomes

Yeast cells were cultured in 5 ml of YPAD medium at 30°C overnight and the optical density was then measured at 660 nm (OD_{660}). Cell cultures were transferred into 5 ml of fresh YPDA media at an initial OD_{660} of 0.1. After incubation at 30°C for 24 hours, cell culture was measured at OD_{660} and the culture was diluted to a concentration of 1×10^3 cells

ml^{-1}. About 100-200 cells were spread on each of three YPAD plates and incubated at 30°C for 24 hours, before being replicated onto YPAD and selective media plates. After incubation at 30°C for 24 hours, colony numbers on the plates were counted and % mitotic stability was calculated by the following equation:

$$\% \text{ Mitotic stability} = \frac{Number\ of\ colonies\ on\ selective\ plate}{Number\ of\ colonies\ on\ YPDA\ plate}\ x\ 100\ \%$$

2.2.7 Estimation of DNA copy number

Genomic DNA of the wild-type BY4742 and segmental aneuploid strains was extracted and treated with restriction enzyme HincII (Takara). The master-mix solution to digest genomic DNA with HincII restriction enzyme contained genomic DNA, 10× buffer and HincII; the solution was incubated at 37 °C for at least 1 hour and subsequently subjected to gel electrophoresis. Southern blot analysis was performed using probe A and probe C or probe B and probe C. Probe A, probe B and probe C were amplified by the primers C8-check-f and C8-check-r, C10-check-f and C10-check-r, and C15-check-f and C15-check-r, respectively (Table 3). Probe labeling, hybridization and hybridization signal detection were performed according to an ECL direct™ nucleic acid labeling and detection system (Amersham Biosciences). The intensity of the hybridization signal was determined by Scion image Beta 4.02 for Windows (Scion Corporation, Frederick, MD, USA) and the copy number was calculated by comparing the signal intensity ratio of the hybridizing band for the segmental aneuploid against that for the parental strain. The experiments were repeated in triplicate.

2.3 Results

2.3.1 PCR-mediated chromosome duplication (PCDup) technology

General scheme of PCDup method is illustrated in Figure 1. The details of preparation of two types of duplicating DNA modules are presented in the Methods section. In general, natural chromosomes are stable and segregate into daughter cells owing to the presence of three essential elements: a telomere at both ends of the chromosome, a single centromere, and an autonomously replicating sequence (ARS). Chromosomes newly created by PCDup must also have these three elements to assure its stable segregation. Therefore, I prepared a duplicating DNA module containing telomere seed sequences and an additional centromere as duplicating DNA module 1 and another duplicating DNA module containing telomere seed sequences as duplicating module 2 (Fig. 1). Since an ARSs are normally distributed about every 40 kb region throughout a natural chromosome (Beach *et al*., 1980), it is, in general, not necessary to add additional ARS sequences in the duplicating module. However, if the target region does not contain an ARS, it is necessary to prepare a duplicating module with additional ARS sequences. If the target region is the terminal region of a chromosome, only one duplicating module is needed to generate the segmentally duplicated chromosome.

The duplicating DNA modules were introduced into a yeast cell by conventional transformation. The selected chromosome region was duplicated through integration of the two introduced DNA modules into each of two targeted sites on the same chromosome simultaneously by homologous recombination. Transformants are obtained by growth on selective medium. Then, the karyotype of transformants was analyzed by using pulsed-field gel electrophoresis (PFGE) and subsequent Southern blot analysis to confirm that targeted chromosomal region was indeed duplicated.

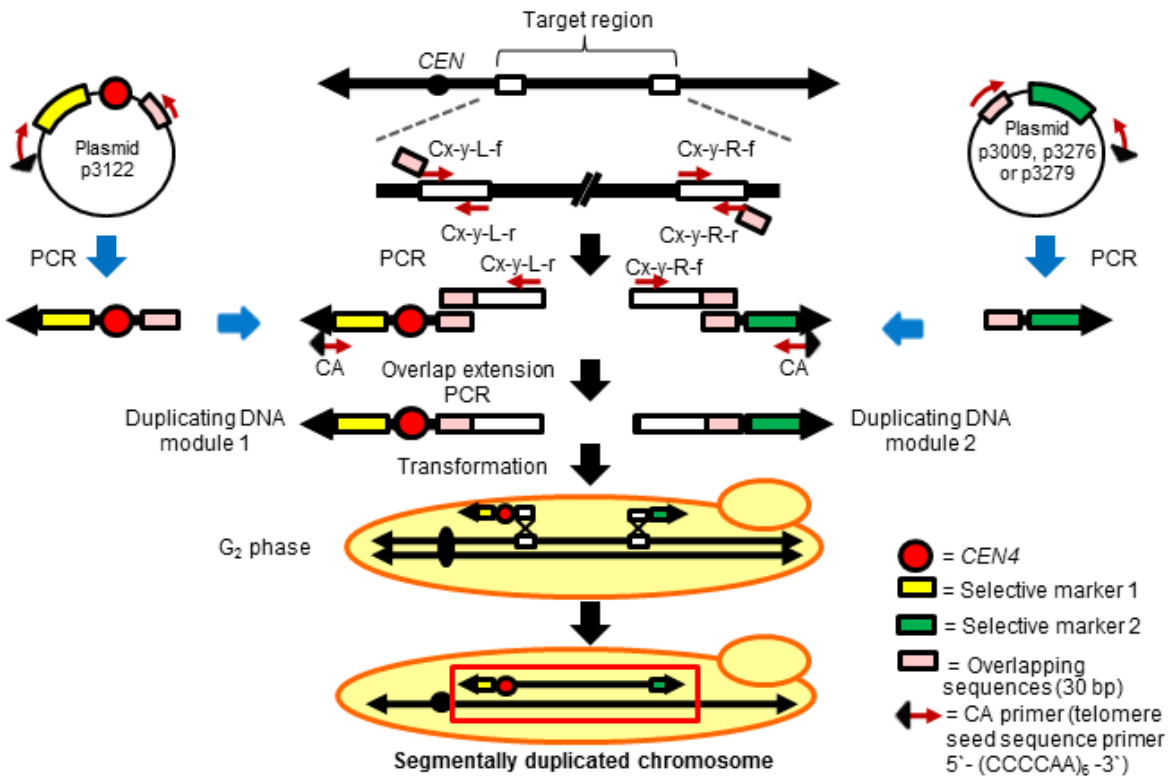


Figure 1. Procedure for construction of a segmentally duplicated chromosome by the PCDup method. Two target DNA fragments with nucleotide sequences corresponding to the left and right ends of the target region (400 bp) were amplified by PCR using genomic DNA as a template and the primers Cx-y-L-f and Cx-y-L-r or Cx-y-R-f and Cx-y-R-r (where x represents chromosome number, y represents size of duplicated chromosome region, L represents left end of sequence of the target region, R represents right end of sequence of the target region, f represents forward primer, and r represents reverse primer). The primer sequences of Cx-y-L-f, Cx-y-L-r, Cx-y-R-f and Cx-y-R-r varied with the target chromosomal region and are listed in Table 2. A fragment containing *CEN4* and selective marker 1 cassette and a fragment containing the selective marker 2 cassette were amplified from the plasmid template using loxP-cas and a CA primer (Tables 1 and 4). Next, one target fragment was combined with the *CEN4* and selective marker 1 cassette, and the other target fragment was combined with the selective marker 2 cassette by overlap extension PCR to form two duplicating modules, designated "duplicating DNA module 1" and "duplicating DNA module 2". The amplified modules were introduced into yeast cells by conventional transformation. The two introduced modules are designed to integrate at the two target sites of the same chromosome by homologous recombination, resulting in duplication of the selected chromosomal region.

2.3.2 Performance of PCDup

To test the performance of the PCDup method, I first tried to duplicate three chromosomal regions that were selected randomly (Table 4); a 50 kb region of chromosome

I, a 145 kb region of chromosome II and a 100 kb region of chromosome X. A DNA duplicating module containing the target sequences, *CEN4* and *CgLEU2* cassettes, and telomere seed sequences, and another duplicating module containing the target sequences, *URA3* cassette, and telomere seed sequences were prepared as described in the Methods section. These two modules were then introduced into the parental strain BY4742. Candidate transformants that harbored segmental duplication were selected by growth on SC-Ura-Leu medium**.** The numbers of transformants obtained for each chromosomal region are shown in Table 4. To analyze the karyotype of candidate transformants, PFGE was performed, followed by Southern hybridization using probes comprising nucleotide sequences corresponding to the target region (Table 3). Hybridization signals for segmental aneuploids were detected at positions corresponding to the intact chromosome and segmentally duplicated chromosome, whereas a hybridization signal for the parental strain was detected only at the position corresponding to the intact chromosome. Our analyses showed that desired duplication was achieved for each of the three regions with a proportion from 10% to 30% (Table 4) based upon the number of transformants having desired karyotype per number of transformants analyzed. Therefore, these initial observations confirmed that the PCDup method could duplicate arbitrarily selected chromosomal regions.

Table 4. Characteristics of segmental aneuploids of chromosomes I, II, IV, VIII and X

Duplicated region[a]	Duplication length (kb)	Plasmid template[b]	Transformants (n)	Proportion of desired karyotype[c]	% Mitotic stability
Chr. I 37,504 -87,735	50	p3122, p3276	55	30.00% (3/10)	100%
Chr. II 360,775-505,293	145	p3122, p3276	11	10.00% (1/10)	100%
Chr. IV 148,203-401,638	250	p3009, p3122	31	7.69% (1/13)	99%
Chr. IV 97,475-401,638	300	p3009, p3122	44	6.25% (1/16)	100%
Chr. IV 50,000-401,638	350	p3009, p3122	39	0.00% (0/39)	ND[d]
Chr. IV 198,996-600,688	400	p3009, p3122	11	0.00% (0/11)	ND[d]
Chr. VIII 294,748- 346,028	50	p3122, p3276	18	21.43% (2/14)	100%

Duplicated region[a]	Duplication length (kb)	Plasmid template[b]	Transformants (n)	Proportion of desired karyotype[c]	% Mitotic stability
Chr. VIII 247,693-346,028	100	p3122, p3276	34	10.00% (1/10)	100%
Chr. VIII 192,203-346,028	150	p3122, p3276	32	10.00% (1/10)	100%
Chr. VIII 145,656-346,028	200	p3122, p3276	6	33.33% (2/6)	100%
Chr. X 225,115-326,063	100	p3122, p3276	18	20.00% (2/10)	100%
		YCp50 (7.8 kb)	NC[d]	NC[d]	85%

*a: Chr. N x-y : Chr. N represents chromosome number, x represents first nucleotide number of chromosomal region and y represents last nucleotide number of chromosomal region.
b: p3009 was used to amplify the *CgHIS3* cassette, p3122 was used to amplify the *CEN4-CgLEU2* cassette, p3276 was used to amplify the *URA3* cassette, p3279 was used to amplify the *CgHIS3-H4ARS* cassette and YCp50 was a *URA3* centromeric plasmid whose length was 7.8 kb.
c: Proportion of desired karyotype in analyzed transformants (number of segmental aneuploids / number of candidate transformants that were analyzed for karyotype).
d: ND means no data. NC means not detected.

2.3.3 Size of the duplicated region

To determine the upper size limit of duplicated regions by PCDup, I attempted to construct a series of segmentally duplicated chromosomes of increasing size (50 kb, 100 kb, 150 kb and 200 kb of chromosome VIII, and 250 kb, 300 kb, 350 kb and 400 kb of chromosome IV) (Table 4). The results showed that 50-kb, 100-kb, 150-kb, 200-kb and 300-kb chromosomal regions could be duplicated while 350-kb and 400-kb chromosomal regions could not. Thus, I concluded that approximately 300 kb was the maximum size of region that PCDup was able to duplicate routinely (Fig. 2 and Table 4). The possible reasons for this size limitation are discussed later.

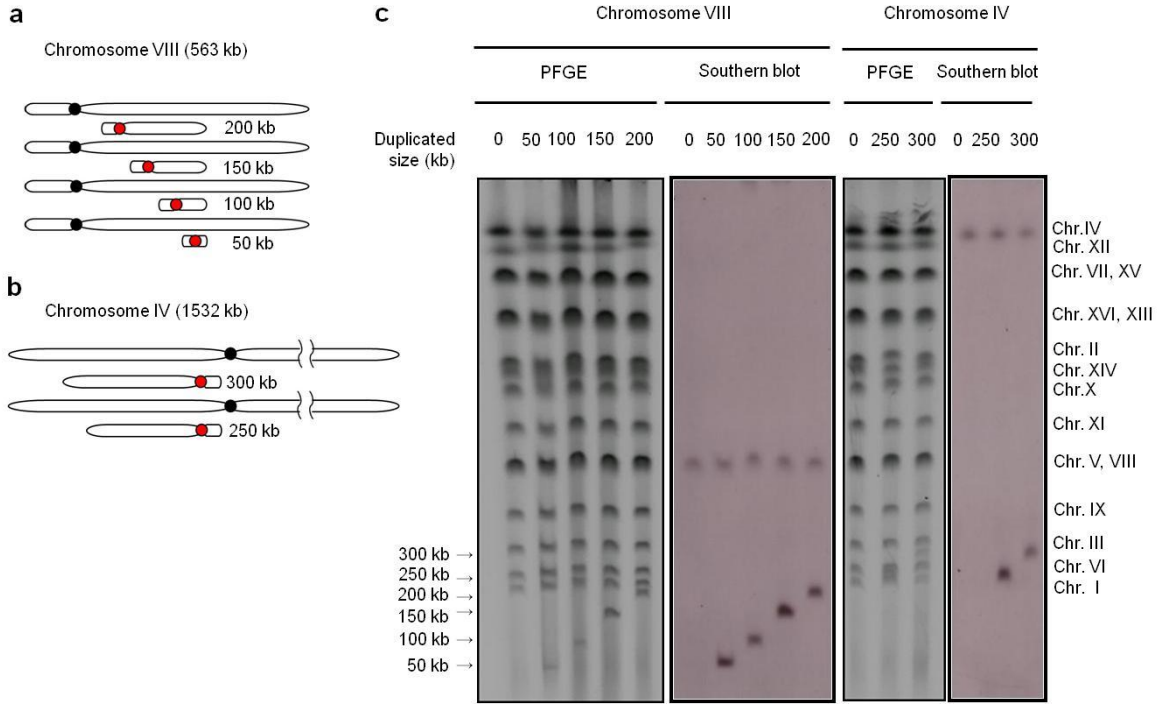


Figure 2. Determination of the maximum size of segmentally duplicated chromosomes by the PCDup method. Segmentally duplicated regions of varying lengths were designed for chromosome VIII (**a**) and chromosome IV (**b**). The probe was prepared by PCR amplification of a 400 bp internal sequence of the target region (red circle represents *CEN4*). (**c**) PFGE and Southern blot analysis of the karyotypes of the 50 kb, 100 kb, 150 kb and 200 kb Chr. VIII segmental aneuploid strains, and the 250 kb and 300 kb Chr. IV segmental aneuploid strains.

2.3.4 Stability of newly generated chromosomes

To investigate whether the segmental duplicated chromosomes were stable during cultivation, the mitotic stability of the strains was evaluated in comparison with that of YCp50, a yeast centromere plasmid. The result showed that YCp50 had 85% mitotic stability, whereas strains carrying a segmentally duplicated chromosome maintained almost 100% mitotic stability. These findings suggested that the segmentally duplicated chromosomes derived by PCDup and ranging from 50 kb to 300 kb can be stably maintained (Table 4).

2.3.5 Estimation of the copy number of segmentally duplicated chromosome

Each segmental aneuploid constructed by PCDup was thought to contain one additional copy of the target region as illustrated in Figure 1. However, the exact copy number had not been confirmed. To determine copy numbers of the segmentally duplicated chromosome, the 50-kb segmentally duplicated chromosome VIII (coordinates: 294,748–346,028) and the 100-kb segmentally duplicated chromosome X (coordinates: 225,115–326,063) was examined (Fig. 3). The genomic DNA of both the parental strain and the segmental aneuploid strain was digested with the restriction enzyme HincII and separated by gel electrophoresis. Southern blot analysis was then performed with the pair of probes A and C for 50-kb Chr.VIII, or the pair of probes B and C for the 100-kb Chr.X. The copy number of the 50-kb segmentally duplicated chromosome VIII was estimated by comparing the signal intensity ratio of Chr.VIII to Chr.XV in the segmental aneuploid against that of Chr.VIII to Chr.XV in the parental strain. The relative signal intensity of the 50-kb segmentally duplicated chromosome VIII was 2.84±1.15, whereas that of the parental strain was 1.45±0.50. Thus, the actual copy number of the segmentally duplicated chromosome was estimated to be 1.94±0.13. Similarly, the copy number of the 100-kb segmentally duplicated chromosome X was estimated comparing by the signal intensity ratio of Chr.X to Chr.XV in the segmental aneuploid against that of Chr.X to Chr.XV in the parental strain. The actual copy number of the 100-kb segmentally duplicated chromosome X was estimated to be 1.71±0.64. Thus, these results showed that the copy number of both the 50-kb Chr.VIII and the 100-kb Chr.X segmentally duplicated chromosomes was approximately two (Fig. 3). These observations suggested that one segmentally duplicated chromosome was constructed by using the PCDup method.

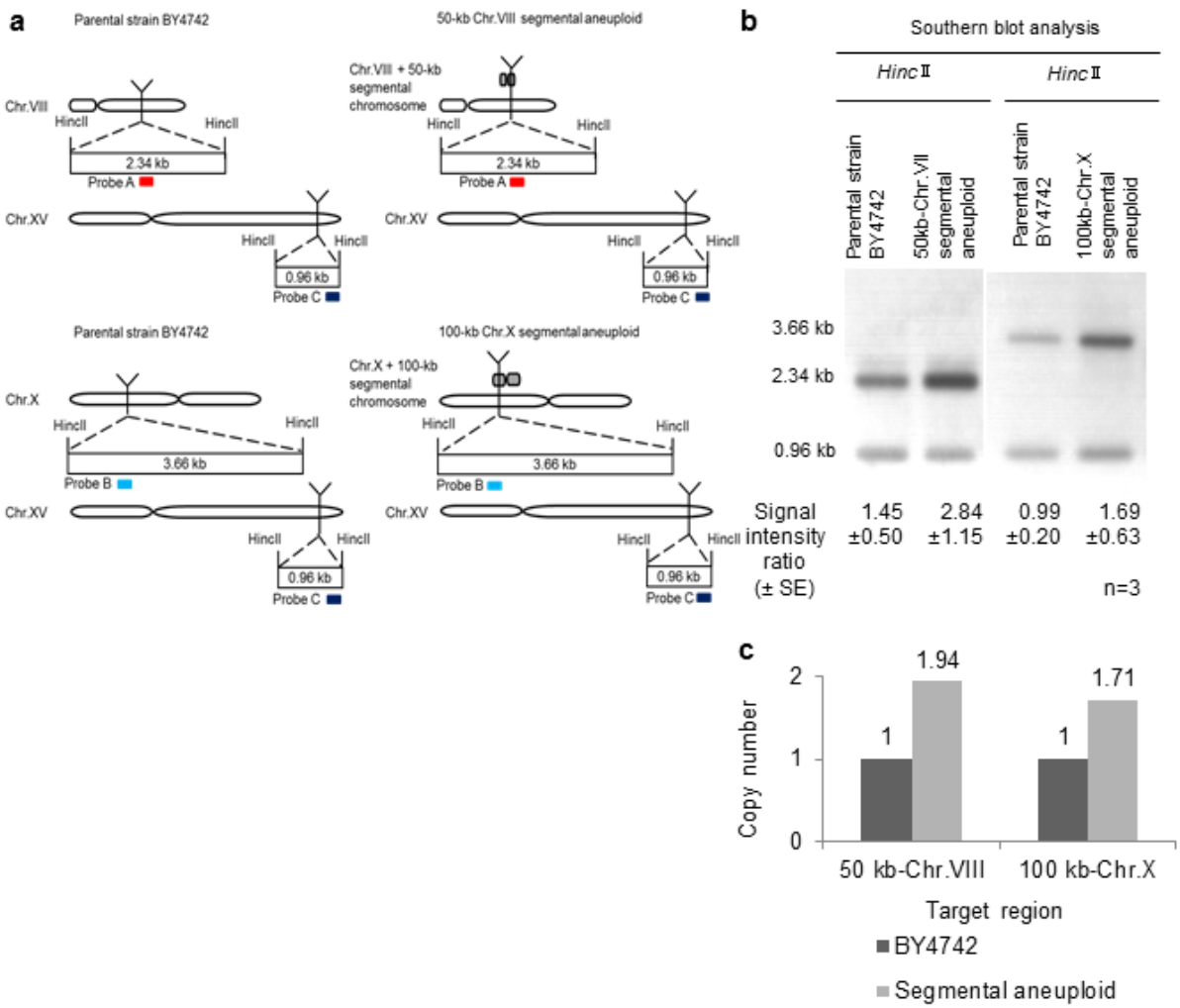


Figure 3. Estimation of the copy number of segmentally duplicated chromosomes. (**a**) Illustration of probe-hybridized location on the HincII-digested fragment of chromsome VIII (restriction site: 324,584–326,928), chromosome X (restriction site: 246,431–250,093) and chromosome XV (restriction site: 1,016,365–1,017,328). Probe A corresponded to the 500-bp fragment of Chr.VIII between coordinates 325,648–326,147, Probe B corresponded to the 500-bp fragment of Chr.X between coordinates 247,685–248,184, and probe C corresponded to the 500-bp fragment of Chr.XV between coordinates 1,016,810–1,017,309 (red box represents probe A, light blue box represents probe B, and dark blue box represents probe C). (**b**) Genomic DNA of the segmental aneuploid strain (50-kb Chr.VIII, [coordinates: 294,748–346,028] and 100-kb Chr.X [coordinates: 225,115–326,063]) and parental strain BY4742 was digested with restriction enzyme HincII and subsequently subjected to Southern blot analysis using probe A and probe C, or probe B and probe C for determining the chromosomal copy number of the 50-kb Chr.VIII or 100-kb Chr.X segmental aneuploid strain, respectively. The signal intensity ratio was measured relative to Chr.XV. (**c**) The copy number of the segmental aneuploid was estimated using the signal intensity ratio of the segmental aneuploid divided by that of the parental strain.

2.4 Discussion

Two possible models might explain how segmentally duplicated chromosomes are generated by PCDup. In the first model (Fig. 4a) is as follows; the duplicating modules recombine with each of their target sites. The regions outside the target area are lost due to the lack of a centromere or telomere. Then, duplicated chromosome is generated. Moreover, the results indicated that was an upper limit to the size of the chromosome region that could be duplicated. This effect may be related to the fact that larger linear chromosomes have a lower frequency of chromosome nondisjunction (Hieter, 1985). Therefore, in the first model (Fig. 4a), chromosome nondisjunction would be expected to occur more frequently for smaller derived chromosomes. The upper size limitation of chromosome duplication here of approximately 300 kb might be determined by the low likelihood of nondisjunction of these newly generated chromosomes.

The second possible mechanism (Fig. 4b) is based on the Break Induced Replication (BIR) model (Morrow *et al*., 1997, Lydeard *et al*., 2007). The distance between two homologous sites is one of the parameters of the recombination execution checkpoint (REC) that regulates the choice of homologous recombination pathway during double strand break (DSB) repair (gene conversion, single-strand annealing or BIR). The signaling for the initiation of new DNA synthesis between DSB ends is lost when the distance between two homologous sites increases. If the distance increases more than 5 kb, the mode of gap repair shifts from gene conversion to BIR (Jain *et al*., 2009). The frequency of BIR depends on the length of template. When the distance is large, complete BIR synthesis is likely limited by the requirement in chromatin remodeling for migration of the D-loop and initiation of lagging stand synthesis (Donnianni and Symington, 2013). Morrow *et al.*, claimed that they could observe duplication events generated by the “break copy” mechanism of up to 365 kb (Morrow *et al*., 1997). Therefore, another explanation for the upper size limit of segmentally

duplicated chromosome here is a possible defect in completion of DNA synthesis due to the increased distance between homologous sites (Fig. 4b) (Donnianni and Symington, 2013).

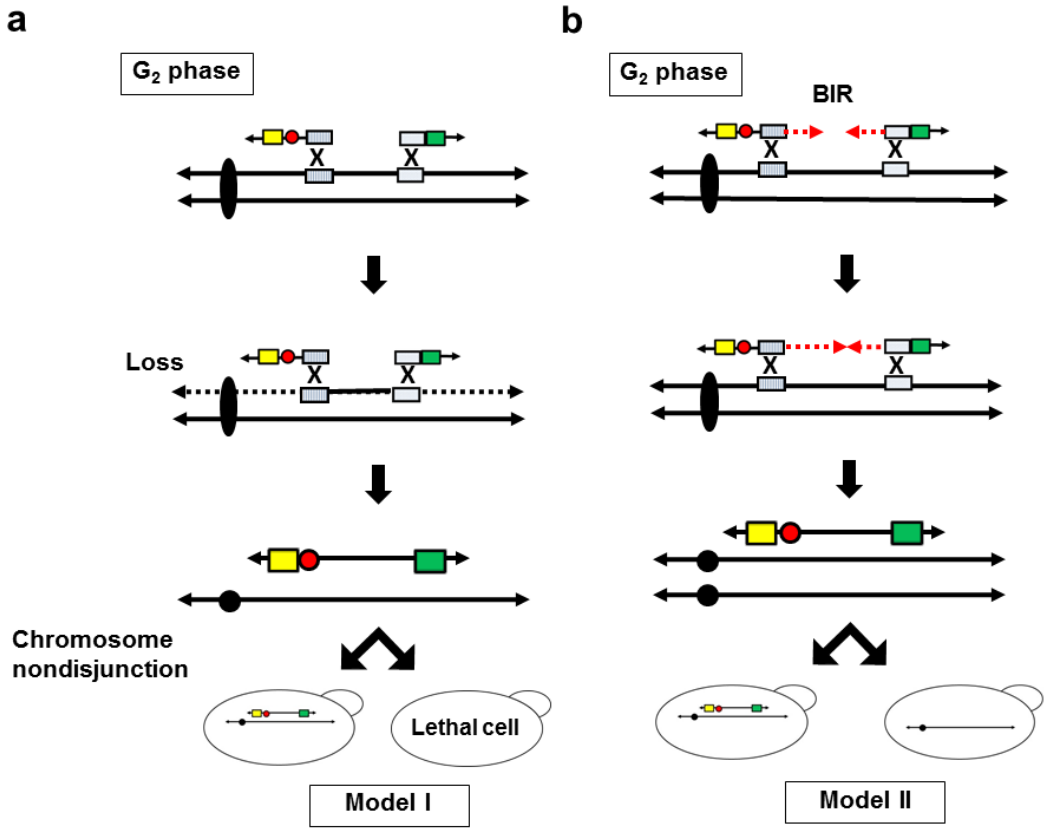


Figure 4. **Possible mechanisms for generation of segmentally duplicated chromosomes.** (**a**) In model I, each of the two duplicating modules is assumed to recombine with two target regions on the same sister chromatid. The target region is then generated as a new chromosome. Sequences outside the target region are lost during mitotic cell division due to the lack of centromere or telomere. If chromosome nondisjunction happens, either the daughter cell or mother cell is expected to have both the targeted natural chromosome and the newly generated segmentally duplicated chromosome, while the remaining cell loses its chromosome. (**b**) Model II is based on the BIR mechanism. In this model, the duplicating module is expected to invade the target chromosome and initiate DNA synthesis from the homologous site of one duplicating module to the homologous site of the other duplicating module. This action generates the segmentally duplicated chromosome.

In conclusion, PCDup technology that was developed in this study could be a promising approach for allowing the duplication of any selected chromosomal region and might be provide a great benefit on the study in segmental aneuploidy in eukaryotic genome.

2.5 Summary

An interesting question is whether, and if so how, segmental aneuploidy is related to phenotypic alterations. However, methodologies to address this issue are limited. This prompted us to design a new technology to overcome this problem. In this chapter, I reported the development of PCDup, a technology that is capable of generating an extra chromosome with segmental duplication of any selected region by means of a PCR, followed by a single transformation. It should be noted that a simple method like PCDup for chromosomal segmental duplication at specific region has not previously been reported for any kind of organism. I first succeed in constructing several types of segmental aneuploid strains of randomly selected chromosomal regions. The results confirmed that the selected chromosomal regions could duplicate arbitrarily by PCDup technology. Next, I also determined the upper size limit of duplicated regions by PCDup technology. The various regions ranging from 50 to 300 kb in different chromosomes were duplicated. Moreover, those newly generated chromosomes were also stable during several rounds of mitosis. These results demonstrated that PCDup technology allows us to create a newly additional chromosome with segmental duplication of any chromosomal region up to 300 kb efficiently. Therefore, PCDup technology might be exploited as a simple genome modification at large scale to contribute both to basic physiological studies and industrial applications.

Chapter 3

Genome-wide construction of segmental aneuploidy by PCDup and the investigation of phenotypes of segmental aneuploidy under stresses

3.1 Introduction

Yeast is a valuable organism with enormous industrial benefits to human life and is also a model organism representing a unicellular eukaryote. The study in chromosome rearrangements in yeast model is one of most suitable strategies to elucidate the molecular mechanisms involved in chromosome rearrangements and the consequences of chromosome rearrangements. One of prominent chromosomal rearrangement that has been found to be related with notable influences on the physiology of eukaryotic cells is segmental aneuploidy. Although segmental aneuploidy usually confers a detrimental effect on a cells (Bigner *et al*., 1988, Warburton, 1991, Crolla, 1998, Viersbach *et al*., 1998, Fuster *et al*., 2004, Makarevitch *et al*., 2008, Lyle *et al*., 2009, Lucas *et al*., 2010, Weischenfeldt *et al*., 2013, Chen *et al*., 2013, Akalin *et al*., 2014), segmental aneuploidy could be an adaptive mechanism of the cell that enables survival and confers a growth advantage in stressful environments (Dunham *et al*., 2002, Infante *et al*., 2003, Selmecki *et al*., 2006, 2008, 2009, Gresham *et al*., 2008, Borneman *et al*., 2011, Brion *et al*., 2013, Chang *et al*., 2013). This raises the interesting question of whether how segmental aneuploidy has the impact on adverse and beneficial effects on cells. As I described in the previous chapter, PCR-mediated chromosome duplication technology (PCDup) have been developed as a novel approach to generate segmental aneuploidy at any desired chromosomal region. Therefore, it should be possible to apply this technology to study the association of segmental aneuploidy and phenotypic alteration in yeast genome.

In this chapter, a series of approximately 100-200 kb segmental duplication covering the genome of *S.cerevisiae* were constructed by PCDup technology. Then, I investigated the

effects of stressful environments, including thermal stress, high contents of ethanol concentration, strong acidic or alkaline pH, osmotic stress and nonfermentable carbon sources on segmental aneuploid strains. Moreover, the correlation between segmental aneuploidy and the observed phenotypes were also verified. The results suggested that PCDup technology might be a promising approach to facilitate the elucidation of the relationship between the presence of duplicated region and stress response phenotype.

3.2 Materials and Methods

3.2.1 Yeast strains, plasmids and DNA preparation.

Saccharomyces cerevisiae strain BY4742 [*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*] was used as the parental strain for the construction of segmental aneuploidy of chromosomes I to XVI and a source of genomic DNA. The plasmids used in this chapter are listed in Table 1. Yeast strains were grown at 30°C in YPAD medium or selective medium (Amberg *et al*., 2005). *E. coli* strains were grown at 37°C in LB medium. The preparation of media, plasmid DNA extraction and Isolation of yeast genomic DNA have been described in Chapter 2.

3.2.2 Preparation of DNA duplicating modules

The DNA duplicating modules were prepared by two rounds of PCR. In the first round of PCR, loxP-cas and CA primers were used to amplify marker cassettes (fragment 1 and 2) from plasmid template **(**Table 1**)**. In parallel, two DNA fragments (400 bp; fragments 3 and 4) with nucleotide sequences corresponding to the left and right ends of the target region were amplified from genomic DNA of strain BY4742. The primers that were used to amplify genomic DNA fragments in this chapter are listed in Tables 5 and 6 (x represents the

chromosome number, y represents the chromosomal region, s represents sub-region, L represents the left end of target region, R represents the right end of target region, f represents a forward primer, and r represents a reverse primer). After that, the PCR products (fragments 1-4) were gel-purified using a Wizard SV Gel and PCR Clean-up System (Promega). Next, overlap extension PCR was conducted to amplify two duplicating DNA module: one target fragment (fragment 3 or 4) was combined with a marker cassette (fragment 1 or 2). After amplification, the two PCR products were ethanol-precipitated. The preparation of PCR mixture and PCR cycling profile has been described in previous chapter.

Table 5. Primers used for construction of segmental chromosome duplications of chromosomes I to XVI

Region name	Primer name	Nucleotide sequences (5'-3')
C1-1	C1-1-R-f	GGCACTAGTTCCCTTCTTAC
	C1-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCAGGGAGAGAAAGGCATTGG
C1-2	C1-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGAATGAGAAGTCGTGTCGTC
	C1-2-L-r	CCTTTAGTAGCTGTTGGGCT
C2-1	C2-1-R-f	TTACATGCGACACCAAGCAG
	C2-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGTCCTCCGAGGCAGGCCCTC
C2-2	C2-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGAATGCAATTCGATACTCG
	C2-2-L-r	CAATCCAGTGATACCCGTGG
	C2-2-R-f	TATAAACGCGCTTGCGATCG
	C2-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGGAGTTTTGAGTTCATCTG
C2-3	C2-3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCACAGCATTTGATCTTGGTC
	C2-3-L-r	CGTGCAAGCAAAAGCATTTG
	C2-3-R-f	TCTCTGAGGGTTATCAAATG
	C2-3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCGTGTGATGTGGACTGTTGC
C2-4	C2-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCTAACCCTTTGATGTCCGAC
	C2-4-L-r	CTTTTCTTCCCTCCAAGATC
C3-1	C3-1-R-f	CTGAGAGAATCCTCCTACGG
	C3-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCATATCACGTTGTGAGCAGCC
C3-2	C3-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGGATCGGGATATGGCTTTG
	C3-2-L-r	CGTGATACCGGGGGTTGAAG
C4-1	C4-1-R-f	AGGGCATCCATCCAACCATC
	C4-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGCTTTGGAGGAGATATTTG
C4-2	C4-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGTGCTCTTCTTGTTAACCCC
	C4-2-L-r	GGCCGCAATTGACGACACAC
	C4-2-R-f	TCTTTTCATTATTGCTAGTA
	C4-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAAAGTAGTTCATGATGCGGG

Region name	Primer name	Nucleotide sequences (5'-3')
C4-3	C4-3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCACTTAACAAGAAGATTAG
	C4-3-L-r	CATACTTGAACCACCTGAAA
	C4-3-R-f	TCGAGGACAAAAAGGCATAT
	C4-3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGAGAATAAAATAGGTCAGGT
C4-4	C4-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAGGATTTTAATCTGTTGGAG
	C4-4-L-r	CCAACCAATATTACTGCTTT
	C4-4-R-f	CCGACCGAGTATTACTCAGT
	C4-4-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGAGTCATCCATATTGCAAAC
C4-5	C4-5-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCAAAAGTTGCCTGTCCAAA
	C4-5-L-r	GAAGGCAAGGCTTACAGGCT
	C4-5-R-f	TTACGGTGGTTGCAAAGGGA
	C4-5-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAAAGAAGACTTCAATAAGTT
C4-6	C4-6-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGTTGACTTGACATACACTAA
	C4-6-L-r	AGGTTAGGACAGGGTACCAT
C4-6	C4-6-R-f	ATGAAATCGATCATAGCGAT
	C4-6-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTCATCGTTTTCATCATAGGT
C4-7	C4-7-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCTTTCAATCTTGTCTCTTGC
	C4-7-L-r	GGAGAAACGCATCTAAGAAA
	C4-7-R-f	AAGGGGACTTTCAGGTGCAT
	C4-7-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCTCATCGTGTGGCTTAACG
C4-8	C4-8-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGAAGATTTTAAACTCACCT
	C4-8-L-r	CGGCCTTATTATGATCCCGA
C5-1	C5-1-R-f	CCCAATCATCTTAAGACAGC
	C5-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGTAGACTCTTTAACACTCG
C5-2	C5-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGCGAACGAGTACATTATTGC
	C5-2-L-r	TGTATTCTACAGTTTGCTCC
	C5-2-R-f	AATAGAAGTGGAGCCTGTGG
	C5-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTATCATGCTGTACCCGCAAG
C5-3	C5-3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGGTAAGGCGTTGTGTTCCT
	C5-3-L-r	CATCTGCATCCACCAATGAA
C6-1	C6-1-R-f	ACGGTGCGCTCCAACGGATG
	C6-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTCCTTCCGATTCTGAAGGTG
C6-2	C6-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCACAAGAAGTAATTACAGG
	C6-2-L-r	TGCAGAGAGTGCCGTAATCC
C7-1	C7-1-R-f	CGGTTGTATGATATAGATCC
	C7-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGCCCAATCGAGCAAATAAG
C7-2	C7-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGCCGAACGTGTACCCGTAAA
	C7-2-L-r	CGCACCATTACAGGGTCAAA
	C7-2-R-f	AGGTTCTCTTCGCATAGTCG
	C7-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTCCAGAAGTTGGCATCTTTG
C7-3	C7-3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGTGGGTCTTGCTGAAAAGA
	C7-3-L-r	GCTTCAGAAAAGAGCCATAG
	C7-3-R-f	CCTACTTGGCGGTGAATTTC
	C7-3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGATAAGCCCAATACACGACA
C7-4	C7-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAAGAAACTTCTCCAGAGGAG
	C7-4-L-r	CCGCCAAGAAGAGACGTAAA
	C7-4-R-f	TAATTACTTCGGTCGTGGCC
	C7-4-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGCTTTACTTAGTATGTCGGG
C7-5	C7-5-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCAATTTGTGGGATGATGACG
	C7-5-L-r	CAAGTCAGATAGCTTTGAGT
C8-1	C8-1-R-f	CGTCGGTTATGCTTGCCTAT
	C8-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGAGGAGGAGAAACGCATAAG

Region name	Primer name	Nucleotide sequences (5'-3')
C8-2	C8-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCTCGTACATTGACTCAAACC
	C8-2-L-r	AGATTATGCACCTATCGGCG
	C8-2-R-f	AATCACCAGAAGCAGCAGCA
	C8-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGCAGCAAGGTTGCCTTTAA
C8-3	C8-3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGTAAAGCAGTTAGAACGTCG
	C8-3-L-r	GACACGGTATGTGGATACTC
C9-1	C9-1-R-f	TTGTTGTTACCTCTCGTGTC
	C9-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGAGATCTAGAGTTAGTCAGG
C9-2	C9-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGACAGTCCAGTACAGTTCA
	C9-2-L-r	GTGGTTCAAATATCCGTACG
C10-1	C10-1-R-f	GCATAATCGGCCCTCACAGA
	C10-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAGTAGTGAGGACAGGCTTAA
C10-2	C10-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTTGCTCGATCTTCTATCCTC
	C10-2-L-r	ACCCCAATAAAGGAAACGAA
	C10-2-R-f	GATTAGCCTACGAGCCATCA
	C10-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCACGGTTGTCATCAAAAAAG
C10-3	C10-3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTAAACATAGATAAGCGAGCC
	C10-3-L-r	TTACGTCTGTTGAAGACGCC
	C10-3-R-f	GTAGAGGTCGATCACCTTCT
	C10-3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTTGAGGTGACTGTGTTAAAC
C10-4	C10-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCGCACTAGCATTTGAAGACC
	C10-4-L-r	CATCAGTGCCAAGTTACACC
C11-1	C11-1-R-f	CAACCATTTCTCAAAGTGCT
	C11-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCATTGGCAATATGTACCAGA
C11-2	C11-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGACTCTAAAACGGCATTTG
	C11-2-L-r	AAAGGGTTAAAGCAATCTCG
	C11-2-R-f	TGGCTTTGAAGAGAAGTCCT
	C11-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTATCGCTAAACAGTTCTTCC
C11-3	C11-3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCAACAGAAAGGTATTCCTT
	C11-3-L-r	CAGCATCAGAAGACCACAAA
C12-1	C12-1-R-f	ATGGATAGGTTTCGAGGGCA
	C12-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCGGTAACGTCAACAGTGGTA
C12-2	C12-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAATCCAAGAAGGAACCTGCG
	C12-2-L-r	CATAACGGTGCAAATACGTA
	C12-2-R-f	CCTGCTCTTATATCCGTTAT
	C12-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAGGCACCTATCGTCATTGTC
C12-3	C12-3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCTTCCCTATGATAAACTTC
	C12-3-L-r	TTCCCTTATAGCAGCAAGGG
	C-12-3-R-f	CCTAACGACGATGATAATAC
	C12-3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTCTTGGAGACGTGTTCAGAA
C12-4	C12-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCATGTCTCCTCTTCACCAAAG
	C12-4-L-r	TCCTCAACAACCTCTAATTC
	C12-4-R-f	GGAAAACGAAGAGCAGCAGC
	C12-4-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGCTATTATCCAGATGAAGGA
C12-5	C12-5-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCGATAAGCTAAGCCATTTTC
	C12-5-L-r	AGAAGACAACCCGTGGCTTG
C13-1	C13-1-R-f	GCCTCTATAGGCTTTTCGGA
	C13-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGTAGCACCTACTTCTCATC
C13-2	C13-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCAGCATTTTGTTATTGGCG
	C13-2-L-r	CCAGTATGTTCCCTTGACAA
	C13-2-R-f	CCAGGAAACGTTCATTCAAT
	C13-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGAGCAAGTTGGCTGAATGTG

Region name	Primer name	Nucleotide sequences (5'-3')
C13-3	C13-3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGACGACAGCCTGAATAATT
	C13-3-L-r	CTCTGATTTCAATGTCGTCT
	C13-3-R-f	TCAGAGGTCTGGAACATGTC
	C13-3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCACGGGAAGTACTAAGGTTGG
C13-4	C13-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGTCTAAAGTCATCCACATG
	C13-4-L-r	AACAGTACTGGGATAGAAGG
	C13-4-R-f	GGGCAAAGGGACAAAATGAA
	C13-4-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTCATGGTTACCGTTACTGGC
C13-5	C13-5-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGAACTTACTTTCCTCTCTGC
	C13-5-L-r	AGAATTTCGAAGGAAAGGGG
C14-1	C14-1-R-f	TCCTCTTCCATCGATATCAG
	C14-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCACAAGTATTGCACGAGACGT
C14-2	C14-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGCACTGGAAATGCTTTTGG
	C14-2-L-r	AGTGCTCTACTGTCCGAGTC
	C14-2-R-f	GAGTCAACATTATAGGGCTG
	C14-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCACGAAACTGTCGGGTTATCA
C14-3	C14-3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTAATGCCATAATGTGGGGAC
	C14-3-L-r	TGCGGTTCTTAAAACTGTCG
	C14-3-R-f	AATACTATGGAGACCTTGGC
	C14-3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCATACGATAGAAGTACTGGGC
C14-4	C14-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTTAGAATGTGGGTCAGGTGG
	C14-4-L-r	GCATAGCCCTCTTTCGCCTC
C15-1	C15-1-R-f	CACCAGGTATTTGCCAATGG
	C15-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCACTTTGCGTAACGCCAAA
C15-2	C15-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCAAATGGAATCGTTGCTGGG
	C15-2-L-r	CGGTTAAGTCGTCTAACGTC
	C15-2-R-f	GTGAGGGATGTCAGTTACTC
	C15-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCGTCTGAAGCCAATTGAGTG
C15-3	C15-3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGCTTACTTAGTCCTTTGGTC
	C15-3-L-r	GCTTTTCCAATAAAGACGCA
	C15-3-R-f	GAAGGGATTGATCTCCGCTT
	C15-3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCAGGATCAAAATCTGGATG
C15-4	C15-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGCGTTATTGAGTGAACCGTC
	C15-4-L-r	CAGATGGTGCAGCCAATAGA
	C15-4-R-f	GATGTCCTCTGCAAGGATCT
	C15-4-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTCACTAGTGGTGCCACACTA
C15-5	C15-5-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCAATTCACAATTTGTCGAT
	C15-5-L-r	TACAGGTCAATGAAAATGCG
C16-1	C16-1-R-f	CACCAAAGGCAAAGAAACTG
	C16-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCATGCCCTTGAACTATGGACC
C16-2	C16-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAGAACAGGTGAGTCAGAAGA
	C16-2-L-r	GTGGATCTTGTGGTTGTCCG
	C16-2-R-f	CATGGATGCTAATCCACTGT
	C16-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGCTAGACATGGTTGAAAATG
C16-3	C16-3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGTCTTGACTGCTGCTTCTTG
	C16-3-L-r	GTAAAGCCATGTTTGATACC
	C16-3-R-f	TAGCCAGAACTTAAGTCAGG
	C16-3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGTTGGTACCCCAAATTATTC
C16-4	C16-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCACGGGTTTCTAGACAGCGAA
	C16-4-L-r	TGCGGCAAATTTTTCTGTGC
	C16-4-R-f	CATCGATTCTAGTCAAGAAG
	C16-4-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCATCAGCCGTTTCACTCAGGT

Region name	Primer name	Nucleotide sequences (5'-3')
C16-5	C16-5-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGAAATTAGACTTGGTACTGG
	C16-5-L-r	CATCCCGACTGATGGTGTAG

Table 6. Primers used for construction of segmental chromosome duplications of sub-regions of unduplicated regions

Region name	Primer name	Nucleotide sequences (5'-3')
C4-2-S1	C4-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGTGCTCTTCTTGTTAACCCC
	C4-2-L-r	GGCCGCAATTGACGACACAC
	4-2-s1-R-f	AGGAACGCTGATCTTGATCT
	4-2-s1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCACTCTTGTATCCCACACAGG
C4-2-S2	4-2-s2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGAAGTCTTTGCATCCGTGG
	4-2-s2-L-r	ACCATCGGAGGGACTTTGA
	4-2-s2-R-f	TTCGTTCCTCAGCGGTGTGT
	4-2-s2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAAGCTGCCAACTACCGTCAG
C4-2-S3	4-2-s3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAGATTTTGTAGTGCTACGGA
	4-2-s3-L-r	AAAGGCTCTACACTCCCAGC
	4-2-s3-R-f	CTACACGGAAGTCAATCTCAC
	4-2-s3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGCGGGTGCAGTCGTGTGCAG
C4-2-S4	4-2-s4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGGCTCTGAACTAGAAACCG
	4-2-s4-L-r	TTCTTGTCTCTGAGAATCGG
	C4-2-R-f	TCTTTTCATTATTGCTAGTA
	C4-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAAAGTAGTTCATGATGCGGG
C4-4-S1	C4-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAGGATTTTAATCTGTTGGAG
	C4-4-L-r	CCAACCAATATTACTGCTTT
	4-4-s1-R-f	CAGAAGACTGAAAGACTGCA
	4-4-s1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCGAATCTTCTCGTCACGGAAG
C4-4-S2	4-4-s2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTCCGTGACGAGAAGATTCGG
	4-4-s2-L-r	AACACTTCACTTTCAAGGCC
	4-4-s2-R-f	GTTGTAGTAATCTCGCGACC
	4-4-s2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCACCAATGGATCGAACGTGAG
C4-4-S3	4-4-s3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGGGAAGTTGCACTAAACGT
	4-4-s3-L-r	CAGATGGAACCAACCTAACC
	4-4-s3-R-f	GAACTGTCTGACTGCCGAAG
	4-4-s3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGCTTGTCACAATTTGCAGA
C4-4-S4	4-4-s4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTAACTCGAAGGGTCACTGCC
	4-4-s4-L-r	CAATACCTACCATTAGCGAC
	C4-4-R-f	CCGACCGAGTATTACTCAGT
	C4-4-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGAGTCATCCATATTGCAAAC
C4-5-S1	C4-5-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCAAAAGTTGCCTGTCCAAA
	C4-5-L-r	GAAGGCAAGGCTTACAGGCT
	4-5-s1-R-f	AGACTATTTTCATTGTTAAT
	4-5-s1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCATTCAATACTTTCACGTGTA
C4-5-S2	4-5-s2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAACATTGTGCGCTCATCTAT
	4-5-s2-L-r	TGATCTAGCAATAATATCAA
	4-5-s2-R-f	AACCAGTGTCCTCGTTAATT
	4-5-s2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTCTAATTTAAGAGATCAGAT
C4-5-S3	4-5-s3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTTAGCCAAAAAGATCAATGT
	4-5-s3-L-r	CTAACATGTGACAATGAATG
	4-5-s3-R-f	CACAGGAATTTCAAGGTAGT
	4-5-s3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGATACTGATCTCCATATAC

Region name	Primer name	Nucleotide sequences (5'-3')
C4-5-S4	4-5-s4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCTACTCATCTTGATTAGTAT
	4-5-s4-L-r	ATCCTATCGTTTCAACTAGA
	C4-5-R-f	TTACGGTGGTTGCAAAGGGA
	C4-5-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAAAGAAGACTTCAATAAGTT
C4-7-S1	C4-7-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCTTTCAATCTTGTCTCTTGC
	C4-7-L-r	GGAGAAACGCATCTAAGAAA
	4-7-s1-R-f	CGGTGAATGGAATGCTGACA
	4-7-s1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGTTGAGCCACTTCCACTTG
C4-7-S2	4-7-s2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAAATGGACCATCGTGGCGAT
	4-7-s2-L-r	GGCTCTATTCTGGCATTTCC
	4-7-s2-R-f	CTGTGTACGAGATTGTGACA
	4-7-s2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTAATGCAAGAGTTGCCAGCG
C4-7-S3	4-7-s3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGCTCTCACATGCTTTTTCTG
	4-7-s3-L-r	CCGAGTGGTTAGCTGCAACT
	4-7-s3-R-f	CTGCGACCGCTTTATTTGAC
	4-7-s3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTAATAACGAGATGTACAGGC
C4-7-S4	4-7-s4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGTATCCTTGCTTGGAAGCAG
	4-7-s4-L-r	ACCGACACCTCCTGCGATAG
	C4-7-R-f	AAGGGGACTTTCAGGTGCAT
	C4-7-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCTCATCGTGTGGCTTAACG
C6-1-S1	SC6-1-R-f	ACGGCACCCTTTGTCAAGAG
	SC6-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAGGGGTGGATATCAACCTAC
C6-1-S2	SC6-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGTGTAGTTGCCTTCTTCACC
	SC6-2-L-r	CAAAGTCATGGGCTTCCCAG
	C6-1-R-f	ACGGTGCGCTCCAACGGATG
	C6-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTCCTTCCGATTCTGAAGGTG
C7-4-S1	C7-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAAGAAACTTCTCCAGAGGAG
	C7-4-L-r	CCGCCAAGAAGAGACGTAAA
	7-4-s1-R-f	GCGTAATTCACGGCGATAAC
	7-4-s1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCTGATTCATGGGCCTCACGC
C7-4-S2	7-4-s2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCAGTGATGTCGGAAACATCG
	7-4-s2-L-r	TTGTCTCACATCTGCATCTG
	7-4-s2-R-f	GCGTTTACCAATACTGGAATC
	7-4-s2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCGCGCTCCTTTGTAGTGCCG
C7-4-S3	7-4-s3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCTTACTGCGCAAGTGGCTCG
	7-4-s3-L-r	ATTGAACCTGACAGAAGCTG
	7-4-s3-R-f	TGTGCCGGCAGAATGTCGCG
	7-4-s3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGTGTACCTATCAGGCCGCTG
C7-4-S4	7-4-s4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGGCTCATTGGACACAACCT
	7-4-s4-L-r	ACCTTAAACGGCTGAACAGG
	C7-4-R-f	TAATTACTTCGGTCGTGGCC
	C7-4-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGCTTTACTTAGTATGTCGGG
C8-2-S1	C8-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCTCGTACATTGACTCAAACC
	C8-2-L-r	AGATTATGCACCTATCGGCG
	8-2-s1-R-f	TATCACAAAAGCCCTCCATC
	8-2-s1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCACCGGCAATATGTCCTGCTTC
C8-2-S2	8-2-s2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGATCCGAGTTTGAAACATCC
	8-2-s2-L-r	ACTGAATAGAAAGCGCTCT
	8-2-s2-R-f	TAAGTGATCACGTGGTCAGA
	8-2-s2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTAGGAACTTCCTTTAGCTGG
C8-2-S3	8-2-s3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCAAAGTAGGAACAGTGCCCG
	8-2-s3-L-r	GCCCTATTGAAGGTGAAGCC
	8-2-s3-R-f	CCCTTCCACCATCATTAC
	8-2-s3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGAAATTCGATGTTCAGGAG

Region name	Primer name	Nucleotide sequences (5'-3')
C8-2-S4	8-2-s4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAGGGTTCAGGAAAATTGCGG
	8-2-s4-L-r	CCTTTCACCAACGTACTCGA
	C8-2-R-f	AATCACCAGAAGCAGCAGCA
	C8-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGCAGCAAGGTTGCCTTTAA
C11-2-S1	C11-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGACTCTAAAACGGCATTTG
	C11-2-L-r	AAAGGGTTAAAGCAATCTCG
	SC11-1-R-f	CCCACATTGGTGTTCAAATG
	SC11-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGCTCGTACCATAGACCTGG
C11-2-S2	SC11-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAAAGTAACGTCTCTGTTCGG
	SC11-2-L-r	GCAAAGTTACAGAACCGGTG
	SC11-2-R-f	GGGCATTGTTCAACATAGGG
	SC11-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTTTAACAGCTGAGCTGAACG
C11-2-S3	SC11-3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCATTTGAAACCGAGTTTGCGG
	SC11-3-L-r	GTTGATTACTGTCGATTCTG
	SC11-3-R-f	TGTCAAACTGCCAAGACGAC
	SC11-3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCACTTCCTTGTCCAGTATGGC
C11-2-S4	SC11-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCTCCAGGATTTTTTTGGCA
	SC11-4-L-r	ACTTTAGGCAAGGTTGTTGC
	C11-2-R-f	TGGCTTTGAAGAGAAGTCCT
	C11-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTATCGCTAAACAGTTCTTCC
C14-2-S1	C14-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGCACTGGAAATGCTTTTGG
	C14-2-L-r	AGTGCTCTACTGTCCGAGTC
	SC14-1-R-f	GGATGATCTGCCGATTTAGG
	SC14-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCTCCTGGAGCTCTTCTAAT
C14-2-S2	SC14-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGATTACGCGTCACAGCTAC
	SC14-2-L-r	ACCCTCAAGTCCTCCCTTGA
	SC14-2-R-f	TCTTCGAGGGGAAAATGTCG
	SC14-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTAGTTTGAGCCAGCACGATG
C14-2-S3	SC14-3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCGGATAAATATTCTTCGAGGGG
	SC14-3-L-r	AGCACGATGGCAGGCCCTTA
	SC14-3-R-f	AGAAGATCTCGTTCATGACTGC
	SC14-3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGTGTCGCCTTAATAGTCAGC
C14-2-S4	SC14-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTCGTCTTGCCGTATCTACAT
	SC14-4-L-r	GACCCAGATAGTGATGCTGA
	C14-2-R-f	GAGTCAACATTATAGGGCTG
	C14-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCACGAAACTGTCGGGTTATCA
C4-2-S3+S4	4-2-s3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAGATTTTGTAGTGCTACGGA
	4-2-s3-L-r	AAAGGCTCTACACTCCCAGC
	C4-2-R-f	TCTTTTCATTATTGCTAGTA
	C4-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAAAGTAGTTCATGATGCGGG
C4-4-S2+S3	4-4-s2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTCCGTGACGAGAAGATTCGG
	4-4-s2-L-r	AACACTTCACTTTCAAGGCC
	4-4-s3-R-f	GAACTGTCTGACTGCCGAAG
	4-4-s3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGCTTGTCACAATTTGCAGA
C4-7-S3+S4	4-7-s3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGCTCTCACATGCTTTTTCTG
	4-7-s3-L-r	CCGAGTGGTTAGCTGCAACT
	C4-7-R-f	AAGGGGACTTTCAGGTGCAT
	C4-7-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCTCATCGTGTGGCTTAACG
C8-2-S3+S4	8-2-s3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCAAAGTAGGAACAGTGCCCG
	8-2-s3-L-r	GCCCTATTGAAGGTGAAGCC
	C8-2-R-f	AATCACCAGAAGCAGCAGCA
	C8-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGCAGCAAGGTTGCCTTTAA

Region name	Primer name	Nucleotide sequences (5'-3')
C11-2-S1+S2	C11-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGACTCTAAAACGGCATTTG
	C11-2-L-r	AAAGGGTTAAAGCAATCTCG
	SC11-2-R-f	GGGCATTGTTCAACATAGGG
	SC11-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTTTAACAGCTGAGCTGAACG
C14-2-S3+S4	SC14-3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCGGATAAATATTCTTCGAGGGG
	SC14-3-L-r	AGCACGATGGCAGGCCCTTA
	C14-2-R-f	GAGTCAACATTATAGGGCTG
	C14-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCACGAAACTGTCGGGTTATCA

3.2.3 Yeast transformation, analysis of karyotype and mitotic stability of chromosome

Yeast cells were transformed according to the method of Gietz and Schiestl (Gietz and Schiestl, 2005). For a selection of yeast transformants, cells were cultured in SC medium without leucine, or without leucine and histidine, or without leucine and uracil at 30°C for 4 days.

To analyse karyotype of transformants by PFGE, chromosome DNA plugs were prepared according to the method of Sheehan and Weiss (Sheehan and Weiss, 1990). Chromosomes were separated on 1% (wt vol^{-1}) pulsed-field gel electrophoresis gels in 0.5× TBE buffer at 14°C using the CHEF DRIII® System (Bio-Rad Laboratories), with a 60-second pulse for 15 hours, followed by a 90-second pulse for 9 hours, at 6 V cm^{-1}. For Southern blot analysis, the specific probes were amplified by primers listed in Table 7 and 8. The procedure for determination of mitotic stability has been described in Chapter 2.

Table 7. Primers used to amplify probes for detection of segmental chromosome duplications of chromosomes I to XVI

Region name	Primer name	Nucleotide sequences (5'-3')
C1-1	C1-1-p-f	TTTTCCGGACCCAAACAACC
	C1-1-p-r	TCTGTGGAGACCAATCGAGG
C1-2	C1-2-p-f	GCCAGTGTAACTCCTCACTG
	C1-2-p-f	AGAACCAGGCCTTCCACTTT

Region name	Primer name	Nucleotide sequences (5'-3')
C2-1	C2-1-R-f	TTACATGCGACACCAAGCAG
	C2-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGTCCTCCGAGGCAGGCCCTC
C2-2	C2-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGAATGCAATTCGATACTCG
	C2-2-L-r	CAATCCAGTGATACCCGTGG
C2-3	C2-3-R-f	TCTCTGAGGGTTATCAAATG
	C2-3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCGTGTGATGTGGACTGTTGC
C2-4	C2-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCTAACCCTTTGATGTCCGAC
	C2-4-L-r	CTTTTCTTCCCTCCAAGATC
C3-1	C3-1-p-f	GCAAGACTCTGGTCTCTTCT
	C3-1-p-r	ACACCTGAGTGGGTCATCAC
C3-2	C3-2-p-f	CTCTTAGCGGACCGTTTTGG
	C3-2-p-r	ATCTCTCCGCAGGGGTAAGC
C4-1	C4-1-R-f	AGGGCATCCATCCAACCATC
	C4-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGCTTTGGAGGAGATATTTG
C4-2	C4-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGTGCTCTTCTTGTTAACCCC
	C4-2-L-r	GGCCGCAATTGACGACACAC
C4-3	C4-3-R-f	TCGAGGACAAAAAGGCATAT
	C4-3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGAGAATAAAATAGGTCAGGT
C4-4	C4-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAGGATTTTAATCTGTTGGAG
	C4-4-L-r	CCAACCAATATTACTGCTTT
C4-5	C4-5-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCAAAAGTTGCCTGTCCAAA
	C4-5-L-r	GAAGGCAAGGCTTACAGGCT
C4-6	C4-6-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGTTGACTTGACATACACTAA
	C4-6-L-r	AGGTTAGGACAGGGTACCAT
C4-7	C4-7-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCTTTCAATCTTGTCTCTTGC
	C4-7-L-r	GGAGAAACGCATCTAAGAAA
C4-8	C4-8-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGAAGATTTTAAACTCACCT
	C4-8-L-r	CGGCCTTATTATGATCCCGA
C5-1	C5-1P-f	ATAAAGCAGCTGAACTTTCC
	C5-1P-r	CATTTTCGTTGTGGGCACAC
C5-2	C5-2P-f	CATATATCAGAGTCACAGCT
	C5-2P-r	CCACCACCCAAAAGAGTGTC
C5-3	C5-3P-f	TGAAGTGTGGAATCTGTCTC
	C5-3P-r	TGGAGGAGATGATGAAGCAA
C6-1	C6-1-p-f	CCACTCGTTGCCGGAGGCAC
	C6-1-p-r	GAACCCTGGCGACTTTTGGA
C6-2	C6-2-p-f	ACGAGCCCTTGACTGAGCAG
	C6-2-p-r	AAGACCGCCTCCAGCAGTTG
C7-1	C7-1-R-f	CGGTTGTATGATATAGATCC
	C7-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGCCCAATCGAGCAAATAAG
C7-2	C7-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGCCGAACGTGTACCCGTAAA
	C7-2-L-r	CGCACCATTACAGGGTCAAA
C7-3	C7-3-R-f	CCTACTTGGCGGTGAATTTC
	C7-3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGATAAGCCCAATACACGACA
C7-4	C7-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAAGAAACTTCTCCAGAGGAG
	C7-4-L-r	CCGCCAAGAAGAGACGTAAA
C7-5	C7-5-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCAATTTGTGGGATGATGACG
	C7-5-L-r	CAAGTCAGATAGCTTTGAGT
C8-1	C8-1 probe-f	TGGATGGTGCATTCTTAGAG
	C8-1 probe-r	TGGGTAAGGAAATGAGAGCA
C8-2	C8-2 probe-f	CACAATCACCGAGCGTCTTT
	C8-2 probe-r	ATATGTGACCAATGCGGGAT

Region name	Primer name	Nucleotide sequences (5'-3')
C8-3	C8-3 probe-f	CCTACAGAGCGTGAAATGCA
	C8-3 probe-r	CGACTCATCGAAGGTTCATA
C9-1	C9-1 probe-f	GGTGTTGTAAACCCCTCAAG
	C9-1 probe-r	ATAACCTTGCCGTCAATGTC
C9-2	C9-2 probe-f	TCAGCAGATTCGATGGATGC
	C9-2 probe-r	GACGAATTCATCAAGACGCA
C10-1	C10-1 probe-r	GTAAAATCGATGAGTGGGGA
	C10-1 probe-f	CAGCACAACGCTCTAACATA
C10-2	C10-2 probe-r	TGACTGACGAATCGTTAGGC
	C10-2 probe-f	CTTGCGATTTCTTCGTATGC
C10-3	C10-3 probe-f	GGGAAACTGCATGTAGTTGT
	C10-3 probe-r	ATACCCGGAAGACAGAATCG
C10-4	C10-4 probe-f	GTCGTTCGGCGAAACCTTAT
	C10-4 probe-r	CAACAGTCGTAGCTAACGAG
C11-1	C11-1P-f	AGATACAGCCTGTTGACCAA
	C11-1P-r	ACCAAACGCGTTTGGCAATA
C11-2	C11-2P-f	GACGAGAATAACCAAGGGCA
	C11-2P-r	GGAGTTGCTTTGTTTTGTTC
C11-3	C11-3P-f	GGCTACAAGAAACTTCGTGC
	C11-3P-r	TCGACATGTGTCCTCCATGT
C12-1	C12-1-R-f	ATGGATAGGTTTCGAGGGCA
	C12-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCGGTAACGTCAACAGTGGTA
C12-2	C12-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAATCCAAGAAGGAACCTGCG
	C12-2-L-r	CATAACGGTGCAAATACGTA
C12-3	C-12-3-R-f	CCTAACGACGATGATAATAC
	C12-3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTCTTGGAGACGTGTTCAGAA
C12-4	C12-4-R-f	GGAAAACGAAGAGCAGCAGC
	C12-4-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGCTATTATCCAGATGAAGGA
C12-5	C12-5-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCGATAAGCTAAGCCATTTTC
	C12-5-L-r	AGAAGACAACCCGTGGCTTG
C13-1	C13-1-R-f	GCCTCTATAGGCTTTTCGGA
	C13-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGTAGCACCTACTTCTCATC
C13-2	C13-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCAGCATTTTGTTATTGGCG
	C13-2-L-r	CCAGTATGTTCCCTTGACAA
C13-3	C13-3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGACGACAGCCTGAATAATT
	C13-3-L-r	CTCTGATTTCAATGTCGTCT
C13-4	C13-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGTCTAAAGTCATCCACATG
	C13-4-L-r	AACAGTACTGGGATAGAAGG
C13-5	C13-5-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGAACTTACTTTCCTCTCTGC
	C13-5-L-r	AGAATTTCGAAGGAAAGGGG
C14-1	C14-p1-f	AACAATGGCACTCATGCAGC
	C14-p1-r	CAGCGCTTCCACGGCATACC
C14-2	C14-p2-f	GACACGTAATCGGAGTTTGC
	C14-p2-r	GCAGTAGGTAAAACGTCACT
C14-3	C14-p3-f	TGGTAACTCTGTTGAAGACG
	C14-p3-r	GCCGAAGAACAAGAGAAAGC
C14-4	C14-p4-f	CAAAGTAGCAAGGTAATCGG
	C14-p4-r	ACGATATCATCGGTTCGCTG
C15-1	C15-1-R-f	CACCAGGTATTTGCCAATGG
	C15-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCACTTTGCGTAACGCCAAA
C15-2	C15-2-R-f	GTGAGGGATGTCAGTTACTC
	C15-2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCGTCTGAAGCCAATTGAGTG

Region name	Primer name	Nucleotide sequences (5'-3')
C15-3	C15-3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGCTTACTTAGTCCTTTGGTC
	C15-3-L-r	GCTTTTCCAATAAAGACGCA
C15-4	C15-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGCGTTATTGAGTGAACCGTC
	C15-4-L-r	CAGATGGTGCAGCCAATAGA
C15-5	C15-5-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCAATTCACAATTTGTCGAT
	C15-5-L-r	TACAGGTCAATGAAAATGCG
C16-1	C16-1-R-f	CACCAAAGGCAAAGAAACTG
	C16-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCATGCCCTTGAACTATGGACC
C16-2	C16-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAGAACAGGTGAGTCAGAAGA
	C16-2-L-r	GTGGATCTTGTGGTTGTCCG
C16-3	C16-3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGTCTTGACTGCTGCTTCTTG
	C16-3-L-r	GTAAAGCCATGTTTGATACC
C16-4	C16-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCACGGGTTTCTAGACAGCGAA
	C16-4-L-r	TGCGGCAAATTTTTCTGTGC
C16-5	C16-5-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGAAATTAGACTTGGTACTGG
	C16-5-L-r	CATCCCGACTGATGGTGTAG

Table 8. Primers used to amplify probes for detection of segmental chromosome duplication of sub-regions

Region name	Primer name	Nucleotide sequences (5'-3')
C4-2-S1	4-2-s1-R-f	AGGAACGCTGATCTTGATCT
	4-2-s1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCACTCTTGTATCCCACACAGG
C4-2-S2	4-2-s2-R-f	TTCGTTCCTCAGCGGTGTGT
	4-2-s2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAAGCTGCCAACTACCGTCAG
C4-2-S3	4-2-s3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAGATTTTGTAGTGCTACGGA
	4-2-s3-L-r	AAAGGCTCTACACTCCCAGC
C4-2-S4	4-2-s4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGGCTCTGAACTAGAAACCG
	4-2-s4-L-r	TTCTTGTCTCTGAGAATCGG
C4-4-S1	C4-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAGGATTTTAATCTGTTGGAG
	C4-4-L-r	CCAACCAATATTACTGCTTT
C4-4-S2	4-4-s2-R-f	GTTGTAGTAATCTCGCGACC
	4-4-s2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCACCAATGGATCGAACGTGAG
C4-4-S3	4-4-s3-R-f	GAACTGTCTGACTGCCGAAG
	4-4-s3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGCTTGTCACAATTTGCAGA
C4-4-S4	C4-4-R-f	CCGACCGAGTATTACTCAGT
	C4-4-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGAGTCATCCATATTGCAAAC
C4-5-S1	4-5-s1-R-f	AGACTATTTTCATTGTTAAT
	4-5-s1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCATTCAATACTTTCACGTGTA
C4-5-S2	4-5-s2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAACATTGTGCGCTCATCTAT
	4-5-s2-L-r	TGATCTAGCAATAATATCAA
C4-5-S3	4-5-s3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTTAGCCAAAAAGATCAATGT
	4-5-s3-L-r	CTAACATGTGACAATGAATG
C4-5-S4	4-5-s4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCTACTCATCTTGATTAGTAT
	4-5-s4-L-r	ATCCTATCGTTTCAACTAGA
C4-7-S1	C4-7-S1	CGGTGAATGGAATGCTGACA
	4-7-s1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGTTGAGCCACTTCCACTTG

Region name	Primer name	Nucleotide sequences (5'-3')
C4-7-S2	4-7-s2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAAATGGACCATCGTGGCGAT
	4-7-s2-L-r	GGCTCTATTCTGGCATTTCC
C4-7-S3	4-7-s3-R-f	CTGCGACCGCTTTATTTGAC
	4-7-s3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTAATAACGAGATGTACAGGC
C4-7-S4	4-7-s4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGTATCCTTGCTTGGAAGCAG
	4-7-s4-L-r	ACCGACACCTCCTGCGATAG
C6-1-S1	SC6-1-p-f	GGAAGATGGATGCCCTTGTT
	SC6-1-p-r	ACTTCCAGACAACACAGGGG
C6-1-S2	SC6-2-p-f	ACTTCCAGACAACACAGGGG
	SC6-2-p-r	GAGCAGCTCTTCTGTTTCTC
C7-4-S1	C7-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAAGAAACTTCTCCAGAGGAG
	C7-4-L-r	CCGCCAAGAAGAGACGTAAA
C7-4-S2	7-4-s2-R-f	GCGTTTACCAATACTGGAATC
	7-4-s2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCGCGCTCCTTTGTAGTGCCG
C7-4-S3	7-4-s3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCTTACTGCGCAAGTGGCTCG
	7-4-s3-L-r	ATTGAACCTGACAGAAGCTG
C7-4-S4	C7-4-R-f	TAATTACTTCGGTCGTGGCC
	C7-4-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGCTTTACTTAGTATGTCGGG
C8-2-S1	8-2-s1-R-f	TATCACAAAAGCCCTCCATC
	8-2-s1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCACCGGCAATATGTCCTGCTTC
C8-2-S2	8-2-s2-R-f	TAAGTGATCACGTGGTCAGA
	8-2-s2-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTAGGAACTTCCTTTAGCTGG
C8-2-S3	8-2-s3-R-f	CCCTTCCACCATCATTAC
	8-2-s3-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCGGAAATTCGATGTTCAGGAG
C8-2-S4	8-2-s4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAGGGTTCAGGAAAATTGCGG
	8-2-s4-L-r	CCTTTCACCAACGTACTCGA
C11-2-S1	SC11-1-R-f	CCCACATTGGTGTTCAAATG
	SC11-1-R-r	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCTGCTCGTACCATAGACCTGG
C11-2-S2	SC11-2-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCAAAGTAACGTCTCTGTTCGG
	SC11-2-L-r	GCAAAGTTACAGAACCGGTG
C11-2-S3	SC11-3-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCATTTGAAACCGAGTTTGCGG
	SC11-3-L-r	GTTGATTACTGTCGATTCTG
C11-2-S4	SC11-4-L-f	CTGCAGCGTACGAAGCTTCAGCTGGCGGCCCCTCCAGGATTTTTTTGGCA
	SC11-4-L-r	ACTTTAGGCAAGGTTGTTGC
C14-2-S1	SC14-1-p-f	GAATACTGGCCTCTGCCTCA
	SC14-1-p-r	AGCGCTGGATACAGAAACGT
C14-2-S2	SC14-2-p-f	CGGAAGTGGTATCCGAACCA
	SC14-2-p-r	CAGATCGGTAAAGGAGACGG
C14-2-S3	SC14-3-p-f	GACTCCCAATGCGATAAACC
	SC14-3-p-r	GGAGATAACCCAGCGGTCTT
C14-2-S4	SC14-4-p-f	GTTGTGACGAAGTGTGTAGG
	SC14-4-p-r	AGGAAGTCCCTGCGAGATCA

3.2.4 Phenotypic analysis under stress conditions

Yeast cells were cultured in appropriate selective media overnight at 30°C. Next day, aliquots of the cell cultures were transferred into fresh selective media and incubated at 30°C

until cell numbers reached the log phase. The cells were then harvested, re-suspended in sterile water, diluted to a concentration of 0.25 x 10^6 cells μl^{-1} and further serially diluted by 1:10. After that, 4 µl aliquots of each cell dilution were spotted onto different plates: YPAD medium supplemented with 4% (wt vol^{-1}), 5% (wt vol^{-1}) and 6% (wt vol^{-1}) lactic acid (pH 2.8, pH 2.7 and pH 2.6, respectively), 4% (vol vol^{-1}), 6% (vol vol^{-1}) and 8% (vol vol^{-1}) ethanol, 0.41% (wt vol^{-1}), 0.44% (wt vol^{-1}) and 0.47% (wt vol^{-1}) sulfuric acid (pH 2.4, pH 2.3 and pH 2.2, respectively), 36 mM formic acid (pH 4.0), 80 mM acetic acid (pH 4.2), 1.2 M NaCl, pH 9 (adjusted by NaOH) and YPA (1% (wt vol^{-1}) yeast extract, 2% (wt vol^{-1}) bacto peptone and 0.04% (wt vol^{-1}) adenine) with 3% (vol vol^{-1}) glycerol (YPEG). The plates were incubated at 30°C. For the temperature stress experiment, cells were incubated on YPAD medium at 13°C, 30°C and 41°C. All plates were incubated for 3-4 days and photographed. Three replicates were carried out for each experiment.

3.2.5 Elimination of the segmentally duplicated chromosome

Yeast strains were cultured in YPAD medium at 39°C for 24 hours and then transferred into fresh medium at an initial OD_{660} of 0.1 followed by culture at 30°C for 24 hours. Approximately 100-200 cells from each cell culture were spread on ten plates of YPAD medium. After incubation at 30°C for 48 hours, the cells were replica plated onto YPAD and appropriate selective media to observe chromosome loss. Colonies that failed to grow on selective media lacking leucine and/or histidine were expected to be those with loss of the segmentally duplicated chromosome during mitotic growth. After confirmation of loss of the segmentally duplicated chromosome by PFGE, serial dilution spot assays were performed to investigate the phenotypes of the segmental aneuploids and the derived strains with the loss of the segmentally duplicated chromosome.

3.3 Results

3.3.1 Genome-wide construction of segmental duplications by PCDup

Following the confirmation of the reliability of the method and the limitation on the size of the duplicated segment, I attempted to construct a complete library of approximately 200 kb fragments that covered the whole *S. cerevisiae* genome. On the basis of nucleotide sequence information in the *Saccharomyces* Genome Database (SGD) (http://www.yeastgenome.org), I designed primers to amplify DNA duplicating modules used for duplication of approximately 200 kb chromosomal regions of each chromosome in a systematic manner (Fig. 5). I designated strains with a segmental duplication of a chromosome region as ScDup(Cx-y): Sc represents *S. cerevisiae*; Dup represents duplication; and (Cx-y) indicates the chromosome number (Cx) and region (-y). I modified the duplication procedure for the three smallest chromosomes as follows; a 100-kb region and a 130-kb region for chromosome I (230 kb), a 158-kb region and a 159-kb region for chromosome III (317 kb) and a 100-kb region and a 171-kb region for chromosome VI (271 kb). The chromosomal region containing the ribosomal DNA cluster (ca. 1500 kb) on chromosome XII was not included in this study. The nucleotide positions of each duplicated region and other details are presented in Table 9.

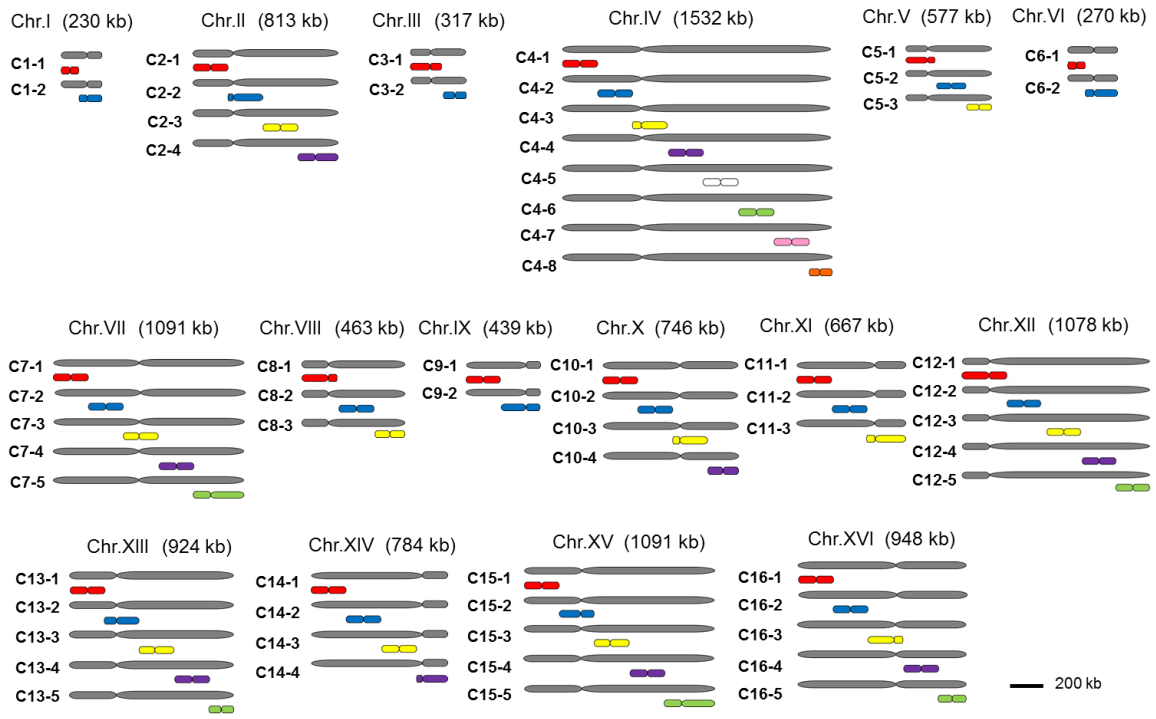


Figure 5. Systematic segmental duplication of chromosomes I to XVI. (**a**) Schematic illustration of a complete set of 62 segmental aneuploid strains covering the whole genome of *S. cerevisiae*. Each chromosome was divided into approximately 200 kb regions and were attempted to duplicate these using the PCDup method.

Analyses of the duplicated regions revealed that 53 out of 62 designated regions were duplicated with desired karyotype with a proportion of 3% to 100% of analyzed transformants (Table 9 and Supplementary Fig. 1). The proportion of desired karyotype in analyzed transformants from 31 terminal regions (54% ± 0.24 s.d.) was higher than those from 22 internal chromosomal regions (19% ± 0.23 s.d.). This difference likely reflected the fact that only one homologous recombination event was required for duplication of the terminal regions. All data of the karyotypes of the segmental aneuploids that was confirmed using PFGE and Southern blots are shown in Figure 6. All of the karyotypic analysis showed the presence of the expected karyotype. Interestingly, remaining 9 designated regions could not be duplicated, i.e., C4-2, C4-4, C4-5, C4-7, C6-1, C7-4, C8-2, C11-2 and C14-2 (Table 9). The possible reason of these results was further analysed in the next section.

Table 9. Characteristics of a complete collection of overlapping segmental aneuploids of chromosomes I to XVI

Region	Strain name	Duplicated region[a]	Plasmid template[b]	Duplication length (kb)	Number of genes	Transformants (n)	Proportion of desired karyotype[c]	% Mitotic stability
C1-1	ScDup(C1-1)	Chr. I 1-100,705	p3122	100	65	7	71% (5/7)	98%
C1-2	ScDup(C1-2)	Chr. I 99,603-230,218	p3008	130	85	16	50% (8/16)	99%
C2-1	ScDup(C2-1)	Chr. II 1-202,750	p3122	200	137	70	67% (6/9)	98.57%
C2-2	ScDup(C2-2)	Chr. II 201,029-401,862	p3008, p3009	200	128	6	17% (1/6)	100%
C2-3	ScDup(C2-3)	Chr. II 400,204-600,988	p3009, p3122	200	124	25	5% (1/22)	99.79%
C2-4	ScDup(C2-4)	Chr. II 599,536-813,184	p3122	213	142	29	100% (9/9)	100%
C3-1	ScDup(C3-1)	Chr. III 1-158,020	p3008	158	139	4	75% (3/4)	99%
C3-2	ScDup(C3-2)	Chr. III 157,543-316,620	p3122	159	110	5	20% (1/5)	100%
C4-1	ScDup(C4-1)	Chr. IV 1-200,732	p3122	200	119	56	78% (7/9)	97.70%
C4-2[#]	ScDup(C4-2)	Chr. IV 198,996-401,638	p3009, p3122	200	128	219	0% (0/219)	ND
C4-3	ScDup(C4-3)	Chr. IV 399,987-600,688	p3008, p3009	200	140	5	20% (1/5)	100%
C4-4[#]	ScDup(C4-4)	Chr. IV 599,793-795,723	p3009, p3122	200	114	134	0% (0/134)	ND
C4-5[#]	ScDup(C4-5)	Chr. IV 795,193-1,000,877	p3009, p3122	200	133	22	0% (0/22)	ND

Region	Strain name	Duplicated region[a]	Plasmid template[b]	Duplication length (kb)	Number of genes	Transformants (n)	Proportion of desired karyotype[c]	% Mitotic stability
C4-6	ScDup(C4-6)	Chr. IV 999,134-1,199,697	p3009, p3122	200	121	13	8% (1/13)	99.56%
C4-7[#]	ScDup(C4-7)	Chr. IV 1,198,183-1,402,247	p3009, p3122	200	134	27	0%(0/27)	ND
C4-8	ScDup(C4-8)	Chr. IV 1,400,770-1,531,933	p3122	130	89	41	89% (8/9)	100%
C5-1	ScDup(C5-1)	Chr. V 1-199,519	p3008	200	146	17	80% (8/10)	100%
C5-2	ScDup(C5-2)	Chr. V 197,812-400,060	p3009, p3122	200	143	5	20% (1/5)	99.77%
C5-3	ScDup(C5-3)	Chr. V 398,496-576,874	p3122	177	127	5	20% (1/5)	100%
C6-1[#]	ScDup(C6-1)	Chr. VI 1-98,498	p3122	100	57	24	0% (0/24)	ND
C6-2	ScDup(C6-2)	Chr. VI 98,213-270,161	p3008	171	128	8	50% (4/8)	100%
C7-1	ScDup(C7-1)	Chr. VII 1-201,147	p3122	200	125	14	57% (8/14)	100%
C7-2	ScDup(C7-2)	Chr. VII 199,564-398,642	p3009, p3122	200	128	3	67% (2/3)	97.45%
C7-3	ScDup(C7-3)	Chr. VII 397,621-599,626	p3008, p3009	200	154	15	7% (1/15)	100%
C7-4[#]	ScDup(C7-4)	Chr. VII 598,443-801,057	p3009, p3122	200	133	156	0% (0/156)	ND
C7-5	ScDup(C7-5)	Chr. VII 799,553-1,090,940	p3122	290	181	10	60% (6/10)	100%

Region	Strain name	Duplicated region[a]	Plasmid template[b]	Duplication length (kb)	Number of genes	Transformants (n)	Proportion of desired karyotype[c]	% Mitotic stability
C8-1	ScDup(C8-1)	Chr. VIII 1-202,241	p3008	200	146	22	44% (4/9)	100%
C8-2[#]	ScDup(C8-2)	Chr. VIII 203,559-401,907	p3009, p3122	200	140	72	0% (0/72)	ND
C8-3	ScDup(C8-3)	Chr. VIII 400,443-562,643	p3122	160	99	27	44% (4/9)	100%
C9-1	ScDup(C9-1)	Chr. IX 1-203,042	p3122	200	116	31	78% (7/9)	99.68%
C9-2	ScDup(C9-2)	Chr. IX 201,284-439,888	p3008	240	175	11	56% (5/9)	100%
C10-1	ScDup(C10-1)	Chr. X 1-195,892	p3122	200	131	7	29% (2/7)	100%
C10-2	ScDup(C10-2)	Chr. X 195,298-403,454	p3009, p3122	200	130	18	11% (2/18)	100%
C10-3	ScDup(C10-3)	Chr. X 401,881-599,357	p3008, p3009	200	142	6	17% (1/6)	100%
C10-4	ScDup(C10-4)	Chr.X 597,731-745,751	p3122	150	87	12	67% (8/12)	100%
C11-1	ScDup(C11-1)	Chr. XI 1-201,168	p3009, p3122	200	116	6	50% (3/6)	100%
C11-2[#]	ScDup(C11-2)	Chr. XI 199,892-399,750	p3009, p3122	200	133	202	0% (0/100)	ND
C11-3	ScDup(C11-3)	Chr. XI 397,819-666,816	p3008	267	153	58	90% (9/10)	100%
C12-1	ScDup(C12-1)	Chr. XII 1-251,980	p3008	250	146	20	10% (2/20)	99.89%
C12-2	ScDup(C12-2)	Chr. XII 250,272-450,039	p3009, p3122	200	117	9	11% (1/9)	100%

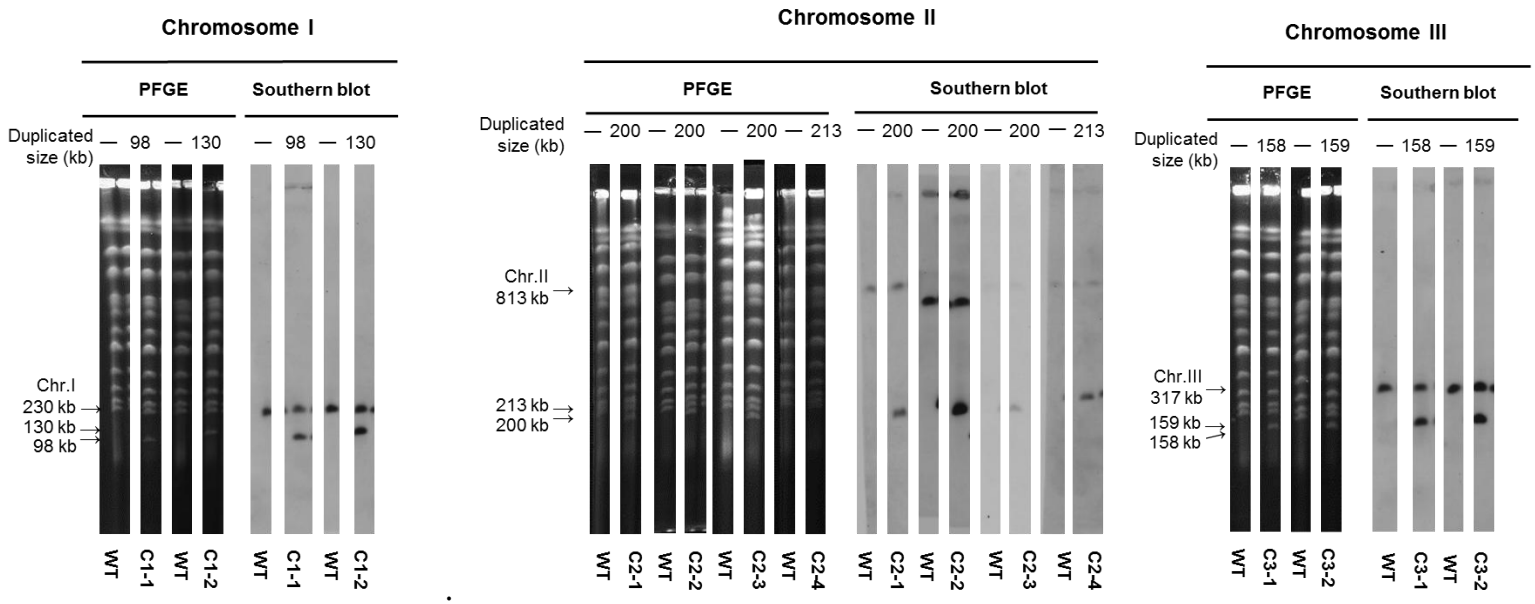
Region	Strain name	Duplicated region[a]	Plasmid template[b]	Duplication length (kb)	Number of genes	Transformants (n)	Proportion of desired karyotype[c]	% Mitotic stability
C12-3	ScDup(C12-3)	Chr. XII 490,862-692,029	p3009, p3122	200	140	11	9% (1/11)	100%
C12-4	ScDup(C12-4)	Chr. XII 690,555-885,764	p3009, p3122	200	139	34	10% (1/10)	99.03%
C12-5	ScDup(C12-5)	Chr. XII 884,258-1,078,177	p3122	200	115	73	70% (7/10)	100%
C13-1	ScDup(C13-1)	Chr. XIII 1-204,690	p3122	200	130	5	20% (1/5)	100%
C13-2	ScDup(C13-2)	Chr. XIII 203,398-402,207	p3008, p3009	200	141	1	100% (1/1)	99.04%
C13-3	ScDup(C13-3)	Chr. XIII 400,538-600,143	p3009, p3122	200	133	33	3% (1/29)	82.02%
C13-4	ScDup(C13-4)	Chr. XIII 598,338-798,915	p3009, p3122	200	120	11	9% (1/11)	100%
C13-5	ScDup(C13-5)	Chr. XIII 797,512-924,441	p3122	120	83	29	60% (6/10)	98.91%
C14-1	ScDup(C14-1)	Chr. XIV 1-200,971	p3122	200	122	21	43% (9/21)	96.67%
C14-2[#]	ScDup(C14-2)	Chr. XIV 199,575-403,514	p3009, p3122	200	132	152	0% (0/152)	ND
C14-3	ScDup(C14-3)	Chr. XIV 401,690-598,530	p3009, p3122	200	130	29	3% (1/29)	99.08%
C14-4	ScDup(C14-4)	Chr. XIV 597,394-784,333	p3008	184	118	7	14% (1/7)	100%
C15-1	ScDup(C15-1)	Chr. XV 1-201,315	p3122	200	125	20	56% (5/9)	99.87%

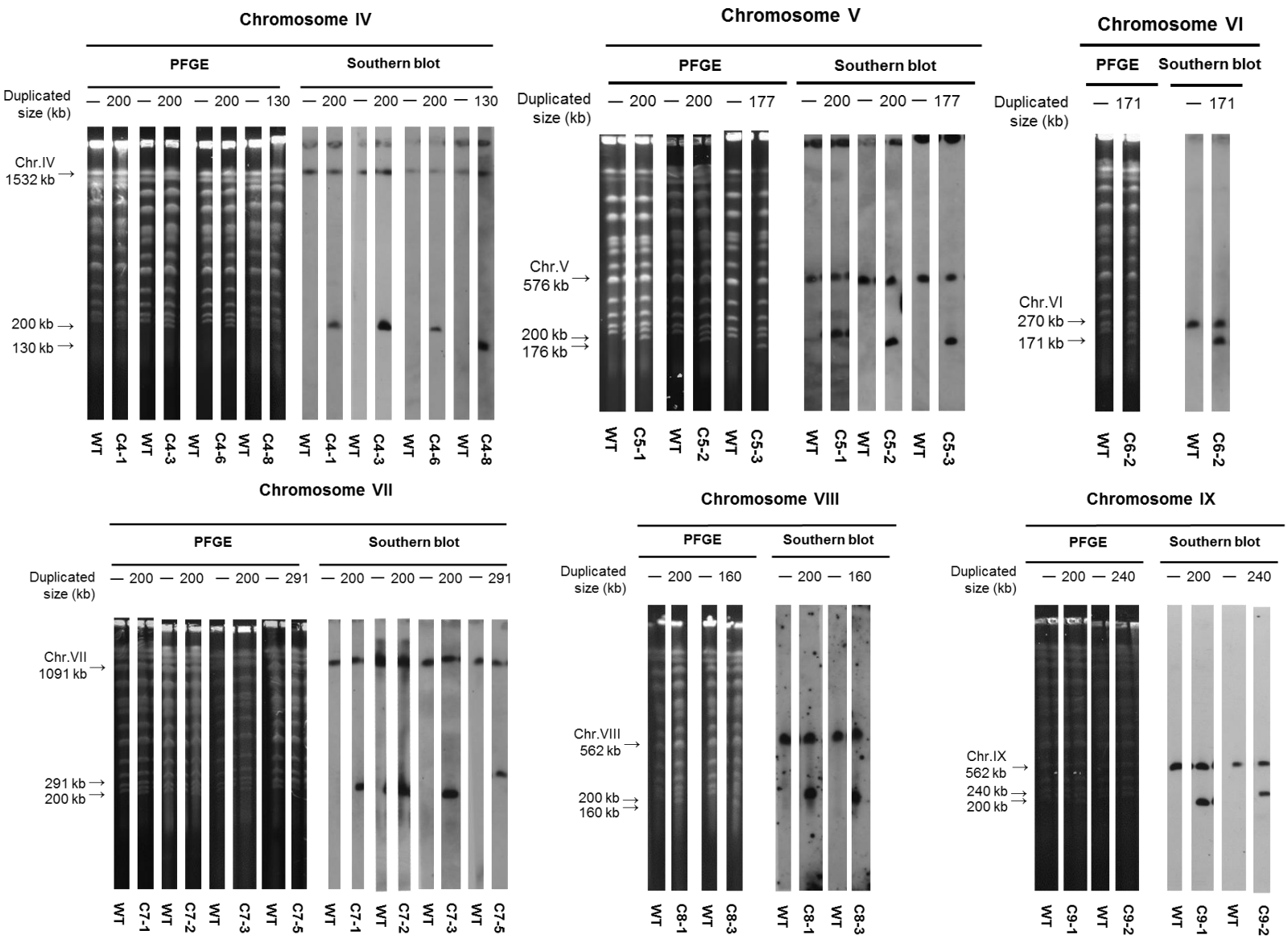
Region	Strain name	Duplicated region[a]	Plasmid template[b]	Duplication length (kb)	Number of genes	Transformants (n)	Proportion of desired karyotype[c]	% Mitotic stability
C15-2	ScDup(C15-2)	Chr. XV 199,377-401,104	p3008, p3009	200	135	17	6% (1/17)	100%
C15-3	ScDup(C15-3)	Chr. XV 399,345-603,357	p3009, p3122	200	128	16	6% (1/16)	99%
C15-4	ScDup(C15-4)	Chr. XV 601,731-801,721	p3009, p3122	200	134	9	11% (1/9)	84.76%
C15-5	ScDup(C15-5)	Chr. XV 799,959-1,091,289	p3122	290	176	67	56% (5/9)	99.45%
C16-1	ScDup(C16-1)	Chr. XVI 1-198,780	p3122	200	124	39	44% (4/9)	99.71%
C16-2	ScDup(C16-2)	Chr. XVI 198,090-399,110	p3009, p3122	200	116	6	17% (1/6)	100%
C16-3	ScDup(C16-3)	Chr. XVI 397,495-597,301	p3008, p3009	200	124	8	13% (1/8)	100%
C16-4	ScDup(C16-4)	Chr. XVI 595,746-799,875	p3009, p3122	200	136	6	17% (1/6)	99.76%
C16-5	ScDup(C16-5)	Chr. XVI 798,248-948,066	p3122	148	112	46	26% (5/19)	100%
YCp50 (7.8 kb)				-				85%

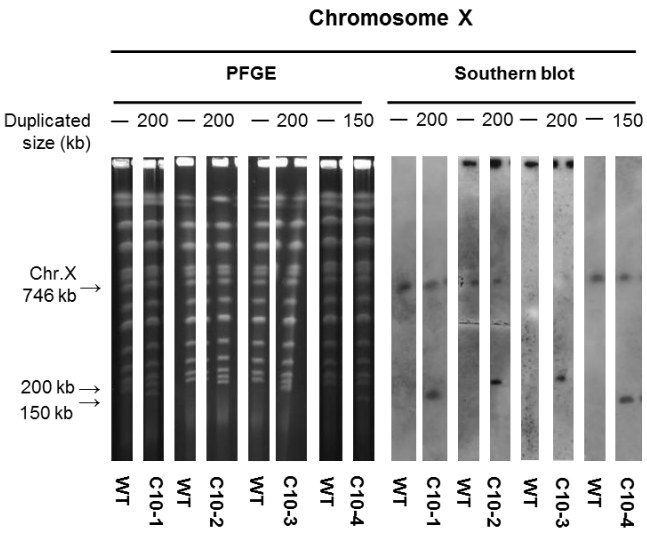
*a: Chr. N x-y : Chr. N represents chromosome number, x represents first nucleotide number of chromosomal region and y represents last nucleotide number of chromosomal region.
b: p3009 was used to amplify the *CgHIS3* cassette, p3122 was used to amplify the *CEN4-CgLEU2* cassette, p3008 was used to amplify the *CgLEU2* cassette and YCp50 was a *URA3* centromeric plasmid whose length was 7.8 kb.
c: Proportion of desired karyotype in analyzed transformants (number of segmental aneuploids / number of candidate transformants that were analyzed for karyotype)
means region that could not be duplicated.
ND. means not determined

Figure 6. Karyotypic analysis of segmental aneuploids for chromosomes I to XVI.

PFGE analysis was performed followed by Southern blot analysis using a probe consisting of nucleotide sequences that corresponded to the target region.




Chromosome IV
PFGE
Southern blot
Duplicated size (kb)
— 200 — 200 — 200 — 130
— 200 — 200 — 200 — 130
Chr.IV 1532 kb
200 kb
130 kb
WT C4-1 WT C4-3 WT C4-6 WT C4-8
WT C4-1 WT C4-3 WT C4-6 WT C4-8
Chromosome V
PFGE
Southern blot
Duplicated size (kb)
— 200 — 200 — 177
— 200 — 200 — 177
Chr.V 576 kb
200 kb
176 kb
WT C5-1 WT C5-2 WT C5-3
WT C5-1 WT C5-2 WT C5-3
Chromosome VI
PFGE
Southern blot
Duplicated size (kb)
— 171
— 171
Chr.VI 270 kb
171 kb
WT C6-2
WT C6-2
Chromosome VII
PFGE
Southern blot
Duplicated size (kb)
— 200 — 200 — 200 — 291
— 200 — 200 — 200 — 291
Chr.VII 1091 kb
291 kb
200 kb
WT C7-1 WT C7-2 WT C7-3 WT C7-5
WT C7-1 WT C7-2 WT C7-3 WT C7-5
Chromosome VIII
PFGE
Southern blot
Duplicated size (kb)
— 200 — 160
— 200 — 160
Chr.VIII 562 kb
200 kb
160 kb
WT C8-1 WT C8-3
WT C8-1 WT C8-3
Chromosome IX
PFGE
Southern blot
Duplicated size (kb)
— 200 — 240
— 200 — 240
Chr.IX 562 kb
240 kb
200 kb
WT C9-1 WT C9-2
WT C9-1 WT C9-2


Chromosome X
PFGE
Southern blot
Duplicated size (kb)
200
200
200
150
200
200
200
150
Chr.X 746 kb
200 kb
150 kb
WT
C-10-1
WT
C-10-2
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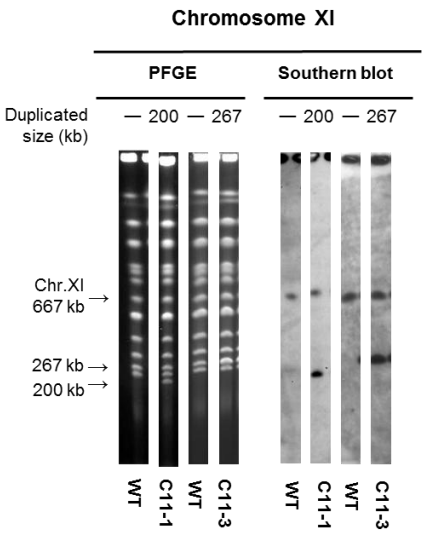
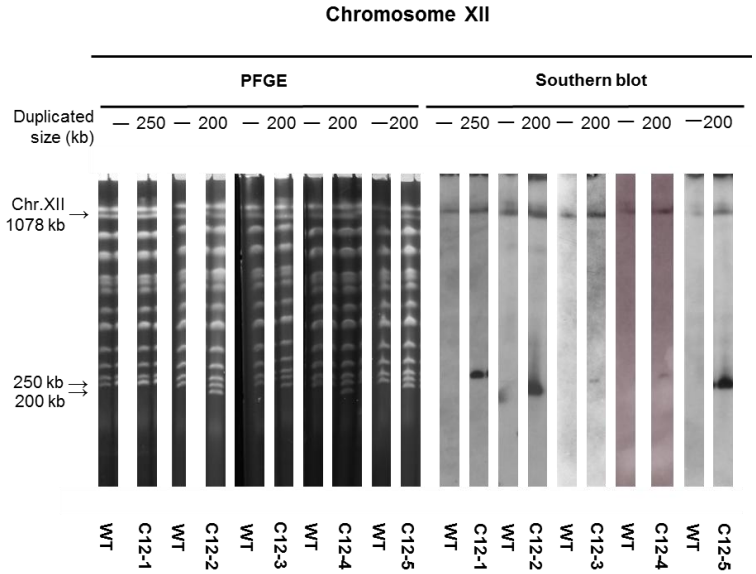
WT
C-10-2
WT
C-10-3
WT
C-10-4


Chromosome XI
PFGE
Southern blot
Duplicated size (kb)
200
267
200
267
Chr.XI 667 kb
267 kb
200 kb
WT
C11-1
WT
C11-3
WT
C11-1
WT
C11-3


Chromosome XII
PFGE
Southern blot
Duplicated size (kb)
250
200
200
200
200
250
200
200
200
200
Chr.XII 1078 kb
250 kb
200 kb
WT
C12-1
WT
C12-2
WT
C12-3
WT
C12-4
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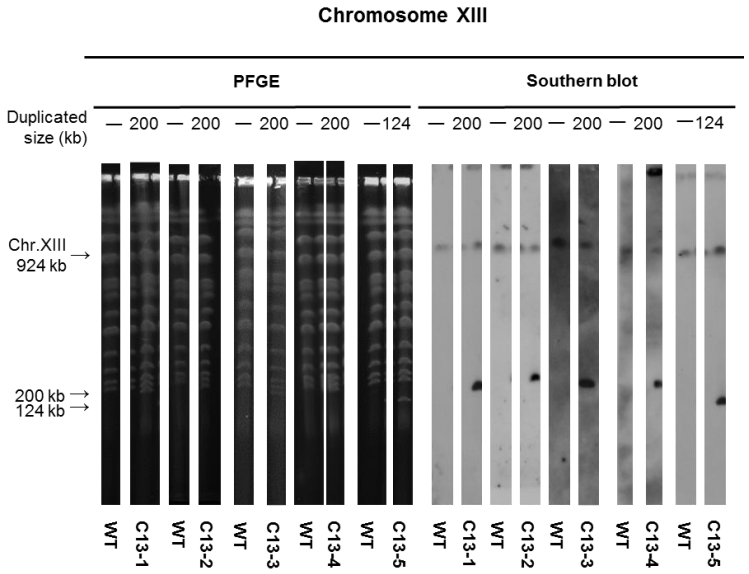
WT
C12-3
WT
C12-4
WT
C12-5


Chromosome XIII
PFGE
Southern blot
Duplicated size (kb)
200
200
200
200
124
200
200
200
200
124
Chr.XIII 924 kb
200 kb
124 kb
WT
C13-1
WT
C13-2
WT
C13-3
WT
C13-4
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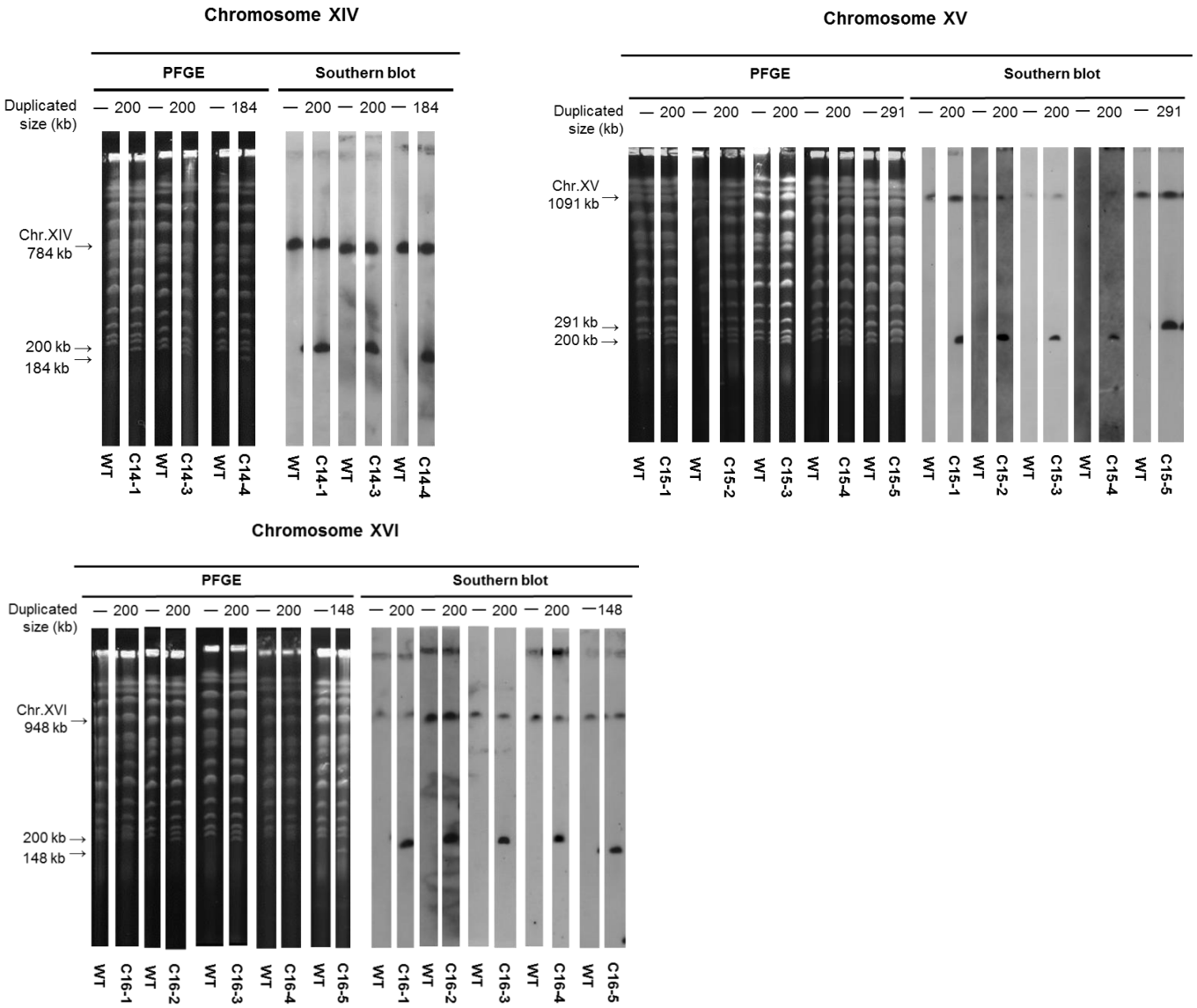
WT
C13-3
WT
C13-4
WT
C13-5


Chromosome XIV
PFGE
Southern blot
Duplicated size (kb)
200 200 184
200 200 184
Chr.XIV 784 kb
200 kb
184 kb
WT C14-1 WT C14-3 WT C14-4
WT C14-1 WT C14-3 WT C14-4
Chromosome XV
PFGE
Southern blot
Duplicated size (kb)
200 200 200 200 291
200 200 200 200 291
Chr.XV 1091 kb
291 kb
200 kb
WT C15-1 WT C15-2 WT C15-3 WT C15-4 WT C15-5
WT C15-1 WT C15-2 WT C15-3 WT C15-4 WT C15-5
Chromosome XVI
PFGE
Southern blot
Duplicated size (kb)
200 200 200 200 148
200 200 200 200 148
Chr.XVI 948 kb
200 kb
148 kb
WT C16-1 WT C16-2 WT C16-3 WT C16-4 WT C16-5
WT C16-1 WT C16-2 WT C16-3 WT C16-4 WT C16-5

3.3.2 Unidentified genes or gene-pairs prevent chromosome duplication

Interestingly, nine of the 62 designated regions of approximately 200 kb could not be duplicated, namely, C4-2, C4-4, C4-5, C4-7, C6-1, C7-4, C8-2, C11-2 and C14-2. To explore this phenomenon, I tried to duplicate these regions after dividing each into 50 kb sub-regions. For C4-5 and C7-4, all 50 kb sub-regions could be duplicated, suggesting that interaction of multiple genes on different 50 kb regions might not allow duplication of the intact 200 kb regions. However, for the remaining seven regions, it was not possible to duplicate one of the four 50 kb sub-regions although the other sub-regions were duplicated. These 50 kb unduplicated regions are including C4-2-S4, C4-4-S2, C4-7-S4, C6-1-S2, C8-2-S3, C11-2-S2, and C14-2-S4 (Table 10). With the exception of C6-1-S2, the 50 kb unduplicated regions did not contain an ARS. It is possible that the duplicating modules did not recombine with its target region but freely replicated in the cell because the duplicating modules in this experiment were prepared by incorporating *H4ARS* with *CgHIS3* and telomere seed sequences. Therefore, I investigated whether a duplicating module with an additional *H4ARS* could recombine with the target site; I attempted to generate C7-4-S4 duplicates that contain an ARS using duplicating modules with *H4ARS* as control experiment. I found that C7-4-S4 could be duplicated even when using duplicating modules with *H4ARS*. Next, I attempted to construct strains with duplication of a 100 kb sub-region, consisted of the 50 kb duplicatable region harboring the resident *ARS* and the adjacent 50 kb unduplicatable region without an *ARS*. These 100 kb sub-regions, designated C4-2-(S3+S4), C4-4-(S2+S3), C4-7(S3+S4), C8-2-(S3+S4), C11-2-(S1+S2) and C14-2-(S3+S4), could not be duplicated, suggesting that the 50 kb unduplicatable sub-region prevented duplication of the 100 kb sub-region (Table 10). These results could be explained if the 50 kb unduplicatable region contained a gene or gene-pairs that induce cell lethality when they are duplicated.

Table 10. Characteristics of duplication of sub-regions in unduplicated regions

Region	Sub-region	Strain name	Chromosome location[a]	Plasmid template[b]	Duplication length (kb)	Number of genes	Transformants (n)	Proportion of desired karyotype[c]
C4-2	S1	ScDup(C4-2-S1)	Chr. IV 198,996-252,217	p3009, p3122	50	31	41	21% (3/14)
C4-2	S2	ScDup(C4-2-S2)	Chr. IV 250,614-301,020	p3009, p3122	50	33	50	14% (2/14)
C4-2	S3	ScDup(C4-2-S3)	Chr. IV 300,644-352,049	p3009, p3122	50	34	42	7% (1/14)
C4-2	S4	ScDup(C4-2-S4)	Chr. IV 350,404-401,638	p3122, p3279	50	30	1280	0% (0/52)
C4-2	S3+S4	ScDup(C4-2-(S3+S4))	Chr. IV 300,644-401,638	p3009, p3122	100	64	4	0% (0/4)
C4-4	S1	ScDup(C4-4-S1)	Chr. IV 599,793-652,548	p3009, p3122	50	34	58	2% (1/58)
C4-4	S2	ScDup(C4-4-S2)	Chr. IV 652,530-700,502	p3122, p3279	50	30	1067	0% (0/42)
C4-4	S3	ScDup(C4-4-S3)	Chr. IV 699,320-751,746	p3009, p3122	50	25	65	7% (1/14)
C4-4	S4	ScDup(C4-4-S4)	Chr. IV 750,633-795,723	p3009, p3122	50	25	22	18% (4/22)
C4-4	S2+S3	ScDup(C4-4-(S2+S3))	Chr. IV 652,530-751,746	p3009, p3122	100	55	17	0% (0/17)
C4-5	S1	ScDup(C4-5-S1)	Chr. IV 795,193-845,861	p3009, p3122	50	31	82	27% (4/15)
C4-5	S2	ScDup(C4-5-S2)	Chr. IV 844,952- 900,006	p3009, p3122	50	34	91	3% (1/30)
C4-5	S3	ScDup(C4-5-S3)	Chr. IV 898,551-951,323	p3009, p3122	50	33	56	13% (2/15)
C4-5	S4	ScDup(C4-5-S4)	Chr. IV 949,563-1,000,877	p3122, p3279	50	36	123	1% (1/104)

Region	Sub-region	Strain name	Chromosome location[a]	Plasmid template[b]	Duplication length (kb)	Number of genes	Transformants (n)	Proportion of desired karyotype[c]
C4-7	S1	ScDup(C4-7-S1)	Chr. IV 1,198,183-1,250,760	p3009, p3122	50	38	15	8% (1/13)
C4-7	S2	ScDup(C4-7-S2)	Chr. IV 1,249,137-1,299,139	p3009, p3122	50	32	12	16% (2/12)
C4-7	S3	ScDup(C4-7-S3)	Chr. IV 1,297,392-1,350,890	p3009, p3122	50	31	39	14% (2/14)
C4-7	S4	ScDup(C4-7-S4)	Chr. IV 1,349,318-1,402,247	p3122, p3279	50	33	822	0% (0/42)
C4-7	S3+S4	ScDup(C4-7-(S3+S4))	Chr. IV 1,297,392-1,402,247	p3009, p3122	100	64	27	0% (0/27)
C6-1	S1	ScDup(C6-1-S1)	Chr. VI 1-48,730	p3122	50	30	8	63% (5/8)
C6-1	S2	ScDup(C6-1-S2)	Chr. VI 47,761-98,498	p3009, p3122	50	27	24	0% (0/24)
C7-4	S1	ScDup(C7-4-S1)	Chr. VII 598,443-651,547	p3122, p3279	50	34	901	2% (1/56)
C7-4	S2	ScDup(C7-4-S2)	Chr. VII 650,314-701,698	p3009, p3122	50	25	39	7% (1/14)
C7-4	S3	ScDup(C7-4-S3)	Chr. VII 701,628-754,816	p3009, p3122	50	39	15	7% (1/15)
C7-4	S4	ScDup(C7-4-S4)	Chr. VII 753,704-801,057	p3009, p3122	50	35	65	21% (3/14)
C7-4	S4	ScDup(C7-4-S4_2)	Chr. VII 753,704-801,057	p3122, p3279	50	35	200	4% (1/28)
C8-2	S1	ScDup(C8-2-S1)	Chr. VIII 203,559-250,652	p3009, p3122	50	46	84	7% (1/14)
C8-2	S2	ScDup(C8-2-S2)	Chr. VIII 250,081-302,950	p3009, p3122	50	27	82	7% (1/14)
C8-2	S3	ScDup(C8-2-S3)	Chr. VIII 301,788-350,205	p3122, p3279	50	21	1500	0% (0/41)

Region	Sub-region	Strain name	Chromosome location[a]	Plasmid template[b]	Duplication length (kb)	Number of genes	Transformants (n)	Proportion of desired karyotype[c]
C8-2	S4	ScDup(C8-2-S4)	Chr. VIII 348,556-401,907	p3009, p3122	50	46	109	7% (1/14)
C8-2	S3+S4	ScDup(C8-2-(S3+S4))	Chr. VIII 301,788-401,907	p3009, p3122	100	67	23	0% (0/23)
C11-2	S1	ScDup(C11-2-S1)	Chr. XI 199,892-246,288	p3009, p3122	50	31	7	14% (1/7)
C11-2	S2	ScDup(C11-2-S2)	Chr. XI 245,144-300,075	p3122, p3279	50	35	961	0% (0/28)
C11-2	S3	ScDup(C11-2-S3)	Chr. XI 298,583-350,129	p3009, p3122	50	35	36	21% (3/14)
C11-2	S4	ScDup(C11-2-S4)	Chr. XI 348,413-399,750	p3009, p3122	50	32	3	33% (1/3)
C11-2	S1+S2	ScDup(C11-2-(S1+S2))	Chr. XI 199,892-300,075	p3009, p3122	100	66	81	0% (0/28)
C14-2	S1	ScDup(C14-2-S1)	Chr. XIV 199,575-251,006	p3009, p3122	50	31	2	100% (2/2)
C14-2	S2	ScDup(C14-2-S2)	Chr. XIV 250,863-302,108	p3009, p3122	50	33	8	13% (1/8)
C14-2	S3	ScDup(C14-2-S3)	Chr. XIV 301,698-349,197	p3009, p3122	50	31	19	5% (1/19)
C14-2	S4	ScDup(C14-2-S4)	Chr. XIV 349,012-403,514	p3122, p3279	50	37	154	0% (0/75)
C14-2	S3+S4	ScDup(C14-2-(S3+S4))	Chr. XIV 301,698-403,514	p3009, p3122	100	68	17	0% (0/17)

*a: Chr. N x-y : Chr. N represents chromosome number, x represents first nucleotide number of chromosomal region and y represents last nucleotide number of chromosomal region.

b: p3009 was used to amplify the *CgHIS3* cassette, p3122 was used to amplify the *CEN4-CgLEU2* cassette, p3279 was used to amplify the *CgHIS3-H4ARS* cassette

c: Proportion of desired karyotype in analyzed transformants (number of segmental aneuploids / number of candidate transformants that were analyzed for karyotype).

3.3.3 Effect of stress on growth of segmental aneuploids

The analyses in Chapter 2 showed that strains with segmental aneuploidies were mitotically stable under normal culture conditions. Documenting the characteristics that are affected by segmental aneuploidy without and with stressful environments may give us knowledge about some aspect of genome function. First, I compared the growth of the 53 segmental aneuploid strains and the parental strain in liquid SC medium. The growth of all segmental aneuploid strains but ScDup(15-4) did not show significantly different from that of the parental strain when cultured at 30°C for 24 hours. However, only ScDup(C15-4) showed slower growth compared to the parental strain (Fig. 7).

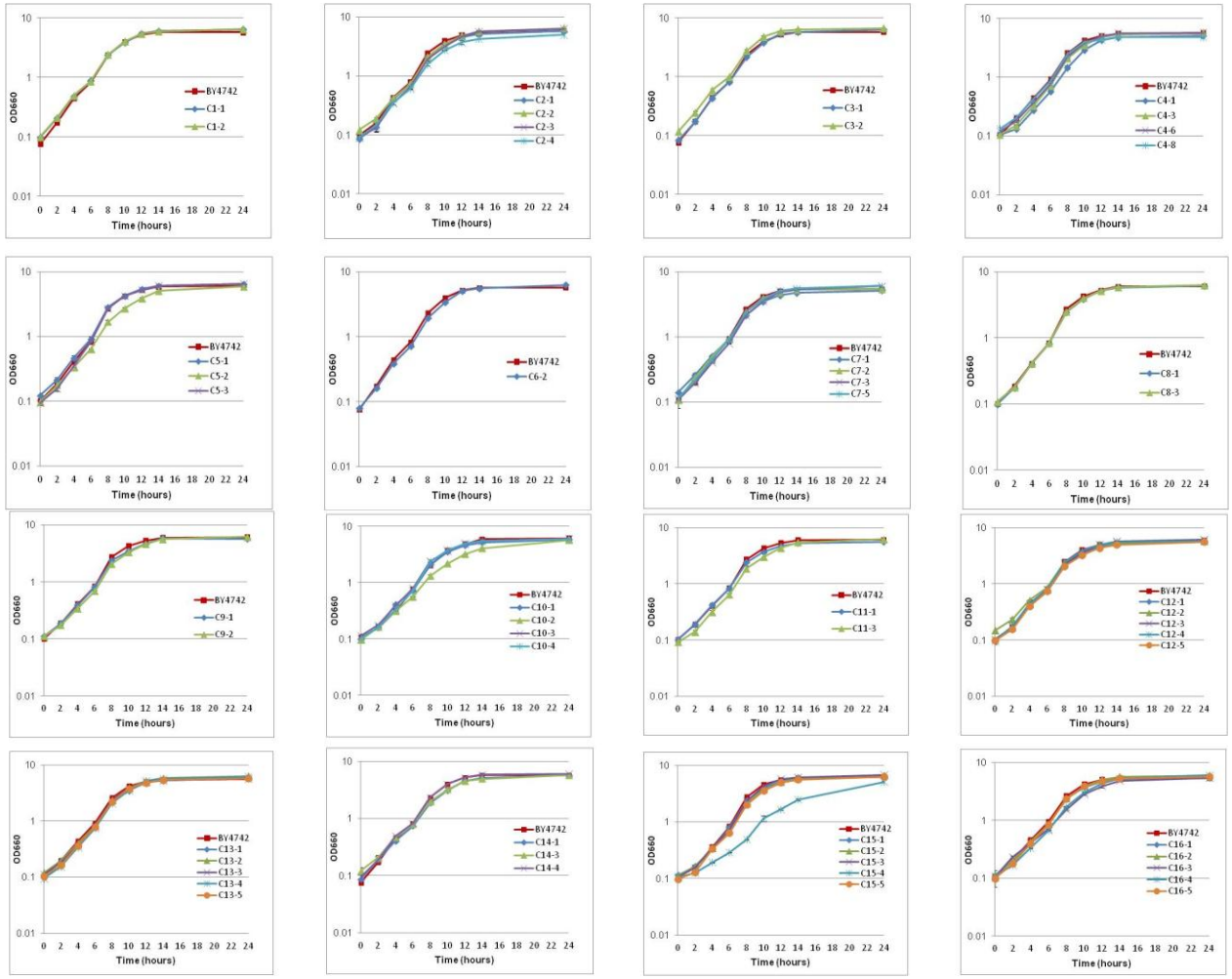


Figure 7. Growth profiles of segmental aneuploid strains in SC medium at 30°C for 24 hours. The OD_{660} of 53 segmental aneuploid strains and the parental strain was measured every 2 hours. Three independent replicate cultures were performed.

To investigate the consequences of segmental aneuploidy under different challenging conditions, the phenotypic examination of 53 segmental aneuploids under various stressful conditions were conducted by serial dilution assays involving lactic acid (4%, 5% and 6% wt vol^{-1}), ethanol (6%, 8% and 10% vol vol^{-1}), sulfuric acid (0.41%, 0.44%, 0.47% wt vol^{-1}); 80 mM acetic acid, 36 mM formic acid, or 3% glycerol as the carbon source; alkaline pH (pH 9); 1.2 M NaCl; high temperatures (39°C, 40°C and 41°C); and low temperature (13°C). The results revealed that all but two strains, ScDup(C7-1) and ScDup(C16-3), showed the same colony formation ability as the parental strain when incubated in YPAD at 30°C (without stress conditions) for 4 days (Fig. 9); these two strains displayed slightly slower growth than the parental strain when incubated for 1 day (Fig. 9g and 9p) although they showed normal growth when incubated for 4 days (Fig. 9). However, under stress conditions, the segmental aneuploids showed different degree of growth competence as compared with the parental strain under stress conditions (Fig. 9a-9p and Table 11). The numbers of strains classified as sensitive or resistant to each stress condition are shown in Figure 8 and all results for the spot assays from all 53 segmental aneuploidy strains under 18 stress conditions are presented in Figure 9 and Table 11. Taken together, our analyses indicated that all segmental aneuploid strains except for ScDup(C10-4) showed a different pattern of response to at least one tested stress compared to the parental strain. Although most of the segmental aneuploidy strains showed stress sensitivity, interestingly, only a few showed increased tolerance of thermal stress, high concentrations of ethanol, acidic conditions or osmotic stress (Table 11, Figs. 8 and 9). We found that segmental aneuploid strains such as ScDup(C2-3), ScDup(C3-1), ScDup(C3-2), ScDup(C5-

3), SCDup(C7-5), ScDup(C12-3), ScDup(C15-2), ScDup(C15-3), ScDup(C16-2) and ScDup(C16-4) showed increased tolerance to multiple stresses. Based on SGD database, we searched genes among those located on these duplicated regions that are required for those stress resistance and found that those chromosomal regions contained several specific genes that may be concerned with resistance against each stress. We also noted that some genes might have conferred tolerance to more than one particular stress (See details in Discussion section). The duplication of specific chromosomal regions might be a mechanism to aid cell survival under stress conditions.

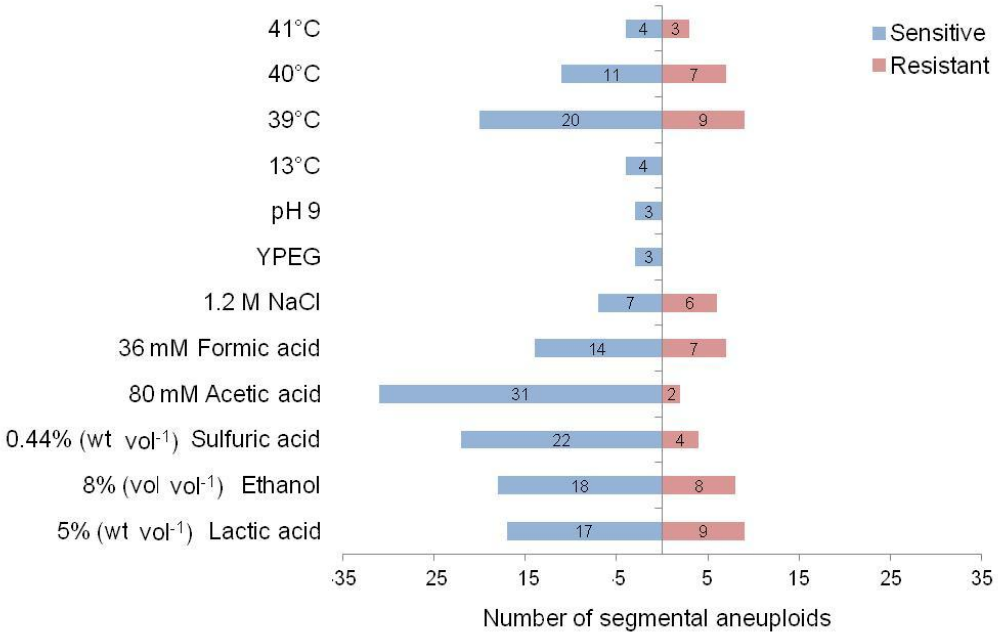


Figure 8. Phenotypic assays of segmental aneuploid strains. The numbers of segmental aneuploids that showed increased sensitivity or resistance to each stress condition. Blue bar represents sensitive phenotype and red bar represents resistant phenotype.

Table 11. Stress sensitive and resistant phenotypes of segmental aneuploids for chromosomes I to XVI

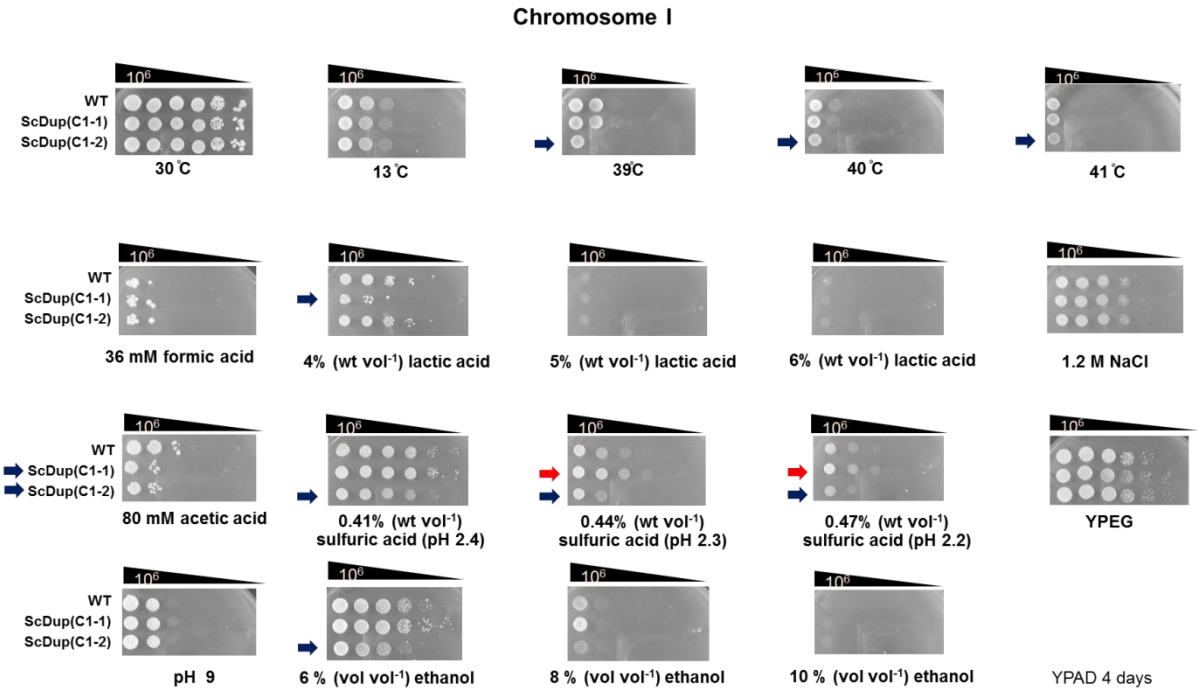
Strain name	Sensitive phenotype[a]	Resistance phenotype[a]
ScDup(C1-1)	4%L, A	S pH 2.3, S pH 2.2
ScDup(C1-2)	S pH 2.3, S pH 2.2, 6%E, A , 39°C, 40°C, 41°C	-
ScDup(C2-1)	4%L, 5%L, S pH 2.3, S pH 2.2, 8%E, A	-
ScDup(C2-2)	4%L, 5%L, S pH 2.3, S pH 2.2, 8%E, G, N, A	F
ScDup(C2-3)	-	F, N, A
ScDup(C2-4)	13 °C, 4%L, 5%L, S pH 2.4, S pH 2.3, S pH 2.2, 8%E, F, A, 39°C	-
ScDup(C3-1)	-	S pH 2.3, S pH 2.2, 6%E, 8%E, N 39°C, 40°C
ScDup(C3-2)	-	S pH 2.3, S pH 2.2, 6%E, 8%E, 39°C, 40°C
ScDup(C4-1)	S pH 2.3, S pH 2.2, A	5%L
ScDup(C4-2)	-	-
ScDup(C4-3)	4%L, 39°C, 40°C, 41°C	-
ScDup(C4-4)	-	-
ScDup(C4-5)	-	-
ScDup(C4-6)	4%L, pH 9	-
ScDup(C4-7)	-	-
ScDup(C4-8)	5%L, S pH 2.3, S pH 2.2, pH 9, 39°C, 40°C, 41°C	A
ScDup(C5-1)	F, 39°C, 40°C	-
ScDup(C5-2)	S pH 2.3, 8%E, G, N, A, 39°C, 40°C	-
ScDup(C5-3)	F, A	39°C, 40°C, 41 °C, 8%E
ScDup(C6-1)	-	-
ScDup(C6-2)	4%L, 5%L, A, pH 9	-
ScDup(C7-1)	13 °C, 4%L, 5%L, S pH 2.3, F, N, A, 39°C, 40°C	-
ScDup(C7-2)	S pH 2.3, 6%E, A	F
ScDup(C7-3)	4%L, 5%L, A	F
ScDup(C7-4)	-	-
ScDup(C7-5)	F, A	6%E, 8%E, N, 39°C, 40°C, 41 °C
ScDup(C8-1)	A	-
ScDup(C8-2)	-	-
ScDup(C8-3)	5%L, F, A	-
ScDup(C9-1)	S pH 2.3, F, 39°C, 40°C	-

Strain name	Sensitive phenotype[a]	Resistance phenotype[a]
ScDup(C9-2)	8%E, 39°C, 40°C	4%L, 5%L
ScDup(C10-1)	4%L, 5%L, S pH 2.4, S pH 2.3, 6%E, 8%E, A, 39°C	-
ScDup(C10-2)	F, G, A	39°C, 40°C, 41°C
ScDup(C10-3)	8%E, F, N, A, 39°C	-
ScDup(C10-4)	-	-
ScDup(C11-1)	S pH 2.3, 6%E, 8%E, N, A	-
ScDup(C11-2)	-	-
ScDup(C11-3)	4%L, 5%L, S pH 2.4, S pH 2.3, S pH 2.2, 6%E,8%E, A, 39°C, 40°C	-
ScDup(C12-1)	13 °C, A, 39°C	4%L, 5%L
ScDup(C12-2)	4%L, 5%L, S pH 2.3, G, A	39°C
ScDup(C12-3)	-	4%L, 5%L, 8%E, N, 39°C, 40°C
ScDup(C12-4)	S pH 2.3, 39°C	-
ScDup(C12-5)	F, A, 39°C	-
ScDup(C13-1)	S pH 2.3, 8%E, F	N
ScDup(C13-2)	S pH 2.3, 6%E, 8%E, 39°C	-
ScDup(C13-3)	S pH 2.3, 8%E, 39°C	F
ScDup(C13-4)	4%L, S pH 2.3, 6%E, 8%E, F, 39°C	-
ScDup(C13-5)	6%E, 8%E	5%L
ScDup(C14-1)	4%L, 5%L, S pH 2.3, S pH 2.2, 6%E,8%E, F, A, 39°C, 40°C, 41°C	-
ScDup(C14-2)	-	-
ScDup(C14-3)	13 °C, 4%L, 5%L, 6%E, 8%E, F, N, A	-
ScDup(C14-4)	13 °C, A	-
ScDup(C15-1)	S pH 2.3, A, 39°C	-
ScDup(C15-2)	-	5%L, 8%E, 39°C, 40°C
ScDup(C15-3)	-	5%L, 39°C
ScDup(C15-4)	4%L, A	-
ScDup(C15-5)	6%E	5%L
ScDup(C16-1)	4%L, 5%L, A	-
ScDup(C16-2)	4%L, 5%L, A	8%E, F
ScDup(C16-3)	41 °C,4%L, 5%L, S pH 2.3, 6%E, 8%E, N, A	-
ScDup(C16-4)	-	5%L, S pH 2.3, 8%E, F, N
ScDup(C16-5)	4%L, 5%L, A, 39°C, 40°C	-

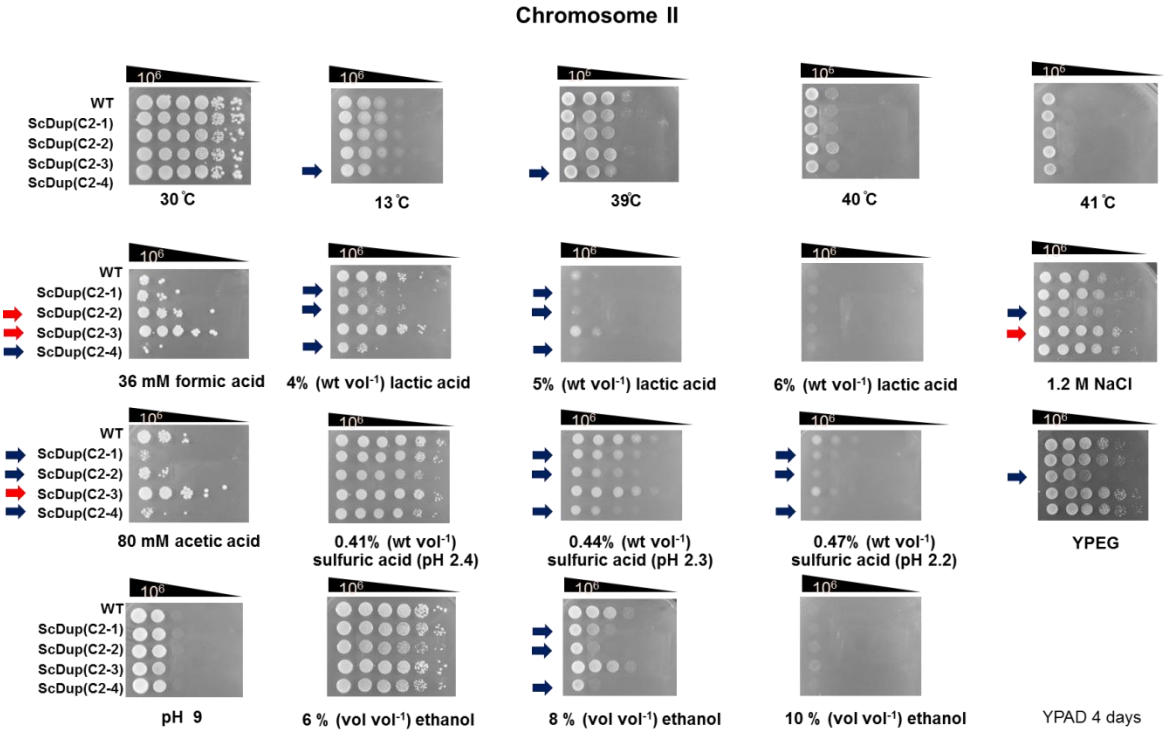
a: 4%L; 4% (wt vol^{-1}) lactic acid, 5%L; 5% (wt vol^{-1}) lactic acid, S pH 2.4; 0.41% (wt vol^{-1}) sulfuric acid pH 2.4, S pH 2.3; 0.44% (wt vol^{-1}) sulfuric acid pH 2.3, S pH 2.2; 0.47% (wt vol^{-1}) sulfuric acid pH 2.2, 6%E; 6% (vol vol^{-1}) ethanol, 8%E; 8% (vol vol^{-1})ethanol, F; 36 mM formic acid, N; 1.2 M NaCl, G; YPEG, A; 80 mM acetic acid

Figure 9. **Phenotypic assays of segmental aneuploid strains for chromosomes I to XVI.** Ten-fold serial dilutions of segmental aneuploid strains of chromosomes I to XVI (a-p, respectively) were spotted on plates and subjected to different stresses including 4% (wt vol^{-1}) lactic acid, 5% (wt vol^{-1}) lactic acid, 6% (wt vol^{-1}) lactic acid, 6% (vol vol^{-1}) ethanol, 8% (vol vol^{-1}) ethanol, 10% (vol vol^{-1}) ethanol, 0.41% (wt vol^{-1}) sulfuric acid (pH 2.4), 0.44% (wt vol^{-1}) sulfuric acid (pH 2.3), 0.47% (wt vol^{-1}) sulfuric acid (pH 2.2), 36 mM formic acid, 1.2 M NaCl, 80 mM acetic acid, YPEG, pH 9, at 13°C, at 39°C, at 40°C, at 41°C. The plates were incubated for 3-4 days before being photographed. Red arrow represents stress resistant phenotype. Blue arrow represents stress sensitive phenotype.

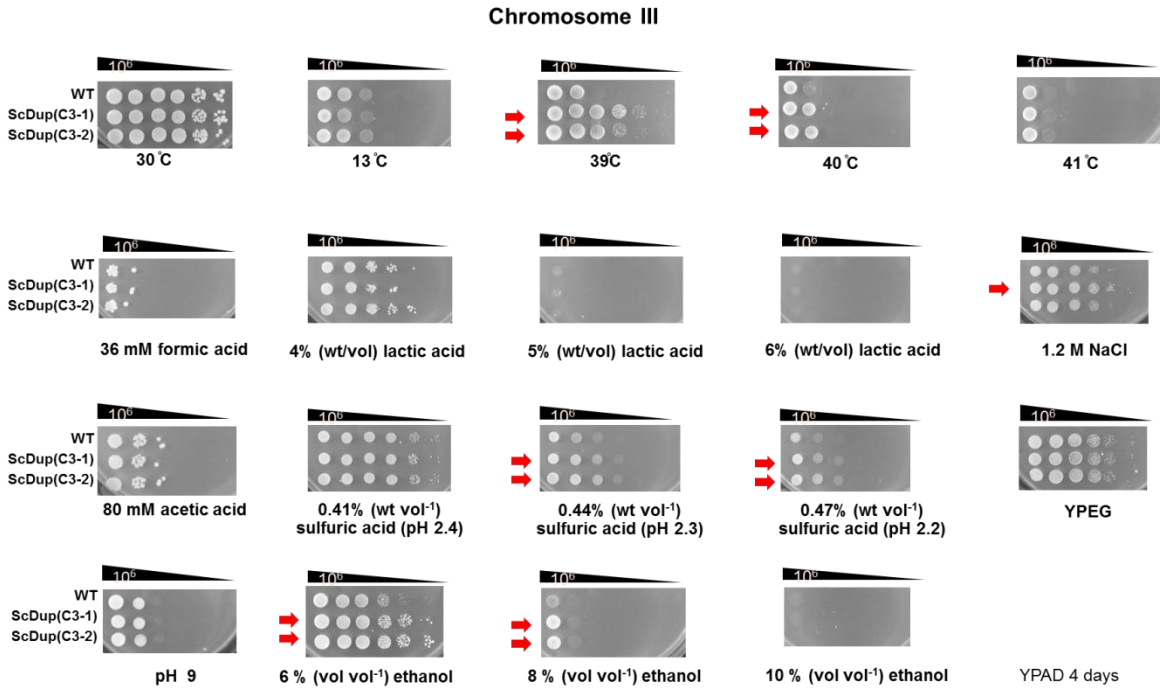
a)



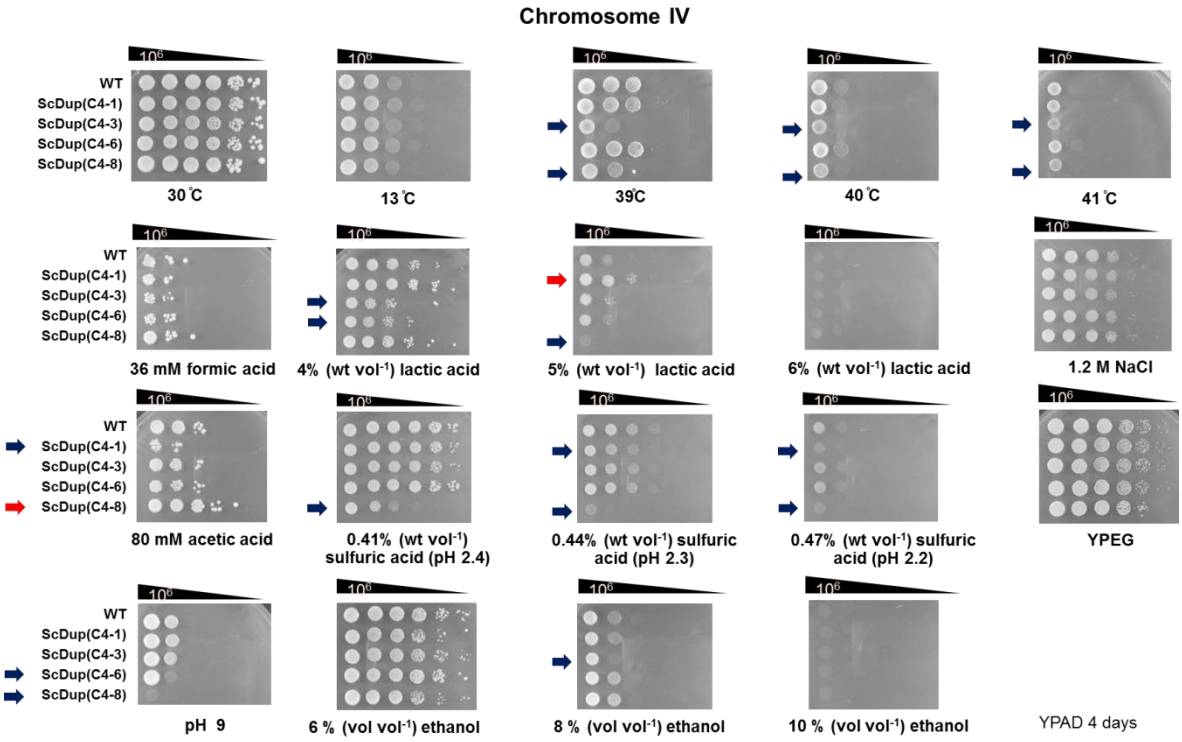
b)


Chromosome II
WT
ScDup(C2-1)
ScDup(C2-2)
ScDup(C2-3)
ScDup(C2-4)
30 °C
13 °C
39°C
40 °C
41 °C
36 mM formic acid
4% (wt vol-1) lactic acid
5% (wt vol-1) lactic acid
6% (wt vol-1) lactic acid
1.2 M NaCl
80 mM acetic acid
0.41% (wt vol-1) sulfuric acid (pH 2.4)
0.44% (wt vol-1) sulfuric acid (pH 2.3)
0.47% (wt vol-1) sulfuric acid (pH 2.2)
YPEG
pH 9
6 % (vol vol-1) ethanol
8 % (vol vol-1) ethanol
10 % (vol vol-1) ethanol
YPAD 4 days

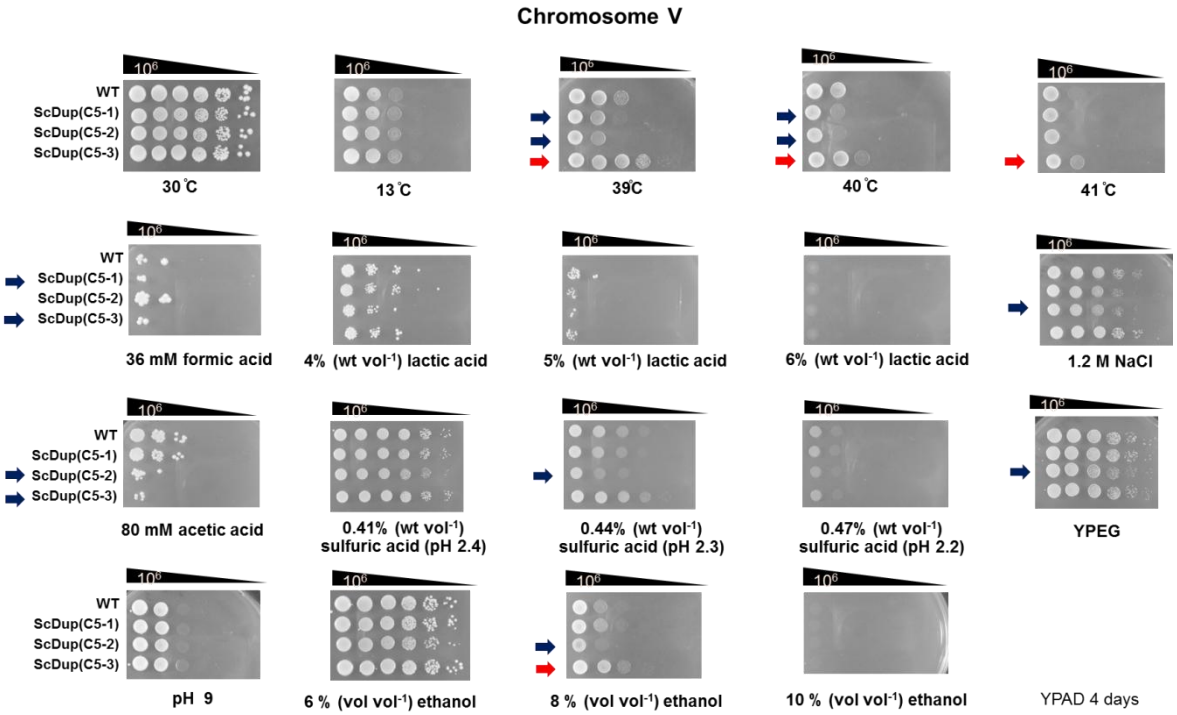
c)


Chromosome III
WT
ScDup(C3-1)
ScDup(C3-2)
30 °C
13 °C
39°C
40 °C
41 °C
36 mM formic acid
4% (wt/vol) lactic acid
5% (wt/vol) lactic acid
6% (wt/vol) lactic acid
1.2 M NaCl
80 mM acetic acid
0.41% (wt vol-1) sulfuric acid (pH 2.4)
0.44% (wt vol-1) sulfuric acid (pH 2.3)
0.47% (wt vol-1) sulfuric acid (pH 2.2)
YPEG
pH 9
6 % (vol vol-1) ethanol
8 % (vol vol-1) ethanol
10 % (vol vol-1) ethanol
YPAD 4 days

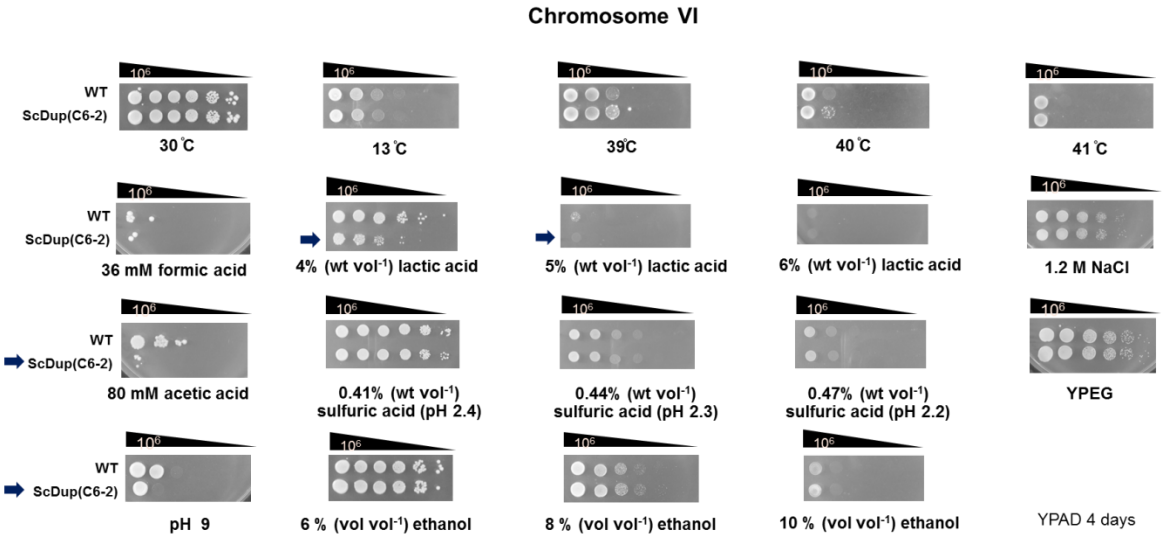
d)


Chromosome IV
WT
ScDup(C4-1)
ScDup(C4-3)
ScDup(C4-6)
ScDup(C4-8)
30 ˚C
13 ˚C
39˚C
40 ˚C
41 ˚C
36 mM formic acid
4% (wt vol-1) lactic acid
5% (wt vol-1) lactic acid
6% (wt vol-1) lactic acid
1.2 M NaCl
80 mM acetic acid
0.41% (wt vol-1) sulfuric acid (pH 2.4)
0.44% (wt vol-1) sulfuric acid (pH 2.3)
0.47% (wt vol-1) sulfuric acid (pH 2.2)
YPEG
pH 9
6 % (vol vol-1) ethanol
8 % (vol vol-1) ethanol
10 % (vol vol-1) ethanol
YPAD 4 days

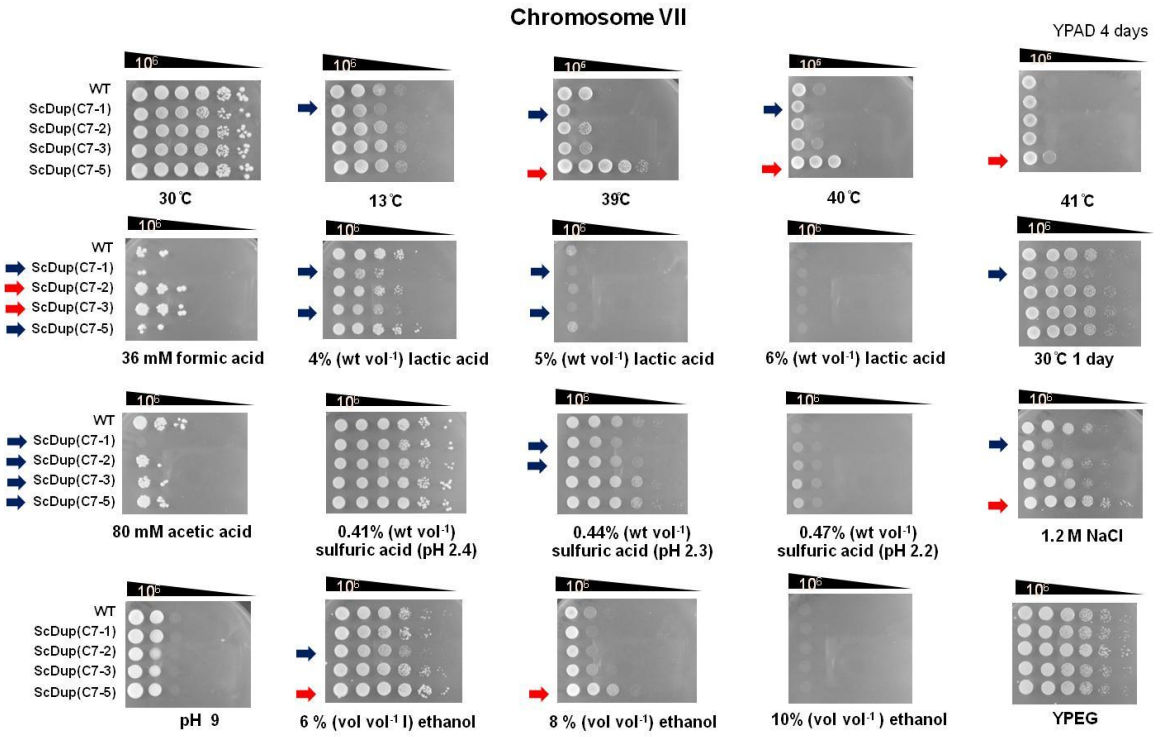
e)


Chromosome V
WT
ScDup(C5-1)
ScDup(C5-2)
ScDup(C5-3)
30 ˚C
13 ˚C
39˚C
40 ˚C
41 ˚C
36 mM formic acid
4% (wt vol-1) lactic acid
5% (wt vol-1) lactic acid
6% (wt vol-1) lactic acid
1.2 M NaCl
80 mM acetic acid
0.41% (wt vol-1) sulfuric acid (pH 2.4)
0.44% (wt vol-1) sulfuric acid (pH 2.3)
0.47% (wt vol-1) sulfuric acid (pH 2.2)
YPEG
pH 9
6 % (vol vol-1) ethanol
8 % (vol vol-1) ethanol
10 % (vol vol-1) ethanol
YPAD 4 days

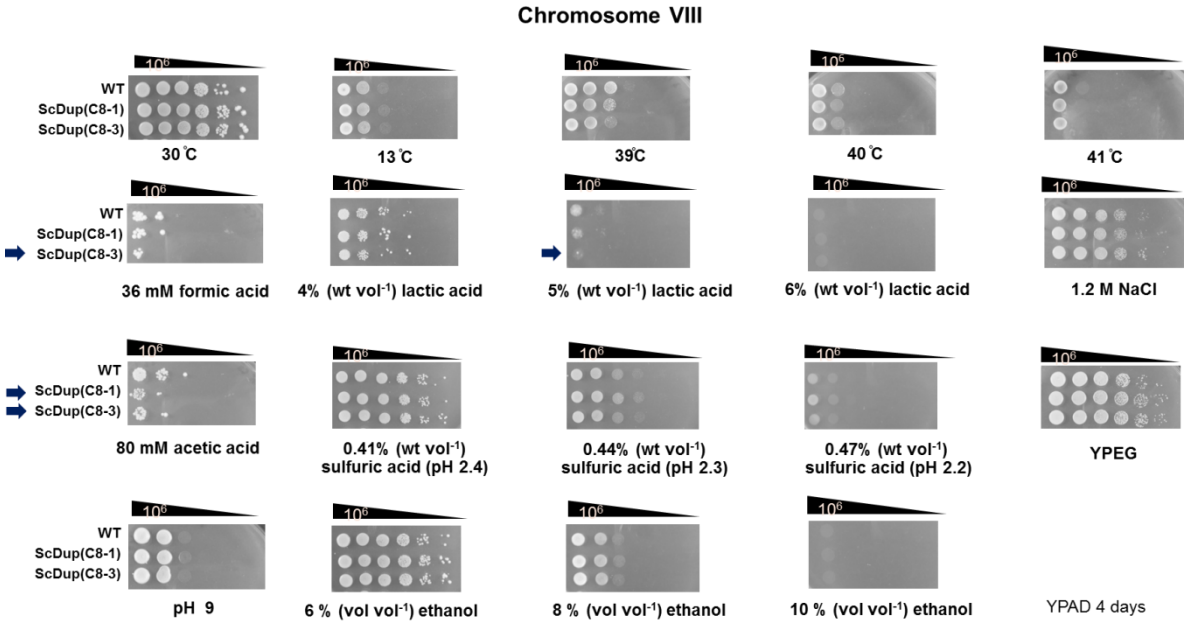
f)


Chromosome VI
WT
ScDup(C6-2)
30 °C
13 °C
39°C
40 °C
41 °C
36 mM formic acid
4% (wt vol-1) lactic acid
5% (wt vol-1) lactic acid
6% (wt vol-1) lactic acid
1.2 M NaCl
80 mM acetic acid
0.41% (wt vol-1) sulfuric acid (pH 2.4)
0.44% (wt vol-1) sulfuric acid (pH 2.3)
0.47% (wt vol-1) sulfuric acid (pH 2.2)
YPEG
pH 9
6 % (vol vol-1) ethanol
8 % (vol vol-1) ethanol
10 % (vol vol-1) ethanol
YPAD 4 days

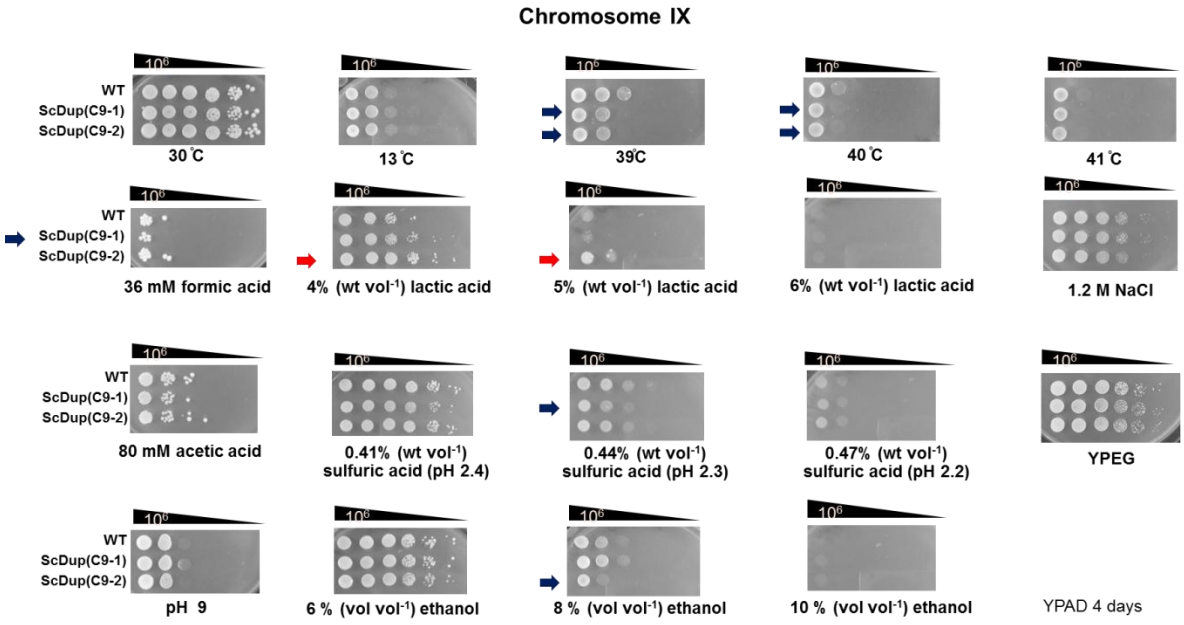
g)


Chromosome VII
YPAD 4 days
WT
ScDup(C7-1)
ScDup(C7-2)
ScDup(C7-3)
ScDup(C7-5)
30 °C
13 °C
39°C
40 °C
41 °C
36 mM formic acid
4% (wt vol-1) lactic acid
5% (wt vol-1) lactic acid
6% (wt vol-1) lactic acid
30 °C 1 day
80 mM acetic acid
0.41% (wt vol-1) sulfuric acid (pH 2.4)
0.44% (wt vol-1) sulfuric acid (pH 2.3)
0.47% (wt vol-1) sulfuric acid (pH 2.2)
1.2 M NaCl
pH 9
6 % (vol vol-1 l) ethanol
8 % (vol vol-1) ethanol
10% (vol vol-1) ethanol
YPEG

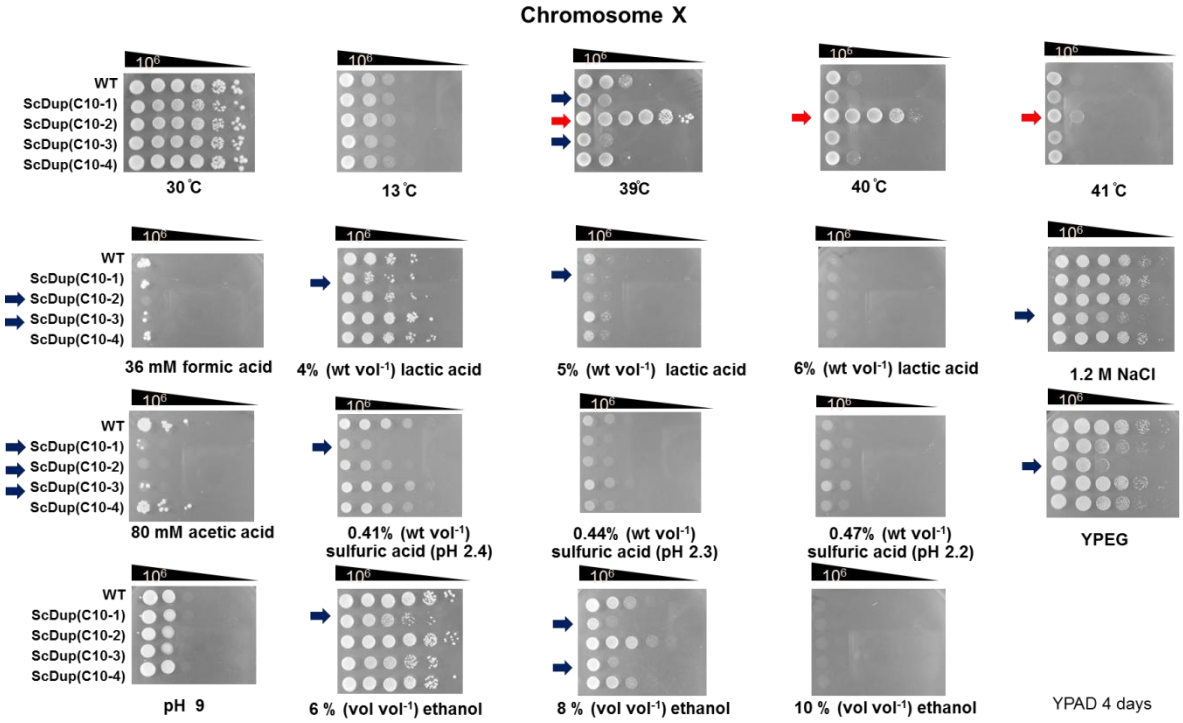
h)


Chromosome VIII
WT
ScDup(C8-1)
ScDup(C8-3)
30 °C
13 °C
39°C
40 °C
41 °C
36 mM formic acid
4% (wt vol-1) lactic acid
5% (wt vol-1) lactic acid
6% (wt vol-1) lactic acid
1.2 M NaCl
80 mM acetic acid
0.41% (wt vol-1) sulfuric acid (pH 2.4)
0.44% (wt vol-1) sulfuric acid (pH 2.3)
0.47% (wt vol-1) sulfuric acid (pH 2.2)
YPEG
pH 9
6 % (vol vol-1) ethanol
8 % (vol vol-1) ethanol
10 % (vol vol-1) ethanol
YPAD 4 days

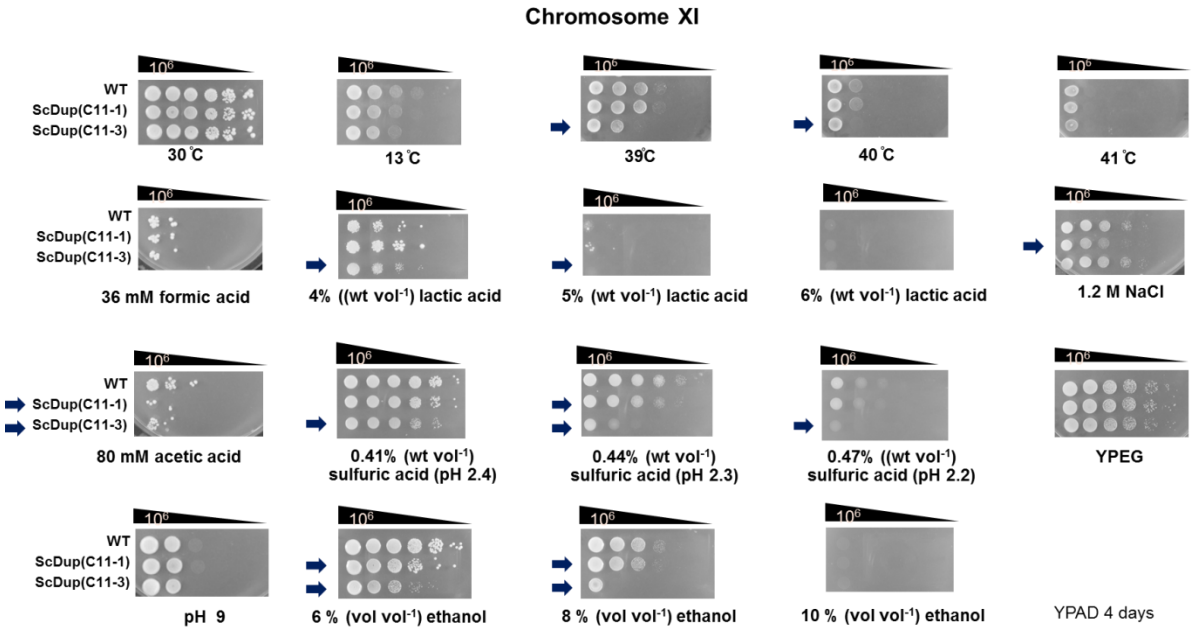
i)


Chromosome IX
WT
ScDup(C9-1)
ScDup(C9-2)
30 °C
13 °C
39°C
40 °C
41 °C
36 mM formic acid
4% (wt vol-1) lactic acid
5% (wt vol-1) lactic acid
6% (wt vol-1) lactic acid
1.2 M NaCl
80 mM acetic acid
0.41% (wt vol-1) sulfuric acid (pH 2.4)
0.44% (wt vol-1) sulfuric acid (pH 2.3)
0.47% (wt vol-1) sulfuric acid (pH 2.2)
YPEG
pH 9
6 % (vol vol-1) ethanol
8 % (vol vol-1) ethanol
10 % (vol vol-1) ethanol
YPAD 4 days

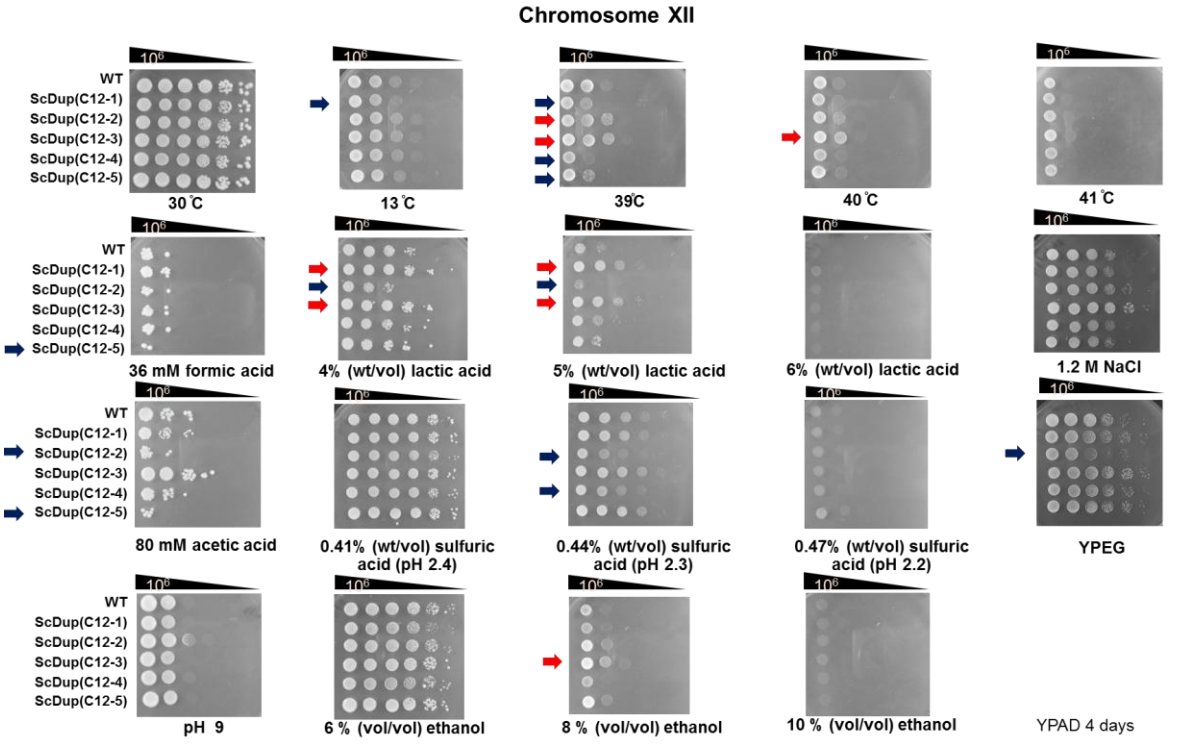
j)


Chromosome X
WT
ScDup(C10-1)
ScDup(C10-2)
ScDup(C10-3)
ScDup(C10-4)
30 °C
13 °C
39°C
40 °C
41 °C
36 mM formic acid
4% (wt vol-1) lactic acid
5% (wt vol-1) lactic acid
6% (wt vol-1) lactic acid
1.2 M NaCl
80 mM acetic acid
0.41% (wt vol-1) sulfuric acid (pH 2.4)
0.44% (wt vol-1) sulfuric acid (pH 2.3)
0.47% (wt vol-1) sulfuric acid (pH 2.2)
YPEG
pH 9
6 % (vol vol-1) ethanol
8 % (vol vol-1) ethanol
10 % (vol vol-1) ethanol
YPAD 4 days

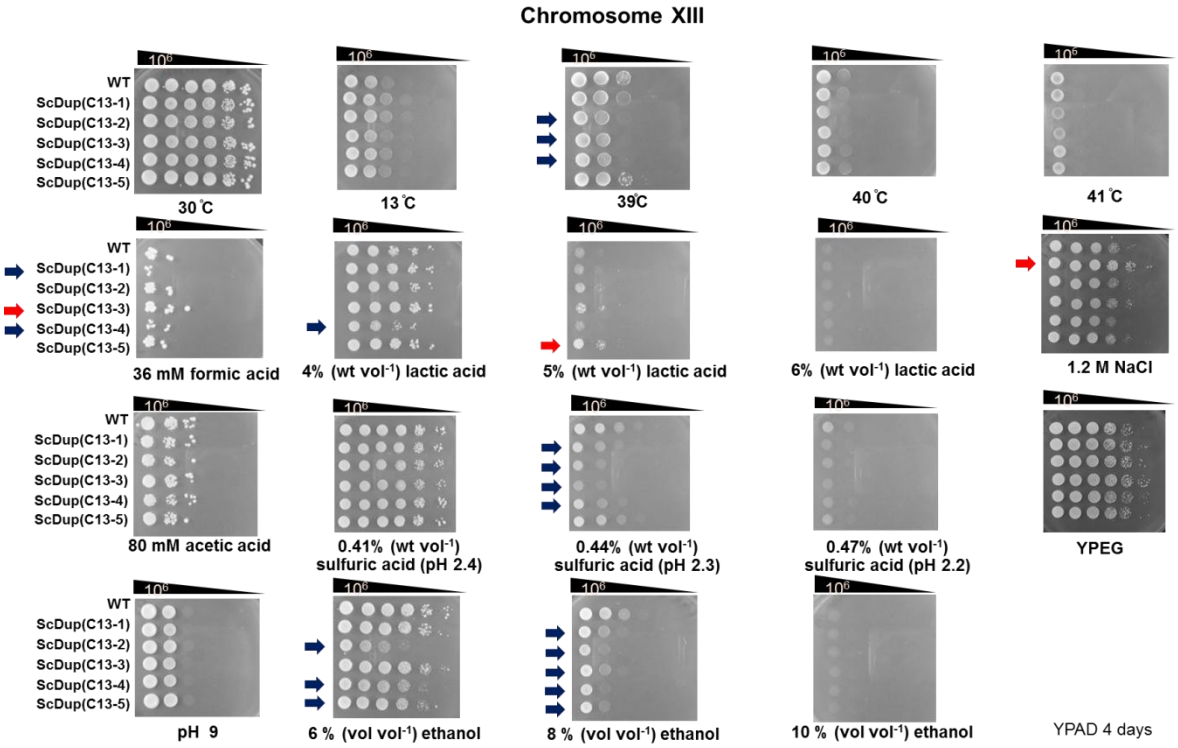
k)


Chromosome XI
WT
ScDup(C11-1)
ScDup(C11-3)
30 °C
13 °C
39°C
40 °C
41 °C
36 mM formic acid
4% ((wt vol-1) lactic acid
5% (wt vol-1) lactic acid
6% (wt vol-1) lactic acid
1.2 M NaCl
80 mM acetic acid
0.41% (wt vol-1) sulfuric acid (pH 2.4)
0.44% (wt vol-1) sulfuric acid (pH 2.3)
0.47% ((wt vol-1) sulfuric acid (pH 2.2)
YPEG
pH 9
6 % (vol vol-1) ethanol
8 % (vol vol-1) ethanol
10 % (vol vol-1) ethanol
YPAD 4 days

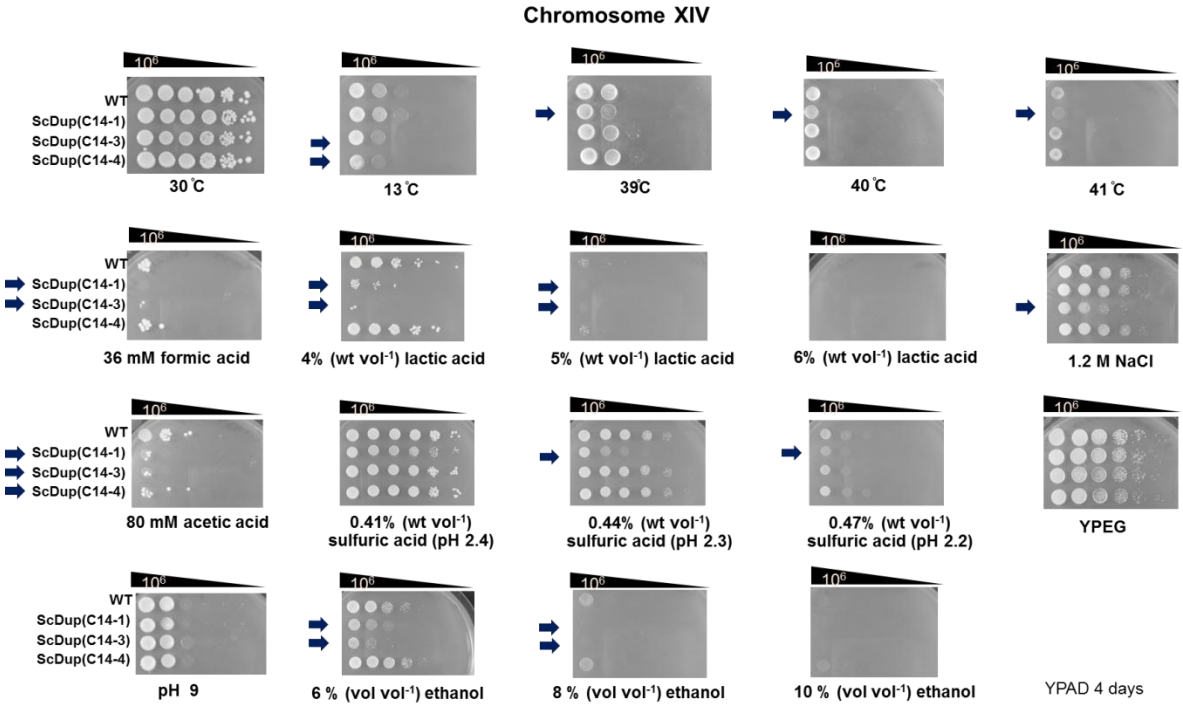
l)


Chromosome XII
WT
ScDup(C12-1)
ScDup(C12-2)
ScDup(C12-3)
ScDup(C12-4)
ScDup(C12-5)
30 °C
13 °C
39°C
40 °C
41 °C
36 mM formic acid
4% (wt/vol) lactic acid
5% (wt/vol) lactic acid
6% (wt/vol) lactic acid
1.2 M NaCl
80 mM acetic acid
0.41% (wt/vol) sulfuric acid (pH 2.4)
0.44% (wt/vol) sulfuric acid (pH 2.3)
0.47% (wt/vol) sulfuric acid (pH 2.2)
YPEG
pH 9
6 % (vol/vol) ethanol
8 % (vol/vol) ethanol
10 % (vol/vol) ethanol
YPAD 4 days

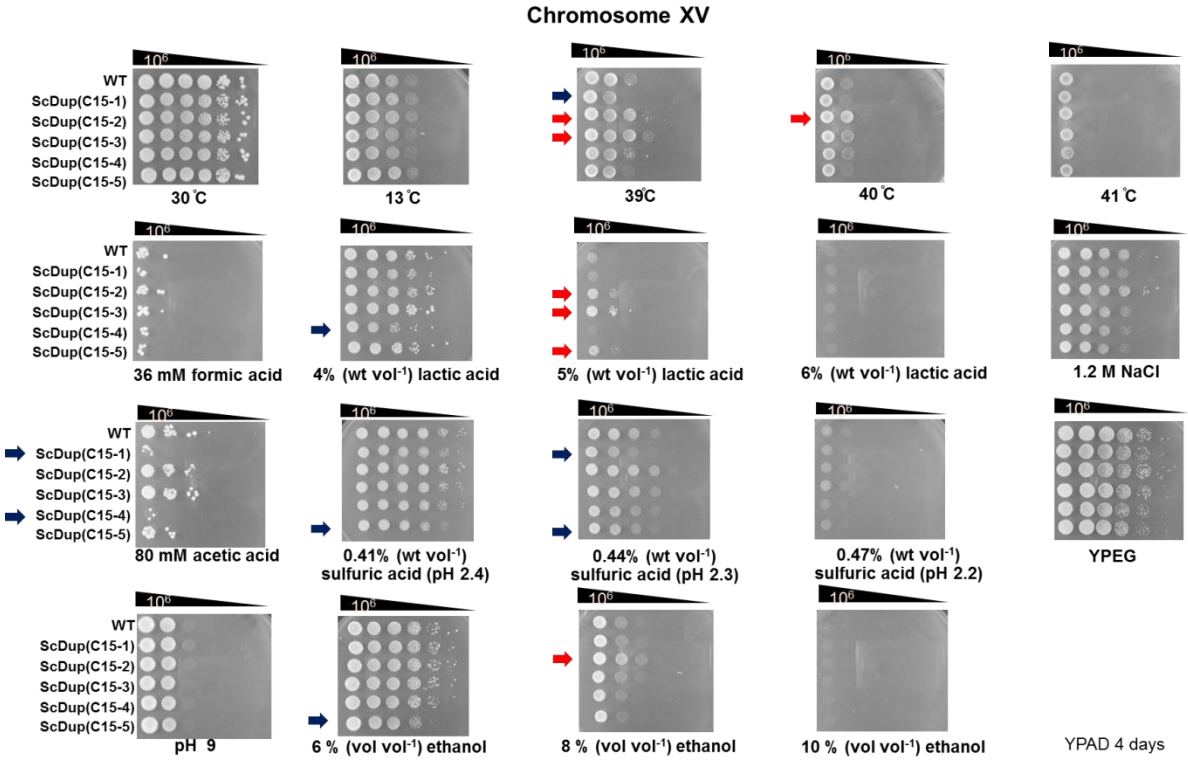
m)


Chromosome XIII
WT
ScDup(C13-1)
ScDup(C13-2)
ScDup(C13-3)
ScDup(C13-4)
ScDup(C13-5)
30 °C
13 °C
39°C
40 °C
41 °C
36 mM formic acid
4% (wt vol-1) lactic acid
5% (wt vol-1) lactic acid
6% (wt vol-1) lactic acid
1.2 M NaCl
80 mM acetic acid
0.41% (wt vol-1) sulfuric acid (pH 2.4)
0.44% (wt vol-1) sulfuric acid (pH 2.3)
0.47% (wt vol-1) sulfuric acid (pH 2.2)
YPEG
pH 9
6 % (vol vol-1) ethanol
8 % (vol vol-1) ethanol
10 % (vol vol-1) ethanol
YPAD 4 days

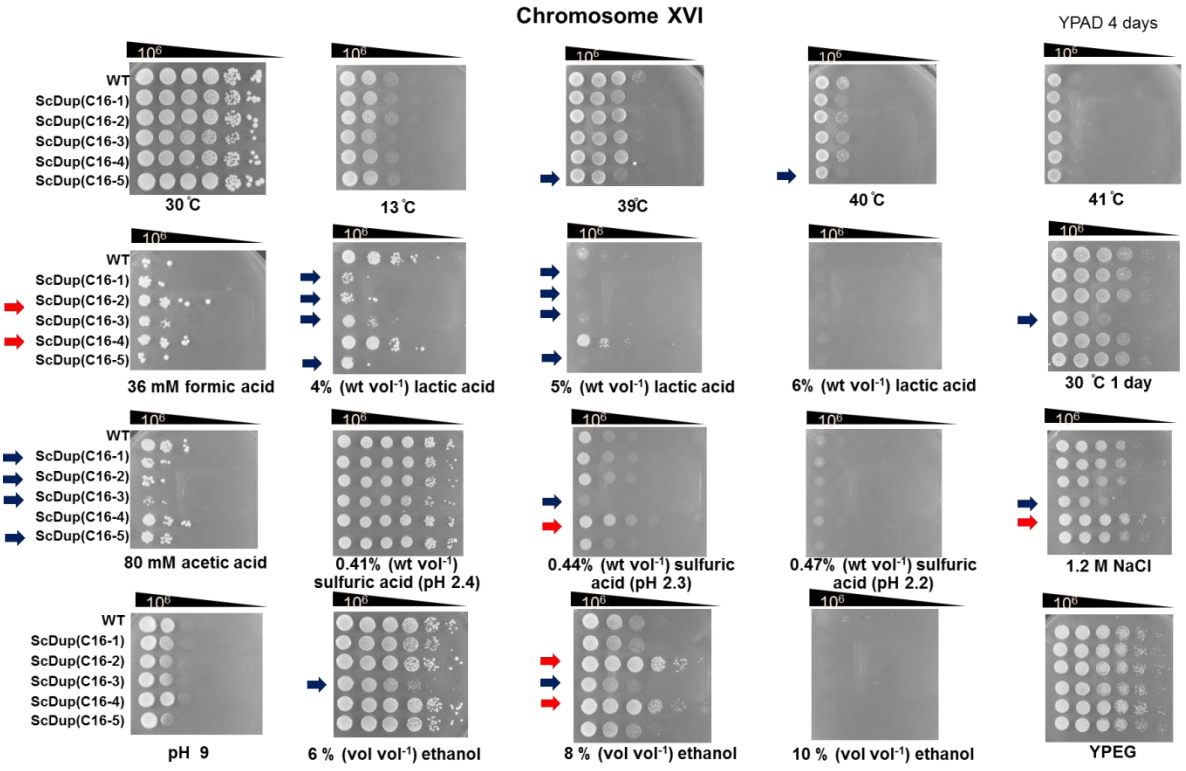
n)


Chromosome XIV
WT
ScDup(C14-1)
ScDup(C14-3)
ScDup(C14-4)
30 °C
13 °C
39°C
40 °C
41 °C
36 mM formic acid
4% (wt vol-1) lactic acid
5% (wt vol-1) lactic acid
6% (wt vol-1) lactic acid
1.2 M NaCl
80 mM acetic acid
0.41% (wt vol-1) sulfuric acid (pH 2.4)
0.44% (wt vol-1) sulfuric acid (pH 2.3)
0.47% (wt vol-1) sulfuric acid (pH 2.2)
YPEG
pH 9
6 % (vol vol-1) ethanol
8 % (vol vol-1) ethanol
10 % (vol vol-1) ethanol
YPAD 4 days

o)


Chromosome XV
WT
ScDup(C15-1)
ScDup(C15-2)
ScDup(C15-3)
ScDup(C15-4)
ScDup(C15-5)
30 °C
13 °C
39°C
40 °C
41 °C
36 mM formic acid
4% (wt vol-1) lactic acid
5% (wt vol-1) lactic acid
6% (wt vol-1) lactic acid
1.2 M NaCl
80 mM acetic acid
0.41% (wt vol-1) sulfuric acid (pH 2.4)
0.44% (wt vol-1) sulfuric acid (pH 2.3)
0.47% (wt vol-1) sulfuric acid (pH 2.2)
YPEG
pH 9
6 % (vol vol-1) ethanol
8 % (vol vol-1) ethanol
10 % (vol vol-1) ethanol
YPAD 4 days

p)



3.3.4 Association of phenotypic changes with segmental aneuploidy

To verify whether these alterations in phenotype were indeed caused by segmental duplication of the respective chromosomal regions, I investigated whether an elimination of the additional chromosome caused a reversion to the parental phenotype (Figs. 10-12). I arbitrarily selected 11 segmental aneuploids, ScDup(C2-3), ScDup(C3-2), ScDup(C4-1), ScDup(C5-3), ScDup(C6-2), ScDup(C7-1), ScDup(C11-3), ScDup(C12-3), ScDup(C14-3), ScDup(C16-2), and ScDup(C16-4), and subjected them to stress assays after removal of the duplicated chromosome. A total of 60 assays were performed with these modified strains and, in 47 cases, removal of the duplicated chromosome resulted in reversion to the parental phenotype. In these segmental aneuploid strains, therefore, the phenotypic changes were caused by the presence of the duplicated region. However, in some assays involving ScDup(C3-2), ScDup(C4-1), ScDup(C11-3), ScDup(C16-2), and ScDup(16-4) (13 of the 60 tests), it was clear that removal of the additional chromosome did not result in reversion to

the parental phenotype indicating that the phenotypes of these segmental aneuploid strains did not show a clear association with the presence of the duplicated region (Fig. 10). Thus, in some cases, the phenotypes may not be due to the segmentally duplicated chromosome.

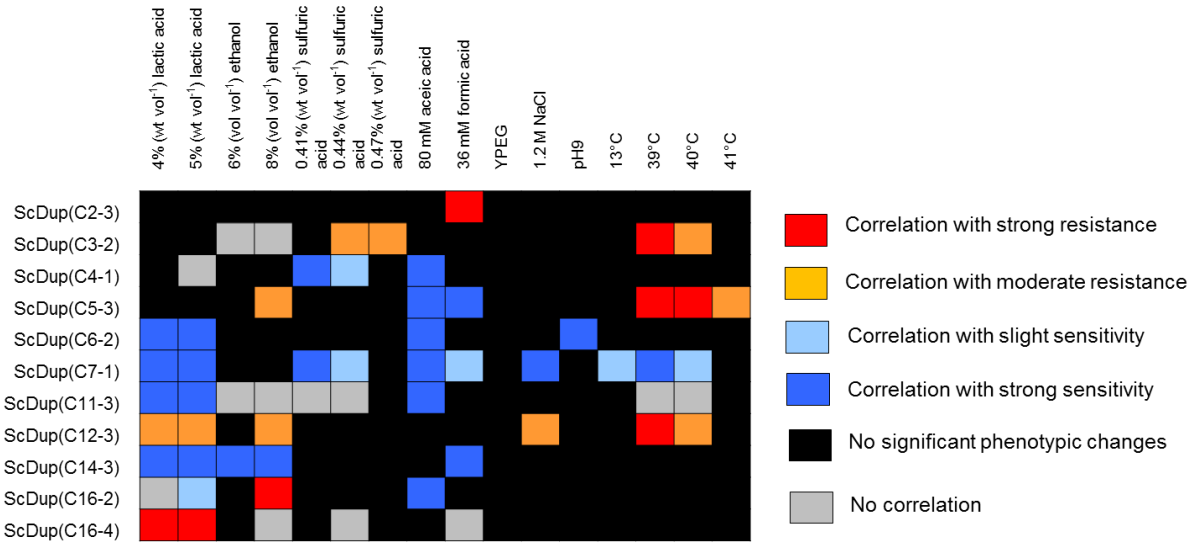
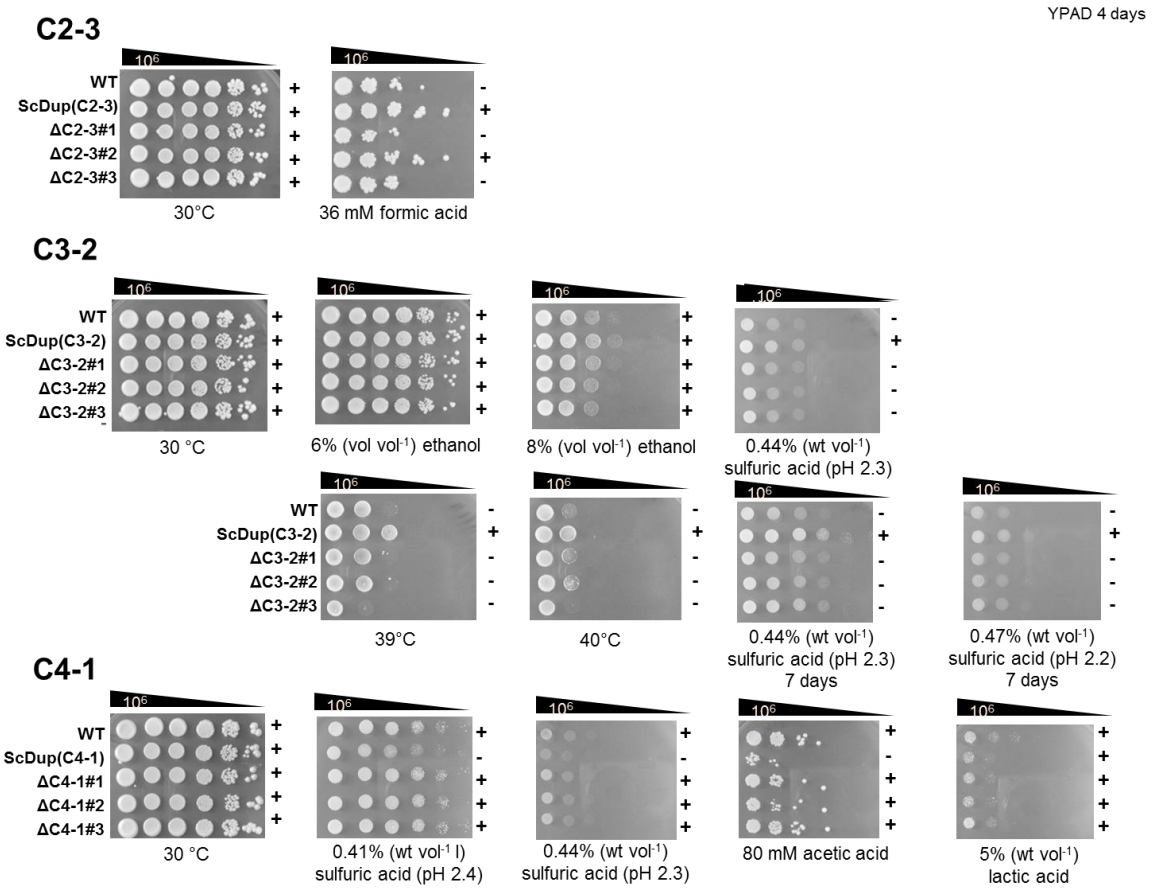
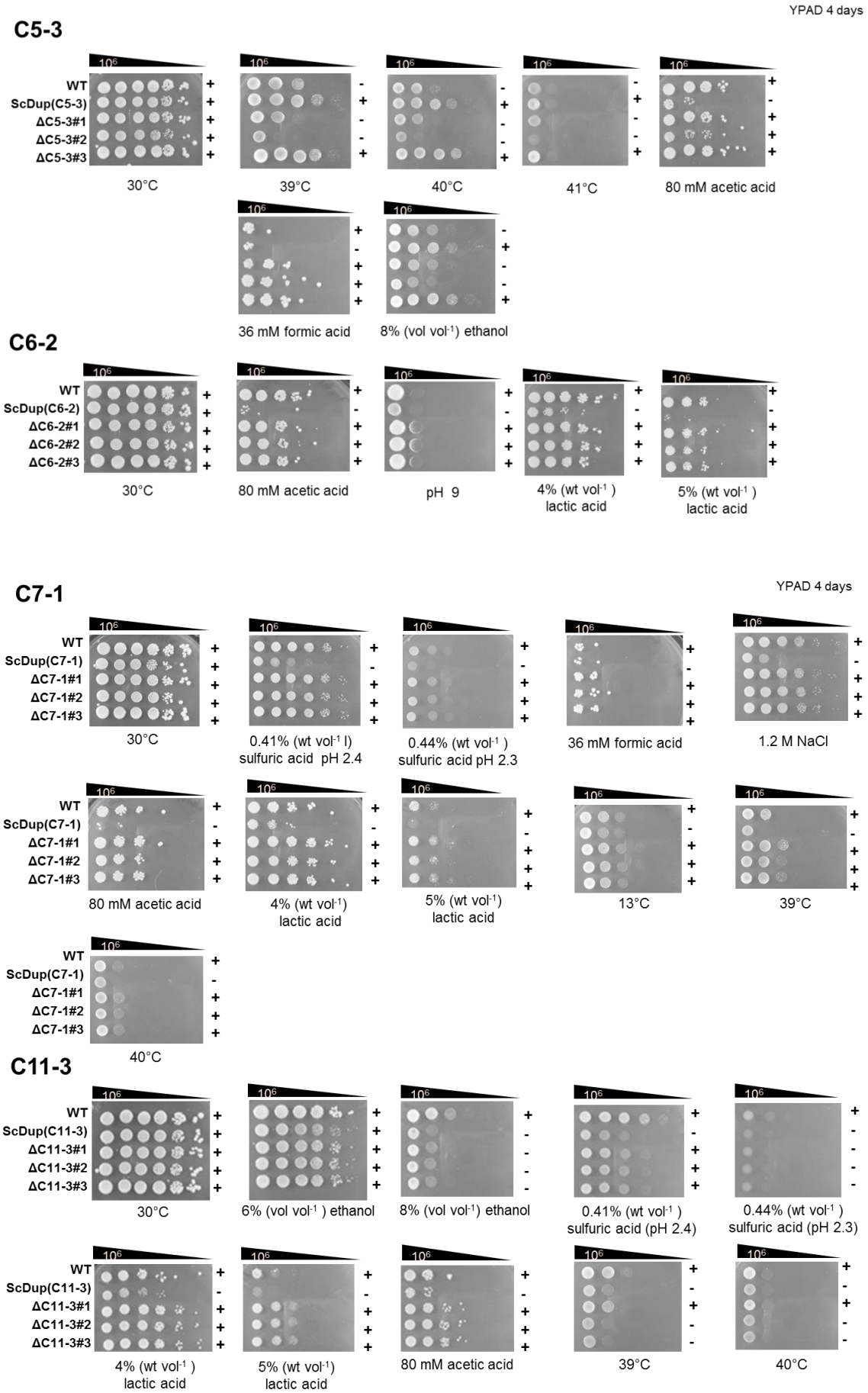
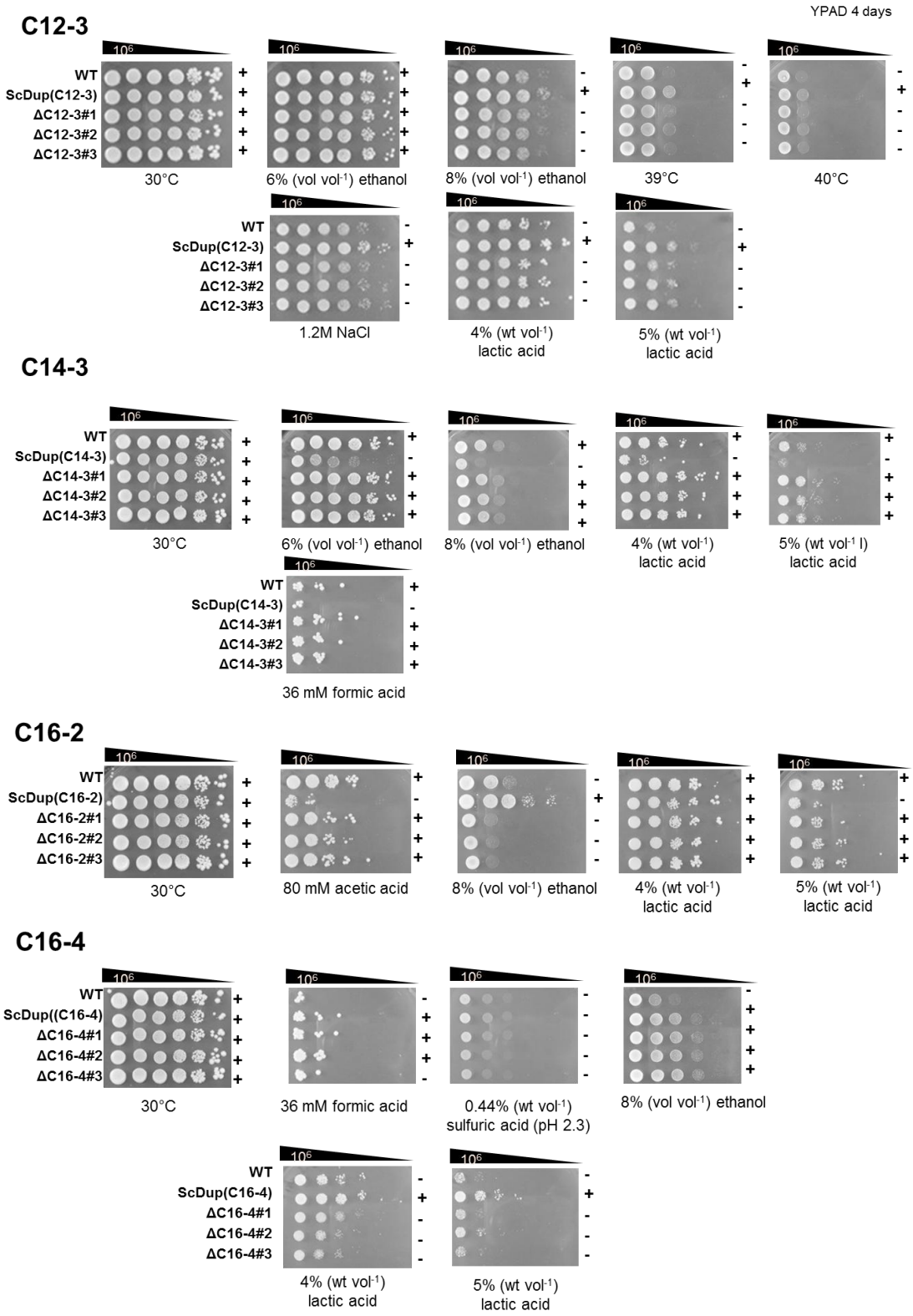


Figure 10. Relationship between segmental duplication of a particular region and phenotype. Effect of loss of the segmentally duplicated chromosome on phenotype. The correlation of phenotypic changes in aneuploids and the presence of a duplicated region is illustrated: red squares, orange squares, light blue and dark blue squares indicate correlation with strongly resistant phenotype, moderately resistant phenotype, slightly sensitive phenotype and strongly sensitive phenotype, respectively. Gray squares represents no correlation of observed phenotype and duplicated chromosome. Black square indicate stress conditions that were not tested as the segmental aneuploid did not show significant growth or other changes compared to the parental strain at the initial phenotypic examination step.

Figure 11. Analysis of the relationship between segmental duplication and phenotype using a chromosome loss strategy. Segmental aneuploid strains were induced to lose their additional chromosome and were then examined phenotypically. ΔCx-y indicates a derivative strains of SCDup(Cx-y) which has lost the duplicated chromosome. x represents chromosome number and y represents chromosome region. "+" and "-" means resistant phenotype and sensitive phenotype, respectively.




YPAD 4 days
C5-3
WT
ScDup(C5-3)
ΔC5-3#1
ΔC5-3#2
ΔC5-3#3
30°C
39°C
40°C
41°C
80 mM acetic acid
36 mM formic acid
8% (vol vol-1) ethanol
C6-2
ΔC6-2#1
ΔC6-2#2
ΔC6-2#3
ScDup(C6-2)
pH 9
4% (wt vol-1) lactic acid
5% (wt vol-1) lactic acid
C7-1
ΔC7-1#1
ΔC7-1#2
ΔC7-1#3
ScDup(C7-1)
0.41% (wt vol-1 l) sulfuric acid pH 2.4
0.44% (wt vol-1) sulfuric acid pH 2.3
1.2 M NaCl
4% (wt vol-1) lactic acid
5% (wt vol-1) lactic acid
13°C
C11-3
ΔC11-3#1
ΔC11-3#2
ΔC11-3#3
ScDup(C11-3)
6% (vol vol-1) ethanol
0.41% (wt vol-1) sulfuric acid (pH 2.4)
0.44% (wt vol-1) sulfuric acid (pH 2.3)


YPAD 4 days
C12-3
WT
ScDup(C12-3)
ΔC12-3#1
ΔC12-3#2
ΔC12-3#3
30°C
6% (vol vol-1) ethanol
8% (vol vol-1) ethanol
39°C
40°C
1.2M NaCl
4% (wt vol-1) lactic acid
5% (wt vol-1) lactic acid
C14-3
WT
ScDup(C14-3)
ΔC14-3#1
ΔC14-3#2
ΔC14-3#3
30°C
6% (vol vol-1) ethanol
8% (vol vol-1) ethanol
4% (wt vol-1) lactic acid
5% (wt vol-1 l) lactic acid
36 mM formic acid
C16-2
WT
ScDup(C16-2)
ΔC16-2#1
ΔC16-2#2
ΔC16-2#3
30°C
80 mM acetic acid
8% (vol vol-1) ethanol
4% (wt vol-1) lactic acid
5% (wt vol-1) lactic acid
C16-4
WT
ScDup((C16-4)
ΔC16-4#1
ΔC16-4#2
ΔC16-4#3
30°C
36 mM formic acid
0.44% (wt vol-1) sulfuric acid (pH 2.3)
8% (vol vol-1) ethanol
WT
ScDup(C16-4)
ΔC16-4#1
ΔC16-4#2
ΔC16-4#3
4% (wt vol-1) lactic acid
5% (wt vol-1) lactic acid

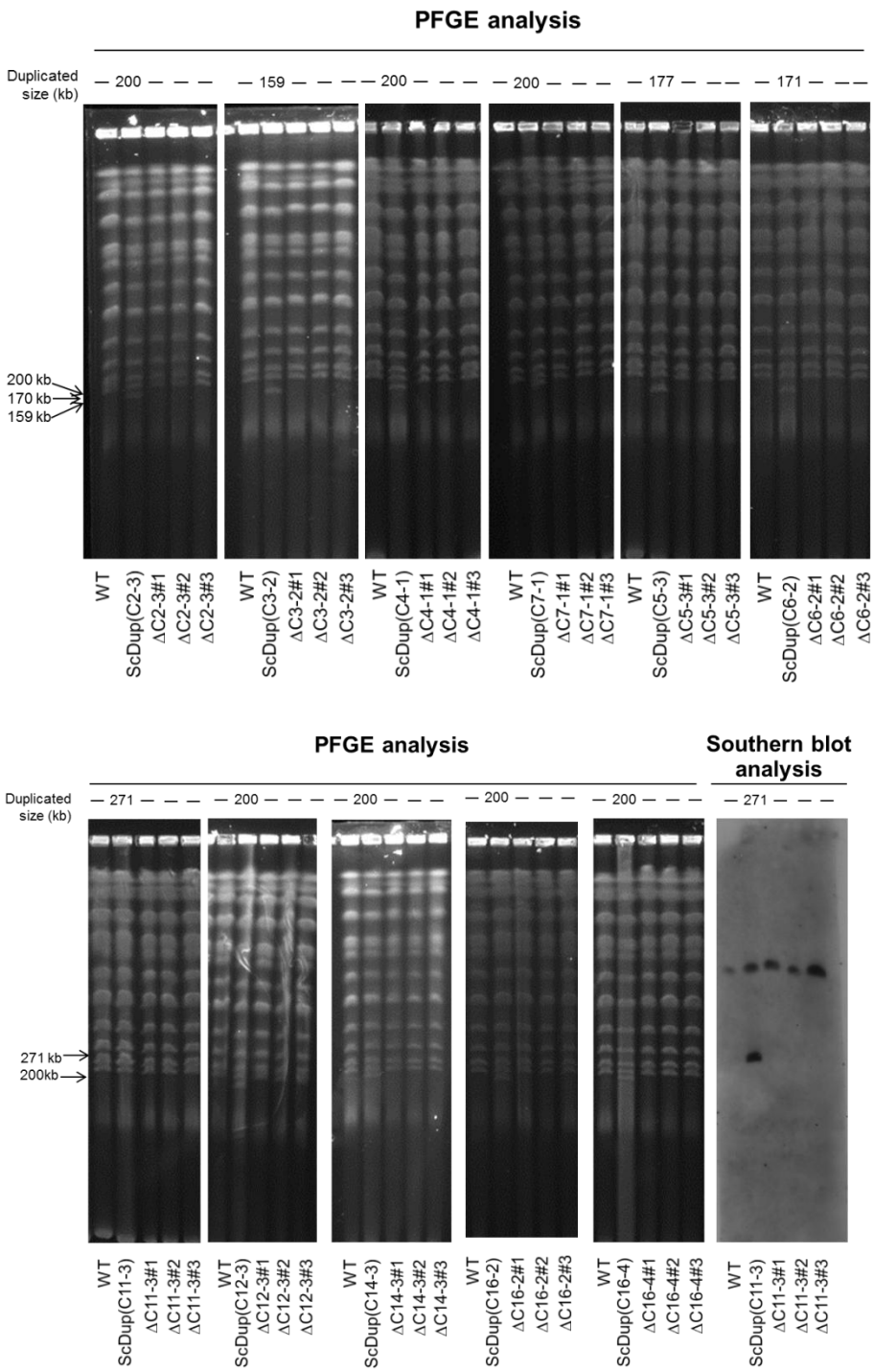


Figure 12. PFGE analysis of segmental aneuploid strains and derivative strains that had lost the duplicated chromosome

In the 53 segmental aneuploids constructed in this study, we noted that only 5 duplicated regions, C3-1, C3-2, C5-3, C12-3 and C15-3, harbored genes based on published data of single-gene overexpression, which confer sensitivity or resistance to a tested stress (Mulet *et al*., 1999, Versele and Thevelein, 2001, Zhang *et al*., 2004, Yang *et al*., 2011,

Anderson *et al*., 2012, Maoz *et al*., 2015) (see Discussion section). Therefore, the phenotypic changes in these segmental aneuploids could be interpreted as being the result of increased expression of particular genes. Interestingly, however, although the strains harboring the other 48 duplicated regions displayed phenotypic changes to stress, the duplicated regions did not contain genes whose overexpression caused the respective change to the tested stress. This suggests that for these 48 regions, an increased dosage of multiple genes might be responsible for the phenotypic alterations.

3.4 Discussion

Interestingly, I found that only the C4-2-S4 region, of the seven 50 kb sub-regions that could not be duplicated, did not contain any gene that might cause cell lethality when it is duplicated. It suggested that the influence of two or multiple genes in the C4-2-S4 sub-region prevented duplication of the 200 kb region. In other 6 sub-regions, the observation suggested the presence of duplicated region containing genes that caused a decrease in cell viability. For example, the C6-1-S2 region carries *TUB2* whose additional copies of *TUB2* cause cell lethality (Katz *et al*., 1990). Likewise, the C4-4-S2, C4-7-S4, C8-2-S3, C11-2-S2 and C14-2-S4 sub-regions harbor one to four genes that cause cell lethality (Liu *et al*., 1992, Sopko *et al*., 2006), toxicity (Douglas *et al*., 2012), or abnormal cell-cycle progression (Stevenson *et al*., 2001, Niu *et al*., 2008) when overexpressed (Table 12). Although these genes may be the cause of severe cell growth defects, there is other evidence that argues against this conclusion. In the reports showing adverse effects, these genes were overexpressed under the control of a strong inducible *GAL1* promoter and/or expressed in multi-copies. However, in the segmental aneuploidy strains here, the genes are regulated by the endogenous promoter with two or three copies at most. Moreover, Makanae *et al.*, catalogued the lowest number of copies of each *S. cerevisiae* gene that caused cell lethality when expressed under the native promoter

(Makanae *et al*., 2013). On the basis of their data, I inspected the genetic contents of the unduplicatable regions and found that none of the 50 kb sub-regions contained genes that cause a severe defect on cell growth when present as two or three copies (Table 13). Therefore, I conclude that combinatorial duplication of two or more genes in these sub-regions might be responsible for cell lethality which prevents duplication of the regions.

Table 12. Genes located in 50 kb unduplicated sub-regions whose overexpression is associated with cell lethality or abnormalities in cell cycle progression or the actin skeleton

Chromosome region	Subregion	Gene	Gene Systematic Name	Chromosome location	Phenotype	Strain Background	Reference
C4-4	S2	*BMH2*	*YDR099W*	Chr.IV 653,607-654,428	actin cytoskeleton morphology: abnormal	Other	Roth *et al*., (1999)
C4-4	S2	*PDS1*	*YDR113C*	Chr.IV 680,617-680,496	fitness defect	S288C	Douglas *et al*., (2012)
C4-4	S2	*PDS1*	*YDR113C*	Chr.IV 680,617-680,496	cell cycle progression: abnormal	W303	Stevenson *et al*., (2001)
C4-4	S2	*PDS1*	*YDR113C*	Chr.IV 681,617-680,496	inviable	S288C	Sopko *et al*., (2006)
C4-4	S2	*KIN1*	*YDR122W*	Chr.IV 694,700 – 697,894	Toxic gene		
C4-4	S2	*INO2*	*YDR123C*	Chr.IV 699,468-698,554	inviable	S288C	Sopko *et al*., (2006)
C4-7	S4	*SPP41*	*YDR464W*	Chr.IV 1,388,872 – 1,393,179	fitness defect	S288C	Douglas *et al*., (2012)
C4-7	S4	*STP1*	*YDR463W*	ChrIV 1,386,816 – 1,388,375	fitness defect	S288C	Douglas *et al*., (2012)
C4-7	S4	*TLG1*	*YDR468C*	Chr.IV 1,398,700-1,398,026	inviable	S288C	Sopko *et al*., (2006)
C4-7	S4	*UGO1*	*YDR470C*	Chr.IV 1,401,214-1,399,706	inviable	S288C	Sopko *et al*., (2006)
C6-1	S2	*ACT1*	*YFL039C*	Chr.VI 54,696-53,260	cell cycle progression: abnormal	S288C	Niu *et al*., (2008)
C6-1	S2	*ACT1*	*YFL039C*	Chr.VI 54,696-53,260	cell cycle progression: abnormal	W303	Stevenson *et al*., (2001)
C6-1	S2	*ACT1*	*YFL039C*	Chr.VI 54,696-53,260	inviable	Other	Liu *et al*., (1992)

Chromosome region	Subregion	Gene	Gene Systematic Name	Chromosome location	Phenotype	Strain Background	Reference
C6-1	S2	*TUB2*	*YFL037W*	Chr.VI 56,336-57,709	fitness defect	S288C	Douglas *et al.*, (2012)
C6-1	S2	*TUB2*	*YFL037W*	Chr.VI 56,336-57,709	inviable	Other	Liu *et al*., (1992)
C6-1	S2	*TUB2*	*YFL037W*	Chr.VI 56,336-57,709	cell cycle progression: abnormal	S288C	Niu *et al*., (2008)
C6-1	S2	*HAC1*	*YFL031W*	Chr.VI 75,179-76,147	cell cycle progression: abnormal	W303	Stevenson *et al.*, (2001)
C6-1	S2	*HAC1*	*YFL031W*	Chr.VI 75,179-76,147	actin cytoskeleton morphology: abnormal	S288C	Sopko *et al.*, (2006)
C6-1	S2	*FRS2*	*YFL022C*	Chr.VI 95,010-93,499	cell cycle progression: abnormal	S288C	Niu *et al*., (2008)
C8-2	S3	*DMA1*	*YHR115C*	Chr.VIII 340,109 – 341,359	fitness defect	S288C	Douglas *et al.*, (2012)
C11-2	S2	*HSL1*	*YKL101W*	Chr.XI 248,920-253,476	inviable	S288C	Sopko *et al.*, (2006)
C11-2	S2	*HSL1*	*YKL101W*	Chr.XI 248,920-253,476	cell cycle progression: abnormal	S288C	Sopko *et al.*, (2006)
C11-2	S2	*YKL100C*	*YKL100C*	Chr.XI 253,697 – 255,460	fitness defect	S288C	Douglas *et al.*, (2012)
C11-2	S2	*MIF2*	*YKL089W*	Chr.XI 273,394 – 275,043	fitness defect	S288C	Douglas *et al.*, (2012)
C11-2	S2	*RRP14*	*YKL082C*	Chr.XI 281,025 – 282,329	fitness defect	S288C	Douglas *et al.*, (2012)
C11-2	S2	*DHR2*	*YKL078W*	Chr.XI 288,845-291,052	cell cycle progression: abnormal	S288C	Niu *et al*., (2008)
C14-2	S4	*TOM22*	*YNL131W*	Chr.XIV 378,767-379,225	cell cycle progression: abnormal	W303	Stevenson *et al.*, (2001)
C14-2	S4	*SPC98*	*YNL126W*	Chr.XIV 387,227-389,767	cell cycle progression: abnormal	W303	Stevenson *et al.*, (2001)

Table 13. Genes whose upper copy number limit is less than 3 (Makanae *et al*., 2013)

Chromosome region	Locus name	Gene name	Chromosome location	Copy number limit
C4-1	*YDL192W*	*ARF1*	Chr.IV 116,321-116,866	1.0
C4-7	*YDR129C*	*SAC6*	Chr.IV 715,379-713,340	2.0
C5-2	*YER040W*	*GLN3*	Chr.V 229,795-231,987	1.5
C6-2	*YFL010C*	*WWM1*	Chr.VI 115,743-115,108	0.6
C6-1	*YFL037W*	*TUB2*	Chr.VI 56,336-57,709	2.7
C6-1	*YFL039C*	*ACT1*	Chr.VI 54,696-53,260	1.2
C6-2	*YFR028C*	*CDC14*	Chr.VI 210,068-208,413	0.9
C7-2	*YGL071W*	*AFT1*	Chr.VII 372,012-374,084	2.9
C7-5	*YGR159C*	*NSR1*	Chr.VII 807,656-806,412	1.7
C9-1	*YIL095W*	*PRK1*	Chr.IX 183,937-186,369	2.1
C10-1	*YJL164C*	*TPK1*	Chr.X 111,159-109,966	0.9
C11-2	*YKL042W*	*SPC42*	Chr.XI 358,475-359,566	1.8
C11-1	*YKL166C*	*TPK3*	Chr.XI 135,705-134,509	0.6
C13-2	*YML016C*	*PPZ1*	Chr.XIII 241,536-239,458	0.3
C14-4	*YNL016W*	*PUB1*	Chr.XIV 602,907-604,268	2.6
C15-2	*YOR008C*	*SLG1*	Chr.XV 342,414-341,278	2.6
C16-2	*YPL145C*	*KES1*	Chr.XVI 279,699-278,395	2.3
C16-2	*YPL154C*	*PEP4*	Chr.XVI 260,931-259,714	0.8
C16-1	*YPL203W*	*TPK2*	Chr.XVI 166,256-167,398	2.1
C16-3	*YPR008W*	*HAA1*	Chr.XVI 573,018-575,102	2.3
C16-4	*YPR080W*	*TEF1*	Chr.XVI 700,594-701,970	0.6
C16-5	*YPR173C*	*VPS4*	Chr.XVI 887,837-886,524	0.7

It has been reported that detrimental effects are proportional to the number of extra genes present in aneuploid cells (Torres *et al*., 2008). Yeast is generally more tolerant to aneuploidy compared to multicellular organisms. Since all but one of the segmental aneuploid strains did not show any effect on growth when cultured in liquid SC medium at 30°C for 24 hours, it appears that the additional copy of genes present in those regions did not influence proliferation. This conclusion is supported by the results of a previous study (Torres *et al*., 2007) in which the a delay in cell division of aneuploid for whole chromosome

is proportional to the number of genes located on the additional chromosome, although disomy for chromosome I (230 kb) does not cause a proliferation delay relative to the euploid genome. The sizes of segmentally duplicated chromosome constructed in this study are quite similar to or even less than (100 kb to 290 kb) that of chromosome I. Therefore, I suppose that the segmental aneuploid strains constructed in this study would not show severe growth defects under non-stressful conditions compared to the parental strain, as their gene dosage imbalance would be similar to or less than that of aneuploidy for chromosome I. However, the growth delay in ScDup(C15-4) might have resulted from the presence of genes whose over-expression interferes with cell proliferation.

It was reported that aneuploid strains of whole chromosome III (ca. 316 kb) acquired thermotolerance at 39°C (Yona *et al*., 2012). I found in this study that segmental aneuploid strains harbouring each of two duplicated region (ca.158 kb and ca. 159 kb) from chromosome III also displayed thermotolerance to 39°C (Fig. 9). This fact suggested that the increased dosages of genes in both sub-regions likely contributed to thermotolerance as in the case of aneuploid for whole chromosome III. Yona *et al.* also reported that the evolved aneuploidy of whole chromosome V (ca. 577 kb) confers alkaline pH resistance (Yona *et al*., 2012). However, in this study any segmental aneuploid of chromosome V did not show tolerance to high pH (Fig. 9). This observation suggested that the combination of increased dosages of gene-pair or multiple genes on a different region of chromosome V might be responsible for high pH resistance. Therefore, supposing that whole duplication of a particular chromosome gives phenotypic change, PCDup method could be exploited to identify a particular region that contributes to the specific phenotypes.

It has been well known that phenotypic changes in aneuploid are conferred by increased copy numbers of either single gene or multiple genes (Selmecki *et al*., 2006, 2008,

2009, Gresham *et al*., 2008, Pavelka *et al*., 2010b, Chen *et al*., 2012, Chang *et al*., 2013). It seems to be that most of the phenotypic changes found here were caused by multiple-gene effects rather than by single genes (Fig. 9 and Table 14). This notion is based on the fact that only a few of the duplicated regions that conferred sensitivity or resistance to environmental stresses contained genes whose overexpression causes such phenotypic alteration. These latter exceptions were *SAT4* (Mulet *et al*., 1999) on C3-1 region and *RSA3* (Anderson *et al*., 2012) on C12-3 region that confer high salt tolerance, *SPT15* (Yang *et al*., 2011) on C5-3 and *RSA3* (Anderson *et al*., 2012) on C12-3 region that confer ethanol resistance, and *LRE1* (Versele and Thevelein, 2001) on C3-1, *HCM1* (Maoz *et al*., 2015) on C3-2 and *LSP1* (Zhang *et al*., 2004) on C15-3 that confer thermotolerance. Moreover, we noted that several segmental aneuploids revealed tolerance to multiple stresses (Table 11 and Figure 4) and by scrutinizing SGD database, we found that some of the duplicated regions contains more than one gene that play a role in resistance to those stresses. For example, ScDup(C12-3) exhibited resistance to ethanol, high salt concentration, lactic acid and high temperature and we found that the duplicated region harbors several specific genes that are essential for tolerance to those stresses as genes whose deletion causes increased susceptibility to each stress. A set of genes that is required for ethanol resistance includes *COQ9*, *LCB5*, *LIP2*, *MSS51*, *QRI5*, *SWI6*, *VPS34*, *VPS63*, *YKE2* and *YLR194C*. A set of genes that is responsible for high salt stress tolerance includes *CLB4*, *DCS1*, *ERF2*, *MAP1*, *RCK2*, *VPS34* and *YLR194C*. A set of genes that play a role for lactic acid resistance include *BUR2*, *VPS34*, *VPS63* and *YPT6*. A set of genes that is required for thermotolerance includes *ARV1*, *BUR2*, *CDD1*, *COA4*, *CPR6*, *CSC1*, *DCS1*, *EST1*, *GSY2*, *HCR1*, *LCB5*, *LIP2*, ,*MAP1*, *MDL1*, *MMR1*, *MSS51*, *PBA1*, *QRI5*, *RFX1*, *RPL37A*, *RPS28B*, *RSA3*, *SAM1*, *SEC22*, *SHH4*, *SWI6*, *TOP3*, *UPS1*, *UPS2*, *UTP13*, *VPS34*, *VPS63*, *YLR169W*, *YLR269C* and *YPT6*. Based upon this information, we recognized that several genes seem to be responsible for resistance to

more than one particular stress. For example, *VPS34* is required for resistance to high salt, high lactic acid and high temperature, *VPS63* is essential for tolerance against high ethanol, high lactic acid and high temperature, *LCB5, LIP2, MSS51, QRI5* and *SWI6* are responsible for ethanol resistance and thermotolerance. *YLR194C* is required for ethanol stress as well as high salt stress resistance. *DCS1* and *MAP1* are essential for high salt along with thermal stress tolerance. *BUR2* and *YPT6* are responsible for resistance to lactic acid and heat stress. These facts suggested that multiple stress resistance observed in those segmental aneuploids might be conferred by the combination of increased dosage of several numbers of individual genes that are required for each particular stress resistance and duplication of gene that is responsible for multiple stress tolerance. However, since increased low dosages (from one copy to two copies) of a single specific gene located in those duplicated regions is not reported to cause multiple phenotypic alterations observed in this study, we think that duplication of only single specific gene is unlikely to cause those observed phenotypic changes but rather suggest that the combined effect resulting from simultaneously increased dosage of multiple genes in duplicated region conferred those observed sensitivity and resistance. Upon these observations, it should be emphasized that generating segmental aneuploidy with desired region could be beneficial approach to study the consequence of change in dosage of multiple genes within contiguous region and to identify possible underlying genes involved in such phenotypic alterations.

Table 14. Genes located in duplicated chromosome regions whose overexpression cause sensitive or resistant phenotypes

Chromosome region	Observed phenotype in this study	Gene	Gene Systematic Name	Chromosome location	Phenotype in previous study	References
C3-1	resistance to 1.2M NaCl	*SAT4*	*YCR008W*	ChrIII 128,470-130,281	resistance to sodium chloride: increased	Mulet *et al*., (1999)
C3-1	tolerance to 39°C and 40°C	*LRE1*	*YCL051W*	ChrIII 35,865-37,616	innate thermotolerance: increased	Versele and Thevelein, (2001)
C3-2	tolerance to 39°C and 40°C	*HCM1*	*YCR065W*	ChrIII 229,310-231,004	innate thermotolerance: increased	Maoz *et al*., (2015)
C5-3	resistance to 8% (vol vol^{-1}) ethanol	*SPT15*	*YER148W*	ChrV 465,303-466,025	resistance to ethanol: increased	Yang *et al*., (2011)
C12-3	resistance to 8% (vol vol^{-1}) ethanol	*RSA3*	*YLR221C*	ChrXII 579,024 - 578,362	resistance to ethanol: increased	Anderson *et al*., (2012)
C12-3	resistance to 1.2M NaCl	*RSA3*	*YLR221C*	ChrXII 579,024 - 578,362	osmotic stress resistance: increased	Anderson *et al*., (2012)
C15-3	tolerance to 39°C	*LSP1*	*YPL004C*	ChrXVI 551,657 - 550,632	innate thermotolerance: increased	Zhang *et al*., (2004)

In 11 arbitrarily selected strains, removal of the duplicated chromosome resulted in reversion to the parental phenotype in the majority of cases when subjected to a stress (47 out of 60 assays; Fig. 10). However, in a few cases, the phenotypes of the segmental aneuploid strains did not appear to be correlated with the duplicated chromosome. I envisage two possible explanations for this observation. First, the duplicated chromosome in the derivative strain might have recombined with the intact chromosome at a homologous or ectopic site and generated a chromosome rearrangement, such as translocation, which would make any linkage between phenotypic change and the segmentally duplicated chromosome unclear. Second, unknown mutations might have occurred by chance in the segmental aneuploid; however, the possibility that a combined effect of the presence of a segmentally duplicated region and unknown mutations is responsible for the phenotype also cannot be excluded. In

conclusion, a discovery of interesting phenotypes here that are indeed affected by the presence of segmentally duplicated chromosome gives us the understanding of genome function to response to stress environment.

3.5 Summary

In this chapter, I have applied PCDup technology to construct a series of segmental aneuplid strains that harbor 100 kb to 200 kb segmental duplications covering the whole genome of *S. cerevisiae*. The results showed that 53 out of 62 designated regions were duplicated with a proportion of desired karyotype of 3% to 100%. Nine remaining regions could not be duplicated possibly because genes or gene pairs located on those regions caused severe defects when they are presented in two copies or more. Moreover, to obtain insights into the function of the duplicated region, the phenotypes of segmental aneuploid strains under stresses were investigated. Interestingly, in some instances, segmental aneuploidy conferred tolerance to stresses such as high temperature, high ethanol content and strong acidic pH, while in others, stress sensitivity and in most severe case lethality presumably as a result of the simultaneous increases in dosages of multiple genes. The associations between the presence of segmentally duplicated chromosome and phenotypic alteration were also verified by whether removing the segmentally duplicated chromosome caused a reversion to the parental phenotype. Removal of the duplicated chromosome resulted in reversion to the parental phenotype in the majority of cases. From these observations, I suggested that PCDup technology will accelerate studies on the effects of changes in the gene dosage balance of multiple genes, enables improvements in desired industrial phenotypes in *S. cerevisiae* for breeding, and also provide insights into adaptive molecular mechanisms in the genome.

Chapter 4

General discussion and conclusion

Although genome rearrangement and alteration could be investigated by the laboratory evolution experiment coupled with whole-genome sequencing, this approach still has some limitations such as the shortage of natural variation in the laboratory, the long period of time in the experiment and the absence of appropriate control for the mutational process (Pál *et al*., 2014). In laboratory evolution experiments, it normally takes more than 200 generations and leads to accumulation of 4-20 independent mutations per populations (Dettman *et al*., 2012, Pál *et al*., 2014). Segmental aneuploidy has been found to cause phenotypic alterations in various kinds of organisms. Most studies about segmental aneuploidy were analysed by CGH and/or whole genome sequencing of samples obtained from natural isolation (Infante *et al*., 2003, Dunn *et al*., 2012, Chang *et al*., 2013) or laboratory evolution experiment (Dunham *et al*., 2002). Data of those studies revealed that additional mutations as well as segmental aneuploidy also occurred. Therefore, it is difficult to conclude which mutation confers the phenotypic changes. Recent development of genome engineering strategies enabled us to facilitate the alteration of targeted genomic regions rapidly and provided insight into the study in genome rearrangement in which natural genetic variation is limited (Sugiyama *et al*., 2005, 2006, 2008, 2009, Dymond *et al*., 2011, Annaluru *et al*., 2014). Thus, the genome engineering to generate segmental aneuploidy of desired region of chromosome could be useful for understanding segmental aneuploidy and its consequences.

In this study, I developed such novel genome engineering technology, PCDup in yeast, which harbors, in addition to one set of haploid genome, an extra chromosome consisting of a specific chromosomal region at the desired site through a single step of transformation. Using this technology, duplication of chromosomal regions up to 300 kb could be generated

efficiently. It should be noted that methodology like PCDup has never been developed in any kinds of organisms. In this study, I used this new technology to generate a set of approximately 200 bp overlapping duplicated regions that covered the 16 chromosomes of *S. cerevisiae* and investigated the phenotypic changes in those segmental aneuploid strains. A small number of regions in the genome could not be duplicated, possibly because they contained genes or gene pairs that cause cell lethality when they are duplicated. Interestingly, segmental duplication of some chromosomal region conferred resistant phenotypes or growth defects if the cells were grown under stresses as a result of the simultaneous increases in dosages of multiple genes. Therefore, I suggest that PCDup technology enables a simple genetic manipulation of the large scale of genome to contribute both to basic physiological studies and industrial applications.

In industrial process, yeast strains are often exposed to several stresses such as high temperature, strong acidic pH or high ethanol concentration. Tolerance traits to those stresses are controlled by multiple genes (Steinmetz *et al*., 2002, van Voorst *et al*., 2006, Patnaik, 2008, Mira *et al*., 2010, Swinnen *et al*., 2012). Therefore, overexpression or deletion of single specific gene cannot confer stress resistance. This fact requires the novel strategies to improve stress tolerance. Segmental anuploidy causes increased gene dosage of multiple genes at the same time. Consequently, it leads to increase in dosage and thus expression of genes located on the duplicated region simultaneously and also may affect expression of target genes on other chromosome(s), if some of them are regulatory genes, to induce preferable traits to survive under stresses. I noted that some segmental aneuploidies, such as ScDup(C2-3), ScDup(C3-2), ScDup(C5-3), ScDup(C12-3), ScDup(C16-2) and ScDup(16-4) as described in Chapter 3 enhanced simultaneous tolerance to several types of stress. If this simultaneous tolerance is proven to be caused by duplication of that particular region, I

believe PCDup could be exploited as a breeding tool to generate superior strains that have desirable industrial phenotypes.

It has been reported that segmental duplication may play an important role in the emergence of stress resistance in yeasts growing in unpleasant environments (Infante *et al*., 2003, Gresham *et al*., 2008, Chang *et al*., 2013). Through integration of the information on spontaneous genome rearrangements in natural and laboratory populations of yeast with the precisely induced segmental duplication constructed by PCDup technology, we will be able to improve our understanding on the biological significance of segmental duplication as an adaptive mechanism in the evolution of the *S. cerevisiae* genome. When whole duplication of a particular chromosome gives phenotypic change, PCDup technology might be exploited to identify an exact region (and more specifically exact gene) that contributes to the specific phenotypes. It should be emphasized that the collection of haploid yeast strains with the duplication of specific regions created in this study will be a valuable resource for studying the biological significance of the association of segmental aneuploidy with particular traits. These strains should help to accelerate research on gene dosage balance and the effects of simultaneously increased dosages of multiple genes on various cell physiologies.

To enhance the efficiency of expected segmental aneuploid strain, the increasing efficiency in homologous recombination and efficiency of target chromosome modification by the induction of the DSB at target site using site-specific endonuclease might be a possible way to improve PCDup technology. It has been reported that overexpression of some genes, i.e., *RAD51* and *RAD54* increase recombination up to 500 fold (DiCarlo *et al*., 2013a). Recently, CRISPR-Cas9 system (DiCarlo *et al*., 2013b) have been developed and speeded up genome engineering in various fields. CRISPR-Cas9 system could generate the DSB at a specific site. Therefore, this system might promote genome modification through the activation of the DNA repair machinery. Moreover, CRISPR-Cas9 system might enable

PCDup technology to generate multiple regions of segmental duplication at once. However, the obstacle for generating multiple regions of segmental duplication is the limitation of numbers of selectable marker for selection of candidate strains (transformants).

Furthermore, regarding the improvement of PCDup technology, the increases in size of segmental duplication should be addressed. According to the model I described in discussion section in Chapter 2 (Fig. 4), the size of segmental duplication is limited by the low frequency of chromosome nondisjunction of large chromosome (Hieter, 1985). Therefore, the induction of mutation of gene that are involved in chromosome nondisjunction might help to increase the proportion of segmental aneuploids with duplication of larger chromosomal region (more than 300 kb). By these improvements of PCDup technology, it could further promote the construction of segmental aneuploid strains with complex genomic diversity and subsequently broaden the knowledge about segmental aneuploidy and its consequences.

Many genetic disorders and cancers in humans are associated with segmental duplication (Bigner *et al*., 1988, Warburton, 1991, Crolla, 1998, Viersbach *et al*., 1998, Fuster *et al*., 2004, Lyle *et al*., 2009, Lucas *et al*., 2010, Chen *et al*., 2013, Akalin *et al*., 2014). However, the relationship between specific segmental duplication in human and its phenotypic consequence has not been clearly understood yet. The development of a technology to generate specific segmental aneuploids in a model organism is a starting point to explore gene(s) or genomic regions that are responsible for pathogenesis and diseases in higher organisms including humans. As demonstrated in this study, segmental aneuploidy occasionally improves the tolerance of cells to stress. This observation suggests that aneuploidy or segmental aneuploidy in human might enable cancer cells to adapt to extreme conditions than normal cells (Pavelka *et al*., 2010a). Information about segmental aneuploidy obtained from the yeast model may give rise to basic understanding of the molecular mechanisms of segmental aneuploidy-derived human diseases and cancer.

In conclusion, PCDup method is a simple, efficient, rapid, and economic tool for generating segmental aneuploidy at any selected region of a chromosome in *S. cerevisiae.* It can be used as a technique not only for deciphering genome function but also breeding novel strains with desired properties for industrial purposes.

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List of publication

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