GENETICS

Rate of spontaneous polyploidization in haploid yeast *Saccharomyces cerevisiae*

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Abstract

Polyploidization is involved in a variety of biological processes. It is one of the principal mechanisms of new species formation since it provides material for gene diversification and subsequent selection. Multiple cases of polyploidization were registered in different branches of the evolutionary tree of eukaryotes. Besides its role in evolution, polyploidization affects gene expression in living cells: pathological genome duplications often happen in cancer cells. The mechanisms and consequences of polyploidization are being studied extensively. However, quantitative determination of the polyploidization rate is challenging due to its low frequency and the absence of selective genetic markers that would phenotypically distinguish between haploids and polyploids. Our work describes a robust and straightforward method for discriminating haploid and polyploid states in the yeast *Saccharomyces cerevisiae*, a model organism for studying different aspects of polyploidization. The measurement of polyploidization rate showed that in yeast cells this process is mainly caused by autodiploidization rather than mating-type switching followed by hybridization.

Keywords: *Saccharomyces cerevisiae*, polyploidization, haploid, diploid, whole-genome duplication

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Introduction

Polyploidization occurs during rounds of duplication of the entire set of chromosomes; thus, polyploid organisms have additional copies of the original genome. Individuals with two copies of the genome are called diploids; with three copies — triploids, etc. Hugo de Vries first described polyploidization as a genomic mutation and suggested the theory of mutations based on polyploid mutants in *Oenothera* (DeVries, 1915). Polyploidy is common in nature — up to 35 % of vascular plants are polyploids (Meyers and Levin, 2006; Wood et al., 2009), and polyploid species were found among all major taxa of animals, in particular, among fishes (Leggatt and Iwama, 2003) and amphibians (Schmid et al., 2015). Ploidy variation is also typical for many yeast strains, including *Saccharomyces cerevisiae* (Zhu et al., 2016). Polyploidization plays a key role in evolution as a mechanism of new species formation; it provides a source of new genomes and material for gene specification (Ohno, 1970; Taylor, Van de Peer and Meyer, 2001; Crow and Wagner, 2006). In somatic tissues, the increased number of all chromosomes, caused by polyploidization, results in the enhancement of gene expression. Some extreme examples of somatic genome amplification are polythene chromosomes

of insects (Zhimulev et al., 2004). Polyploid cells have been revealed in higher vertebrates in hepatocytes, cardiac myocytes, arterial smooth muscle cells, lactating mammary glands, and decidual cells in the uterus during embryo implantation. Besides contributing to normal physiological processes, polyploidization is involved in some pathological situations: ploidy of cells increases during chronic inflammation, wound healing, or viral infection. Polyploidization also contributes to tumor progression (Sroga, Ma and Das, 2012; Orr-Weaver, 2015; Rios et al., 2016; Matsumoto et al., 2021). Since the increase in chromosome number leads to the increase of both the nucleus and whole-cell size, polyploid organisms are typically larger than their lower-ploidy ancestors. This phenotype is widely exploited in plant breeding, and most modern cultivated plants are polyploids (Feng et al., 2021; Ferrão et al., 2018).

One of the mechanisms of polyploidization is whole-genome duplication (WGD), or autodiploidization, when the replicated chromosomes fail to separate during cell division, resulting in polyploid progeny. New WGD rounds of a polyploid genome lead to further increase of ploidy. Autopolyploidization is supposed to have happened repeatedly in the evolutionary history of vertebrates and plants (Meyer and Shartl, 1999; Soltis, Marchant, Van de Peer and Soltis, 2015). Another source of polyploidy is interspecies hybridization, occasionally occurring in animals, plants, and microorganisms such as yeast (Whitney et al., 2010; Morales and Dujon, 2012; Cahill et al., 2015; Zhung et al., 2016; Figueiro et al., 2017). Most interspecies hybrids are initially sterile because they produce chromosome-imbalanced gametes, but after further genome autoduplication fertile individuals emerge, which can evolve into a new species (Karpechenko, 1928; Charron et al., 2019). Allopolyploidization accompanied by WGD is supposed to be one of the main mechanisms of speciation among eukaryotes (Villanea and Schraiber, 2019; Marcet-Houben and Gabaldón, 2015).

The yeast *S. cerevisiae* is a relevant model organism to investigate mechanisms of polyploidization because haploid, diploid, and polyploid forms represent natural, industrial, and laboratory *S. cerevisiae* strains. The *S. cerevisiae* life cycle includes the alternation of haploid and diploid stages: haploid cells of the opposite mating types (a and α) form non-mating (a/α) diploid hybrid cells, which further undergo meiosis and thus restore the haploid state. Typically, meiosis is stimulated by starvation (Kerr et al., 2012).

There are homo- and heterothallic strains of *S. cerevisiae*, and they differ in the relative duration of the haploid and diploid stages. Homothallic strains are diploids for almost the entire life cycle, with the haploid state represented mainly by ascospores. Haploid cells originating from ascospores in homothallic strains can easily switch their mating type to the opposite, and then mate to form diploid cells. The mating type of *S. cerevisiae* is controlled by the *MAT* locus mapped in the right arm of chromosome III. The *MAT* locus contains one of two alternative sequences — *MAT*a or *MAT*α determining a and α mating types, respectively (Lee and Haber, 2015). Besides the *MAT* locus, chromosome III contains two silent cassettes (*HML*α and *HMR*a) also bearing information about the mating type. Cells of the homothallic yeast strains possess endonuclease HO, which makes a double-strand break in a specific site of the *MAT* locus. During the subsequent programmed gene conversion between the active *MAT* locus and one of two silent cassettes, *HML*α or *HMR*a, genetic material in the *MAT* locus is replaced by one of the two cassettes containing information about the opposite mating type (Haber, 1992; Klar, 2010). As a result, the cells undergo mating type switching and become able to mate with neighboring cells of the opposite mating type. Unlike homothallic strains, heterothallic ones lack endonuclease HO activity and thus can maintain their haploid state for an unlimited time. This capacity makes them a suitable model for studying the mechanisms of polyploidization unrelated to the normal life cycle of yeast.

Since the cells of heterothallic strains are haploid, the simplest and the most frequent variant of polyploidization for them is diploidization. It is known that yeast heterothallic haploid cells can diploidize by two mechanisms — hybridization and WGD (Fig. 1) (Edgar and Orr-Weaver, 2001; Lee and Haber, 2015).

The mechanism of heterothallic strains hybridization is based on the mating type switching, which in the absence of HO activity has a very low frequency (Meiron, Nahon and Raveh et al., 1995). This rare event can be detected due to the appearance of "illegitimate" hybrids during the crossing of the two strains with the same mating type. It can happen due to various genetic changes in the *MAT* locus (Repnevskaya, 1987; Inge-Vechtomov and Repnevskaya, 1989). α→a switching occurs when the *MATα* locus is lost, inactivated due to mutations or temporary lesions, or replaced with the *HMR*a cassette — in all these cases a-specific genes are expressed constitutively. a→α switching occurs when *MAT*a is replaced by the silent cassette *HML*α. The frequencies of different genetic events leading to mating type switching α→a were studied previously in the α-test (Kochenova et al., 2011). According to our previous results, the frequency of spontaneous "illegitimate" hybridization caused by the mating type switching α→a in heterothallic strains is approximately 10^{-6} (Inge-Vechtomov et al., 1986; Kochenova et al., 2011; Stepchenkova et al., 2011; Zhuk et al., 2020).

The frequency and mechanism of WGD remains unclear. Establishing the polyploidization rate in *S. cerevisiae* requires screening of thousands of individual colonies,

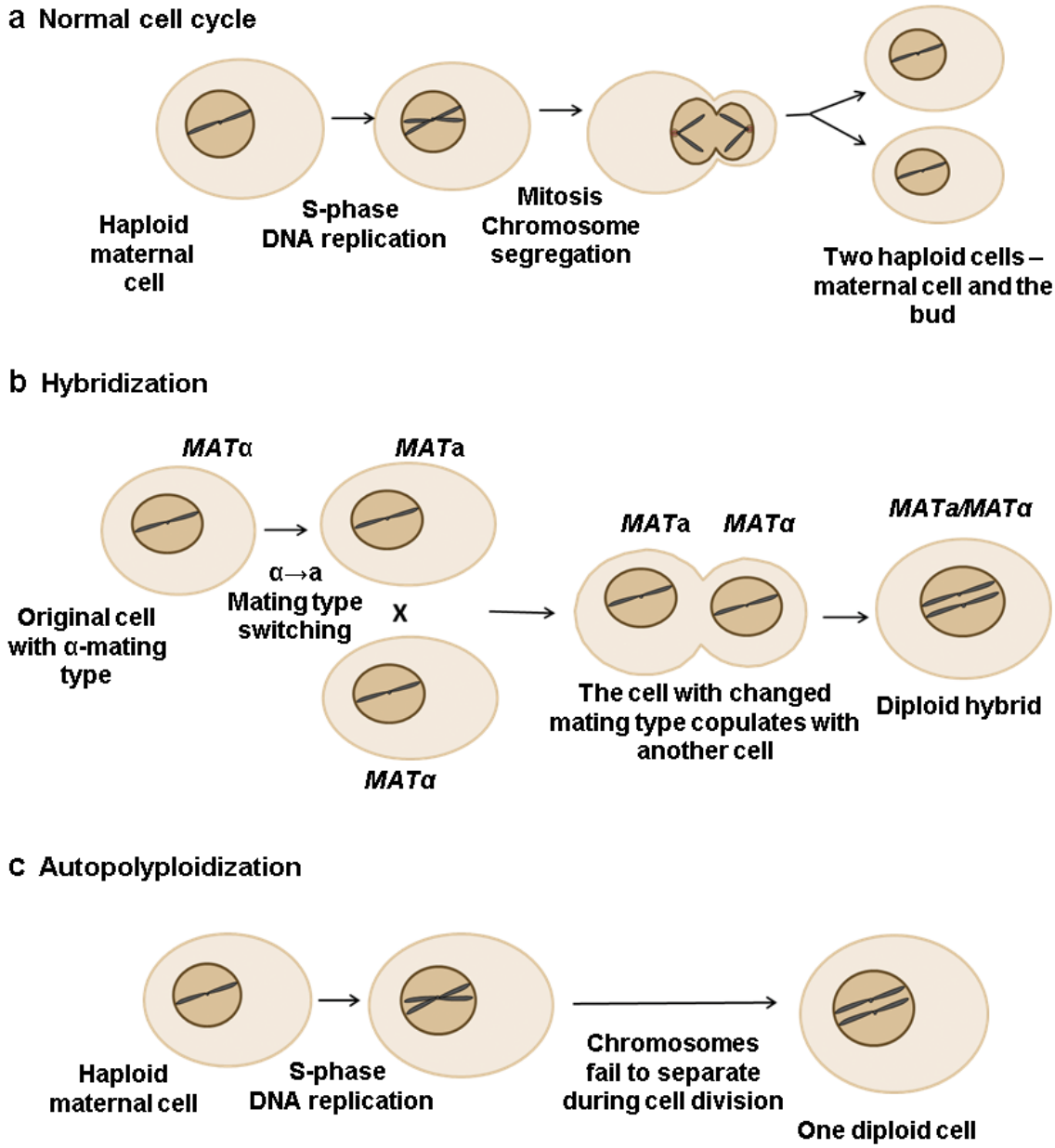

Fig. 1. The mechanisms of diploidization in haploid yeast. **(a)** Normal cell cycle. During the normal cell cycle, the chromosomes are replicated during the S-phase, and then doublet chromosomes equally separate into the daughter cells in mitosis. **(b)** Hybridization. One of the maternal cells switches its mating type due to different genetic events related to the *MAT* locus, copulates with a neighbor cell, and produces a diploid hybrid. As a result, the hybrid contains two copies of genomes, heterozygous at the *MAT* locus. **(c)** Autopolyploidization. The chromosomes are replicated during the S-phase but fail to separate during cell division. During WGD, the maternal haploid cell produces diploid progeny, which is the absolute homozygote.

which is challenging because there are no direct selective markers of polyploids. Though several methods of ploidy identification have been described, not all of them are relevant for measuring the polyploidization rate (Table 1). Recently, Harari et al. suggested an indirect statistical approach and estimated the polyploidization rate in yeast in a long-term in-lab evolutionary experiment by monitoring the genome size of cells in several cultures during 100 generations using flow cytometry (FC) (2018). The value of the WGD rate varied from 5.3×10^{-5} to 9×10^{-5} in *MAT*a and *MATα* strains, correspondingly. The disadvantage of this approach is that the polyploids in yeast culture could be revealed only when their proportion was high enough to be detected by FC (a minimum of several percent). Another problem is the duration of the experiments. The relatively high ratio of polyploids in the culture might mean that polyploids had an adaptive advantage over haploids, or it could be the result of their appearance at an early stage of cultivation and the resulting production of numerous progeny. Therefore, the value of the polyploidization rate obtained in long-term evolutionary experiments may be modified by indirect factors.

Table 1. Methods of identification of ploidy states in *S. cerevisiae*

Method of polyploid detection	Selective criteria	Frequency or rate of polyploidization	Reference
Flow cytometry	Genome size	≈10^{-4}	Harari et al., 2018
Sensitivity to γ-radiation	The efficiency of DNA-strand breaks repair by homologous recombination	ND	Mortimer, 1958; Lobachevskii, Cherevatenko and Mishonova, 1988
Mutation accumulation test	Decrease in survival after mutagen exposure due to accumulation of recessive mutations in the genome	ND	Sharp, Sandell and Otto, 2018
Colony color essay Red/Pink/ White selection	The degree of colony pigmentation in a strain containing the *ade3* null mutation in its genome and the *ADE3* allele on a centromere plasmid depends on the number of plasmid copies in relation to the number of genomic copies of the *ade2*.	ND	Baum, Yip, Goetsch and Byers, 1988
Loss of heterozygosity	Frequency of mutant colonies' appearance due to loss of heterozygosity	0–75 % during plasmid transformation	Karpova et al., 1984
Canavanine papillation ploidy test	Frequency of appearance of recessive canavanine-resistant colonies on a selective media after UV irradiation	ND	Schield, Ananthaswamy and Mortimer, 1981
Genetic analysis	Ability to produce viable spores	ND	Schield, Ananthaswamy and Mortimer, 1981

ND — not determined.

Here we suggest a simple, rapid, and straightforward method for the identification of polyploids in a culture of a haploid *S. cerevisiae* strain of the α mating type, building on the approach of Shield et al. (1981). This method undeniably discriminates between haploids and polyploids by the phenotype of yeast colonies. It is based on the difference in the frequency of recessive UV-induced Canavanine-resistant mutants in haploid and polyploid strains bearing the wild-type *CAN1* gene. In our work, we have estimated the rate of polyploidization in overnight *S. cerevisiae* cultures and concluded that most diploids originated as a result of autodiploidization since the frequency of "illegitimate" hybridization was dramatically lower.

Materials and methods

Strains. All the strains used in this work were from the Peterhof yeast collection. Strain K5-35V-D924-*ade1-14* (*MATα lys5::KanMX ura3Δ leu2Δ met15Δ ade1-14 cyh*^{r} [*PIN*+]) was used to measure polyploidization rate; strains 2G-P2345 (*MAT*a *his5*) and 78A-P2345 (*MATα his5*) were used as mating-type testers. Strain D926 (*MAT*a//*MATα ade1Δ*//*ADE1 lys2Δ*//*lys2Δ his3Δ*//*his3 Δura3Δ*//*ura3Δ leu2Δ*//*leu2Δ thr4Δ*//*thr4Δ*) was used as a diploid control.

Media. Yeast strains were incubated in YEPD, rich broth (1 % yeast extract, 2 % Peptone, 2 % glucose). Solid YEPD media contained 2 % agar. The selective media for discrimination between haploid and polyploid cells was minimal media (MD) (Zakharov et al., 1984) containing L-canavanine (40 mg/L) and uracil, adenine, lysine, leucine, methionine at corresponding final concentrations 20 mg/L, 20 mg/L, 30 mg/L, 60 mg/L, 150 mg/L.

Identification of polyploid strains. The analyzed cultures were grown overnight at 30 °C, then diluted to the appropriate concentration and placed on YEPD plates to obtain individual colonies. Each colony was then transferred to a fresh YEPD plate at a square about 0.5 cm^2 and grown overnight. Then the plates were replicated on the selective media containing L-canavanine and exposed to UV at a dose of 40 J/m^2. After five days of incubation at 30 °C, colonies unable to form canavanine-resistant (Can^r) colonies (non-mutable phenotype) were selected (see Fig. 2). Later, the ploidy of the selected colonies was confirmed by the FC method.

Measuring polyploidization rate. The rates of polyploidization were measured by the fluctuation test in experiments with sets of six independent cultures. The polyploidization rate was calculated from polyploidization frequency (the ratio of the polyploids to the total amount of living cells in each culture) using the Drake equation (Drake, 1991). Data from six independent cultures were used to calculate the median and 95 % confidence intervals for the median as described (Dixon and Massey, 1969).

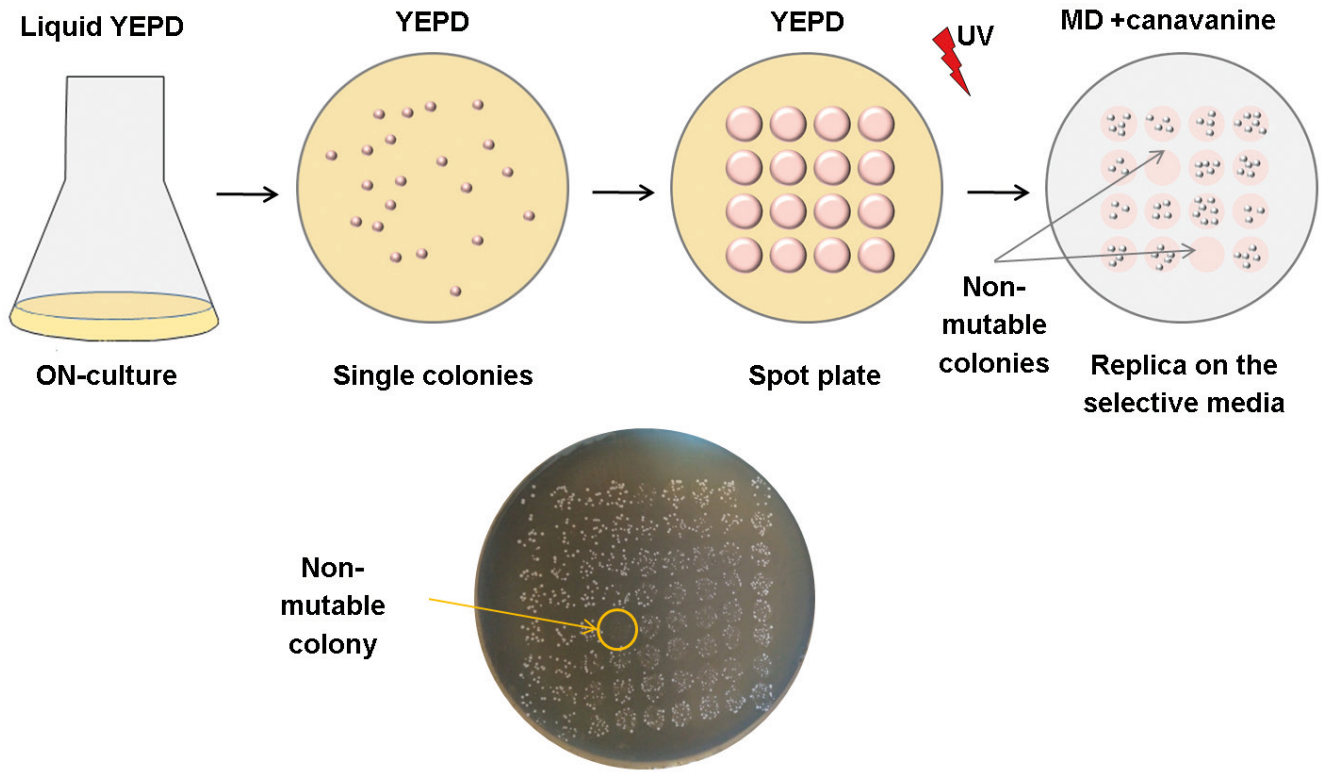

Fig. 2. The scheme of the experiment. The upper part: A suspension of cells (overnight culture) was diluted and plated on a YEPD media to obtain individual colonies, which were then plated as small patches on a fresh YEPD plate. After one day of incubation at 30 °C, the plates were replicated on the selective media containing canavanine and exposed to UV irradiation. In five days, haploid colonies form Can-resistant colonies at the replica, while no resistant colonies could be seen in polyploids. The lower part: the appearance of Can^r colonies after UV irradiation. Patches of haploid clones have multiple Can^r papillae, potential di- or polyploid clone does not produce recessive Can^r mutations (circulated by the yellow line).

Flow cytometry. FC analysis was performed in the Saint Petersburg State University Research Park in the Center of Molecular and Cell Technologies using a flow cytometer and sorter BD FACS AriaIII. The cells were collected and fixed in 70 % ethanol overnight at 4 °C. Fixed cells were washed with water and then treated by RNase for 6–15 hours at 37 °C and Proteinase K for 1 hour at 50 °C. The pellet cells were diluted in 50 mM Tris buffer pH = 7.5 to an OD 600 nm of 0.1–0.2, sonicated for 2–5 min at 40 kHz, and stained by Propidium Iodide (PI) at a final concentration of 5 μg/ml. Samples were stored in the dark at 8 °C until analysis. The 100,000 cells per sample were analyzed with a laser at 488 nm using filter 616/23 nm to detect the PI fluorescence. The Flow Jo software was used to create the histograms of fluorescence intensity.

Results and discussion

The method we used in this study to evaluate the diploidization rate in yeast cells is based on a qualitative difference in the frequency of recessive Can^r mutations after UV-irradiation in clones of haploid and polyploid strains of *S. cerevisiae*. The *CAN1* locus determines sensitivity to the toxic arginine analog L-canavanine. This genetic marker is a widespread standard reporter gene in mutational tests. The haploid strains with a single wild-type allele of the *CAN1* gene form Can^r colonies with low frequency (~10^{-7}). The presence of the second wild-type copy of the *CAN1* gene in homozygous diploids drastically decreases the frequency of Can^r colony formation, because it demands two steps of inactivation of both *CAN1* copies. The first step is the inactivation of one of the two copies of *CAN1* due to mutation (base substitutions or frameshift mutations). Inactivation of the second copy of *CAN1* usually happens through allelic crossover, gene conversion, or chromosome loss with the rate of about 10^{-4}, so the predicted rate of appearance of mutation in diploids is ~10^{-11} (Gordenin and Inge-Vechtomov, 1981; Pavlov et al., 1988; Ohnishi et al., 2004; Lada et al., 2013). The frequency of Can^r mutations in polyploid strains bearing three and more copies of the wild-type *CAN1* is even lower. Thus, the mutant Can^r colonies derive more frequently from haploid cells than from polyploids. The difference between haploid and diploid strains becomes more visible after UV irradiation: in haploids the frequency UV-induced Can^r mutations increases by several orders of magnitude, while diploid and polyploid colonies produce almost no Can^r mutants.

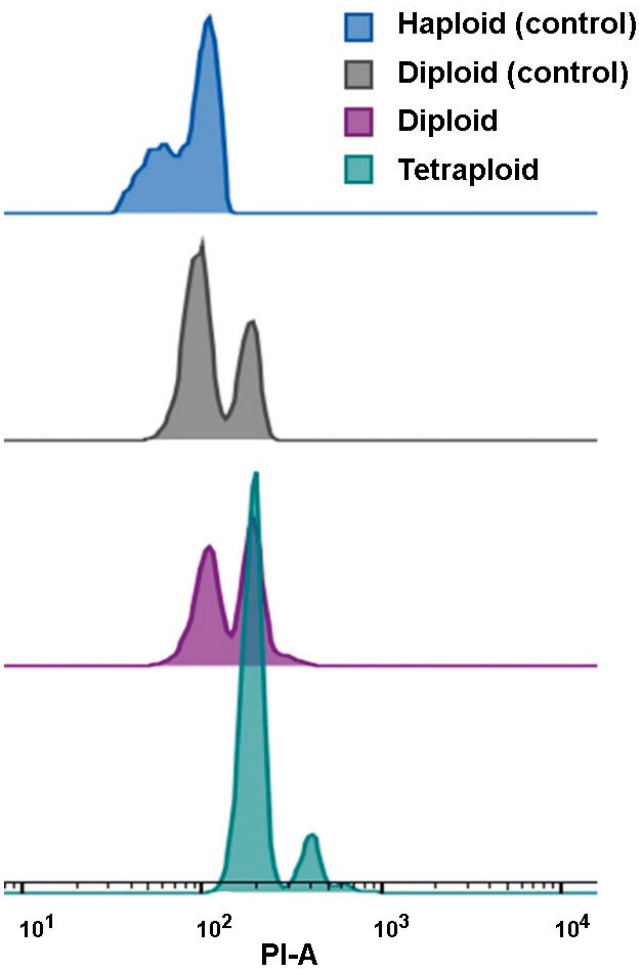

Fig. 3. FC profiles of yeast strains. Histogram of DNA content in cells stained by propidium iodide (PI-A). The histogram reflects the total number of cells in a sample with PI fluorescence corresponding to 1c, 2c, 4c or 8c DNA content.

Figure 2 represents the scheme of the experiment revealing the ploidy of yeast cells in the overnight culture. Using this technique, we analyzed approximately 1500 colonies from each of 6 independent cultures (9266 colonies total) of the haploid strain K5-35B-D924-ade1-14 and selected 48 UV non-mutable colonies. The ploidy of the selected colonies was checked by FC, and 39 of them turned out to be diploids, 1 — tetraploid, and 8 — haploids; i.e., polyploids were detected in 83 % of immutable clones. Considering only confirmed diploids and one tetraploid, we calculated the spontaneous rate of polyploidization in each of the six cultures and estimated that the median and 95 % confidence interval of diploidization rate was 6.2×10^{-5} (5.1×10^{-5} — 36.4×10^{-5}). The FC profiles of two polyploids obtained in the experiment, diploid and haploid controls, are presented in Fig. 3.

The rate of polyploidization measured in our study is very close to the value obtained by Harari et al. (2018). The method used in our work considers the phenotypes of colonies that originated from single cells, thus excluding the weakness of long-term experiments discussed in the introduction. The few non-mutable colonies which turned out to be haploids might have appeared due to duplication of the *CAN1* locus or mutation in the genes encoding for the mutasome components (Halas et al., 2021). Thus, though the suggested method looks robust enough for rapid routine analysis of ploidy, it should be used in combination with FC when high accuracy of polyploid identification is needed.

Two processes may lead to the appearance of polyploid cells in a haploid yeast culture: WGD and mating-type switching followed by hybridization with a neighboring cell. During WGD, polyploids contain identical genome copies, including the *MAT* locus. Consequently, such polyploids have the same mating type as maternal haploid cells (*MAT*a//*MAT*a or *MAT*α//*MAT*α). "Illegitimate" hybridization demands preceding mating-type switching, so the hybrids that appeared in the yeast culture of α mating type may have either α- (*MAT*α//*mat*a and *MAT*α//*mat*aΔ) or non-mating phenotype (*MAT*α//*MAT*a). To assess the relative contribution of WDG and hybridization to polyploid formation, we identified the mating type of selected non-mutable colonies (Table 2).

Table 2. Mating type of selected non-mutable clones of different ploidy

ploidy \ mating type	α	a	n/m
haploid	3	5	0
diploid	38	0	1
tetraploid	1	0	0

Most selected diploids, as well as the tetraploid, had an α mating type. Thus, they could appear as a result of autoduplication or "illegitimate" hybridization. Moreover, only one diploid was non-mating, originating due to heritable mating type switching. Among the selected non-mutable haploids there were cells with a-mating type. They might have appeared due to the mating type switching and incomplete mating. According to our previous results (Inge-Vechtomov et al., 1986; Kochenova et al., 2011; Stepchenkova et al., 2011), the frequency of "illegitimate" hybridization in the same strain is approximately 10^{-6}, which is about 100 times lower than the polyploidization rate. Thus, we can assume that mating type switching and successive hybridization is a much rarer event than genome autoduplication. The results shown in Table 2 do not contradict this assumption. Harari et al. (2018) made the same conclusion. Considering two different mechanisms of the origin of diploids, they analyzed the sequences of the *MAT* locus from isolated diploid cells to estimate the rate of both autoduplication and mating type switching and subsequent hybridization. The estimated values were $\approx 10^{-4}$ and $\approx 10^{-7}$, respec-

tively, and the authors reasonably concluded that polyploidization occurred more likely via autoduplication.

Multiple environmental factors can influence the polyploidization rate in a yeast culture. Haploids have a selective advantage over polyploids in the presence of caffeine and hydroxyurea (Harari et al., 2018). Ethanol is a well-known factor provoking polyploidization, and many industrial yeast strains, selected to produce beverages with up to 12 % ethanol, are polyploids (Turanli-Yildiz et al., 2017). The other polyploidy inducers are high hydrostatic pressure and KCl (Hamada et al., 1992; Harari et al., 2018). It is known that stressful factors can trigger polyploidization in the hepatocytes of mammals (Gentric and Disdouets, 2014). The mechanism and the consequences of stress-induced polyploidization are still not well understood. Some researchers suppose that polyploidization can protect cells from genotoxic damaging factors (Lin et al., 2020), while others suggest that proliferating polyploid cells can provoke cancer development (Davoli and de Lange, 2011; Matsumoto et al., 2021).

As all organisms using sexual reproduction have an alternation of haploid and diploid phases in their life cycle, ploidy is under strict genetic control. A robust and straightforward method of polyploid identification, which we have used in our work, can help to reveal the genes controlling ploidy and to study the molecular mechanisms of polyploidization. The genetic changes leading to the increased rate of polyploidization should be associated with the genes controlling different steps of cell division. To date, 16 genes contributing to the increase of polyploidization level have been found in the *S. cerevisiae* genome. *BBP1*, *CDC31*, *ILP1*, *KAR1*, *MPS1*, *MPS2*, *MPS3*, and *NDC1* encode for proteins participating in the control of spindle polar body duplication (Shield et al., 1981; Rose and Fink, 1987; Winey et al., 1991; Chan and Botstein, 1993; Xue et al., 1996; Chen et al., 2000; Jaspersen et al., 2002; Jaspersen et al., 2006); *MOB1* is required for cytokinesis and cell separation (Luca and Winey, 1998); *BEM2* is involved in the control of cytoskeleton organization (Chan and Botstein, 1993); *WHI3* is involved in the cell-cycle control (Shladebeck and Mosch, 2013); *ESP1* and *BIR1* regulate chromosome segregation (Baum et al., 1988; Li et al., 2000). The other genes encode for proteins indirectly participating in chromosome duplication and segregation: *DEF1* is RNAPII degradation factor (Stepchenkova et al., 2018), *KAP123* mediates nuclear import of ribosomal proteins and import of histones H3 and H4 (Ptak et al., 2009), and *BFR1* is necessary for nuclear secretion (Xue et al., 1996).

The molecular mechanisms controlling the genome size are still unclear and require new methods for further investigation. Establishing a direct, simple method for polyploid identification in the *S. cerevisiae* model organism may contribute considerably to elucidating the mechanisms of cancer development, drug resistance of pathogens, and the evolution of species.

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