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Genetic and phenotypic diversification of yeast upon meiotic reversion : ubiquity, mechanism and applications

Simone Mozzachiodi

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THÈSE DE DOCTORAT

Diversification génétique et phénotypique de la levure par réversion de la méiose: ubiquité, mécanisme et applications

Genetic and phenotypic diversification of yeast upon meiotic reversion: ubiquity, mechanism and applications

Simone MOZZACHIODI

IRCAN-CNRS UMR7284-INSERM U1081 Génomique des populations et traits complexes

Meiogenix

**Présentée en vue de l'obtention
du grade de docteur en** Sciences de la
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Devant le jury, composé de :
Martine BASSILANA, Directeur de
Recherche CNRS, iBV Nice,
Président du Jury
Duncan GREIG, Professeur
University college London, London
Rapporteur
Kenneth H. WOLFE, Professeur
University college Dublin, Dublin
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Université Paris-Saclay, Paris
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Alain Nicolas, Professeur émérite
Institute Curie, Paris
Membre invité
Gianni LITI, Directeur de Recherche CNRS,
Institut de Recherche sur le Cancer et le
Vieillessement, Nice

- Résumé -

Les hybrides constituent une source de variabilité génétique et phénotypique importante qui résulte de combinaisons alléliques uniques issues du mélange de génomes distincts. L'humanité a exploité pendant des milliers d'années l'hybridation des plantes, des animaux et des microbes pour leurs performances améliorées par rapport à celles des espèces endogènes. Cependant, les hybrides sont dans la plupart des cas des « impasses » évolutives en raison de la très faible viabilité de leur progéniture, empêchant de ce fait l'amélioration des caractéristiques d'intérêt industriel par croisements. Dans cette thèse, j'ai profité de la capacité de la levure bourgeonnante *Saccharomyces cerevisiae* à interrompre la méiose et à restaurer la croissance mitotique - par l'ajout soudain de nourriture abondante - pour palier à la stérilité des hybrides. Ce processus, dénommé « Return to Growth » (RTG), induit des cassures double-brin d'ADN générant des régions de perte d'hétérozygotie (LOH), et mène à la recombinaison du génome et à la création de variabilité phénotypique sans avoir recours à la reproduction sexuée. Bien qu'il ait déjà été démontré que des hybrides fertiles dérivés de souches de laboratoire peuvent effectuer le RTG, on ne connaît pas le degré d'application de celui-ci chez des souches d'origines diverses présentant des structures génomiques et un niveau de stérilité différents. Pour aborder ce problème, j'ai soumis plusieurs hybrides artificiels et industriels au RTG et j'ai caractérisé son impact au niveau génomique et phénotypique.

Dans la première partie de ma thèse, j'ai déterminé dans quelle mesure le RTG peut aider à surmonter les barrières de stérilité post-zygotiques décrites dans les espèces de *Saccharomyces*. Tout d'abord, j'ai analysé des données de séquençage d'échantillons issus d'une souche hybride stérile soumise au RTG, et j'ai confirmé que le génome était recombiné, bien que la machinerie méiotique soit défectueuse. Ensuite, j'ai mis au point un système génétique permettant de mesurer le taux de recombinaison du génome à un locus donné et j'ai séquencé plusieurs individus afin de démontrer l'efficacité du RTG chez plusieurs hybrides présentant une stérilité aiguë. De plus, j'ai caractérisé l'impact de fortes divergences génomique sur le RTG en étudiant des hybrides entre *S. cerevisiae* et *S. paradoxus*, et j'ai observé que le système de réparation des mésappariements de l'ADN inhibait la recombinaison génétique induite par le RTG. Enfin, j'ai mis en évidence la capacité du RTG à générer de la diversité phénotypique que j'ai utilisée pour décortiquer l'architecture génétique des caractères complexes de lignées isolées sur le plan reproductif. Dans la

Summary

deuxième partie de ma thèse, j'ai testé l'efficacité du RTG sur deux souches hybrides polyploïdes stériles de *S. cerevisiae* utilisées dans le domaine industriel. En utilisant le système génétique mis au point dans la première partie, j'ai pu démontrer que ces souches étaient compétentes au RTG et j'ai mis en évidence la présence de nombreux événements de recombinaison génétique après séquençage du génome. Ensuite, j'ai développé une méthode n'impliquant pas de modifications génétiques pour sélectionner les cellules soumises au RTG et j'ai généré deux bibliothèques d'échantillons recombinants. L'analyse de leurs génomes a révélé une grande variabilité, comprenant de vastes régions de LOH. Finalement, j'ai mesuré la variabilité phénotypique existante au sein de ces bibliothèques et j'ai trouvé que certaines souches recombinantes étaient plus performantes que les souches industrielles originales dans des environnements spécifiques.

Dans l'ensemble, mes travaux présentent le RTG comme une nouvelle stratégie permettant de surmonter la stérilité post-zygotique et offrant la possibilité de développer des souches hybrides de *Saccharomyces* d'intérêt industriel sans avoir recours à des modifications génétiques.

Mots clés: évolution du génome, hybrides stériles, RTG, LOH, méiose, génie génomique, hybrides industriels, caractères quantitatifs.

- Summary -

Hybrids are a source of genetic and phenotypic variability as they derive from the merging of two different genomes producing new allelic combinations. Humankind exploited for thousands of years hybrid plants, animals and microbes for their improved performances compared to those of the respective parental species. However, often hybrids represent evolutionary dead ends because of the extremely low viability of their offspring. Thus, hybrid sterility is a significant issue because it limits the improvement of relevant hybrids through classical breeding approaches and more broadly, it hampers hybrid evolution. In this thesis, I took advantage of the remarkable property of the budding yeast *Saccharomyces cerevisiae* to abort meiosis and Return-To-Growth (RTG) when starved cells in the early meiotic phase encounter a rich nutrient environment. This process induces a reshuffle of the genome generating genome-wide regions of loss-of-heterozygosity (LOH). LOH regions can have a substantial impact on hybrid fitness and can also drive adaptation to stressful conditions. While it was already shown that fertile hybrids derived from the mating of lab strains could perform RTG, we do not know whether strains with different ecological origins, genome structures and level of sterility can evolve through RTG, and if so, to what extent. To answer these questions, I applied the RTG paradigm to several artificial and industrial hybrids and characterized its impact at the genomic and phenotypic level.

In the first part of my thesis, I determined to what extent RTG can overcome common post-zygotic sterility barriers described in *Saccharomyces*. First, I re-analyzed sequencing data of RTG samples derived from a sterile hybrid with defective meiotic machinery and confirmed that the hybrid recombined its genome through RTG despite being incapable of completing meiosis. Following, to explore RTG recombination in different hybrids, I took advantage of a genetic system to measure recombination rates at a single locus during RTG and to isolate samples for whole-genome-sequencing. By using this approach, I showed that intraspecific hybrids with different ecological origins and extreme sterility due to genome structure variation could evolve through RTG. Then, I explored how extreme sequence divergence between *S. cerevisiae*/*S. paradoxus* hybrids affects RTG recombination, and I found that recombination is mostly reduced by the mismatch repair system, whose inactivation partially increased the recombination efficiency. Finally, I showed that RTG induced phenotypic diversity in the evolved samples, and I used the phenotypic variability generated for dissecting complex traits between reproductively isolated lineages.

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In the second part of my thesis, I worked with two industrial polyploid *S. cerevisiae* intraspecific hybrids and explored RTG in an industrial setting. To demonstrate that these strains were RTG competent, I engineered their genome with the genetic system developed in the first project using CRISPR-Cas9 technology. By analyzing whole-genome-sequencing of the evolved genomes, I found that also industrial polyploid genomes recombine through RTG. Then, I developed a GMO-free method for selecting RTG strains and generated two RTG libraries of recombinant samples. Analysis of their genomes revealed high variability between samples with some having vast regions of LOH. Last, I measured phenotypic variability in the RTG libraries and found that some RTG recombinants were fitter than the original industrial strains in specific environments. This proof of concept showed that RTG represents a new avenue for inducing genetic and phenotypic novelty in sterile industrial hybrids.

Overall, my work proved that RTG represents a novel path through which *Saccharomyces* hybrids can evolve and overcome common post-zygotic sterility barriers and that RTG constitutes a novel GMO-free strategy to generate improved industrial hybrid strains.

Keywords: *Genome evolution, hybrid sterility, RTG, LOH, meiosis, genome engineering, industrial hybrids, quantitative traits.*

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List of abbreviations

LOH: Loss of heterozygosity
RTG: Return to growth
5-FOA: 5-fluorootic acid
CFU: colony forming units
SNV: Single nucleotide variant
CNV: Copy number variant
MAL: Mutation accumulation line
BDMI: Bateson Dobzhansky Muller incompatibility
HMS: Haldane male sterility
CO: Crossover
NCO: Non crossover
NHEJ: Non homologous end joining
BWA: Burrow-Wheeler transform
Chr: chromosome
DNA: Deoxyribonucleic acid
FACS: Fluorescence activating cell sorting
GMO: Genetic modified organism
HGT: Horizontal gene transfer
OD600: Optical density at 600 nm
ORF: Open reading frame
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
PI: Propidium iodide
QTL: Quantitative trait locus
Sc: *S. cerevisiae*
Sp: *S. paradoxus*
SNP: Single nucleotide polymorphism
WGD: Whole genome duplication

Introduction

1. Hybrids and hybridisations

- In this chapter, I report a summary of the fundamental concepts developed from studies on hybrids and hybridisation made in different organisms. This theoretical framework will be further discussed in the following chapters (2 and 3) in the light of hybridisation in *Saccharomyces* yeasts.

1.1 Identification of hybridisation and hybrids across different taxa

A hybrid is an organism generated through sexual reproduction of two diverged species or varieties. The word "hybrid" stems from the Latin word "*hybrida*" (person born of a Roman father and a mother with non-Roman ancestry or a slave), which has its root in ancient greek. Although the word hybrid has an ancient etymological origin, and hybridisation events being even more ancient, formal recognition of what is a hybrid and how it forms is recent.

The first formal identification of hybrids in plants is thought to date back almost 300 years when, in 1716, Cotton Mather observed natural hybrids occurring between different corn variates (Zirkle, 1934). In 1742, Carolus Linnaeus rejected his thesis published seven years earlier in '*nullae dantur species novae*' (there are no new species) and acknowledged that new species might form as a result of hybridisation (Baack, Rieseberg 2007). In the following century, Darwin (Darwin, 1860) described hybridisation in the context of speciation and evolution proposing that the sterility of hybrids is driven by misformed reproductive systems compared to "pure" species.

In the following decades, zoologists and botanists argued about the role of hybridisation in promoting species evolution. The first proposed that hybridisation was limited (Dobzhansky 1940). Their argument stems from the biological definition of species that define species as reproductively isolated such that their breeding produces sterile offspring (Dowling, Secor 1997. Schwenk 2008). In contrast, botanists, have suggested that hybridisation is a significant evolutionary force capable of generating new species with novel phenotypes (Smocovitis 2006). The extent of hybridisation and its evolutionary significance has remained controversial until more recent advances in molecular phylogenesis that have unravelled ancient hybridisation events occurred in many taxa, and led to reconsider the impact of hybridisation on species evolution (Chapman et al, 2007, Taylor and Larson 2019, *Figure 1*). Such signatures of ancient hybridisation can be detected in the genomes of extant species through whole-genome sequencing.

Genome analyses enable to detect extant hybrids species as well as ancient events of hybridisation by genotyping positions that differentiate the parental genomes of the hybrid. Recent hybridisation events can be easily detected because several regions in the hybrid genome would have retained the genetic information of both parents. In contrast, the fingerprint of ancient hybridisation events are introgressed genomic regions derived from a different species, which are retained in an otherwise completely homozygous genome. These regions consist in genetic material that resisted the erosion of genetic drift or that has been maintained by selection and segregated in the population of the recipient species.

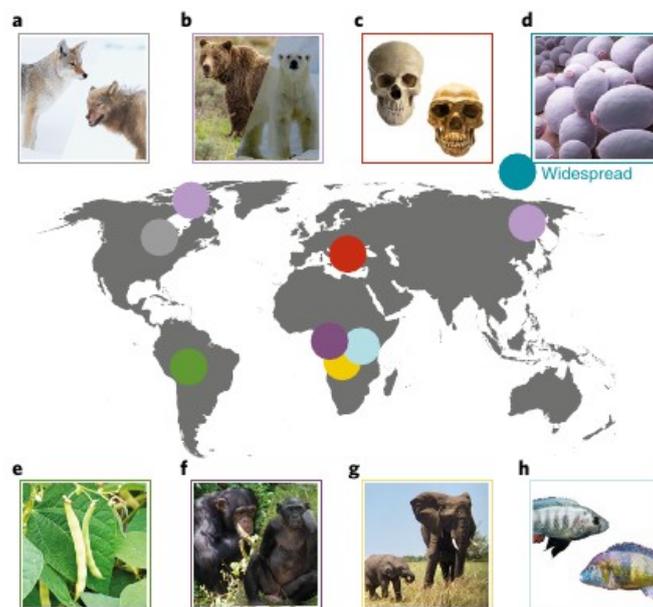


Figure 1: Example of detected hybridisation across different taxa. Each circle is color coded according to the color of the species reported in the squares above or below the figure (a-h)(Taylor and Larson 2019)

The evolutionary events leading to the origin of introgression initiate with a hybridisation event followed by repeated backcrossing with a parental species (Harrison, Larson 2018). This genomic legacy is found often non randomly distributed in the genome. According to the concept of differential introgression, some introgressed regions are retained more often because they bear genes promoting adaptation (Hedrick 2013). In contrast, genes that have a neutral effect or even a harmful impact on the fitness will tend to introgress rarely, and then get lost, or never at all (Martin and Jiggins, 2017). Several studies (Mallet 2005, Twyford and Ennos 2012) proposed that hybridisation occurred in at least 25 % of plant species and between 1 and 10 % of animal species. These percentages are likely to change as the number of available genomes is dramatically

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increasing, and methods to detect hybridisation events are becoming more sophisticated (Taylor and Larson 2019). Although researchers have identified many introgressed regions, it is still challenging to prove that those regions had an adaptive role and are not just genomic legacy left for neutral genetic drift. In fact, this process is also regulated by the genetic location of the introgressed material and its propensity to experience recombination. It has been shown that recombination rate can be a predictive properties of a region being introgressed and maintained (Schumer et al. 2018, Martin et al. 2019). These two studies showed that in butterfly (*Heliconius spp*) and swordtail fish (*Xiphophorous spp.*), regions with high recombination rates had maintained a higher amount of foreign genetic materials compared to regions with lower recombination rate. That finding is in line with regions that experience higher recombination would easily remove deleterious epistatic interactions uncoupling deleterious alleles from neutral or beneficial ones, whereas regions of lower recombination rate would remove such negative genetic interactions together with genes in the same linkage group. Therefore, selection of what is “left over” as a legacy of an ancient hybridisation event is not merely the result of selection of an adaptive role but it has a more complex nature. Nevertheless, recent studies have demonstrated an adaptive function of introgressed genes in different organisms supporting an adaptive role of introgressions (Jones et al. 2018, Taylor and Larson 2019).

1.2 Reproductive barriers in hybrids

The process of hybridisation and speciation is regulated by several reproductive barriers between diverged species or populations. Initially, pre-zygotic barriers can preclude the process of mating and fertilization (*Figure 2*) but they can also hamper further backcross of the hybrid with the parents and thus leading to speciation (Schumer et al. 2017). One well known example of pre-zygotic barrier is the mate choice, which result in assortative mating by which “the similar tend to mate with the similar” (Seehausen 2014), thus diminishing the likelihood of hybridisation. Another strong pre-zygotic barrier consists of geographical isolation between different species (Ma et al. 2015). According to it, species that do not share overlapping reproductive area (called hybrid zone) are reproductively isolated, although this does not exclude that if the species are forced to mate they can generate viable offspring. Nevertheless, even species with overlapping niches can be reproductively isolated by asynchrony in their reproductive timing (Monteiro et al. 2016). Several studies have described similar pre-zygotic barriers across different organisms. Indeed as pre-

zygotic barriers largely depends on the environments they can be more flexible, for instance, the pollution of the water might have caused the collapse of assortative mating between two fish species and lead to the formation of an hybrid population (Schumer et al. 2017).

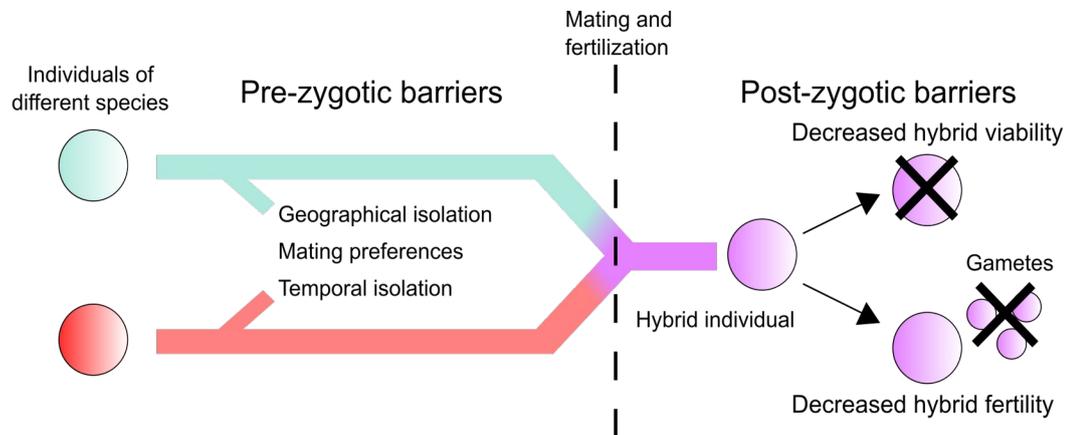


Figure 2: Diagram showing major sources of pre-zygotic and post-zygotic reproductive barriers.

If fertilization between two different species occur the formed zygote will either die out for potential incompatibilities between the subgenomes or developed in a novel hybrid organism. This hybrid might face intrinsic fitness defects, to the point that its not capable to produce viable offspring. This post-zygotic barrier (*Figure 2*) is commonly named “hybrid sterility”, and it has been studied in many viable hybrids (Wu and Ting 2005, An example from rice: Ouyang et al. 2010). Interestingly, some mechanisms of hybrid sterility are common among organisms separated by broad evolutionary distances. For instance, karyotypic differences in the number or structure of chromosomes is a significant factor contributing to sterility in diverse organisms such as the sunflower (*Heliantus spp*) (Lai et al. 2005), the fruit fly (*Drosophila spp*) (Noor et al. 2001) or the most notorious case of the donkey, which, once again, was known since ancient time but the biological causes remained elusive until more recent times.

Post-zygotic reproductive isolation can arise also as a result of the incompatibility between diverged genes (Maheshwari and Barbash. 2011, Figure 3). This theory was proposed and developed by William Bateson (1909), Theodosius Dobzhansky (1936) and Herman Muller (1942) (Orr. 1996), and therefore is often referred to as Bateson-Dobzhansky-Muller incompatibility (BDMI).

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The BDMI model states that following a two-gene model there is a minimum of two interacting genes, which evolved independently in separated species, and have lost the ability to properly interact (deleterious epistasis) when combined in a hybrid organism. BDMI does not develop as a consequence of natural selection but rather as a byproduct of the independent evolutionary processes acting on different genes in the diverging species.

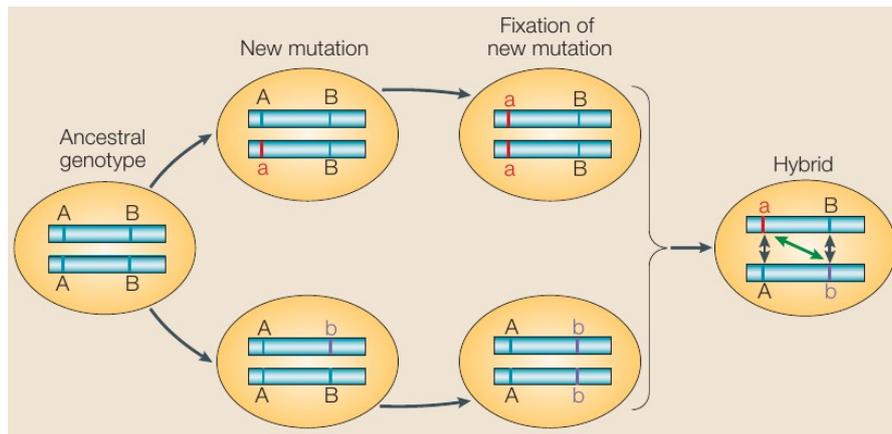


Figure 3: Drawing depicting the theory explaining how BDMI incompatibilities arise and act. Adapted from Wu and Ting 2004

In eukaryotes, examples of BDMI have been found in the fruit fly (*Drosophila spp*) (Brideau et al. 2006, Masly et al. 2006), in mouse (*Mus spp*) (Mihola, 2009) and fish (*Xiphophorus spp*) (Powell et al. 2020). Among the previously reported cases only two BDMI genes are known in vertebrates. One gene, *Prdm9*, encodes for a H3 lysine 4 trimethyltransferase (Mihola, 2009) expressed in the testis. This gene was detected by analysing mouse interspecific hybrids that were affected by hybrid male sterility (HMS), a specific case of hybrid sterility proposed by Haldane. The study revealed that in the mouse interspecific hybrid used, the HMS phenotype could be rescued by introducing a gene encoding the fertile allele of *Prdm9*. The only other example known of BDMI in vertebrates is the gene *xmrk* that encodes a tyrosine kinase in swordtail fishes (Powell et al. 2020). However, it is predicted that many more genetic incompatibilities pair exists (Schumer et al. 2014), although such incompatibilities can have a variable degree in causing reproductive isolation (Cutter et al. 2012) and might in fact affect the fitness of the organism without precluding sexual reproduction.

Examples of genetic conflict caused by BDMI are not limited to nuclear genes, but such deleterious interactions can take place also between nuclear and mitochondrial genomes. For instance, Lee and colleagues described a cytonuclear incompatibility in yeast (Lee et al. 2008) between the *AEP2*

allele inherited from *S. bayanus* and the *OLII* allele inherited from *S. cerevisiae*. This negative interaction resulted from the lack of regulation of *OLII* mediated by the *AEP2* allele causing inefficient respiration and gametogenesis. Other studies have described many cases of hybrid sterility also in plants, where the best-known examples are derived from hybrids between subspecies of rice (*Oryza sativa* L.) (Ouyang 2010) and *Arabidopsis* hybrids (Chen et al. 2016). Finally, hybrid sterility can also be due to meiotic defect in the development of the gametes as shown in mouse (Bhattacharyya, 2013), or in the genus *Saccharomyces*, where the high sequence divergence between the species impairs correct chromosome segregation during meiosis (Hunter et al. 1996, Liti et al. 2006, Greig 2009).

1.3 Hybrid vigour and niche adaptation

Despite the different sources of sterility can significantly decrease the reproductive capacity and fitness of hybrids, many hybrid organisms have ancient origin. This suggest that despite having to face the challenge of having two diverse genomes cooperating in a single individual, the interactions of the hybrid subgenomes are not always harmful but can be, more or less often, beneficial. This phenomenon is called “hybrid vigour” or heterosis (Shull, 1908) according to which, hybrids display improved traits compared to either of the two parental species. Often, hybrid vigour has been the main driver of selection for artificially bred hybrids, as the breeders were crossing parental species to obtain a mixture of beneficial traits present in the parents. Heterosis has been selected, for instance, in many corn varieties (Hochholdinger and Baldauf, 2018) that are hybrids displaying different morphological variations useful for agricultural applications such as plant height and cob size. To date have been proposed three principal mechanisms to explain heterosis in different organisms. Briefly, according to the theoretical framework developed from studies on *Saccharomyces* (Shen et al. 2014, Shapira et al. 2014), heterosis may arise as a result of:

- i) Complementation of a weak recessive allele by a dominant allele present in the other parental species.
- ii) Positive interaction between heterozygous loci.
- iii) Positive epistatic interaction between pairs of non-allelic genes.

1. *Hybrids and hybridisation*

Moreover, hybrid vigour might have other beneficial effects promoting the colonisation of new ecological niches which were inaccessible to both parents (Rieseberg 2006). Thus, hybridisation can promote niche colonisation and adaptation and might also explain why hybrids survive once they form (for a review supporting hybridisation and invasiveness see Hovick and Whitney, 2014). Therefore, hybrids are not just the “average” of their parents but can have wide phenotypic variability depending on the complex interactions between the hybrid subgenomes and between these and the environment.

2. Hybridisation in the *Saccharomyces* genus

- In this chapter, I further expand the general concepts related to the hybrid biology introduced in the previous chapter and I discuss them in the light of hybridisation between *Saccharomyces* yeasts.

2.1 Identification of *Saccharomyces* hybrids

Hybrids do form also between microbes. However, their identification lagged in comparison to the characterisation of hybrids in plants and animals because of the technical difficulties associated with recognising hybrids in microorganisms. Currently, eight pure species and at least two hybrid species have been identified in the *Saccharomyces* genus (Figure 4). However, the number of hybrid species identified is rapidly evolving (Figure 5). One of the first pure yeast cultures ever obtained, was an interspecific hybrid which was isolated by Dane Emil Hansen in 1883 at Carlsberg and named after the brewery *Saccharomyces carlsbergensis* (Walther et al. 2013). Lately, taxonomists rejected the name proposed by Hansen and adopted the name *Saccharomyces pastorianus*, proposed by Max Rees in honour of Louis Pasteur, but the two terms are often used as synonyms. Despite that being a milestone in yeast biology, it was not possible at the time to

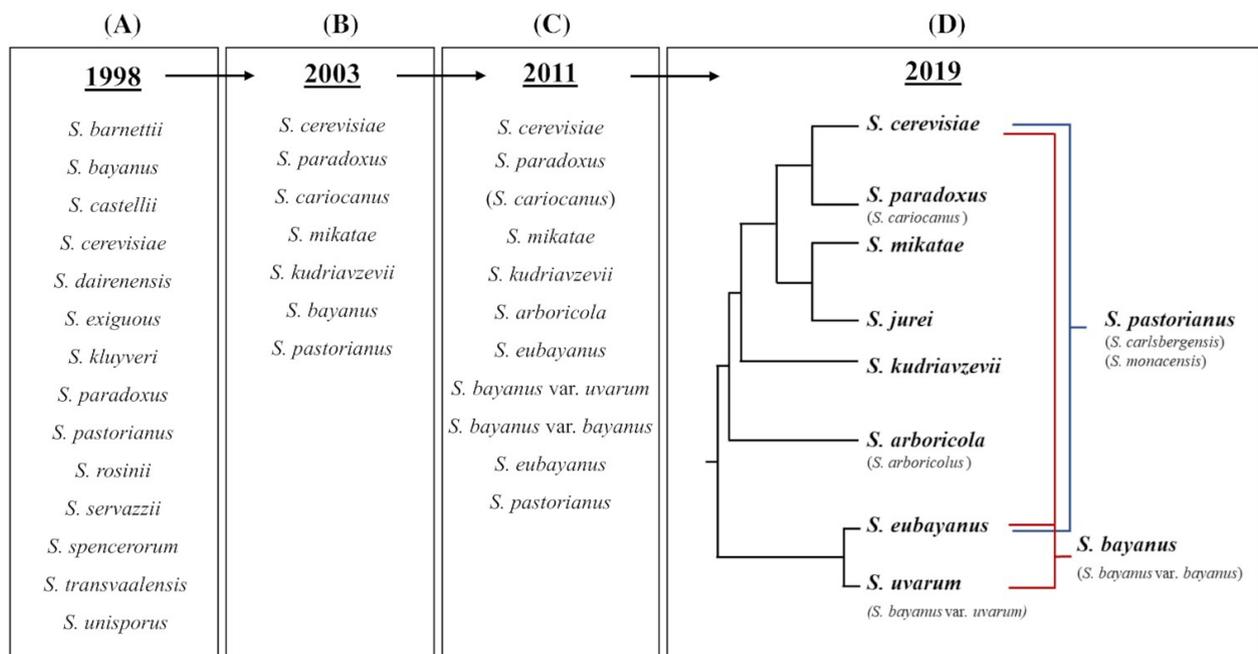


Figure 4: Classification of *Saccharomyces* species from 1998 (A) to 2019 (D) with highlighted the hybrid origin of the lager strain *S. pastorianus* (blue line) and the triple hybrid *S. bayanus* (red). From (Alsammar and Delneri 2020)

2. Hybridisation in the *Saccharomyces* genus

identify that this microbe was an hybrid. Indeed, its classification as a hybrid species arrived only after almost one century in 1985 when DNA-DNA reassociation studies revealed that *S. pastorianus* was an hybrid between *S. cerevisiae* and *S. bayanus* (Martini and Kurtzman. 1985).

This classification was revisited in the past decades upon the discovery of the new species *S. eubayanus* in Patagonia (Libkind et al. 2011) from which is derived the non-*S. cerevisiae* subgenome of *S. pastorianus* (Figure 4). To conclude the long history of the classification of this microbe, the recent isolation and sequencing of a new *S. eubayanus* isolates, traced back the origin of the non-*S. cerevisiae* genome to the *S. eubayanus* population in the Tibetan plateau in Asia (Bing et al. 2014) which would be supported by a more ancient history of trade and geographical connection of Asia and Europe.

Recognising *Saccharomyces* hybrid yeasts was also complicated because only metabolic or morphological criteria were available to identify different species for most of the past century. In more recent times, different species were recognised by assessing the reproductive capacity and gamete viability of different crosses of *Saccharomyces* isolates (Naumov 1987) or by using DNA-DNA reassociation (Martini and Kurtzman 1985). However, as in other organisms, the booming of new sequencing technologies allowed to rapidly increase the number of sequenced genomes making it easier to recognise hybrids (Taylor and Larson. 2019), and thanks also to the small genome of this microorganism (12 Mb, haploid genome), *S. cerevisiae* was the first eukaryote having its genome completely sequenced in 1996 (Goffeau et al. 1996). The knowledge of the entire genetic sequence of this model organism immediately revealed to be an important resource and allowed to infer a whole-genome duplication being at the origin of this microbe (Wolfe and Shields. 1997). Later it has been proposed that this whole-genome duplication followed an ancient hybridisation event suggesting once again the fundamental role of hybridisation in evolution (Marcet-Halbon and Gabaldon. 2015). The data accumulated so far has revealed that hybridisation is pervasive in the *Saccharomyces* genus and several isolates carry in their genomes the traces of recent or old hybridisation events (Morales and Dujon 2012, Barbosa et al. 2016, Monerawela and Bond 2017, Dujon and Louis 2017, Peter, De Chiara et al. 2018, Alsammar and Delneri 2020, D'Angiolo 2020). Extant hybrids between different *Saccharomyces* yeasts have been isolated both in domesticated and wild environments (Sipickzi 2008, Morales and Dujon 2012, Leducq et al. 2015, Gallone et al. 2019, Langdon et al. 2019, Alsammar and Delneri 2020, D'Angiolo et al. 2020, Figure 5).

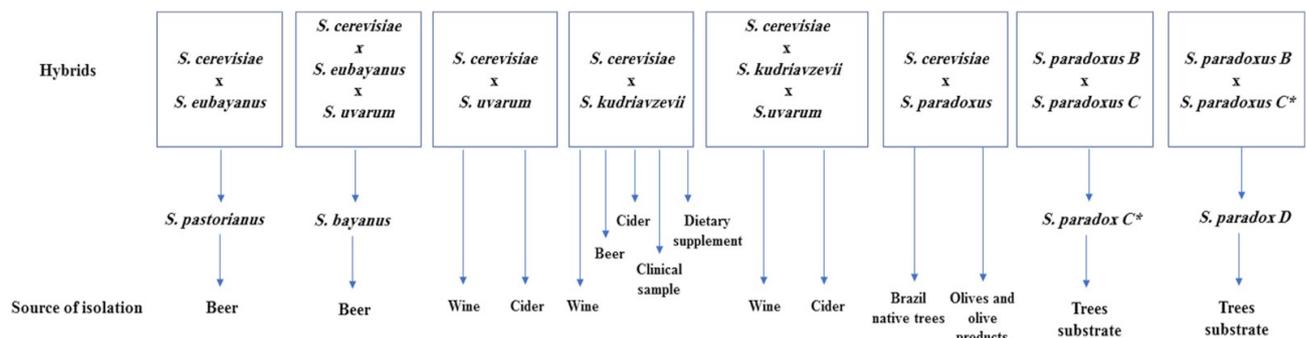


Figure 5: Summary of interspecific hybrids in the *Saccharomyces* genus isolated from domesticated (first five blocks) and wild or semi-wild (last three boxes). *S. paradox C** = *S. paradoxus C**, *S. paradox D* = *S. paradoxus D* From (Alsammar and Delneri 2020)

Domestication appears to have triggered hybridisation either by bringing together species that do not have overlapping ecological niches in the wild, or by providing harsh environmental conditions that promote the hybridisation process or select its outcome. Interestingly, most of the domesticated interspecies hybrids share *S. cerevisiae* as a common parental species. It has been proposed that this preference is driven by the strong fermentation performances of *S. cerevisiae* that can ferment the sugar maltotriose an ability that is often absent in other non-*S. cerevisiae* species (Gibson et al. 2013). The other parental species has often the capacity to better ferment at lower temperatures and better resist at cold temperatures, a characteristic shared between *S. kudriavzevii*, *S. uvarum* and *S. eubayanus*, which would confer an advantage to the growth of the hybrid in cold conditions of lager, wine or cider fermentations (Li et al. 2019, Gallone et al. 2019).

Hybrids have been isolated also from many wild habitats such as oak forests (Eberlein et al. 2019) where *S. cerevisiae* and *S. paradoxus* coexist (Sniegowski et al. 2002, although the hybrids isolated by Eberlein are between different *S. paradoxus* populations), olive tree and associated environments (Peter et al. 2018, Pontes et al. 2019, D'Angiolo et al. 2020) or insects guts (Stefanini et al. 2016). The finding of yeasts in the gut of insects suggests a possible role of insects in facilitating mating between different species of yeasts and the dispersal of the resulting hybrids. This explanation might be supported by the ecological relationship between yeasts and social insects, in particular social bees and social wasps (Madden et al. 2018). Another possibility proposed to explain outbreeding into the wild is that haploid spores form microcolonies and bud for a few generations before engaging in sex and mate with other spores (McCluire et al. 2018). Although hybrids have been frequently isolated, it is not yet known what is the frequency of

2. Hybridisation in the *Saccharomyces* genus

outcrossing between *Saccharomyces* species in wild and domesticated niches and what is the impact of environmental conditions in facilitating it.

The frequent isolation of hybrids suggests that pre-zygotic barriers are weak in the genus. In lab experiments, spores of *S. cerevisiae* and *S. paradoxus* showed a partial mating preference, indicating the existence of pre-zygotic barriers although they might be weak (Greig 2009). *S. cerevisiae* had a stronger mating preference than *S. paradoxus*, at least in lab condition, and mating speed and differential germination time appear to drive the preference in mating choice (Murphy and Zeyl 2012). Interestingly, differences in the germination time are also regulated by environmental conditions such as the presence of microelements in the growing media, suggesting that also in yeast, pre-zygotic barriers are modulated by the environment (Plante and Landry 2020). Taken together, it is likely that the spatial separation of the parental species might represent the most substantial pre-zygotic barrier in the *Saccharomyces* genus due to the punctuate and patchy nature of yeast populations into the wild and this could also explain why hybrids are often retrieved in domesticated environments where human activity might play an important role in favouring hybridisation. More data on yeast natural demography are necessary to further investigate the rates of *Saccharomyces* hybridisation and characterise how environmental factors modulate it.

2.2 Hybridisation and niche adaptation

As reported above, many of the *Saccharomyces* hybrids isolated so far are associated to human activities such as brewing, winemaking and other industrial fermentations (Morales and Dujon 2012, Gallone et al. 2019, Alsammar and Delneri 2020). The recurrent isolation of hybrids in related human environments suggests that the unique merging of the parental traits in one hybrid have allowed it to thrive in the harsh fermentative conditions. In recent studies (Langdon et al. 2019, Gallone et al. 2019), the authors found that different *Saccharomyces* interspecific hybrids clustered in diverse phenotypic classes according to the industrial process from which they were isolated. Therefore, niche adaptation exerts an intense selective pressure on the newly formed hybrids by selecting loss of undesired traits and resistance to specific stresses. The authors also showed that the hybridisation itself immediately promoted phenotypic improvement in *de novo* lager hybrids similar to results obtained previously (Krogerus et al. 2016), which is consistent

with those hybrids enjoying hybrid vigour. Therefore, niche adaptation and the hybrid unique genetic makeup drive the successive evolutionary phases after the initial hybridisation process.

However, for microbes, these findings need to be carefully taken as most of the extant domesticated hybrids derived from closely related strains that have been isolated as pure cultures in the late nineteenth century and rapidly spread. This aspect is particularly significant for the lager *S. pastorianus* hybrids whose genome analyses (Gallone et al. 2019) show a very low degree of genetic divergence consistent with a small population bottleneck occurring during the evolution of these strains. Moreover, experiments in which *de novo* lager hybrids were evolved for few generations in conditions mimicking the lager beer fermentation did not find any sequenced clone with a genetic makeup resembling the complex genomic architecture of the extant *S. pastorianus* hybrids (Gorter de Vries et al. 2019). This suggests that the ecological niche might have selected specific traits such as cold-tolerance or sugars utilisation, and the beer style (in the case of beer yeasts) has promoted the selection of specific genetic alterations that removed the phenolic-off-flavours undesired in the lager brewing style. However, the current genetic makeup might have resulted solely by genetic drift, and a narrow population bottleneck when the first pure cultures of this microbes were obtained and exported across different breweries.

2.3 Post-zygotic reproductive barriers in *Saccharomyces* hybrids

Saccharomyces yeasts are facultative asexual organisms, thus to propagate their genetic material they do not require a sexual reproduction as obligate sexual organisms. Nevertheless, in certain occasions these yeasts can engage in sex and eventually if outbreeding occurs, form a new hybrid organism. The hybrid itself can either propagate its genomic information through asexual reproduction or reproduce sexually generating gametes that carry new allelic combinations shaped by the meiotic recombination.

Saccharomyces hybrids are generally fit and often display heterosis for different phenotypes (Shapira et al. 2014, Bernardes et al. 2017). A study performed on wild *S. paradoxus* populations (Charron and Landry 2018) showed that there is no evidence of strong extrinsic post-zygotic barriers as the F1 hybrids were often showing over-dominance or partial-dominance in the conditions tested. Thus, hybrid inviability and fitness defect seem not to play a major role in determining post-zygotic isolation in the genus. In contrast, *Saccharomyces* hybrids face strong post-zygotic barriers that have an important role in promoting homoploid hybrid speciation in the

2. Hybridisation in the *Saccharomyces* genus

genus (Greig et al. 2002). There are three post-zygotic barriers identified in *Saccharomyces* yeast hybrids (Greig 2009, Ono et al. 2020) among which, high sequence divergence is the most important in regulating species barriers.

Karyotypic divergence:

Studies on *Saccharomyces* hybrids have looked at karyotypic divergence as a source of evolution and speciation in both *S. cerevisiae* and *S. paradoxus* species (Charron et al. 2014, Hou et al. 2014). Genomic translocations that alter the collinearity between hybrid subgenomes can induce sterility as a result of unfaithful segregation of chromosomes during meiosis. In intraspecific hybrids, a non-reciprocal translocation decrease the viability of the offspring of 25% while a reciprocal translocation decrease the viability by 50% (Ono et al. 2020), with additional translocations further promoting reproductive isolation. One extreme case is the Malaysian lineage of *S. cerevisiae* that although has maintained a low sequence divergence, comparable with other *S. cerevisiae* isolates, it is reproductively isolated from other *S. cerevisiae* populations due to 5 large scale chromosomal rearrangements (Yue et al. 2017). Interestingly, other *Saccharomyces* species harbour chromosomal rearrangements if compared to the karyotype of *S. cerevisiae*. For instance, *S. eubayanus* has two reciprocal translocations between chromosome II and IV and between chromosome VIII to XV, which further exacerbate the reproductive isolation of this species from *S. cerevisiae* (Baker et al. 2015). However, engineering of chromosomal translocations to reestablish the colinearity between *S. cerevisiae* and *S. mikatae*, which also harbour genomic rearrangements compared to *S. cerevisiae*, did not restore complete fertility (Delneri et al. 2003). Therefore, other mechanisms must play role in establishing reproductive isolation in extant *Saccharomyces* species; nevertheless, chromosomal structure variations represent a fast way to generate reproductive barriers between sympatric populations.

Sequence divergence:

Variability at the level of the nucleotide sequence between hybrid subgenomes, a feature known as sequence divergence or heterozygosity, is the strongest and more common cause of post-zygotic barrier to sexual reproduction in *Saccharomyces* hybrids. Essentially, the high sequence divergence triggers an anti-recombination activity of the mismatch repair system (Hunter et al. 1996) leading to a defective recombination pathway, which does not produce crossover molecules. Ultimately, the lack of proper physical contact and recombination between chromosomes leads to missegregation

of the homologous chromosomes in the gametes that die for the high rates of aneuploidy (Rogers et al. 2018). The deletion of the genes *MSH2* and *PMS1*, that encode proteins of the mismatch machinery complex, partially rescued the sterility of *S. cerevisiae/S. paradoxus* hybrids supporting the model that the anti-recombination mediated by the mismatch repair is at the basis of the sterility (Hunter et al. 1996). Interestingly, it appears to be a linear relationship between sequence divergence and hybrid sterility (Liti et al. 2006). In recent work, the deletion of *MSH2* and *SGS1*, two genes involved in the antirecombination process, allowed to retrieve the full set of viable gametes from a meiosis of a *S. cerevisiae/S. paradoxus* hybrid (Bozdag et al. 2019). This approach further supported previous results of antirecombination acting in meiosis preventing recombination between diverged genomes and allowed for the first time to study more extensively gene incompatibilities in interspecific *Saccharomyces* hybrids.

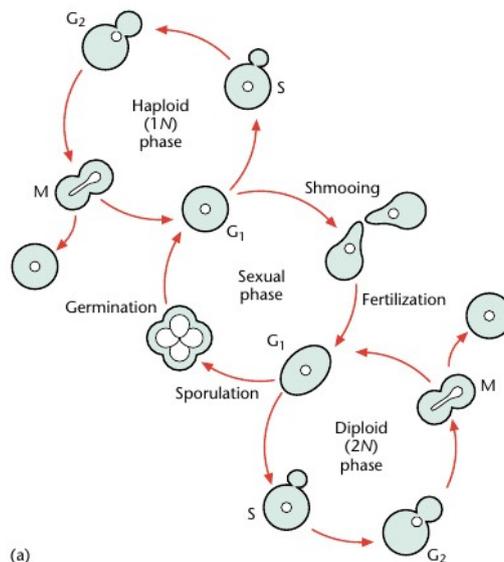
Gene incompatibilities:

As reported in chapter 1, incompatible genes can cause sterility in hybrids as a result of a lack of proper interaction between two or more genes. Studies on gene incompatibilities have also been performed in *Saccharomyces* hybrids, although it seems that their role in hybrid sterility and speciation might be limited. An experiment in which nine chromosomes of *S. paradoxus* were introduced one at a time in a complete set of *S. cerevisiae* chromosomes did not detect any incompatibility that killed the gametes (Greig 2007). However, gene incompatibilities may affect the fitness of the formed F2 hybrids rather than just killing the gametes (Xu and He. 2011). Therefore, despite a case in which the researchers identified a strong genetic incompatibility between *S. cerevisiae* and *S. bayanus* (Lee et al. 2008), other gene incompatibilities may have weaker effects and affect hybrid viability rather than hybrid sterility. In a recent study (Bozdag et al. 2019) aimed at identifying BDMI acting between *S. cerevisiae* and *S. paradoxus*, whole genome sequencing of a full viable set of gametes (tetrads) revealed at least 3 combinations of genomic regions that were underrepresented, suggesting that genes within these regions have incompatible interactions. However, the large linkage size prevented so far a fine mapping and the underlying molecular basis of yeast BDMI remain to be elucidated.

3. *Saccharomyces* hybrid genomes evolution

- In this section I will describe the hallmarks of sexual reproduction, asexual reproduction and how these phases of the lifecycle contribute to genome evolution in *Saccharomyces*. I will mainly focus on studies made on *Saccharomyces* hybrids and occasionally refer to studies made on homozygous backgrounds. I also introduce the paradigm of RTG and report evidence accumulated on other mechanisms of genome evolution in hybrids such as polyploidisation and genome instability.

As *Saccharomyces* yeasts are facultative sexual organisms, they can reproduce both sexually and asexually (Figure 6); thus, both phases of the lifecycle can shape the evolution of the genome. Moreover, polyploidisation and genome instability which can occur during asexual propagation, and genome shuffling through an abortive meiosis (RTG, see later) can have a profound impact on the genome evolution of yeast hybrids.



(a)
 Figure 6: Lifecycle of *Saccharomyces*. G_1 , S , G_2 and M represent different phases of the lifecycle. Sporulation (Meiosis) in *Saccharomyces* generate 4 haploid spores enclosed in an ascus. Asexual reproduction by mitosis generate an identical daughter cell. (from Melt et al. 2003)

Recently, with the rapid increased capability of whole-genome-sequencing, several studies have started to characterize the mutational landscape of hybrids evolved through several asexual propagations (As example: Dutta et al. 2017, Tattini et al. 2019, Charron et al. 2019).

3.1 Evolution through asexual reproduction

Mitosis is triggered in *Saccharomyces* yeasts by favourable conditions and abundance of nutrients in the external environments. These yeasts double their genomic content in the mitotic S-phase, generate a bud which will grow in size and form a newborn daughter cell, in which the duplicated genome segregates together with organelles and other biological structures necessary for its development (Duina et al. 2014). Through this process, the mother cell produces an identical daughter cell which can also reproduce through the same mechanism. However, due to errors occurring during the replication of the DNA or during the chromosomal segregation and the cell division, several genetic alterations can arise, which can have a profound evolutionary impact (*Figure 7*).

The most known and studied event occurring during asexual growth is the introduction of mutations, which is at the basis of genetic variability. However, not all mutations have necessary an effect, and that depends on the genomic location affected and the variation produced. Synonymous mutations do not supposedly have major impact in most cases as they do not change the aminoacid coded by the codon affected; however, there are known cases in which synonymous mutations do impact the fitness of yeasts (She and Jarosz, 2018).

In contrast, non-synonymous mutations that alter the aminoacid they affect can have a stronger effect by disrupting active enzymatic sites, modify the structure of the protein or affect the general transcription of the gene by affecting the promoter, the start-codon or the stop-codon. An experiment in which non-synonymous aminoacid substitutions were introduced in a conserved region of 9 aminoacids of the protein Hsp90 has shown that non-synonymous substitutions can have either deleterious effect on the fitness or a neutral effect in the conserved region analysed (Hietpas et al, 2011). However, also non-synonymous substitutions that impact aminoacids not relevant for the function of the protein are likely to have little impact. The rate with which mutations occur in *Saccharomyces* genomes has been estimated in mutation accumulation lines (MALs) in which cells are propagated through several bottlenecks on solid agar plate aiming at minimising selective pressure.

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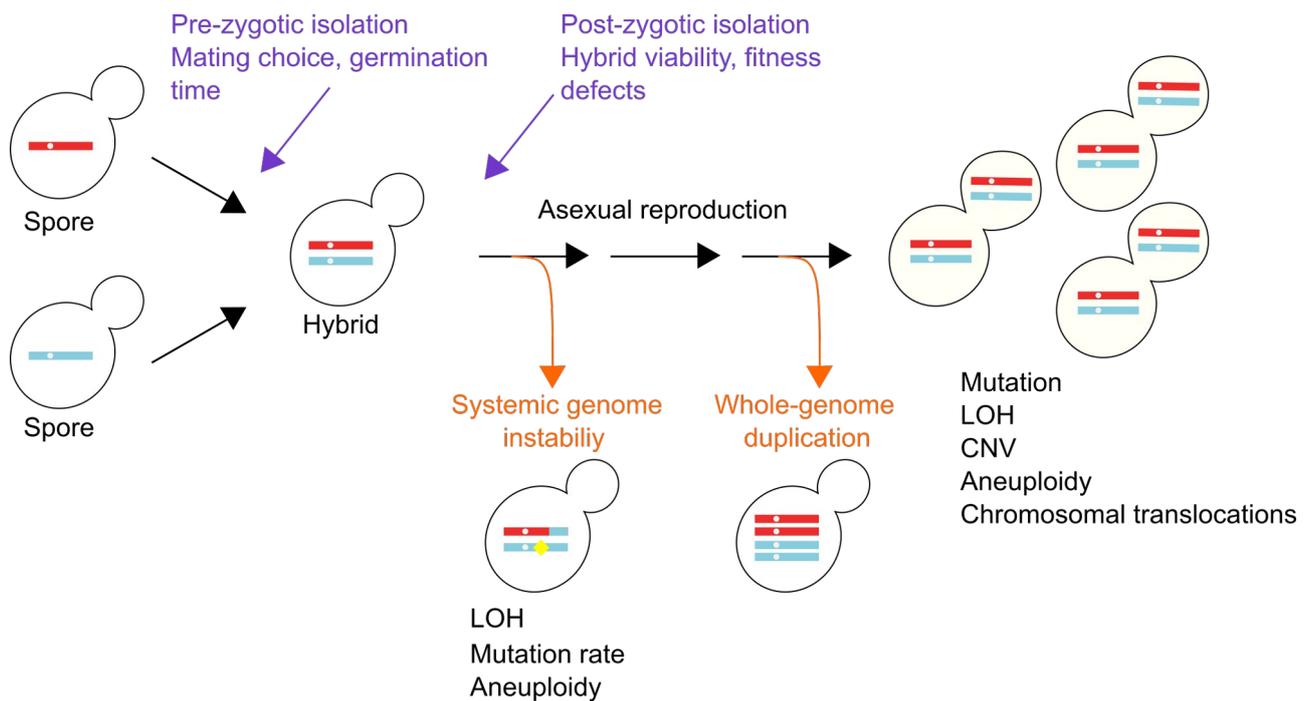


Figure 7: Main events that can lead to genome evolution of yeast hybrids. Red and cyan rectangles symbolise the haploid genome. The orange arrows represent events that can occur in part of the growing population and have a dramatic impact on the genome of the hybrid. The purple arrows represent potential pre and post zygotic isolation mechanisms described in chapter 2. Below each event is reported the genomic alterations found. LOH: Loss of heterozygosity. CNV: copy number variations. Spore: haploid gamete. The yellow rectangle represent increased in mutation rate in the systemic genome instability.

Different homozygous haploid and diploid genetic backgrounds (Lynch et al. 2008, Zhu et al. 2014, Sharp et al. 2018) as well as hybrids (Dutta et al. 2017, Tattini et al. 2019, Pankajam et al. 2020, Loeillet et al. 2020, D'Angiolo et al. 2020, Sui et al. 2020) have been evolved through MAL so far which enabled not only to assess the mutation rate but also to broadly characterize the mutational landscape. The study of Sharp and colleagues (Sharp et al. 2018) reported that haploids had a higher mutation rate compared to diploid homozygous strains, however they reported that mutations in diploids were more likely to be more detrimental in contrast with the expectations that in a diploid genetic background such mutations would be masked. A similar mutation rate to the diploid homozygous strain use in that work was obtained in another study using a different diploid homozygous genetic background (Tattini et al. 2019). The same study detected a lower level of mutation rate in the *S. paradoxus* homozygous genetic background compared the mutation rate of *S. cerevisiae*. Moreover, the hybrid between the Wine European *S. cerevisiae* and the European *S. paradoxus* showed an increased mutation rate suggesting that the genetic background

affects the mutation rate (Tattini et al. 2019). It is possible that a higher mutation rate might have helped the adaptation of *S. cerevisiae* to different ecological niches or at least promoted a faster evolution compared to *S. paradoxus*, but more studies on different populations of *S. cerevisiae* and *S. paradoxus* are needed to confirm that. The advantage that fast mutator strains might experience a fast transient adaptation has been confirmed in a study where two incompatible alleles of *MLH1* and *PMS1*, involved in the mismatch machinery repair, were inserted in the same genetic background, where they induced a mutator phenotype and allowed a faster adaptation to stressful conditions (Bui et al. 2015). However, the advantage gained was transient because of the accumulation of other possible deleterious mutations. Interestingly, in a follow-up study (Bui et al. 2017) the same combination of incompatible alleles was found in natural *S. cerevisiae* yeasts but in these cases it was not contributing to an increased mutation rate. The authors proposed that suppressor mutations emerged to buffer the phenotypes which would not be more advantageous once the strain has adapted to the stress. Therefore, the mutation rate is not constant across hybrid backgrounds and pure species, at least in lab conditions, and interaction between the genetic backgrounds can alter the rate of accumulation of mutation. In addition, the genome content can influence the mutation rate, and a study performed on haploid, diploid and tetraploid homozygous strains showed that the tetraploid strains acquired mutations faster than lower ploidy strains (Selmecki et al. 2015) although the protocol adopted in this study was different from the MAL approach used in the previously cited works and these results still need to be replicated in hybrids. Environmental conditions also regulate the mutation rate. For instance, in haploid backgrounds, ethanol increases the mutation rate (Voordeckers, 2020) compared to normal lab conditions and it might explain the higher mutation rate predicted for industrial beer yeast strains that spend part of their lifecycle in an harsh environment in which ethanol is present at different concentrations (Gallone et al. 2016), however also the hybrid nature of these strains might affect the mutation rate. Therefore, the pace with which mutations arise in hybrids genomes is affected by complex interaction between the subgenomes and is likely to be affected by environmental conditions. Furthermore, once mutations arise their fate will be modulated by the size of the reproductive population as in small populations newly arised mutations even if deleterious will be more likely to be fixed by genetic drift.

Other genomic alteration can fuel the evolution of hybrid genomes when hybrid yeasts reproduce through mitotic division. A recurrent event is the formation of stretches of sequence homology between subgenomes called loss-of-heterozygosity (LOHs). In a diploid hybrid genome, LOH

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results in the genetic information of one hybrid subgenome being permanently replaced by the genetic information deriving from the other subgenome. This event can be traced by genotyping nucleotide positions that would differentiate the two parental genomes and its detection is therefore dependent on the sequence dissimilarity of the two subgenomes. LOHs can have a profound evolutionary impact. For instance, they can influence fitness in a specific environment by increasing the copies of beneficial alleles (Heil et al. 2017, Lancaster et al. 2019, Heil et al. 2019, James et al. 2019). Interestingly, LOHs can arise in hybrids even in conditions that minimise selection (Dutta et al. 2017, Tattini et al. 2019) suggesting that LOHs arise spontaneously, and advantageous phenotypes are then selected by the environment. However, the formation of LOH in mitosis is constrained by the sequence similarity so that high diverged sequences will be less likely to produce long tracts of LOH than more similar sequences has shown in MALs of intraspecific and interspecific hybrids (Tattini et al. 2019). Moreover, an early study showed that mutants in DNA replication and chromatin assembly lead to an increased formation of LOH tracts (Andersen et al. 2008) thus, as for mutations, also the specific genetic background can influence the formation of LOH. In addition, LOH formation is also influenced by environmental cues as for instance when hybrid cells grow in hydroxyurea they experience replication stress leading to an increased LOH rate (Vazquez-Garcia et al. 2017). Despite, LOH predominantly maintains the copy number of the genes involved, it can also result in copy number variations (CNVs) when non-homologous tracks of subgenomes recombine by increasing or decreasing the copies of genes with non matching homologous (Tattini et al. 2019).

Copy number variations can arise through different mechanisms mediated by aberrant homologous recombination or non homologous recombination or aberrant recombination initiated by environmental stresses (Steenwyk and Rokas. 2018) leading to gene duplication or deletion. A more global copy number variation of entire chromosomes, called aneuploidies, can arise as a result of defective chromosome segregation during budding. In addition environmental stresses can promote and eventually select the formation of aneuploidies as they often have a strong phenotypic impact by increasing or decreasing gene dosage (Gilchrist, Stelkens. 2019). Therefore, chromosomal copy number variation can be a rapid mechanism for fast adaptation by facilitating the survival of yeast cells evolved under selective pressure such as heat (Yona et al. 2012), or drugs (Linder et al. 2017). On the same line, also chromosomal translocations can confer a rapid fitness advantage in specific environments as shown by a work on a *S. cerevisiae/S. uvarum* hybrid in which a recurrent translocation was retrieved in evolved samples under ammonium limitation (Dunn

et al. 2013). However, in a recent work aiming at mimicking lager beer adaptation of the lager strain *S. pastorianus*, the researchers also detected aneuploidies and LOHs (Gorter de Vries et al. 2019) but surprisingly the number of these events was lower than what expected given the extant genetic make-up of the lager hybrids (Monerawela et al. 2017, Gorter de Vries et al. 2019) suggesting that possible alternative mechanisms might be at the basis of this complex genetic architecture and might not be necessarily involved in adaptation to that environment. An alternative route to explain such complex genomes is that mutations, LOH and CNVs, might arise in bursts of genomic instability, called systemic genome instability (SGI). In a recent work, the authors managed to capture SGI events in a diploid hybrid reproducing asexually and showed that there were clones bearing more than expected aneuploidies and LOH in apparently unlinked regions and proved that this was not the outcome of an aborted meiosis (return-to-growth) but was linked to events occurred during the asexual propagation (Sampaio et al. 2020). Intriguingly, a genome instability episode might have been the catastrophic event that allowed an ancestral hybrid between *S. cerevisiae* and *S. paradoxus* to overcome post-zygotic reproductive barriers and generates through backcrossing the extant Alpechin lineage of *S. cerevisiae* found in environments related to the olive oil production (D'Angiolo et al. 2020). Therefore, genomic instability could also have a beneficial role and helps hybrids to rapidly adapt to specific environmental challenges or even promote the rescue of hybrid sterility by producing regions of LOH that mitigate the sterility barrier caused by antirecombination (D'Angiolo et al. 2020). On a similar line, it has been shown that punctuate events of whole-genome-duplication (WGD) can restore fertility (Greig 2003) in hybrids with high sequence divergence and extreme sterility, and recently it has been shown that such events can occur during asexual propagation in non-selective conditions (Charron et al. 2019). Based on a similar work of Charron and colleagues it has been proposed recently (Marsit et al. 2020) that these WGD events are dependent on the genetic background and that hybridisation itself can promote WGD. Thus, it seems that hybrid *Saccharomyces* genomes can be highly dynamic, however in two recent works (Hénault et al. 2020, Smukowski Heil et al. 2020) on different interspecific hybrids, the authors have shown that in contrast with what observed in other species, transposable elements do not have a major activation burst upon hybridisation and that their activation depends mainly on the genetic background rather than the hybridisation itself.

All the findings reported so far suggest that it is not trivial to infer the evolutionary histories of extant hybrids just from experimental evolution, although such experiments give precious

information of which mechanisms might have had a role in shaping the genetic architecture of extant hybrids.

3.2 Evolution through meiosis and sexual reproduction

3.2.1 Meiosis in *Saccharomyces* yeast: initiation, recombination and outcome

The sexual reproduction in the *Saccharomyces* genus generates four haploid gametes, called spores, enclosed in an ascus which essentially enable the cell to resist the harsh environmental conditions. Several signalling pathways act upstream the gametogenesis in *Saccharomyces*, and they orchestrate the beginning of this developmental process that is initiated in a diploid cell, heterozygotic for the sex determinant locus *MAT*, encountering starvation for nitrogen and glucose (Van Werven, F. J. and Amon. 2011). The beginning of meiosis is regulated by the master gene *IME1* (inducer of meiosis 1) (Tam and Van Werven. 2020). This gene has a long promoter to which at least 30 transcription factors bind and induce or repress the expression of *IME1* thus accurately regulating its expression to avoid that cells trigger meiosis when it is not necessary. However, only the lack of PKA and TORC1 activity can lead to meiosis in rich media suggesting that some

controllers of meiosis initiation have a more significant role than others (Weidberg et al. 2016). Moreover, although the same population can encounter the same environmental stresses triggering meiosis initiation cells will respond individually to such signals and that will regulate what fraction of the population will engage in meiosis and sporulation (Nachman et al. 2007). Once *IME1* is transcribed and translated, it induces the transcription of the early meiotic genes (Figure 8) and sets the stage for the events leading to meiotic recombination and progression to the

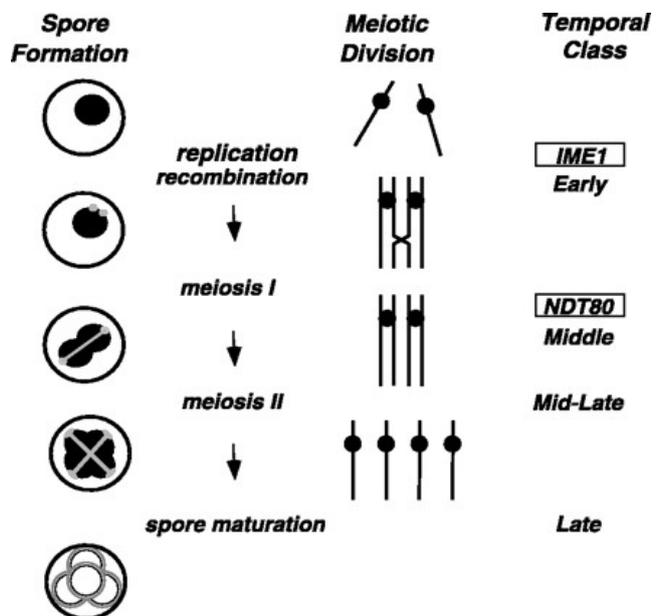


Figure 8: Landmark events of meiosis in *Saccharomyces* with highlighted when *Ime1* and *Ndt80* are active and the different phases of meiosis (right column). (from Chu et al 1998)

middle phase of meiosis. Once the cell has started to replicate its genome in the premeiotic S-phase generating a copy of each homologous chromosome (*Figure 8*) the next landmark event in meiosis is the induction of DSBs. It has been proposed that DNA replication is highly interconnected with the induction of double-stranded breaks (DSBs) by Spo11 (Borde et al. 2000). The gene *SPO11* encodes for an evolutionarily conserved topoisomerase, which bounds the DNA as a dimer through a Tyrosine residue, conserved in other Spo11p orthologous and generates genome-wide DSBs by binding the DNA molecule forming a transient intermediate (de Massy 2013). How, and where, DSBs are induced and repaired, has been extensively studied and has led to the formulation of alternative models (de Massy 2013, Kaniecki et al. 2018). In *Saccharomyces* DSBs are not randomly formed but are preferentially induced in regions called htopspots. This results has been achieved by two different methods: by collecting intermediate Spo11-oligo molecules that form when DSBs are induced by Spo11 (Pan et al. 2011), and by mapping COs and NCOs from several gametes (Mancera et al. 2008).

The repair of the DSBs through homologous recombination (HR) can lead to the formation of non-crossovers (NCO) and crossover (CO) molecules (de Massy 2013, *Figure 9*).

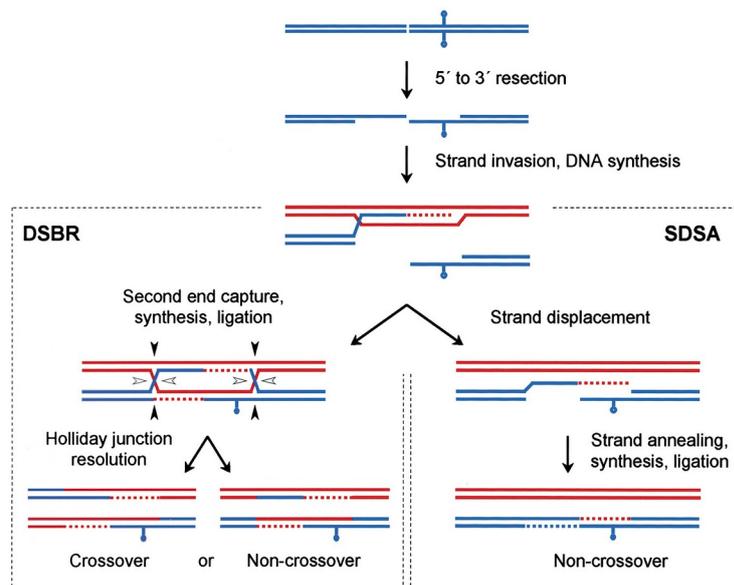


Figure 9: Models of resolution of recombinant molecules produced to repair DSBs induced by Spo11. In the Double strand break repair model the synthesis of the invading strand (blue) displace the read strand which bind to the opposite blue strand and form a Holliday junction, whose resolution produce CO or NCO. In the strand displacement model the synthesis of the invading strand is terminated by its displacement and then produce a NCO. From Allers et al 2001.

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However, other pathways repair DSBs through recombination with the sister chromatid (called sister chromatid exchange), which is generally preferred during the mitotic division, or through non-homologous-end-joining (NHEJ), which uses the segments of the broken DNA molecule to repair the gap (Andersen and Sekelsky 2010) but both are less active in meiosis where the formation of CO is favoured. The formation of the recombinant molecules start when cells enter in the meiotic prophase and chromosomes get closer to engage in homologous recombination and the synaptonemal complex, a protein structure that keep the homologous chromosomes together, is assembled. This moment of the meiotic progression is of central importance as it marks the turning point after which the cell become committed to complete meiosis even if the sporulation signaling stops (Winter. 2012, *Figure 10*). Once the cascade of molecular events that leads cells to complete

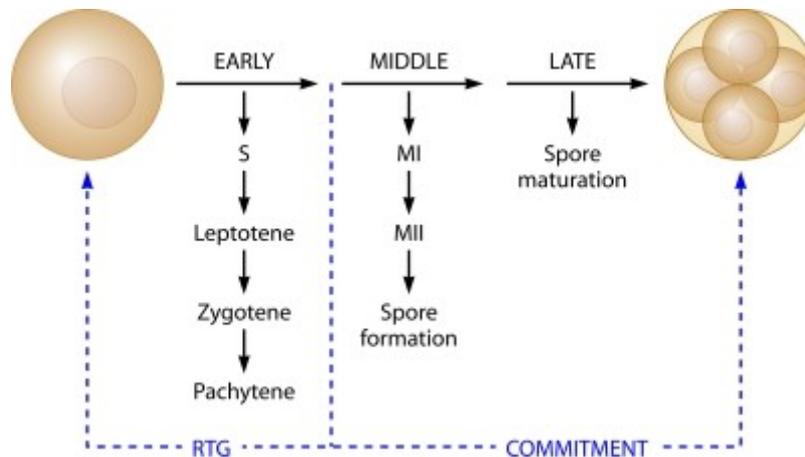


Figure 10: The main phases of sporulation in Saccharomyces are reported below the black lines according to their temporal occurrence. The dotted colored lines represent the different time windows of commitment to meiosis and RTG. This time window coincide with the expression of Ndt80.

the first meiotic division has started, the cells cannot reverse their developmental fate and will carry on the meiotic genetic program being genetically committed (Winter. 2012). The process of meiotic commitment is strictly regulated by a master regulator gene called *NDT80* whose activation and transcription is self-induced and sustained in a positive feedback-loop (Chu and Herskowitz 1998, Winter. 2012, Tsuchiya et al. 2014). The expression of *Ndt80* is strictly regulated by the early meiotic genes *IME1* and *UME6* that promote the expression of *Ime2* whose activity, together with other kinases, is necessary to decrease the repression of the protein *Sum1* on the *Ndt80* expression (Neiman. 2011). *Ndt80* is responsible of inducing key middle and late meiotic genes that dictate the fate of the next steps of the meiotic progression and thus, is essential for exit from pachytene (Tung et al. 2000, Winter. 2012, *Figure 10*) and complete the meiotic

developmental program. Interestingly, if uncommitted cells encounter again nutrients they interrupt the meiotic development program and revert to vegetative growth in a process called Return-To-Growth (RTG, Figure 10, explained more in details in chapter 3.3). If the process is not interrupted by environmental changes the cells carry on the first meiotic division (MI) in which the replicated homologous chromosome are segregated into two cells (Neiman. 2011). Next, the meiotic cells engage in the last meiotic division without an additional DNA replication round thus producing haploid cells, through a reductional division. Then, the last step of sporulation is the maturation of the spores and the formation of an ascus in which all the haploid spores are enclosed.

3.2.1 Sexual reproduction impact on the evolution of *Saccharomyces* hybrids

As reported above, sporulation is a necessary step to form gametes that by outcrossing generate *Saccharomyces* hybrids. Thus, the first contribution of sporulation to hybrid evolution is obviously to form hybrids. Besides that, during meiosis homologous chromosomes of the hybrid subgenomes can engage in recombination and create novel allelic combinations. However, unsuccessful recombination due to high sequence divergence is frequently observed in interspecies *Saccharomyces* hybrids and is also a leading cause of sterility, reducing the spore viability of the F1 hybrid to very low level (< 1%) (see Chapter 2.2). Nevertheless, the produced viable spores can either form F2 hybrids by haplo-selfing due to mating type switch, engage in intra-tetrad mating, which is rare in highly sterile hybrids due to the low spore viability, or engage in inter-tetrad mating (Figure 11). The haplo-selfed meiotic spores have higher fertility compared to F1 hybrids of *S. cerevisiae* and *S. paradoxus*, and they are a route to overcome the post-zygotic reproductive isolation because in doing so the spores homogenise their genomes along its entire length (Greig. 2002). Moreover, viable F1 spores could drive the generation of introgressions if repeatedly backcrossed with one of the two parents. It has been shown that F1 spores backcrossed with one of the parental species have in general increased fertility compared to the initial hybrid, which might promote further backcrosses and homogenisation of the genome (Greig. 2002). However, because the high sequence divergence impair meiotic recombination, often the viable gametes have several aneuploidies, and very low level of genomic recombination (Kao et al. 2010). In F1 spores derived from *S. cerevisiae/S. paradoxus* hybrids, Kao and colleagues identified an average of 2.7 recombination events and common aneuploidies in 58 sequenced spores and they also showed that the lower recombination rate increased in a *msh2* mutant (Kao et al. 2010).

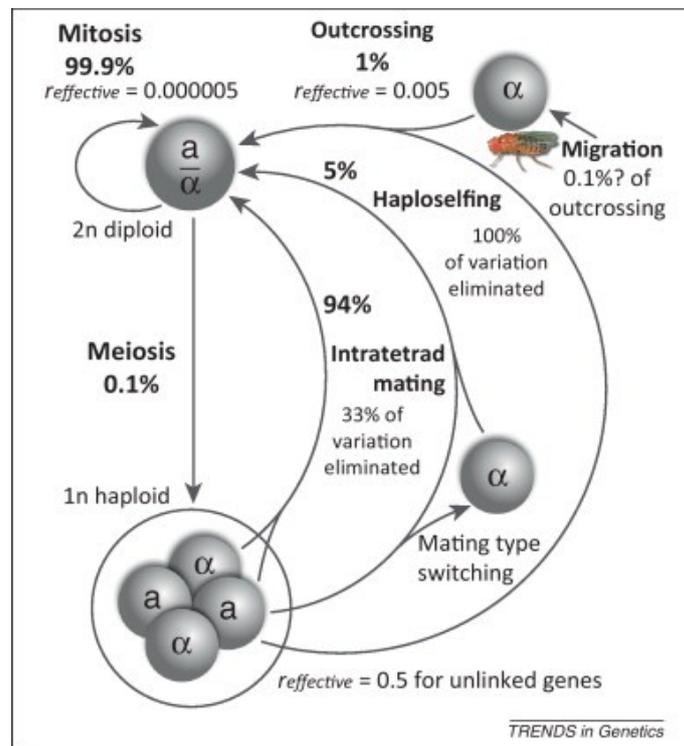
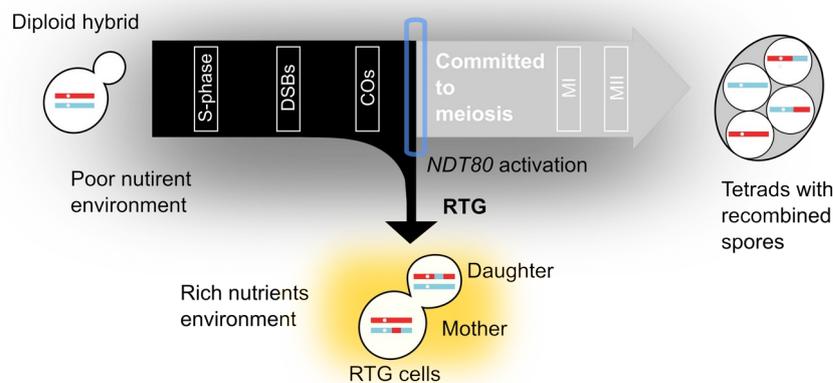


Figure 11: Main lifecycle parameters estimated from *S. paradoxus* lifecycle by Tsai et al. 2008 with highlighted the effective recombination rate ($r_{effective}$) and the estimated frequency of each event in the lifecycle (percentage reported). From Hittinger 2013.

Although intra-tetrad mating is rare in highly sterile interspecific hybrids, in intraspecies *S. cerevisiae* hybrids with lower sequence divergence ($< 1\%$), repeated cycles of intra-tetrad mating rapidly homogenise the genome and increased the fertility of the hybrid. By the fifth round of meiosis, an intraspecific hybrid has increased its fertility to similar levels of the homozygous parental strains, and the sequence divergence between the original hybrid subgenomes has reduced by 90% (Dutta et al. 2017). However, hybrids may not engage in sex so often in the wild as often as when they are induced to do in the lab. From estimations based on recombination rate, Tsai and colleagues (Tsai et al. 2008, Figure 11) have proposed that wild *S. paradoxus* yeasts engage in meiosis once every ~ 1000 asexual generations. However, these estimates are based on assumptions that might not resemble the actual yeast behaviour into the wild. Thus, the impact of sexual reproduction on genome evolution in hybrids into the wild is still not fully understood and more data on natural demography of yeasts might help in further elucidating it.

3.3 The return-to-growth (RTG) paradigm

Mitosis and meiosis play a fundamental role in genome evolution, and they alternate in the *Saccharomyces* lifecycle. Cells replicate by mitosis in rich environments where they thrive and enjoy nutrient abundance. When nutrients are scarce, cells enter in meiosis and sporulate waiting for a more favourable environment. Cells can also enter in a status of reduced metabolic activity called quiescence (De Virgilio. 2012) and stop dividing. Intriguingly, *Saccharomyces* yeast cells can also engage in another process during their lifecycle, at least under laboratory conditions, called return to growth (RTG) (Figure 12).



*Figure 12: Example of the RTG paradigm applied to a *Saccharomyces* hybrid. The black/grey arrow report the major event occurring after and before the meiotic commitment which takes place with *NDT80* expression. Once cells encounter an environment rich of nutrients they buds upon and generate a daughter cell in which the recombined chromatids are segregated.*

The first observations on the return to growth (RTG) phenomenon date back to the past century when researchers found that sporulating cells could revert to mitotic growth if supplied with rich media (Ganesan et al. 1958). Sherman and Roman (Sherman and Roman 1963) used this property to investigate when commitment to meiosis was taking place in a sporulating culture. They found that cells withdrawn from sporulating cultures and plated onto a selective media were maintaining their genome content, and in doing so they measured an increased frequency of recombinant colonies. The increase frequency of recombinants held, until a certain time of sporulation

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induction, after which the retrieved cells were only spores, thus they hypothesized that commitment to sporulation took place after a specific timepoint in the sporulating culture.

The RTG time-window lays between the expression of *Ime1* and early meiotic genes, and the induction of *Ndt80*, which promotes the transcription of middle meiotic genes and meiotic commitment. Cells that encounter nutrients during the early sporulation readily abort the meiotic developmental program by turning off the expression of meiotic transcripts and inducing the expression of genes associated with mitotic growth (Friedlander et al. 2006). However, only cells that have formed DSBs and eventually recombinant molecules will have to face something unexpected; repair the DSBs and resolve recombinant molecules when the cell aborts the meiotic genetic program. During RTG, DSBs repair takes place through at least two different pathways compared to the one operating in meiosis; a first in which *RAD51* but not *DMC1* (that act in meiosis) promotes inter homologue recombination, and a second pathway that promotes recombination using the sister chromatid (Zenvirth et al. 1997). At the same time, RTG cells degrade the proteins of the synaptonemal complex (SC) that rapidly disappear upon RTG (Zenvirth et al. 1997); thus this proteinaceous structure is not necessary to resolve the recombinant molecules in RTG. As during meiosis, the inter homologue recombinant molecules can form CO and NCO when repaired during RTG. Dayani and colleagues (Dayani et al. 2011) showed that in RTG in an *ndt80Δ* background (that is blocked before the meiotic commitment and can accumulate recombinant molecules) there were two "waves" of events that resolve the recombinant molecules. During the first wave, in early RTG, the helicase Sgs1 promotes resolution of recombinant molecule by a dissolution mechanism which promotes the production of mainly NCO. In the last wave, the remaining recombinant molecules are resolved by a Mus81-Mms dependent mechanism that produces both CO and NCO. Thus, RTG tend to minimise the generated homozygosity. Finally, the recombinant chromatids segregate without an additional round of replication in a mother and a daughter cells that therefore maintain the original genome content of the pre-meiotic cells (Dayani et al. 2011, Laurea et al. 2016, *Figure 12*). The recombination events produced by RTG can be detected in diploid hybrid cells as stretches of loss-of-heterozygosity (LOH) (Laureau et al. 2016) where the cell has retained the same allelic information derived from the same parent on both chromosomes.

To briefly summarise:

- i) In a non synchronised RTG culture, there will be a mixture of cells at different stages of the meiotic progression. Some will have accumulated DSBs and partially resolved them; others might not have even started the meiotic genetic program.
- ii) The repair of DSBs during RTG takes place through two mechanisms, one dependent on Rad51 and one that uses the sister chromatid.
- iii) Resolution of recombinant molecules after RTG does not depend by the SC, and most of the recombinant molecules will generate NCO while only a small fraction will produce COs compared to a normal meiosis.
- iv) RTG mother-daughter cells are generated for each cell that performs RTG, and they maintain the original genome content of the pre-meiotic cell.
- v) The product of RTG recombination can be visualized as regions of LOHs in diploid hybrid cells.

3.3.1 Studies of RTG in *Saccharomyces* hybrids.

Given what reported above, it is clear that RTG is an alternative sexual cycle resulting from environmental modifications that deregulate the meiotic genetic program resulting in an abortive meiosis. A recent work (Laureau et al. 2016) produced the first whole-genome-sequencing map of RTG cells isolated from an S288C/SK1 hybrid (*S. cerevisiae*) and showed that the recombination generated during RTG produces genome-wide regions of loss-of-heterozygosity (LOHs) in the hybrid genome. The recombination induced by RTG was also used to perform a controlled linkage analysis in the hybrid background. The same authors also started to explore RTG in a S288C/SK1 *ndt80Δ* mutant, and that work is part of the collaboration reported in chapter 6.

Interestingly, a recent study proposed that even a yeast species which diverged millions of year ago from *Saccharomyces cerevisiae* have retained the ability to abort meiosis and recombines its genome in an RTG-like mechanism (Brion et al. 2017, Discussed in chapter 8).

LOHs generated through RTGs can have the same consequences as those generated during asexual reproduction (see Chapter 3.1) with the only exception that in the case of RTG many LOHs can arise at the same time (Although the LOH formation is minimised in RTG). Thus, hybrid cells can explore different genetic combinations in a burst of recombination and potentially evolve through this mechanism.

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However, so far there is only one study available on *Saccharomyces* hybrids that have investigated its impact on genome evolution (Laureau et al. 2016) and data on different hybrid backgrounds, in particular non-lab strains and sterile hybrids, are missing.

Graphical summary chapters 2-3

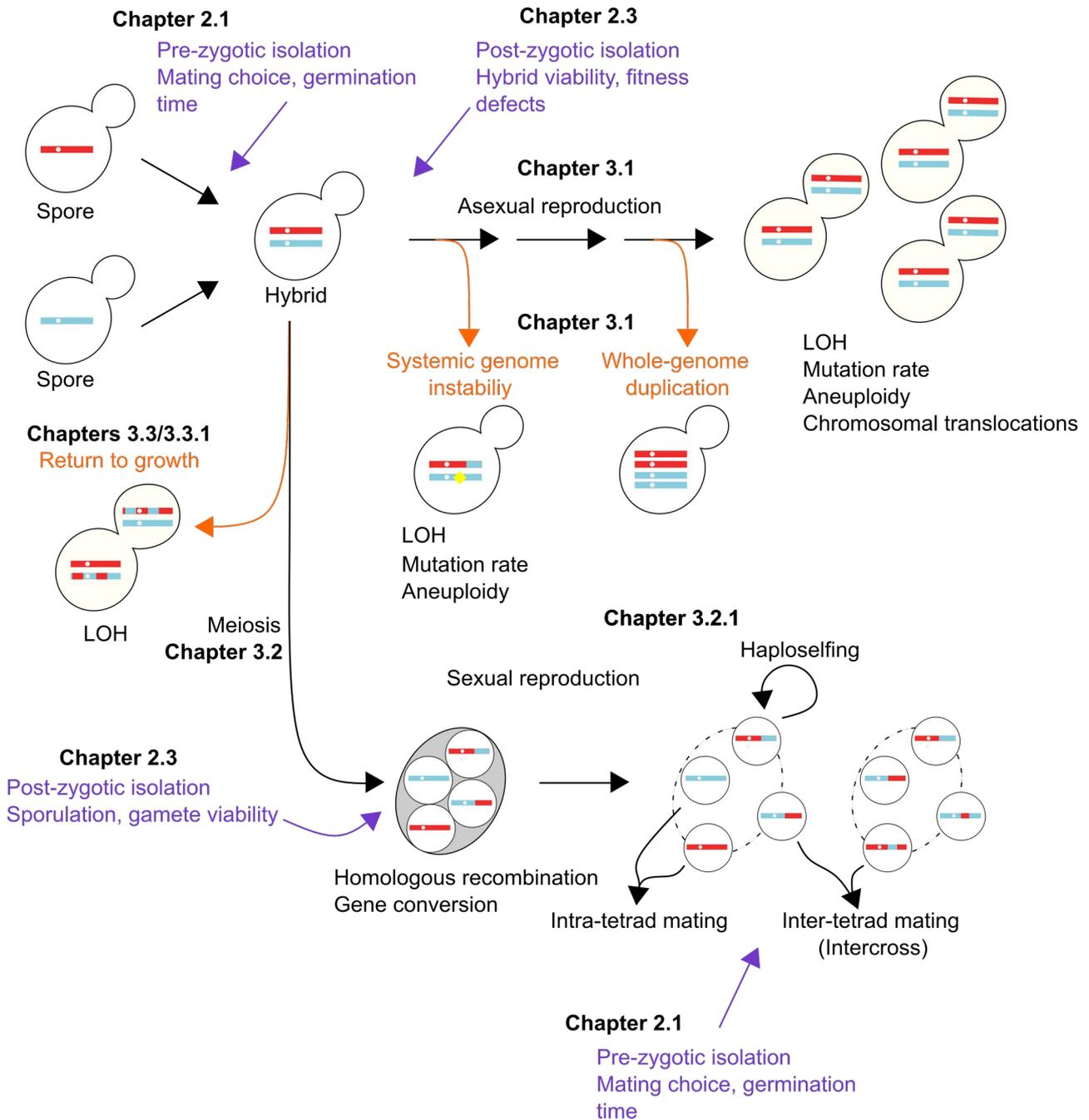


Illustration 1: Summary illustration of chapters 2 and 3 relative to the evolution of hybrid genomes. Each text box reports the chapter information in which that argument has been described. Purple arrows report reproductive barriers, orange arrows report events that can have massive impact on genome evolution although it is difficult to quantify how often they arise. The red and cyan rectangles represent a haploid genome derived from different parents. Spore: Haploid gamete.

4. Improving industrial *Saccharomyces* strains

- In this section I briefly summarise the main findings on how the process of domestication has shaped *Saccharomyces* yeasts diversity and I continue presenting examples of the main approaches used for improving industrial yeasts.

4.1 Domestic *Saccharomyces* yeasts diversity

Besides being a simple and powerful model systems to answer fundamental biological questions, *Saccharomyces* yeasts are also important microbes that play a crucial role in many industrial processes (Parapouli et al. 2020). The domestication of *Saccharomyces* yeasts started even before the first microorganisms were observed and occurred independently on several occasions (Gallone et al. 2016, Duan et al. 2018). However, these domestication events are radically different from other well-known cases of domestication as humankind was not directly aware of it. Nevertheless, the study of the genomes and phenomes of these domestic yeasts showed that the outcome of the domestication process was, in principle, similar to other domestic organisms (Steensels et al. 2019). As results of decades or centuries of continuous growth on favourable conditions, where nutrients were readily available, many domestic *Saccharomyces* have partially, or totally, lost the capacity to reproduce sexually (De Chiara et al. 2020), they have become more resistant to specific stresses, which are commonly encountered in fermentative conditions and use some sugars more efficiently than wild strains (Steensels et al. 2019). All these characteristics resemble what is called a "domestication syndrome", already described by Darwin back in 1868 (Gregory et al. 2009) according to which, organisms during domestication tend to lose unselected or undesired traits and to acquire traits which make them successful in the human-made environment. Besides these conceptual similarities, there are also some important differences in the domestication process of microbes and higher eukaryotes partly due to the size differences. During the domestication of microbes, the selective pressure acted simultaneously on millions of individuals and promoted competition between subpopulations selecting the fitter cells each round of fermentation. In contrast, the domestication of crops or animals was more controlled and breeder meticulously selected improved individuals mainly for observable phenotypic traits, such as size, colour or yield. Domestic yeasts are well-known for their importance in the beer and wine industries (Marsit and Dequin 2015, Gallone et al. 2016) and in bread-making (Carbonetto et al. 2018). However, these microorganisms do more. For instance, they are crucial in the coca fermentation (Jespersen et al.

2005) where they are essential for the production of ethanol, higher alcohols and esters and to maintain the quality of the chocolate, are involved in the fermentation of molasses in the biofuel industry and produce a great variety of sake, cheese, liquors and numerous other products.

Beyond the fact that yeasts are unicellular microorganisms and thus are obviously different from domestic multicellular organisms, the core questions related to their exploitation are partly similar as the aim is that of improving their resistance to specific stresses, increase the fermentation rate (process efficiency) or produce desired molecules which can, for instance, impact the flavour of fermented beverages. In the past centuries, before the refrigeration was adopted to conserve yeast stocks, bakers, brewers and other artisans were used to perform sequential fermentations using microbes from an old batch following a procedure called back-slopping (Gallone et al. 2016). This procedure was essential to maintain the stability of the final product that ultimately was regulating the fate of each economic activity. The refrigeration and the usage of pure cultures for starting the fermentation further improved the stability of the final product (Gorter de Vries et al. 2019). Paradoxically, once brewers became aware of the vital role of yeasts in the fermentation and started to isolate them as pure cultures, the genetic diversity of yeasts in industrial fermentations reduced dramatically as pure cultures were used to start the fermentation and clonally expanded batches were conserved through refrigeration. In some styles of fermentation, most of the strains are strictly related, for example, the lager beer fermentation is dominated by *S. cerevisiae*/*S. eubayanus* hybrids (Gallone et al. 2019, Langdon et al. 2019, Gorter de Vries et al. 2019). However, alternative brewing styles are still a treasure of genetic diversity harbouring strains with peculiar fermentation activities (as example see: Norwegian kveik beer, Preiss et al. 2018, Lambic and Trappist beers, Gallone et al. 2019, *Brettanomyces* yeast used in fermentation, Colomer et al. 2020). However, other genetic backgrounds, which are often hybrids, are used in other fermentation industries such as in the production of cider or wine where *S. cerevisiae*/*S. kudriavzevii* hybrids have been found (Morales and Dujon 2012, Alsammar and Delneri. 2020, see Chapter 2.1) Interestingly, most yeasts used today, in particular, those involved in beer and wine fermentations, are often used more for historical reasons rather than on practical grounds (Steensels et al. 2014). Moreover, since the request of the industry and the customers has changed and kept changing, there is still room for improvement of industrial strains despite their long domestication process.

Broadly, the strategies used for generating novel and improved yeast strains can be grouped based on the source of genetic diversity. One group of approaches relies on exploiting the natural genetic

diversity, whereas another category includes strategies that exploits artificial genetic diversity generated by using different techniques. This latter group can be further divided into methods generating GMOs or non-GMOs yeast strains variants, though sometimes defining a GMO or non-GMO approach depends on the specific legislation of the country. The two strategies can also interconnect, as it is possible to isolate a particular interesting strain from the wild and then further optimize interesting traits with artificial selection.

4.2 Exploiting natural genetic diversity.

Researchers have started to extensively study the natural genetic diversity of *S. cerevisiae* and other species in the genus thanks to the groundbreaking improvement of sequencing technologies which helped in identify genetic differences that were otherwise difficult or impossible to spot. Seminal (Liti et al. 2009, Schacherer et al. 2009) and more recent works (Peter, De Chiara et al 2018, Duan et al 2019) have expanded our knowledge of the natural diversity existing between different *S. cerevisiae* populations. Similar works have been carried out also for other species of the genus such as *S. eubayanus* (Nespolo et al. 2020, Langdon et al. 2020) or *S. paradoxus* (Liti et al. 2009, Barbosa et al. 2016, Leducq et al. 2016, for a review see Alsammar and Delneri 2020), and have helped to identify subpopulations of the respective species. One clear message emerged from these studies is that the natural diversity is still largely unexplored, and interesting strains may exist in the wild or even novel species (Naseeb et al. 2017).

Other than enriching our knowledge of the evolution of this genus, the proliferation of novel natural isolates has a double importance for the industry. First, natural isolates may harbour desired traits for the industry, but they have never been domesticated just by chance as the first domestication events were unconscious and there is no historical track that records which strains were used, as humans were not yet aware that the fermentation was driven by microbes. Second, by phenotyping extensive libraries of natural strains, desired phenotypic properties can be spotted and transferred to a specific industrial strain by hybridisation, genetic engineering or other strategies. Interestingly, recent work on wild isolates of *S. paradoxus* showed that these strains could produce beer with specific organoleptic properties, which were also not rated as unpleasant by a panel of tasters (Nikulin et al. 2020). However, these strains could not metabolize any maltotriose present in the wort, had a long fermentation time and no vigorous flocculation activity, but it could be adapted through serial repitching in the wort.

4.3 Exploiting artificial genetic diversity

Despite the vast genetic diversity of wild isolates, it is possible that they will not ultimately perform well in industrial fermentations and therefore either the trait of interest is directly transferred to a domesticated strain or the wild strains need to be adapted to the new niche. Regardless of which approach industry and researchers adopt, artificial manipulation seems likely to be an essential bridge for completely exploit the natural genetic diversity. The vast array of techniques used for improving industrial strains are usually grouped as stated above based on the outcome strain; some of these methods will generate GM strains (GMO methods), whereas others will generate GM-free strains. Moreover, GMO-free methods can further be divided on those exploiting the sexual or “asexual” hybridisation from those exploiting the asexual reproduction (Figure 13).

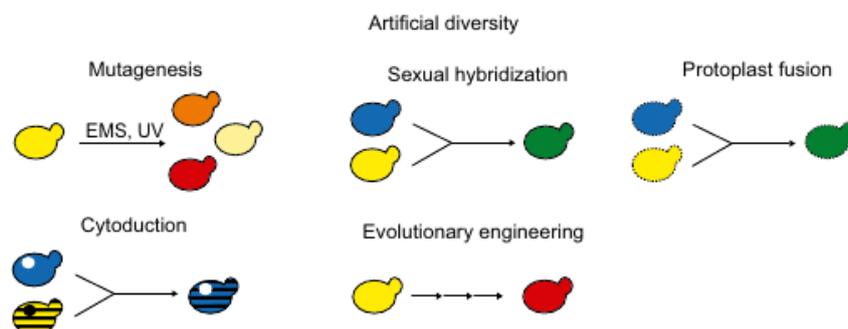


Figure 13: Improvement methods of industrial strains aiming at generating artificial diversity. Protoplast fusion and Cytoduction are often referred to as asexual hybridisation. (Adapted from Steensels et al 2014)

4.3.1 Methods based on sexual or asexual hybridisation

Controlled sexual reproduction and breeding (selective breeding) have been widely used by humans to generate fit hybrids with desired traits. Not surprisingly, a similar practice has been used in yeast biotechnology to create yeast strains with specific phenotypes with the first attempts dating back to the past century (Lindegren and Lindegren. 1943). Generally, the controlled mating can take place between two single cells with opposite mating types (a and α) by direct mating (Sipiczki

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2008), or pools of haploid *MATa* and *MATα* cells derived from different parents are mated in dense cell cultures resulting in "mass mating". The first approach (direct mating) has the advantage of allowing more comfortable isolation of improved hybrids by selecting directly the fitter hybrids on the plate, but it requires to screen many formed hybrids to find the one with the desired quality. In contrast, mass mating enables to generate many individual hybrids at the same time, and it can be advantageous when the parental species have low sporulation efficiency or mating efficiency. However, after mass mating, might be more challenging to identify and collect single hybridised clones among the population as there will be a mixture of mated and non mated cells. To overcome this limit, researchers have developed techniques that enable to isolate single improved strains derived from mass mating by plating the mass-mated population on selective media where the desired clones can easily grow (Shi et al. 2009, Zheng et al. 2014). However, often industrial strains are polyploids with unstable and complex mating-type genotype and may be completely sterile; thus, seriously hampering improvement strategies based on sexual reproduction. An alternative is to exploit the natural change of mating-type occurring during a mating-type switch event in which diploid a/α cells become either a/a or α/α and thus become mater. This approach called rare mating has been successfully used, for instance, to generate interspecific *S. cerevisiae/S. mikatae* hybrids for the wine industry (Bellon et al. 2013). However given the intrinsic "rare" nature of this event, the isolation of candidate hybrids relies, as in mass mating, on plating the population in selective conditions in order to identify mated strains through selection of specific genetic markers, such as auxotrophies, or phenotypic properties.

Another array of artificial hybridisation techniques are based on the "asexual hybridisation" because they still rely on the fusion of two different cells, although they do not exploit the sexual phase of *Saccharomyces*. In the cytoduction approach, one parental cell knocked-out for the *KAR1* gene and mated with another cell derived from another parent generates a heterokaryon. The *KAR1* mutant is defective for nuclear fusion upon hybridisation and therefore after budding will segregate the haploid nuclei of the two parental species in different cells that will inherit a mix of cytoplasmatic determinants from both parents. This technique has been exploited to transfer cytoplasmatic determinants from one parental strain to another (Yoshiuchi et al. 2000). In the protoplast fusion, yeast cells from different parents are grown to high density, then the cell wall is removed and the fusion between different cells is enhanced by using lipids. Once the cells are fused they are left resting in buffering conditions to reconstitute the cell wall (Scheinbach. 1983). The power of this approach is that it does not require meiosis at all and thus is easily exploitable for

sterile industrial strains, but once again, being a sort of "mass approach", it requires a strong selection to isolate the successfully merged strains. To conclude, it is important to note that cytoduction requires genome editing, thus generating GMOs, and protoplast fusion is also classified as a GMO approach in some countries.

4.3.2 Methods based on the asexual reproduction

The short generation time, high population size and ease with which *S. cerevisiae* grows, make it an excellent organism for improvement approaches based on direct evolution. Although this procedure was already exploited more than a hundred years ago (Reviewed in Bennett and Huges, 2009) when a first experimental evolution was carried out on protists for over 7 years, only recently it has become clear its relevance in the fields of evolution (Elena and Lenski. 2003, Buckling et al. 2009) and industrial strain improvements (Dragosits and Mattanovich. 2013). Therefore, in the last

c Serial transfer

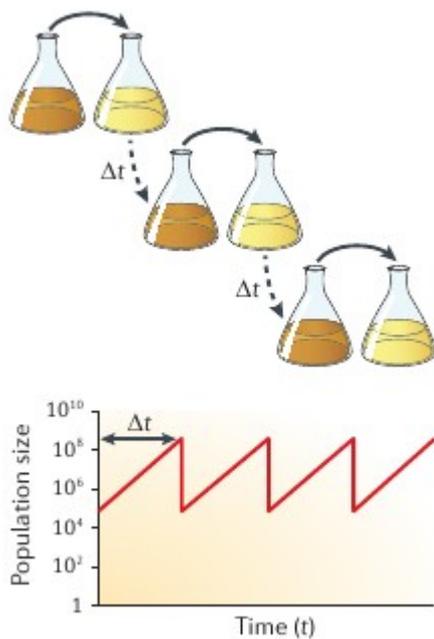


Figure 14: Example of the rationale of serial transfer where cells are transferred between flasks at periodical time intervals. On the bottom is depicted the variation in population size according to the time. Each transfer coincide with a lower peak in the population size. (From Barrick and Lenski 2013)

decades this strategy has been widely adapted to evolve several industrial microbes such as bacteria (As example see López-González et al. 2018) and yeasts (As example see Mangado et al. 2018). The core concept of this approach relies on growing a dense cell population in selective conditions in which clones bearing mutations or other genomic modifications that offer a selective advantage gain an edge over the other clones and can expand rapidly. After established time-windows the population is transferred to a new flask containing the same environmental stress starting back the of selection and growth (Figure 14).

This principle has been exploited in adaptive laboratory evolution experiments (ALE), where cells are grown continuously in flasks and exposed to a specific stress, which can eventually increase over the time of the experiment to further select fitter clones emerging in the population. In more recent studies, this approach have been automatized in sequential batch reactors (SBRs) that allow controlling

with high precision several parameters of the process such as CO₂ concentration or pH (For a review see Mans et al. 2018). Interestingly, it has been shown that the adaptation to the stress and fitness gain is not linear along the experiments and tend to decrease at later stages. In contrast, the fitness gain seems to be higher in the first 100-500 generations (Dragosits and Mattanovich. 2013); thus, a long adaptation time might not promote a better adaptation to the stress. Although this approach succeeded in improving several yeast strains that optimised sugars utilisation or resistance to diverse stresses, it has become evident that tradeoffs in unselected traits can emerge at the end of the adaptation (Mans et al. 2018). For instance, *S. cerevisiae* cells adapted to grow at high temperature partially changed the lipidic composition of their membranes and thus were more adapted to grow at a higher temperature but not anymore in standard lab conditions and also experienced defective respiration (Caspeta and Nielsen 2015). Nevertheless, it has been developed an alternative design for ALE which aims at minimising eventual tradeoffs emerging during the experiment by exposing the yeast cells to alternate cycles of different stresses or nutritional conditions (Reviewd in Mans et al. 2018). This strategy has been adapted, for instance, to overcome a tradeoff in sugars utilisation that often arises when evolving yeast are exposed to a single sugar source. The trick consistend in propagating the yeasts in a different mixture of sugars at different concentrations which enabled to recovere a strain with improved utilisation of a mix of sugars instead of a “crippled” strain that was highly efficient in consuming only one type of sugar (Wisselink et al. 2009). To conclude, this field offers a vast range of options to customise the selective regime under which improve industrial strains and overcome eventual setbacks and it will be crucial in developing novel industrial strains.

4.4 Methods based on genome editing or mutagenesis

Genetic modification using mutagenesis aim at increasing the natural rate with which genetic alterations occur in the genome. In the first phase of "exposure", the cells are treated with a mutagenic agent, which can be either a chemical or physical agen, that induces a different spectrum of modifications, such as mutations, indels or gene conversions (Rowlands. 1984). Subsequentially, cells are left recovered from the mutagenic treatment, and finally, mutated clones fitter for a specific trait are selected among the population. Although technically simple, several critical points need to be considered when designing such experiments. For instance, it is essential to choose the right type of mutagen and to do not overstress the cells with high doses which can be lethal, and it

is also advised to alternate the mutagen used (Rowlands. 1984). Mutagenesis, as the previous method reported above, does not encounter barriers due to the GMO legislation and thus it shares this benefit with the other approaches already reported but this classification may change according to the country. Moreover, as the induced mutagenesis occurs randomly, it does not require an *a priori* knowledge of the genetic pathway regulating the trait, which can be definitely intricate for complex industrial phenotypes.

The ability to directly modify a single gene, group of genes or entire pathways is, without doubts, a milestone in the field of strain improvement. Although genetic engineering in particular in the food sector is not widely accepted and still controversial, many studies have been conducted in which industrial microbes were modified to achieve improved or novel functions (Overview on genome editing in Fraczek et al. 2018). This method offers endless customisation possibilities; however, it requires a knowledge of the genetic architecture regulating the trait of interest, which is not trivial despite the characterisation of the genes of the *S. cerevisiae* genome. To bypass this constraint, the researchers have developed an approach named "inverse-engineering" (Alkim et al. 2014). In inverse-engineering, first, the genetic determinants regulating the traits are decomposed by analysing the variability of the phenotype landscape in a collection of natural strains or using strategy that generates artificial variability such as genome shuffling. Second, the information obtained regarding the genetic basis of the phenotype is exploited to modify the desired industrial strain. Moreover, the emerging field of synthetic biology is providing an increasingly number of tools and techniques to modify the genome of selected industrial strains. The basic concept of synthetic biology is that different "biological parts" like terminators, promoters or other genetic elements can be assembled and modified in different ways to achieve, for instance, a faster or longer expression of a transcript (Liu et al. 2019) or the expression of genes derived from other species in yeast (Liu et al. 2018). For example, it has been possible to modify strains to make them produce different flavours in the beer (Denby et al. 2018) a feature which is often technically challenging to improve using the previously reported methods. Eventually, these new tools will enable to scale-up the customization and improvement of industrial yeast and so called "biofoundries" are already bringing this approach at an high-throughput scale.

5. Scientific background and aims of the thesis

The study of hybridisation is a fundamental research topic in biology, and it has become clear that it is more widespread than what thought in the past (Taylor and Larson 2019). Since Darwin's studies on hybrids, several researchers and breeders have continued to study and breed new hybrids for their relevance in many fields of our economy and for fundamental research. Through these studies, we have become more aware of several properties governing the biology of hybrids such as heterosis, hybrid vigour and hybrid sterility. Strikingly, despite hybrids often enjoy beneficial traits from both parents and thus are selected by humans, they are often evolutionary dead-ends because of their sterility. The extreme sterility of hybrids essentially impeded them to propagate favourable genetic information to the offspring and to further optimise it through sexual reproduction. This has a major economic relevance when sterile hybrids such as crops or microbes are associated with human activities. Hybrid yeasts of the *Saccharomyces* genus are not an exception as those hybrids isolated from domesticated environments are often sterile precluding any improvement strategy based on sexual reproduction. Lab-made hybrids have been fundamental to study mechanisms regulating hybrid sterility in the genus, and have revealed that antirecombination and chromosomal structure divergence are the main drivers of sterility (Ono et al. 2020). By studying population genomics of wild and domestic *Saccharomyces* (Gallone et al. 2016, Peter, De chiara et al. 2018, De Chiara et al. 2020) it has become clear that in some populations this sterility can be caused by a relaxed selection of genes regulating the yeast gametogenesis leading to pathway decay. Although some examples of gene incompatibilities have been reported (Lee et al. 2008) gene incompatibilities may play a minor role in causing sterility in *Saccharomyces* hybrids. Therefore, the *Saccharomyces* genus harbour a profound paradox. The species of this genus have extreme levels of sequence divergence (12-15% to 30-35%) that ultimately hamper meiotic recombination and gametes formation (Hunter et al. 1996, Greig 2009). Yet, hybrid strains with a signature of loss-of-heterozygosity (LOH) and recombination are frequently isolated and historical patterns of LOH are observed in different populations (Morales and Dujon 2012, Peter, De Chiara et al. 2018). These LOHs can have a profound evolutionary impact as they can promote phenotypic diversification, promote adaptation and confer stretches of sequence homology that can promote recombination between highly diverged genomes and rescue sterility (Marsit et al. 2017, D'Angiolo et al. 2020).

5. *Scientific background and aims of the thesis*

I joined Gianni Liti's team and MeioGenix in 2017, and since then I worked on the fascinating field of hybrid evolution by using both experimental and bioinformatic approaches. During my PhD, I mainly focused on an unconventional sexual cycle, called meiotic reversion or return-to-growth (RTG), in which, uncommitted meiotic cells can abort the meiotic developmental program and return to mitotic growth upon exposure to rich nutrients.

Recently, it has been shown that RTG can reshuffle a diploid genome of a fertile diploid lab hybrid (Laureau et al. 2016). However, the RTG paradigm has been tested only on a handful of lab strains so far, and the only genome-wide recombination data available are derived solely from the fertile *S. cerevisiae* hybrid S288C/SK1. Therefore several questions are still unanswered, such as how conserved is this mechanism in wild and domesticated strains? Can sterile hybrids evolve through RTG, thus having access to an unconventional pathway for genome recombination? Can RTG promote genome evolution of industrial strains and improvement of industrial relevant traits?

Broadly, the common aim behind the two projects of my PhD was to verify to which extent RTG can help sterile hybrids to overcome different reproductive barriers. Since my PhD developed in the framework of an industrial collaboration with MeioGenix, I could explore the RTG outcome from two different perspectives that culminated in the two works reported here.

In **chapter 6**, I report the submitted version of an article in which we wanted to answer to different questions related to the evolution through RTG of sterile diploid hybrids. First, we tested to what extent RTG allows hybrids with a diverse degree of sterility to further evolve by recombining their genomes. To do so, we recreated three common causes of sterility in the *Saccharomyces* genus: chromosomal structure divergence between hybrid subgenomes, sexual pathway decay and high sequence divergence between hybrid subgenomes. Second, we further characterised the RTG recombination landscape by comparing its signature genome-wide to the meiotic recombination signature. Third, we evaluated the potential of RTG recombination in inducing phenotypic diversification in a sterile hybrid and exploited that to perform linkage analysis in an RTG library in order to acquire a genetic understanding of the phenotypic diversity between sterile lineages.

In **chapter 7**, I report the draft paper of a work in which I explored the application of RTG to promote the evolution and improvement of sterile industrial strains. In this work, my first objective was to show that these industrial hybrids can perform RTG, which was unknown. The second objective was to develop a method to isolate non-GMOs RTG variants from industrial strains. I

validated the method I developed, by whole-genome-sequencing of candidate RTGs and taking advantage of different molecular biology approaches that enabled me to collect enough evidence of RTG recombination in such complex genomes. As the last objective, I evaluated the potential of RTG in generating phenotypic diversity for relevant industrial traits in sterile polyploid hybrids.

In **chapter 8**, I conclude with a discussion of the main results obtained during the thesis and provide a more general overview of still open questions relative to the field. For each chapter is reported my individual contribution to the work.

5.1 References

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Title: Aborting meiosis overcomes hybrid sterility

Authors:

Simone Mozzachiodi^{1,2,†}, Lorenzo Tattini^{1,†}, Agnes Llored¹, Agurtzane Irizar¹, Neža Škofljanc¹, Melania D'Angiolo¹, Matteo De Chiara¹, Benjamin P. Barré¹, Jia-Xing Yue^{1,#}, Angela Lutazi¹, Sophie Loeillet³, Raphaele Laureau^{3,§}, Souhir Marsit^{3,4,^}, Simon Stenberg⁵, Benoit Albaud⁶, Karl Persson⁵, Jean-Luc Legras⁴, Sylvie Dequin⁴, Jonas Warringer⁵, Alain Nicolas^{2,3} and Gianni Liti^{1,*}

Affiliations:

¹Université Côte d'Azur, CNRS, INSERM, IRCAN, Nice, France.

²Meiogenix, 38, rue Servan, Paris 75011, France.

³Institut Curie, Centre de Recherche, CNRS-UMR3244, PSL Research University, Paris 75005, France.

⁴SPO, Université Montpellier, INRAE, Montpellier SupAgro, Montpellier, France.

⁵Department of Chemistry and Molecular Biology, University of Gothenburg, Gothenburg, Sweden.

⁶Institut Curie, ICGEX NGS Platform, Paris 75005, France.

Notes:

[†]These authors contributed equally to this work.

[#]Current address: State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Sun Yat-sen University Cancer Center, Guangzhou, China.

[§]Current address: Department of Genetics and Development, Hammer Health Sciences Center, Columbia University Medical Center, New York, NY, USA.

[^]Current address: Institut de Biologie Intégrative et des Systèmes (IBIS), Université Laval, Québec, QC, Canada.

*Correspondence and requests for materials should be addressed to G.L. (gianni.liti@unice.fr)

One sentence summary:

Aborting meiosis followed by a return to mitotic growth promotes evolution by genome wide-recombination in sterile yeast hybrids.

Abstract:

Hybrids between species or diverged lineages contain fundamentally novel genetic combinations but an impaired meiosis often makes them evolutionary dead ends. Here, we explored to what extent and how an aborted meiosis followed by a return-to-growth (RTG) promotes recombination across a panel of 20 yeast diploid backgrounds with different genomic structures and levels of sterility. Genome analyses of 284 clones revealed that RTG promoted recombination and generated extensive regions of loss-of-heterozygosity in sterile hybrids with either a defective meiosis or a heavily rearranged karyotype, whereas RTG recombination was reduced by high sequence divergence between parental subgenomes. The RTG recombination preferentially occurred in regions with local sequence homology and in meiotic recombination hotspots. The loss-of-heterozygosity had a profound impact on sexual and asexual fitness, and enabled genetic mapping of phenotypic differences in sterile lineages where linkage or association analyses failed. We propose that RTG gives sterile hybrids access to a natural route for genome recombination and adaptation.

Introduction

Meiotic recombination is a primary source of genetic diversity in species undergoing sexual reproduction. During meiosis, crossovers ensure that haploid gametes receive one copy of each chromosome and prevent a genetic imbalance in the offspring that often decreases fitness (1). However, reproductive barriers acting before (pre-zygotic) or after (post-zygotic) zygote formation (2) can arise during species evolution, with post-zygotic barriers leading to hybrid sterility (3) across the tree of life (4).

Saccharomyces hybrids have served as models to elucidate the mechanisms contributing to post-zygotic reproductive isolation (5). Sterile *Saccharomyces* intraspecies and interspecies hybrids have been repeatedly isolated in both wild and domesticated environments (6). Relaxed selection on sexual reproduction in domesticated *S. cerevisiae* populations has led to the accumulation of loss-of-function mutations in genes involved in gametogenesis, i.e. “sporulation” in yeast biology, and to severe sterility (7)

. Chromosomal rearrangements between the subgenomes of a hybrid can also lead to sterility due to aberrant chromosome pairing and segregation (8, 9). The Malaysian *S. cerevisiae* lineage represents the most dramatic chromosomal speciation example as it contains 5 rearranged chromosomes that isolate it reproductively from other *S. cerevisiae* lineages, despite retaining high levels of sequence similarity (10, 11). In contrast, *Saccharomyces* interspecies hybrids produce inviable gametes because of the extreme DNA sequence divergence between parental subgenomes, namely heterozygosity, which suppresses recombination and leads to chromosome mis-segregation (12). Therefore, many *Saccharomyces* intraspecies and interspecies hybrids have only very limited possibilities to evolve through meiosis and some are

completely sterile. Moreover, wild yeasts reproduce sexually approximately only once every 1000 asexual generations, thus further limiting the role of meiosis in both intraspecies and interspecies evolution (13).

We showed that aborting the meiosis in a fertile *S. cerevisiae* lab hybrid and returning the diploid cells to mitotic growth, a process known as “return-to-growth” (RTG) (14), reshuffles the diploid genome and produces extensive regions of loss-of-heterozygosity (LOH) (15). LOHs resulting from single crossing-over events generate terminal events that extend to the chromosome ends, whereas interstitial LOHs in chromosome cores can be ascribed to gene-conversions or double crossovers. Meiosis in *S. cerevisiae* is initiated by starvation and chromosomes are replicated during the meiotic S-phase (**Fig. 1A**). Next, as in most eukaryotes (16), a DNA topoisomerase, Spo11p in yeast, generates genome-wide double-strand breaks (DSBs) (17). The repair of these DSBs produces joint DNA molecules and ultimately leads to recombination through chromosomal crossover (CO) and non-crossover (NCO) molecules. Yeast cells brought back to a nutrient-rich environment before the commitment to complete meiosis, abort the meiotic program and instead express genes promoting mitotic division, thus entering the RTG process (18, 19). RTG cells repair the DSBs, bud likewise mitotically dividing cells, and segregate their recombined chromatids with no further DNA replication (20). This process generates a mother and a daughter cell that maintain the parental diploid state, as they do not complete the two chromosomal segregation events that occur in a normal meiosis, but contain different rearranged genotypes. Here, we show that RTG allows sterile *Saccharomyces* hybrids to overcome common reproductive barriers, and we propose RTG as a powerful alternative recombination mechanism that may play an unanticipated role in the evolution of hybrid genomes in nature.

Results

An incomplete meiosis supports recombination in a sterile hybrid

Deleterious variants in meiotic genes can lead to various meiotic defects and sterility. However, mutations that impair mid or late meiotic progression should not prevent RTG, if cells can resume mitotic-like chromosome segregation and budding (20, 21) (**Fig. 1A**). Therefore, we probed whether RTG allows *S. cerevisiae* (Sc) to overcome mutational barriers in meiosis by deleting *NDT80*, the master regulator of middle and late meiotic genes, in an otherwise fertile ScS288C/ScSK1 intraspecies lab-hybrid. First, we confirmed by DAPI staining that *ndt80Δ* cells were arrested before the first meiotic division (MI) after 8 hours of sporulation induction, whereas $\geq 50\%$ of the wild-type ScS288C/ScSK1 cells had already completed MI (≥ 2 nuclei). Then, we evolved the hybrid through the RTG protocol and sequenced the genome of 12 mother-daughter *ndt80Δ* RTG pairs ($n=24$) isolated 8 hours after sporulation induction. As a comparison, we re-analysed the mother-daughter RTG genomes ($n=22$) of the wild-type

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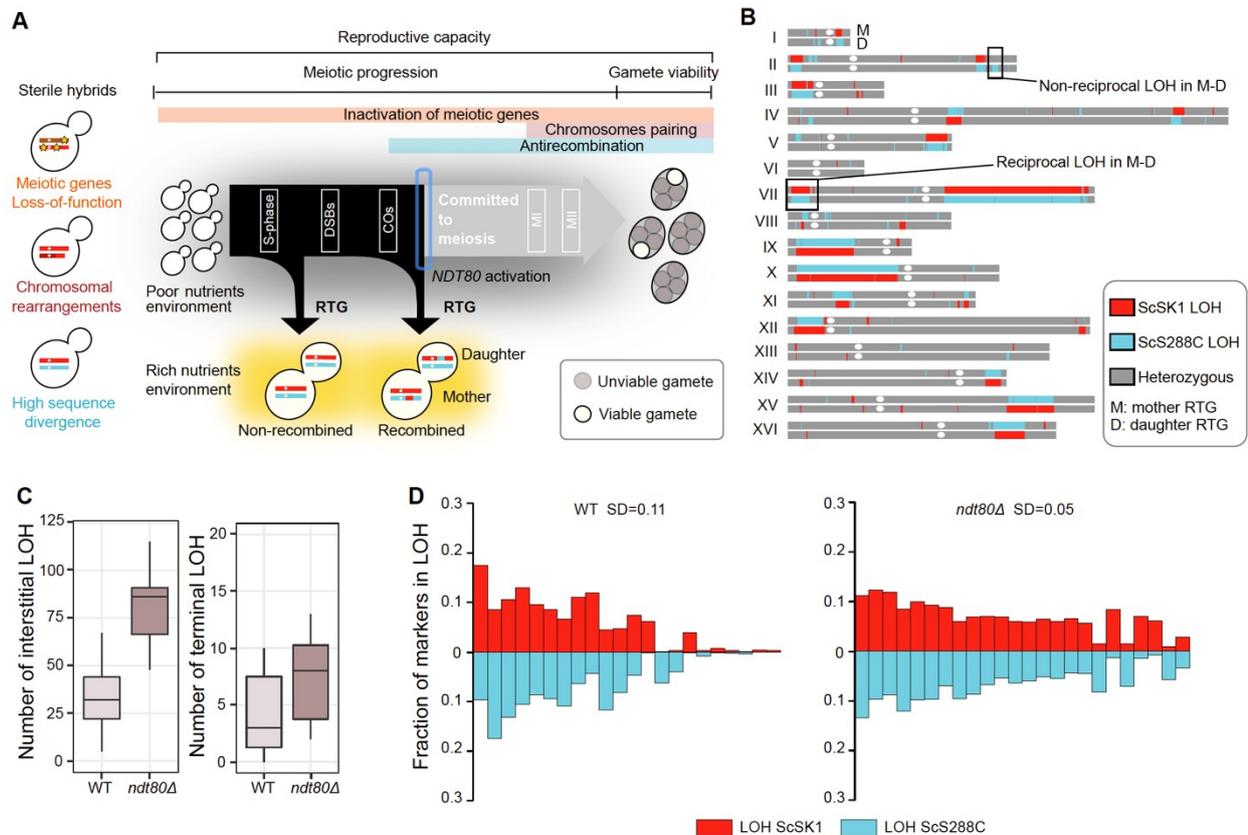


Figure 1. RTG paradigm and genomic landscape of *ndt80Δ* evolved RTG clones. (A) Inactivation of essential meiotic genes (yellow stars), genomic rearrangements and high levels of heterozygosity in hybrid genomes can impair meiotic progression or reduce gamete viability. RTG represents an alternative route to hybrid evolution that creates LOH blocks. (B) Example of LOH map in a mother (top)-daughter (bottom) RTG pair derived from the *ndt80Δ* hybrid. LOH blocks can be tracked by genotyping single-nucleotide marker in the two parental subgenomes, which give a homozygote readout if the position is in a LOH block. (C) Boxplots of the number of interstitial (left) and terminal LOH events (right) in the WT ($n=22$) and *ndt80Δ* ($n=24$) RTG clones derived from ScS288C/ScSK1. *ndt80Δ* RTGs have more interstitial (p -value= 3.9×10^{-8}) and terminal (p -value=0.001) LOHs. Both tests are one-tailed Wilcoxon rank-sum test, with continuity correction. We performed the same statistical test by taking into account only the mother cell in both datasets ($n=12$ *ndt80Δ*, $n=11$ WT) and confirmed that *ndt80Δ* mothers accumulated more interstitial (p -value= 5.033×10^{-8}) and terminal (p -value=0.0053) LOHs than the WT mothers. No LOH parental bias was detected by comparing the average number of events towards one of the two subgenomes in WT (Welch's t-Test, p -value = 0.67) and *ndt80Δ* datasets (Welch's t-Test, p -value = 0.25). (D) Fraction of homozygous markers for both parental alleles for the WT and *ndt80Δ* datasets. Each bar represents a sequenced clone. The *ndt80Δ* RTGs show a lower LOH variation compared to the wild-type, both if considered as a pooled dataset ($n=24$) (Fligner-Killeen test, p -value = 0.0008) and if considering only the mothers from each datasets (Fligner-Killeen test, p -value=0.02).

ScS288C/ScSK1 derived from 4-5 hours of sporulation induction (15). Short-read sequencing data were analysed by an integrated framework that enables a high-resolution view also of LOHs supported by only a single marker (22). The *ndt80Δ* clones had highly recombined diploid genomes (Fig. 1B) with an average of

88 LOHs per clone, when combining both terminal ($n=181$) and interstitial ($n=1933$) LOHs. The number of interstitial LOHs was higher in the *ndt80Δ* clones (**Fig. 1C, S1A**), which also had more markers lying in non-reciprocal recombined regions (p -value = 3.663^{-06} , Welch's t-test) (**Table S13**). Consistently, the *ndt80Δ* background accumulated more NCOs (23). In contrast, fewer large LOHs (> 10 kbp) were detected compared to the wild-type (**Fig. S1A, S2A**). Despite the LOH size variability, the two genetic backgrounds showed a similar median fraction of markers in LOH (**Table S6**), but the distribution was more homogeneous in the *ndt80Δ* population compared to the wild-type population (**Fig. 1D**), in line with cells accumulating at the same stage of meiosis by arresting at prophase I. The frequency of LOH events increased from the centromere towards chromosome ends in both datasets, in accordance with centromeres being cold meiotic recombination regions (**Fig. S1C**). Finally, we found no bias in LOH formation towards either of the two parental subgenomes in the datasets, consistent with RTG generating complementary recombined genomes with few non-reciprocal events (15) (**Fig. 1D**). Overall, we demonstrated that RTG allows a hybrid that is unable to complete meiosis due to the lack of a functional key meiotic gene to generate highly recombined diploid genomes.

Profiling RTG efficiency across hybrid diversity

Next, we asked to what extent RTG promotes recombination in hybrids with variable degrees of sterility due to heterozygosity and structural differences between the subgenomes. We exploited natural variation to generate a panel of 19 diploid genetic backgrounds, comprised by 4 fully homozygous *S. cerevisiae* (Sc), 7 *S. cerevisiae* intraspecies hybrids (Sc/Sc) and 8 interspecies hybrids between *S. cerevisiae* and its sister species *S. paradoxus* (Sp/Sc) (**Fig. 2A-B, Table S1 and S9**). Then, we engineered a simple genetic system to measure RTG-induced LOH rates at the *LYS2* locus on chromosome II (**Fig. 2C**). We replaced one of the *LYS2* alleles with a *URA3* gene and compared how often this *URA3* marker was lost due to LOH, in cells returned to growth after 6 hours of meiosis induction (T6) but before commitment to meiosis of the fastest sporulating strain (**Fig. S3A**), to control mitotic cells (T0). We derived the efficiency of RTG-induced LOH (T6/T0) (**Fig. 2D and Tables S4**) and observed pronounced genetic background effects with fast sporulating strains promoting efficient recombination upon RTG (**Fig. 2E, Tables S2**). Among the homozygous diploid, the ScNA/ScNA showed the largest LOH rate increase (21-fold) (**Fig. 2D**) consistent with its faster meiotic progression, while the 3 other homozygous diploids had a poor sporulation efficiency and synchrony (**Fig. S3B**), preventing the production of a significant number of recombinant RTGs. All the intraspecies hybrids with a ScNA subgenome also showed a significant increase of recombination upon RTG, ranging from 3 to 42-fold, whereas other intraspecies hybrids showed no significant increase (**Fig. 2D**). In particular, we detected a 23-fold increase in LOH

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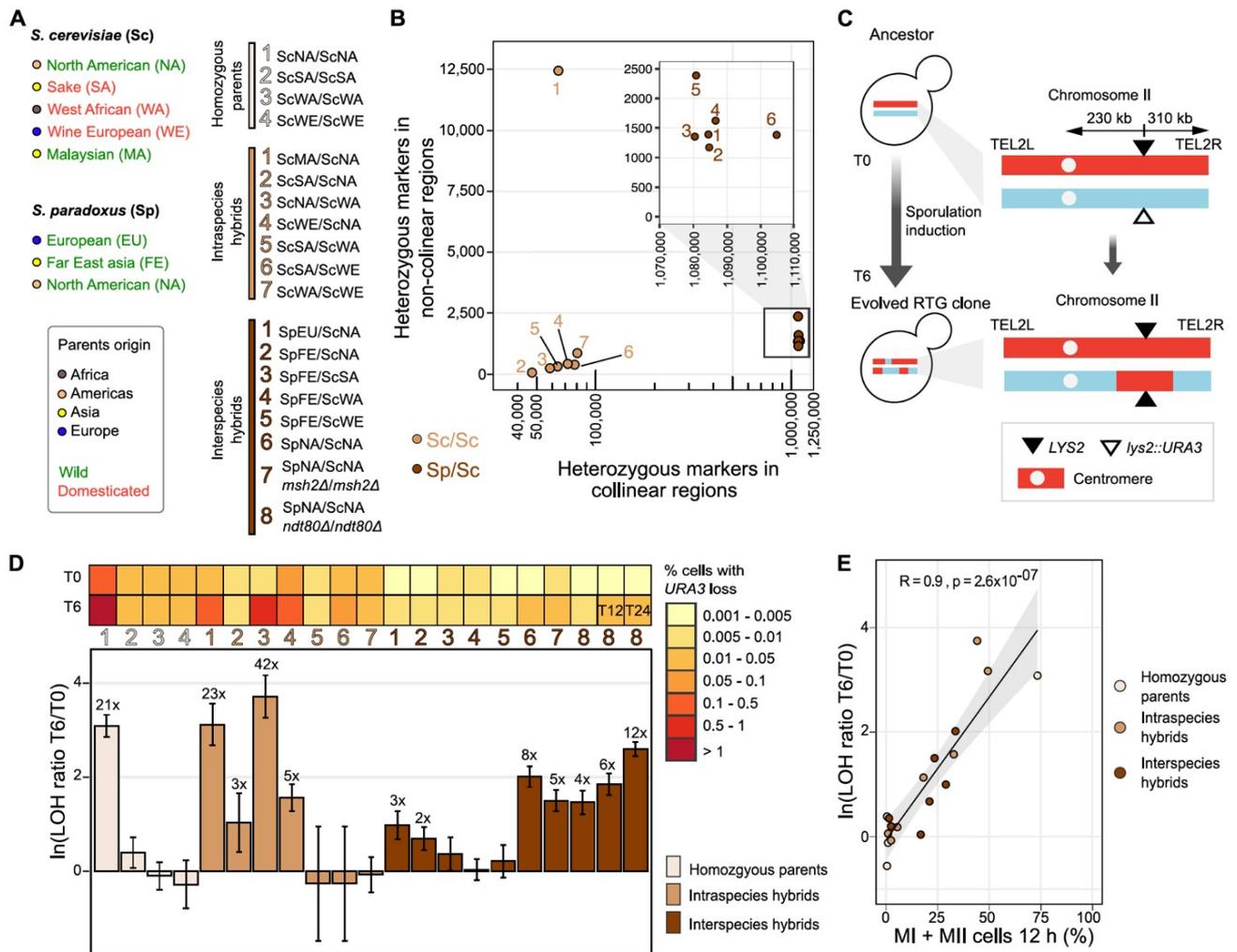


Figure 2. Quantifying RTG-induced recombination across hybrid diversity. (A) Geographical (coloured circle) and ecological (coloured name) origins of the parental strains (left) used for generating the homozygous diploids, intraspecies hybrids and interspecies hybrids (right). Diploids are grouped according to their level of heterozygosity as: homozygous parents (light brown), intraspecies hybrids (orange) and interspecies hybrids (dark brown) and the same colour code apply to panels B, E and E. (B) Level of heterozygosity across the hybrid panel with number of heterozygous markers detected in non-collinear and collinear regions. Each data point is labelled with a number/colour encoded according to the strains list on panel A. The four homozygous parents are not reported. (C) *URA3*-loss assay used for measuring RTG-induced recombination rates. (D) Top plot: percentage of cells growing on 5-FOA measured with the *URA3*-loss assay at two time points (or more, if specified): T0 = no meiosis induction, T6, T12 and T24 indicate 6, 12, and 24 hours of meiosis induction respectively. Each square is coloured accordingly to the average of the replicates as indicated in the right scale. The percentage of cells with *URA3*-loss was calculated as the ratio of colony forming units (CFU) CFU/mL in 5-FOA and CFU/mL in YPD. Bottom: bar plot showing the natural logarithm ratio of number of cells growing on 5-FOA at the RTG induction time (T6, or more) and control (T0). Error bars represent the standard deviation for each ratio. The number on the top of each bar plot indicates the linear fold increase (e.g. 24x) of the LOH rate, comparing T6 to T0 as specified above. (E) RTG efficiency as a function of meiotic progression after 12 hours measured as cells that passed first (MI) and second (MII) meiotic divisions. R is the Pearson's correlation coefficient. the fraction of cells engaged in RTG increasing with time (Fig. 2D).

upon RTG in the ScMA/ScNA hybrid, showing that the non-collinearity between several homologous chromosomes other than chromosome II, did not reduce RTG-induced LOH at the *LYS2* locus.

All the interspecies *S. cerevisiae/S. paradoxus* hybrids had a lower basal LOH rate during mitosis (10-fold average) and RTG enhanced the LOH rate less than in intraspecies hybrids (**Fig. 2D, Table S4**). Nevertheless, all three interspecies hybrids with a ScNA subgenome showed significant LOH rate increases (**Fig. 2D**). The lower LOH rates at both T0 and T6 suggest that very high heterozygosity strongly inhibits recombination in interspecies hybrids through an antirecombination mechanism, as it does during meiosis (24)(21). To test this conjecture, we deleted the gene *MSH2*, which encodes a key protein involved in the mismatch machinery, in both subgenomes of the SpNA/ScNA hybrid. Indeed, we observed increased LOH rates in both T0 (3.6-fold) and T6 (2-fold) samples compared to the wild-type SpNA/ScNA hybrid (**Fig. 2D, Table S4**), despite meiotic progression being slower in the *msh2Δ* mutant (**Fig. 2E and S3B**). This is consistent with the mismatch repair machinery acting to prevent RTG recombination in hybrids with highly diverged subgenomes. Finally, we generated a SpNA/ScNA *ndt80Δ* with both high heterozygosity and meiotic progression defects, and we measured LOH rates at 6 (T6), 12 (T12) and 24 (T24) hours after sporulation induction. We observed that RTG recombination increased with the time spent in gametogenesis, consistently with the fraction of cells engaged in RTG increasing with time (**Fig. 2D**).

Hence, genetically very similar cells in clonal populations are nevertheless quite heterogeneous in their meiotic progression, with only a minor fraction of cells having committed to recombination at 6 hours. Thus, the absence of RTG induced recombination in a hybrid may reflect that cells had not progressed sufficiently in their meiosis to engage in recombination and produce recombinant RTGs. We therefore focused our follow-up whole-genome-sequencing analyses on hybrids for which the RTG did increase recombination. Overall, our experimental system showed that RTG efficiency varies broadly across genetic backgrounds and is strongly influenced by their meiotic progression.

RTG recombination in hybrids with extensive chromosomal rearrangements

We performed whole-genome sequencing of RTG-evolved clones derived from the fertile ScWE/ScNA hybrid ($n=24$, T6) and the sterile ScMA/ScNA hybrid ($n=123$ T6, $n=2$ T4, $n=3$ T0), which have similar levels of heterozygosity but different genome structures (**Fig. 2B and Table S9**). The ScMA/ScNA hybrid represents sterility driven by chromosomal rearrangements, with 5 homologous chromosomes for which the parental subgenomes are not collinear over long stretches. Our analyses revealed that the RTG genomes of ScWE/ScNA and ScMA/ScNA clones recombined equally often (average number of LOH per clone=65 and 69, respectively; **Fig. 3A-B and Table S6**). This underscores that genome-wide RTG recombination is not hampered by extensive non-collinearity between subgenomes, in line with that the sterility caused missegregation of the non-collinear chromosomes rather than a lack of recombination (25). Moreover,

neither parental subgenome was favoured over the other in terms of the created homozygosity (**Fig. S2C**) consistent with no parental bias in the accumulation of Spo11p-induced DSBs, which occur at highly conserved sites across the *S. cerevisiae* and *S. paradoxus* lineages used here (26).

Both hybrids showed that the genomic intervals encompassing LOH breakpoints on the right arm of chromosome II were characterised by lower heterozygosity (**Fig. 3C, Fig. S4A-B**). Moreover, LOH breakpoints on the right arm of chromosome II were associated both with known meiotic hotspots (27) and sites where Spo11p induces DSBs during meiosis (28) (**Fig. 3D, Fig. S4C, Table S10**). Since this implies that RTG recombination relies on DSBs created during the aborted meiosis, we probed whether

LOH breakpoints induced by RTG recombination were associated with meiotic recombination hotspots genome-wide and found this to be the case (**Fig. S4C, Table S11**). We found that centromeres were always maintained in a heterozygous state, consistent with mitotic like segregation in RTG, and constituted RTG-induced recombination coldspots (**Fig. S1C**). We found no increase in single nucleotide variants (SNVs), aneuploidies or copy number variations (CNVs) and conclude that RTG-induced recombination does not cause global genome instability (**Fig. S5A**). This is consistent with the major role of the error-free homologous recombination pathway in repairing meiotic DSBs.

Four ScWE/ScNA and nine ScMA/ScNA RTG clones carried a much larger fraction of markers in LOH (15-34% and 14-35% respectively) than the median per RTG clone (1% and 1.7%, respectively) (**Fig. S2C**). We hypothesized that extensive LOH could homogenise subgenomes sufficiently to alleviate the sterility of the ScMA/ScNA hybrid. Thus, we probed the capacity of three ScMA/ScNA RTG clones with extensive LOH to produce viable gametes. Indeed, these clones had up to 3-fold increased gamete viability compared to the ancestral ScMA/ScNA hybrid (**Fig. S6A and Table S3**), confirming that RTG can help restore meiotic fertility by homogenising subgenomes and making them more collinear.

We also detected LOHs encompassing the *MAT* locus on chromosome III in 2 of the ScWE/ScNA and in 1 of the ScMA/ScNA extensively recombined clones (**Fig. S6B**). We found that although this *MAT* homozygosity led to complete sterility and prevented further RTG cycles, it enabled these hybrids to pass from a diploid mating deficient behaviour to a haploid-like mating proficient, thereby providing a direct route to polyploidization (**Fig. S6 C-D**). Overall, these results showed that sterile intraspecies hybrids can evolve through RTG and overcome reproductive barriers caused by a divergent chromosomal structure, with even a single RTG cycle having a dramatic impact on the genome evolution.

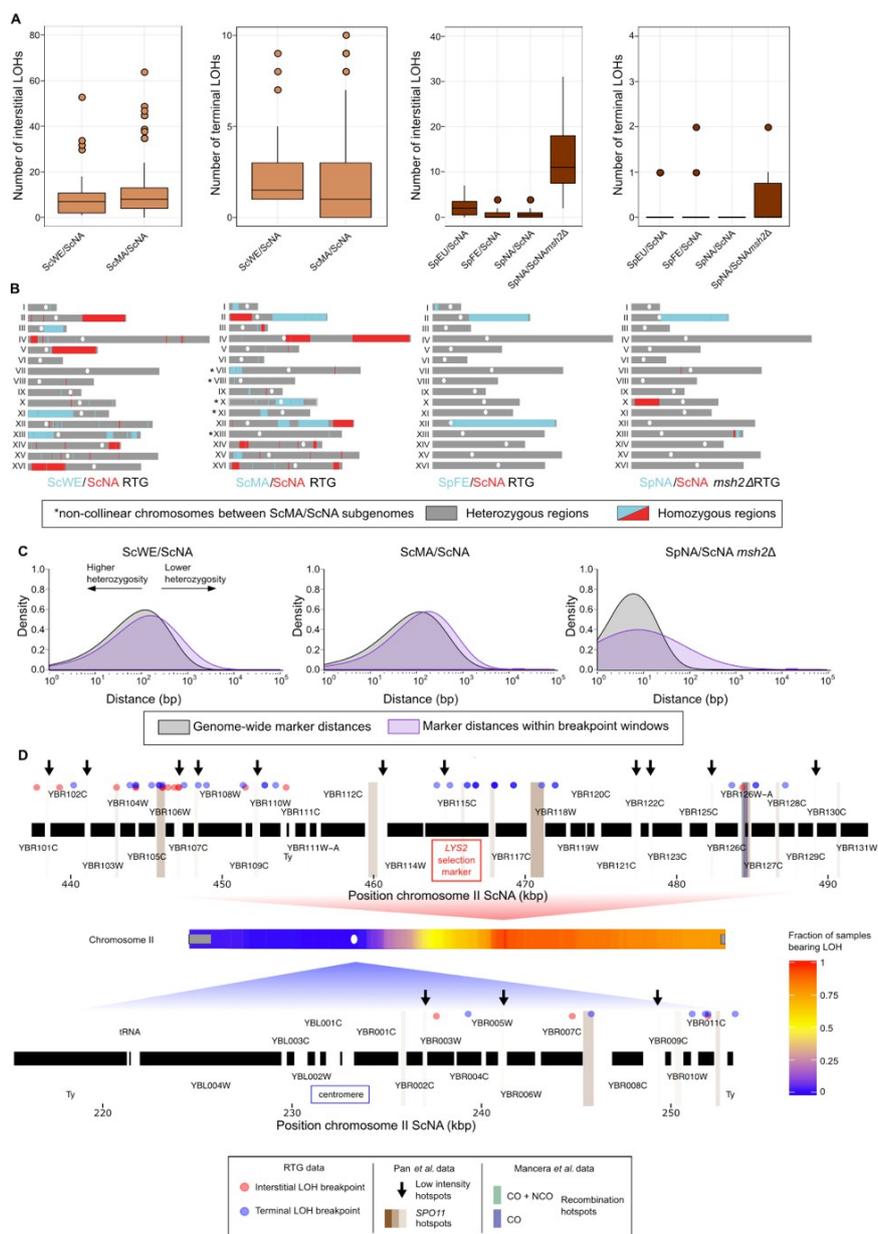


Figure 3. LOH landscape of hybrids evolved through RTG. (A) Left panels: boxplots of the number of interstitial and terminal LOHs in intraspecies hybrids. We detected no significant difference in the number of interstitial or terminal events comparing the ScWE/ScNA and the ScMA/ScNA datasets (two-sided Wilcoxon rank-sum test, p -value = 0.5 and p -value = 0.1, respectively). Right panels: boxplots of the number of interstitial and terminal LOHs in interspecies hybrids. Colour code as in figure 2A. (B) Genome-wide view of the LOH landscape for two RTG-evolved intraspecies hybrids (left) and two evolved interspecies hybrids (right). All plots are based on the ScNA reference genome. (C) Distribution of marker distances genome-wide (grey) and in LOH breakpoint windows (purple) across three different hybrids. LOH breakpoint windows comprise the 5 heterozygous markers and the 5 homozygous markers closer to the breakpoint. (D) Association between meiotic recombination and LOH breakpoints within two regions of

chromosome II. Low-intensity Spo11p hotspots are highlighted with black arrows. The heatmap of the LOHs (i.e. two copies of ScNA alleles) resulting from the breakpoints is also reported. Grey boxes represent subtelomeric regions.

Local homozygosity enables RTG recombination in interspecies hybrids

To shed light on how extremely high heterozygosity shapes the RTG recombination landscape, we sequenced the genomes of evolved clones ($n=53$ T6, $n=5$ T12, $n=4$ T24) and non-evolved clones ($n=28$ T0) derived from three interspecies *S. paradoxus/S. cerevisiae* hybrids (**Table S9**). Genome-wide RTG recombination was less efficient in the interspecies hybrids compared to intraspecies hybrids, generating fewer and smaller LOH (**Fig. 3A-B, Fig S2, Table S6**). Nevertheless, 22 RTG isolates out of 34 had at least one additional recombination event, beside the one selected on chromosome II, and one evolved clone, derived from the SpFE/ScNA hybrid, carried LOH on 3 different chromosomes and spanning 9% of its genome (**Fig. 3B**). This suggested that highly recombined RTG clones do arise in interspecies hybrid populations, but at very low frequencies. To test this possibility, we constructed a SpNA/ScNA hybrid carrying a second unlinked selectable heterozygous marker (*CANI*), which enabled us to screen for additional, independent LOH events. Genome sequencing of 4 RTG-clones (T6) selected for loss of *URA3* and *CANI* revealed clones with multiple LOHs, supporting the scenario that RTG can reshuffle also highly heterozygotic genomes but does so only rarely (**Fig. S7**).

We probed the role of the mismatch repair machinery in suppressing RTG recombination by sequencing SpNA/ScNA evolved and control clones lacking *MSH2* and found ~1000-fold increase in the median fraction of genome in LOH (2.10×10^{-4} vs 1.58×10^{-7} in the wild-type). Indeed, LOH formation is hampered by heterozygosity and the inactivation of the mismatch-repair system can mitigate this effect. LOH breakpoints coincided with low local heterozygosity, underscoring that islands of local sequence homology facilitate RTG recombination also in highly heterozygotic hybrids (**Fig. 3C, Fig. S4, Table S7**). Although the scarcity of such islands in highly heterozygous hybrids reduced RTG recombination, the impact of even a single RTG cycle on LOH formation was truly massive compared to the conventional vegetative growth, both in interspecies and intraspecies hybrids (**Fig. S5B**). Interspecies RTG clones were characterised by remarkably stable genomes (**Fig. S5A**), with the only exception of a clone that carried an inversion underlying a complex CNV (**Fig. S7C**). Overall, our results showed that RTG recombination in interspecies hybrids is strongly influenced by heterozygosity, and that inactivation of the mismatch repair machinery mitigates the effect of high sequence divergence.

Mapping quantitative traits in a sterile hybrid

We probed whether recombination induced by return-to-growth can generate beneficial allelic combinations by measuring the fitness (population doubling time and yield) of 125 ScMA/ScNA recombined RTG clones and 3 T0 samples as controls across 82 environments (**Table S5**). The fitness of a subset of RTG clones was often inferior to that of the non-evolved hybrid across a wide range of environments. Nevertheless, all RTG recombinants were fitter than their parent in at least some niches and a few were broadly superior, showing that return-to-growth can generate new beneficial allelic combinations capable of driving local adaptation (**Fig. 4A and Fig. S8**).

between non-collinear segments of the North American chromosome VII and the Malaysian chromosome VIII (**Fig. 4B, 4C, Fig. S8**). Recombinants missing the right arm of Malaysian chromosome VIII ($n=7$) grew slower in all the 13 environments, while those missing the corresponding North American ($n=2$) chromosome arm grew faster or equally fast as non-recombinant samples (**Fig. S8**).

Next, we asked whether QTL mapping by RTG can be sufficiently resolved to generate a biological understanding of the genetic variants underlying the traits of sterile hybrids. First, we focused on an arsenic resistance QTL located just before the subtelomere of the right arm of chromosome XVI (**Fig. 4B and Fig. S9**), a region that harbours the *ARR* locus, a gene cluster controlling arsenic exclusion from the cell. This locus is absent in the ScMA subgenome (*11*) and recombinants not inheriting the *ARR* locus were highly sensitive to arsenic (**Fig. S9**). Then, we explored a major QTL on chromosome XV associated to fitness in presence of the antifungal drug cycloheximide and found near its peak the transcription factor *YRR1* that mediates drug resistance. Since the Malaysian *YRR1* contains two aminoacid substitutions predicted to be deleterious (**Fig. 4D, Table S8**), we tested whether *YRR1* drives variation in cycloheximide resistance by reciprocal hemizyosity. We found hemizygous strains carrying the Malaysian *YRR1* to be less fit than those carrying the North American allele (**Fig. 4E**), demonstrating that RTG can generate a genetic understanding of the traits of sterile hybrids.

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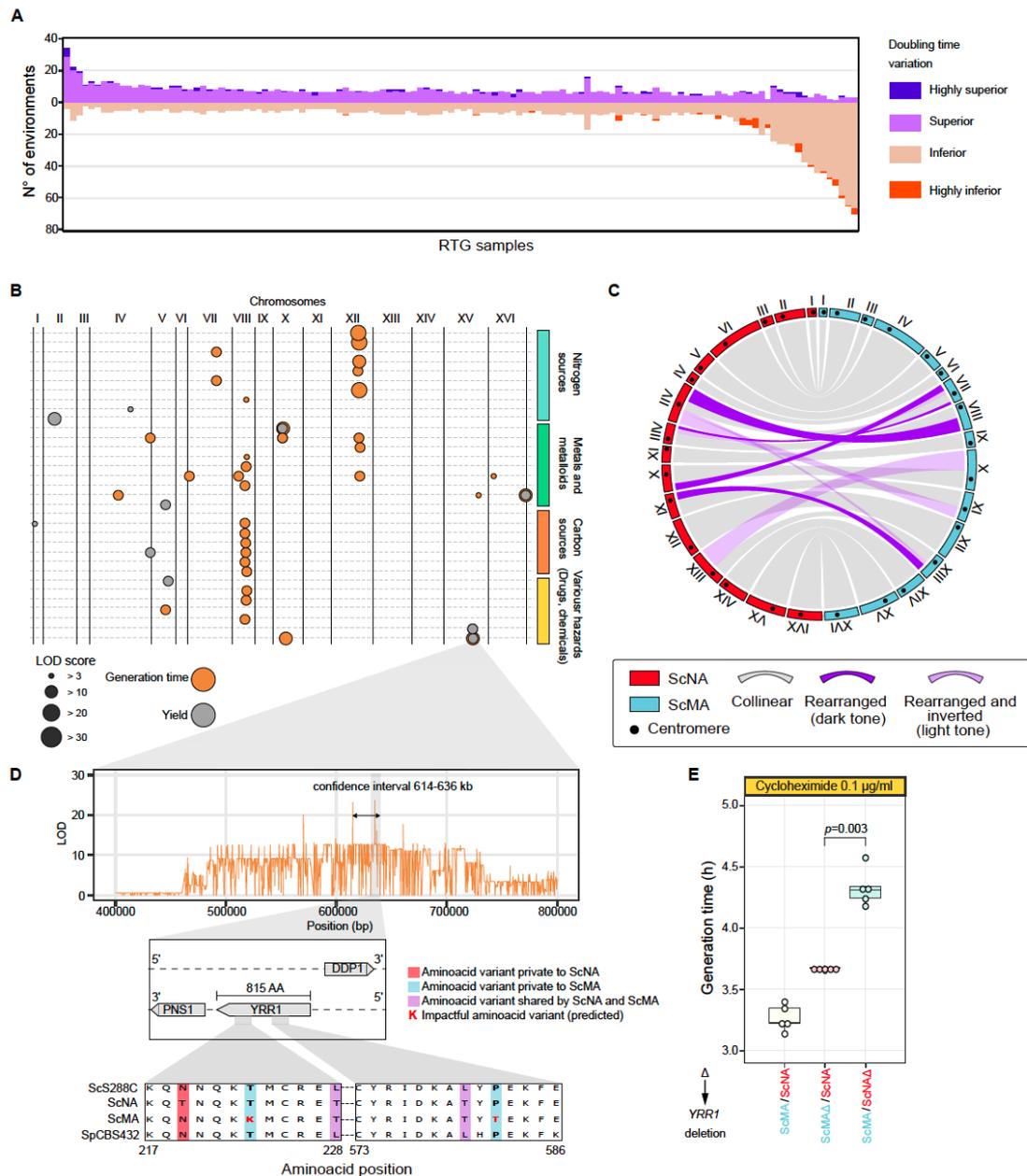


Figure 4. Fitness diversification of RTG clones and QTL mapping. (A) Extent of fitness variation upon RTG in the ScMA/ScNA dataset. y-axis shows the number of environments in which the cell doubling time of the RTG clone is superior or inferior to that of the ScMA/ScNA parent hybrid. (B) QTLs mapped across the environments. Only conditions where QTLs were detected are reported. (C) Circular plot representing the rearrangements between the two subgenomes of the ScMA/ScNA hybrid. Regions are considered inverted if they do not resemble the ancestral centromere-telomere orientation. (D) Linkage scan for growth in media containing cycloheximide. Zoom-in on chromosome XV QTL and the two highly conserved regions of *YRR1* with two aminoacids substitutions predicted to be deleterious. (E) Boxplot of doubling times in presence of cycloheximide for the parental ScMA/ScNA strains (5 replicates) and the two

hemizygous strains (5 replicates). The deletion of either allele reduces growth in cycloheximide compared to the WT, underlying *YRR1* haploinsufficiency in this background. The deletion of the ScNA allele is significantly worse (one-tailed Wilcoxon rank-sum test p -value = 0.003) than the ScMA.

Discussion

We evolved 20 diploid yeast genetic backgrounds with varying levels of sterility through RTG, challenging the dogma that sterile hybrids are evolutionary dead ends. We showed that aborting meiosis after its initiation and returning cells to mitotic growth allowed sterile hybrids to overcome reproductive barriers due to mutational defects in late meiosis, structural differences between subgenomes and extreme levels of heterozygosity, and to produce viable recombinant fit clones. These mechanisms constitute some of the post-zygotic reproductive barriers that most commonly underlie speciation in nature, in yeast and other species (3). The capacity of one RTG process to drive recombination was substantially lower in interspecies hybrids due to their high heterozygosity and we showed that this barrier to RTG recombination originated in the mismatch repair system. However, abolishing the mismatch repair by removing the mismatch binding protein Msh2 promoted RTG recombination mirroring the increased meiotic recombination observed in gametes of a similar interspecies hybrid (29).

The LOH regions generated by recombination between hybrid subgenomes can have profound evolutionary consequences. We have previously shown that the homozygous blocks produced by LOH can mediate meiotic recombination between highly diverged subgenomes, which in turn can rescue hybrid fertility and initiate interspecies introgressions (30). Here, we showed that RTG-induced LOHs can make the mating type locus homozygotic, and thereby give rise to mating proficient diploid hybrids. This provides an obvious direct route to polyploidization, and such polyploidization can restore the fertility of sterile hybrids by whole genome duplication (31, 32). This RTG driven polyploidization is similar to what is observed in plants, where polyploidization can result from the mating of endoreplicated gametes with an unreduced genome content (33), and may help to explain the abundance of yeast polyploids in nature (34). LOH regions might be selected by adaptation under specific selective regimes (35-38) but may also be constrained by incompatibility between allele pairs located in different subgenomes (39). Furthermore, the maintenance of a stable diploid state in RTG clones might promote unique evolutionary trajectories, such as the ability to tolerate large CNVs that would otherwise kill haploid gametes. In concert, the impact of RTG recombination on yeast natural evolution has the potential to be quite substantial, and this is underscored by that is arguably quite common, being induced simply by a brief starvation that is followed by cells encountering again nutrients, which must be a occurrence in fluctuating wild habitats (40). . Whether the role of RTG recombination also extends across broader swaths of the tree of life is unknown, but yeasts separated by hundreds of millions years of evolution from *S. cerevisiae* experienced LOH with a pattern compatible with the RTG signature without a specific tailored protocol (41, 42). We see no evident mechanistic reasons for why the RTG process would not extend also across more distantly related sexual organisms, in which meiosis is induced by starvation.

Deviations from the paradigm of sexual reproduction have been repeatedly reported across the eukaryotic tree of life. A meiotic-gene mutant that skips the second meiotic division and produces gametes with unreduced genomic content and complementary recombined genomes was reported in *Arabidopsis thaliana* (43). Furthermore, other organisms such as *Candida albicans* (44) or the rotifer *Adineta vaga* (45) experience genetic recombination without a conventional sexual cycle. The recent characterization of the lifecycle of more than 1000 *S. cerevisiae* strains revealed an independent loss of sexual reproduction in several lineages (7).

Population genomics data revealed pervasive genome-wide signatures of historical LOHs (46) suggesting that RTG might have contributed to the genome evolution of sterile strains. The access to this non-conventional sexual cycle provides a powerful alternative path for genome evolution that breaks the paradigm of sterile hybrids as evolutionary dead ends.

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Supplementary Materials

Materials and Methods

Construction of hybrid yeast strains

All the *Saccharomyces cerevisiae* (Sc) and *Saccharomyces paradoxus* (Sp) strains constructed and used in this study are reported in **Supplementary Table 1**. The diploid hybrid ScS288C/ScSK1 *ndt80Δ* was generated by mating the heterothallic (*ho*) ScS288C and ScSK1 commonly used laboratory strains deleted for *NDT80* (*ndt80::KanMX*) and diploid complementing for histidine auxotrophy were selected. The haploid parental heterothallic (*ho::HygMX*) strains ScNA, ScWA, ScWE, ScSA, ScMA used for generating the intraspecies and interspecies hybrids were previously described (1, 2). In each Sc *MATa* and *MATα* haploid background, the native *URA3* on chromosome V was deleted (*ura3::KanMX*). Subsequently, the *URA3* gene was inserted at the *LYS2* locus in the *MATα* haploid background (*MATα, ura3::KanMX, lys2::URA3*). For generating the intraspecies hybrids, *MATa* cells and *MATα* cells of the two Sc parental species were mated and prototroph diploids (*MATa/MATα, ura3::KanMX/ura3::KanMX, LYS2/lys2::URA3*) were selected on minimal media lacking both lysine and uracil. The haploid parental heterothallic (*ho::HygMX*) strains SpEU, SpFE, SpNA were generated for this work following the genetic engineering scheme explained above for *MATa* and *MATα* strains. For generating the interspecies hybrids, *MATα* cells and *MATa* cells from the Sc and Sp parental species were mated and prototroph diploids (*MATa/MATα, ura3::KanMX/ura3::KanMX, LYS2/lys2::URA3*) were selected on minimal media lacking both lysine and uracil. The ScNA/SpNA *ndt80* and *msh2* diploid mutants were generated by deleting each gene in the respective Sc or Sp haploid background (*ndt80::NatMX* or *msh2::NatMX*) using the lithium acetate/PEG transformation protocol. The haploid strains were mated and diploid prototrophs (*MATa/MATα, ura3::KanMX/ura3::KanMX, LYS2/lys2::URA3, msh2::NatMX/msh2::NatMX*) or (*MATa/MATα, ura3::KanMX/ura3::KanMX, LYS2/lys2::URA3, ndt80::NatMX/ndt80::NatMX*) were selected on minimal media lacking both lysine and uracil. The *CAN1* deletion in the SpNA background was obtained using the lithium acetate/PEG transformation protocol and the diploid hybrid was obtained by mating with ScNA (*MATa/MATα, ura3::KanMX/ura3::KanMX, LYS2/lys2::URA3, can1::NatMX/CAN1*). All the deletions made were verified by polymerase chain reaction (PCR). All the PCR primers used in this study are reported in **Supplementary Table 12**.

Selection of clones before and after RTG

Each hybrid was patched from the -80 °C glycerol stock on YPD solid media (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar) and incubated overnight at 30 °C. The following day the strain was streaked to minimal solid media not supplemented with uracil and the plate was incubated at 30 °C for 48 hours. Five single colonies of the hybrid strain were taken and inoculated separately in 10 mL of YPEG pre-sporulation medium (1% yeast extract, 2% peptone, 3% ethanol, and 3% glycerol) for 15 hours at 30 °C with shaking at 220 rpm. Each pre-sporulation culture was washed twice with sterile water and resuspended in 2% potassium acetate to reach an $OD_{600} = 0.5$ in a 250-mL flask. One mL was immediately collected from the starving culture to generate the T0 sample (before meiosis). The flasks were incubated at 23 °C, with shaking at 220 rpm, and after 6 hours of incubation the T6 sample were taken. The T0 and T6 samples were washed twice with 1 mL of YPD and incubated in 1 mL of nutrient rich YPD for 18 hours at 30 °C without shaking, thereby aborting meiosis in the T6 samples and returning cells to growth (RTG). The following day the YPD liquid cultures were vortexed and, depending on the strain and the density of the culture, 20 to 200 μ L of 10-fold diluted culture were plated on minimal medium containing 1 mg/mL of 5-fluoroorotic acid (5-FOA) and spread with glass beads (3). In parallel, cells from the same YPD liquid culture were serially diluted up to 10^{-5} and spotted on YPD plates in at least two replicates for each biological replicate. The YPD and 5-FOA plates were then incubated at 30 °C for 48 hours. After that, colonies growing on 5-FOA (and therefore having lost their *URA3* marker through loss-of-heterozygosity) and YPD plates were counted and used to infer CFU/mL in each condition. RTG clones from the S288C/SK1 hybrids were obtained using the mother-daughter protocol as previously described (4).

Meiosis dynamics and spore viability

The meiosis of diploid hybrids engineered with the *LYS2/lys2::URA3* system was induced with the same protocol used for RTG but the flasks were kept at 23 °C with shaking at 220 rpm and monitored for the formation of viable spores (germinated gametes). In order to analyse the sporulation efficiency (fraction of cells having passed through meiosis), at least 200 cells were counted for each sample and estimated the percentage of those that had sporulated (formed dyads, triads or tetrads of spores). To estimate spore viability (gametes that are viable and capable of germinating), complete tetrads were dissected and deposited spores on YPD plates where they were allowed to germinate and grow for four days at 30 °C. The spore viability was calculated as the fraction of spores that had germinated and formed visible colonies. To monitor meiotic progression by DAPI staining 2 mL of T0, T6 and T12 culture were collected. Each of the 2 mL samples were put in a separate tube with 5 mL of EtOH 70% and stored at -20 °C. Frozen samples were washed twice with 1 mL of sterile water, resuspended in 200 μ L of water and stained with 2 μ L of DAPI

(4',6-diamidino-2-phenylindole) for 30 minutes in the dark. Cells were counted using a fluorescence microscope.

RTG selection by *URA3*-loss assay and *URA3/CANI*-loss assay

We designed the 5-FOA assay to detect the increase of recombination upon RTG at the heteroallelic locus *LYS2/URA3* on chromosome II. We deleted the copy of *URA3* from its native location on chromosome V in all the haploid parental strains. We replaced one *LYS2* allele on chromosome II with one copy of *URA3* in one of the two parents used to generate each diploid hybrid, and performed the assay as already reported in the literature (5). The growth in YPD for 18 hours ensured that all the cells that were in the early phase of meiosis performed RTG. We used the same incubation time in liquid YPD for the sporulating cultures (T6) and the controls (T0) to take into account LOH occurring during the growth in YPD. We confirmed by whole-genome-sequencing of single clones that *de novo* mutations and aneuploidies were not impacting the heteroallelic assay, and all events that lead to *URA3* loss were LOHs. Therefore, we concluded that T0 cells growing on 5-FOA were due to mitotic LOH whereas T6 cells had a composite effect of mitotic and RTG-induced recombination. Sequencing of single clones supported this scenario, and T6 RTGs had a single LOH event on chromosome II (mitotic recombination or low efficiency RTG), or additional LOHs (RTG) in the genome. This difference must be carefully considered also for interspecies hybrids, although in those backgrounds the lack of additional LOH events can result from anti-recombination mechanism due to the high sequence divergence. We expanded this protocol to introduce an additional selection step as already reported in the work by Coelho *et al.* in which multiple selection steps were used (6) and also based on an early work in which RTG cells had recombination at unlinked genetic markers (7)

. We introduced an additional selection marker by deleting one copy of *CANI* on chromosome V in one of the two parents of the hybrid tested, so that two independent LOHs could be selected for, one on chromosome II, at the *LYS2/URA3* locus, and one on chromosome V, at the *CANI/CANI::NatMX* locus. Briefly, after 18 hours of YPD incubation RTG cells were diluted 1:10 to an OD of ~0.5 in SD media supplemented with canavanine and grown for 10 hours. Finally, cells were plated on 5-FOA plates following the protocol described in the paragraph “Selection of clones before and after RTG” in the Materials and Methods section. The canavanine liquid incubation enriches for cells bearing LOH at the *CANI* locus and the passage on 5-FOA plates selects for cells carrying LOH at the *LYS2/URA3* locus.

Sequencing data analysis

All the samples were sequenced with Illumina paired-end technology at the NGS platform of Institut Curie according to the manufacturer's standard protocols. Short-read sequencing data were processed by means of the MuLoYDH pipeline using default parameters and the assemblies/annotations embedded in MuLoYDH (5). All the data-sets consisted of the hybrids evolved under the RTG protocol and analysed against the corresponding parent hybrid before RTG as control samples (**Supplementary Table 9**). The pipeline required as input: (1) a data set of short-read sequencing experiments from yeast diploid hybrids, and (2) the two parental genomes which were used to produce the hybrids in FASTA format as well as the corresponding genome feature annotations in the "general feature format" (GFF). The availability of reference-quality genome assemblies for all the parents used to generate the panel of hybrids, allowed a highly accurate tracking of the mutational landscape. Reads from sequencing data were mapped against the assemblies of the two parental genomes separately (standard mappings) and against the union of the two aforementioned assemblies (namely a multi-FASTA obtained by concatenating the two original assemblies) to produce the competitive mappings. In the latter case, reads from parent 1 were expected to map to the assembly of parent 1 on the basis of the presence of single-nucleotide markers. Conversely, reads from parent 2 were expected to map to the assembly of parent 2.

Standard mappings were used to determine the presence of CNVs. The latter were also exploited to discriminate LOHs due to recombination from those resulting by deletion of one parental allele. The markers between the parental assemblies were determined by the NUCmer algorithm (v 4.0.0 beta) and were exploited to map LOH segments. Markers were genotyped from standard mappings. Marker positions characterized by nonmatching genotype or alternate allele were filtered out, as well as multiallelic sites, whereas those lying in subtelomeric and telomeric regions were masked. Remarkably, MuLoYDH provided LOH calls without filtering out small events using an arbitrary threshold based on the number of supporting markers. Indeed, we are able to detect events supported by a single marker and we previously demonstrated that such events are genuine LOH (5). Stretches of consecutive markers showing homozygous genotypes were grouped in LOH regions. The genomic coordinates of each LOH event were determined using both the "first/last" coordinates and the "start/end" coordinates. First/last coordinates were determined using the coordinates of the first and the last markers of the event. Start/end coordinates were calculated using the average coordinate of the first (last) marker and the last (first) marker of the adjacent event. LOH regions were annotated as terminal/interstitial. Interstitial LOHs were defined as homozygous segments flanked on both sides by heterozygous markers. Terminal LOHs were defined as homozygous regions extended to the end of the chromosomal arm, i.e. the last non-masked marker.

Due to low coverage that did not match the standard requested by MuLo, we excluded two WT RTG derived from ScS288C/ScSK1. We also removed one sample that went through two cycle of RTG in order to have only samples that performed one RTG cycle in WT and *ndt80Δ*.

De novo single-nucleotide variants and indels were determined from both competitive and standard mappings. Competitive mapping allowed for direct variant phasing in heterozygous regions. Variant calling from competitive mapping was performed setting ploidy = 1 in heterozygous regions and ploidy = 2 in LOH blocks. Regions characterized by reads with low mapping quality (MAPQ < 5 in the competitive mapping) were assessed from standard mapping using arbitrarily the assembly from parent 1. All the *de novo* variants detected were checked by visual inspection using IGV (8).

Testing for association between LOH breakpoints and Spo11p-induced DNA double-strand breaks (DSB) or recombination hotspots

The association between LOH breakpoints and DSB/recombination hotspots was tested using the *regioneR* package by means of the function *overlapPermTest*, setting the parameters: *ntimes* = 10000, and *alternative* = “greater”. The *fasta* suite (9) was used to convert the coordinates of the hotspots regions detected in the original reference genomes (SGD_R62-1-1_20090218 and SGD_R58-1-1_20080305 for the hotspots reported by Pan *et al.* (10) and Mancera *et al.* (11) respectively) to the corresponding coordinates of the hybrid subgenome engineered with the *lys2::URA3* selection system.

Genome content analysis of polyploid strains

The DNA content of the mating proficient diploid RTGs was analysed upon mating with tester strains using a propidium iodide (PI) staining assay. Cells were first pulled out from glycerol stocks on YPD solid media and incubated overnight at 30 °C. The following day a small portion of each patch was taken with a pipette tip, transferred in 1 mL of liquid YPD and incubated overnight at 30 °C. Then, cells were washed with water, resuspended in 1 mL of cold 70% ethanol and fixed overnight at 4 °C. Finally, the samples were washed twice with phosphate-buffered saline (PBS), and 100 μL of each sample was resuspended in 900 μL of staining solution (15 μM PI, 100 μg/mL RNase A, 0.1% v/v Triton-X, in PBS) and incubated for 3 hours at 37 °C in the dark. Ten thousand cells for each sample were analysed on a FACS-Calibur flow cytometer. Cells were excited at 488 nm and fluorescence was collected with a FL2-A filter. The data were analysed using the R packages *flowCore* (12) and *flowViz* (13), and were plotted with *ggplot2* (R version 3.6.1).

Long reads sequencing and structural variant analysis

Yeast cells were grown overnight in liquid YPD media. Genomic DNA was extracted using Qiagen Genomic-Tips 100/G according to the manufacturer's instructions. The MINION sequencing library was prepared using the SQK-LSK109 sequencing kit according to the manufacturer's protocol. The library was loaded onto a FLO-MIN106 flow cell and sequencing was run for 72 hours. Long read basecalling and scaffolding were performed using the pipeline LRSDAY (v 1.6) (14) and the dotplot of chromosome III was generated using mummerplot (15). The inversion on chromosome III was detected using sniffles (16) implemented within the Varathon framework (<https://github.com/yjx1217/Varathon>).

Estimating growth during mitotic reproduction

All yeast strains were stored at -80 °C in 20% glycerol and cultivated at 30 °C in temperature and humidity-controlled cabinets. Yeast strains were revived from frozen 96-well stocks by robotic transfer (Singer RoToR; long pins) of a random sub-sample of each thawed population (~50,000 cells) to a Singer PlusPlate in 1536 array format on solid Synthetic Complete (SDC) medium composed of 0.14% Yeast Nitrogen Base (CYN2210, ForMedium), 0.50% (NH₄)₂SO₄, 0.077% Complete Supplement Mixture (CSM; DCS0019, ForMedium), 2.0% (w/w) glucose, pH buffered to 5.80 with 1.0% (w/v) succinic acid and 0.6% (w/v) NaOH. In every fourth position, fixed spatial controls (genotype: YPS128, *MATa/MATa*) were introduced to account for spatial variation across plates in the subsequent experimental stage. Controls were similarly sub-sampled from a separate 96-well plate and introduced to the pre-culture array (Singer RoTor; long pins). Populations were pre-cultivated for 72 hours at 30 °C. For pre-cultures of nitrogen-limited environments, the background medium was modified to avoid nitrogen storing and later growth on stored nitrogen: CSM was replaced by 20 mg/L uracil (not converted into usable nitrogen metabolites) and (NH₄)₂SO₄ was reduced to growth-limiting concentrations (30 mg/L of nitrogen). We cast all solid plates 24 hours prior to use, on a levelled surface, by adding 50 mL of medium in the same upper right corner of the plate. We removed excess liquid by drying plates in a laminar air-flow in a sterile environment. Pre-cultured populations were mitotically expanded until stationary phase (2 million cells; 72 hours), were again subsampled (~50,000 cells; short pins) and transferred to experimental plates, containing the medium of interest (**Supplementary Table 5**). Synthetic grape must (SGM) was prepared as previously described. We tracked population size expansion using the Scan-o-Matic system, version 1.5.7 (<https://github.com/Scan-o-Matic/scanomatic>) (17). Plates were maintained undisturbed and without lids for the duration of the experiment (72 hours) in high-quality desktop scanners (Epson Perfection V800 PHOTO scanners, Epson Corporation, UK) standing inside dark, humid and thermostatic cabinets with intense air circulation. Images were analysed and phenotypes were extracted and normalized against the fourth position controls using Scan-o-Matic. We extracted the normalized, relative population size doubling time, D_r , by subtracting the control value for that position, $D_r = \log_2(D) -$

$\log_2(D_{\text{control,local}})$. D_r is reported as output data. Growth phenotypes were qualitatively classified as inferior, when D_r normalized to the initial hybrid was between 0.25 and 1, highly inferior when D_r was greater than or equal to 1, superior when D_r was between -0.25 and -1 and highly superior when the D_r was less than or equal to -1.

QTLs mapping and phenotype analysis

QTL analysis was performed with the package R/qtl (v1.46-2) (18) using mitotic growth data normalized with the growth data of the parental hybrid for 125 RTG clones and 3 T0 samples of the ScNA/ScMA hybrid as the phenotype variable and the genotype of heterozygous markers extracted from the sequence data of the same RTG clones as the genotype variable. We removed markers from the dataset to which the pipeline did not assigned any genotype with the function “drop.nullmarkers”, and we also removed markers for which only one sample was found to be homozygous. We estimated a genome-wide LOD statistical threshold performing 1000 random permutations of both the phenotype and genotype rows and extracting the 95th percentile of LOD values as threshold. The confidence interval of the QTL was calculated using the “lodint” function of R/qtl (18).

Reciprocal hemizyosity assay and functional mutation prediction

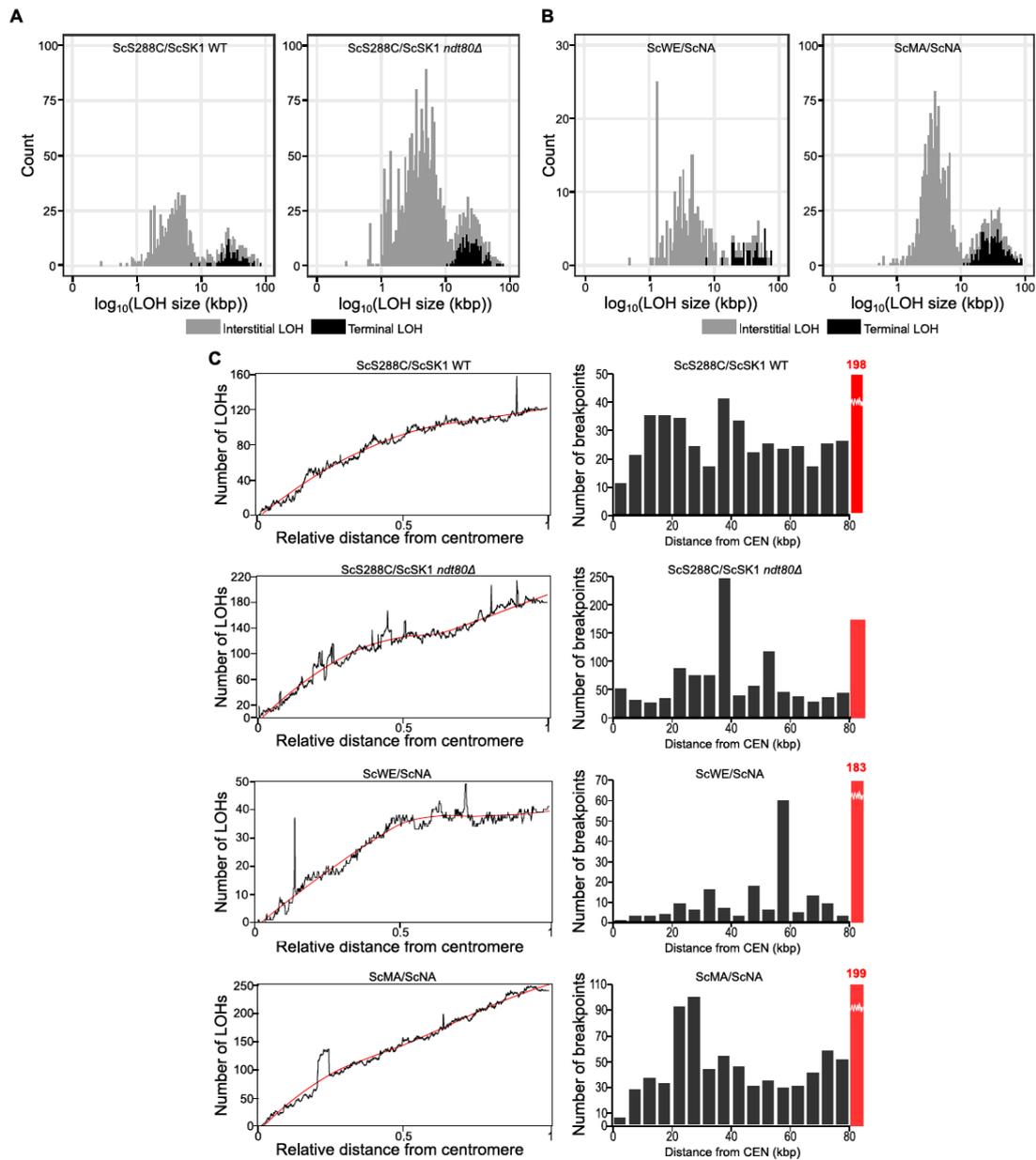
Start to stop *YRR1* gene deletions was engineered in the parental haploid strains CC407 (ScNA) and YGL1027 (ScMA) by replacing the open reading frame with the NatMX cassette. The haploid strain CC407 *yrr1::NatMX* was then crossed with a wild-type haploid YGL1027 to obtain a hemizygous diploid carrying only the MA *YRR1* allele, and the haploid YGL1027 *yrr1::NatMX* strain was crossed with a wild-type haploid CC407 to obtain a hemizygous diploid carrying only the NA *YRR1* allele.

Cells from the wild-type ScMA/ScNA hybrid and the two hybrids reciprocally hemizygous for *YRR1* were pre-grown in a 96 well-plate containing YPD for 16 hours in 5 biological replicates for each strain background. The following day 20 μL of cultures were taken from each well and transferred to another 96 well-plate containing 180 μL of YPD with cycloheximide at a concentration of 0.1 $\mu\text{g}/\text{mL}$. The growth was monitored by measuring OD changes over 72 hours on a Tecan plate reader (infinite F-200 pro). The generation time was extracted from the OD profile using the software PRECOG (19) and then plotted and analysed in Rstudio. The prediction of deleterious variants was obtained from the mutfunc suite (20).

Supplementary references

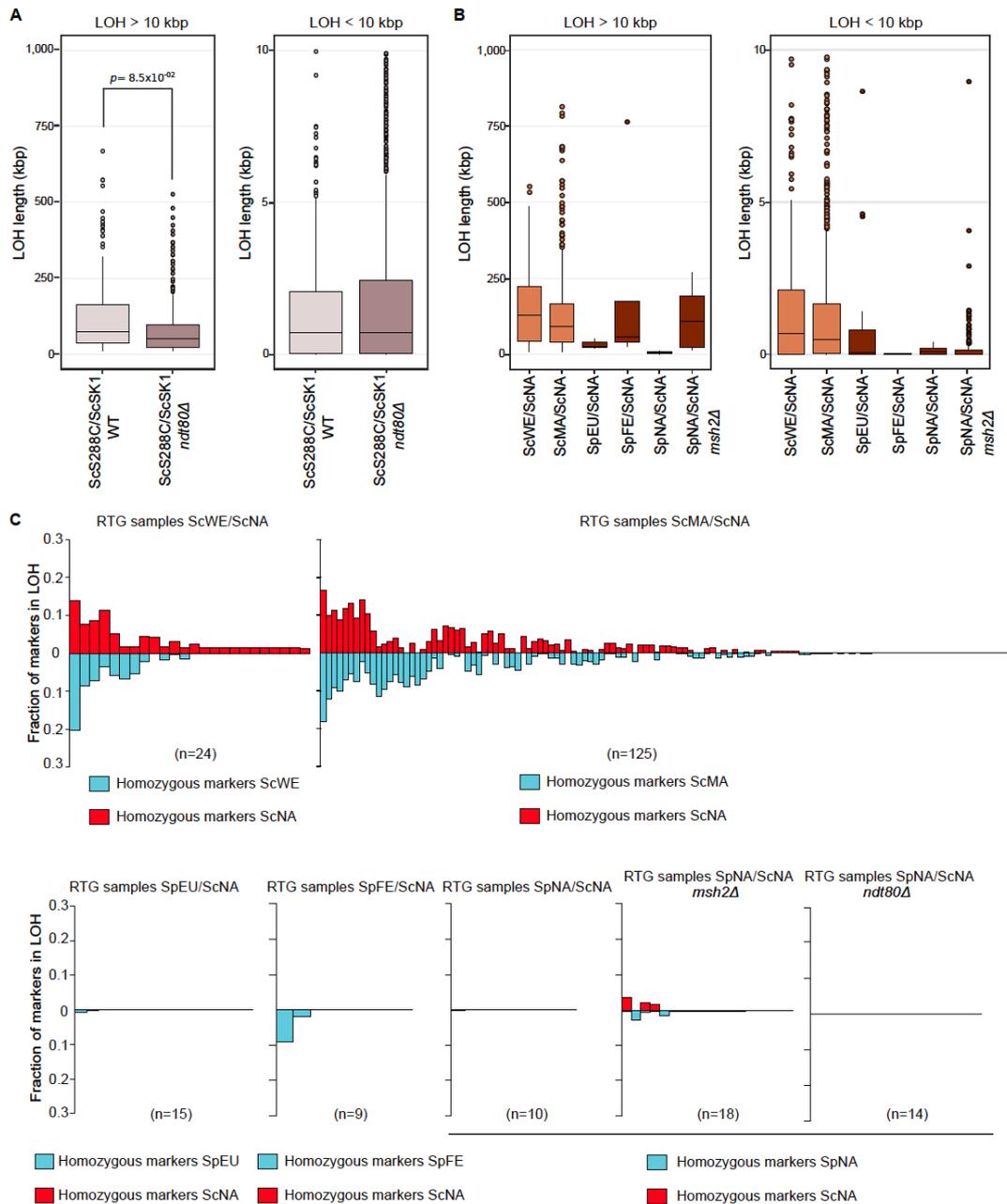
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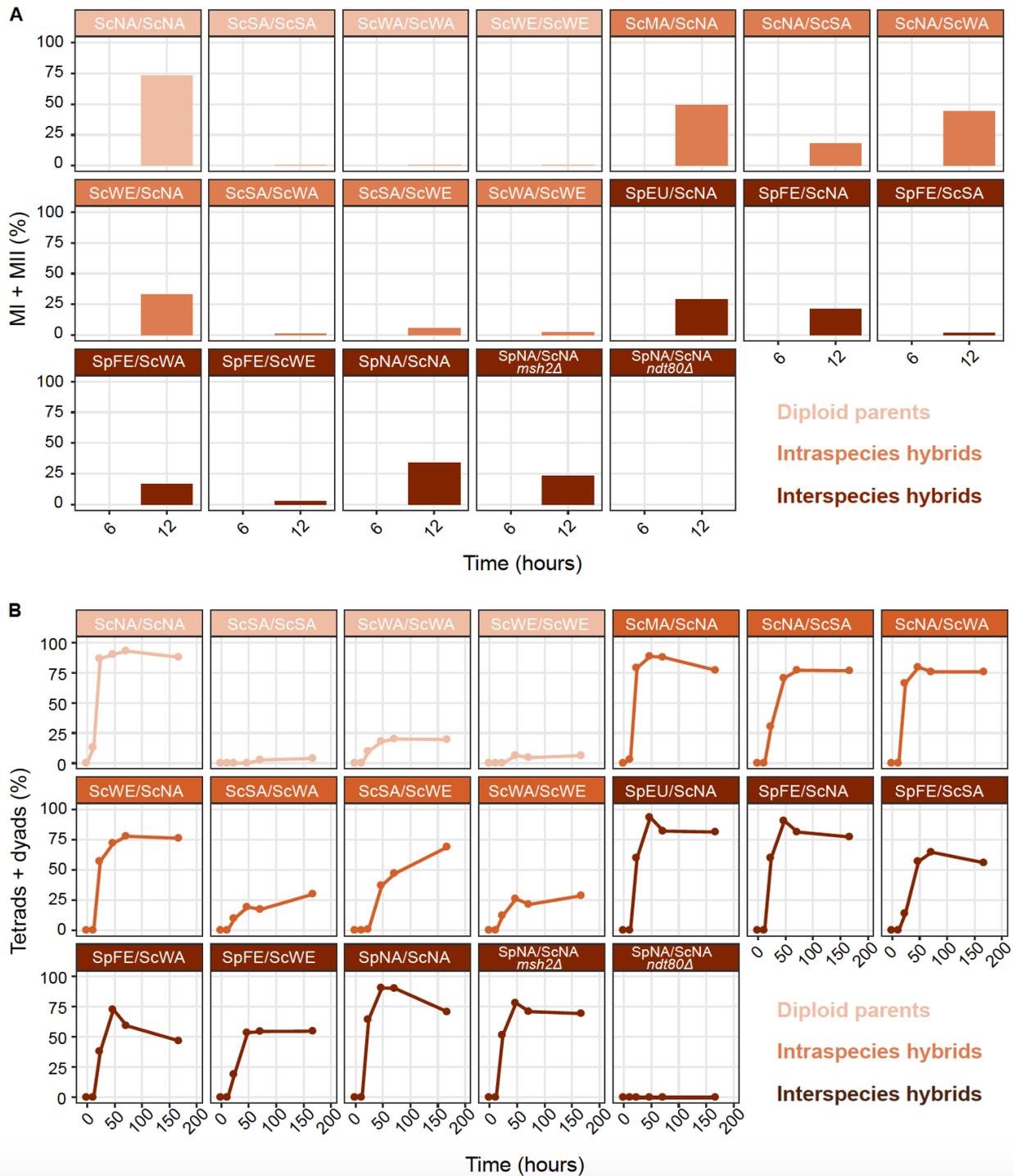


Supplementary Figure 1. LOH size and chromosomal position. (A) Size distribution of interstitial (grey) and terminal (black) LOH events for the ScS288C/ScSK1 WT and *ndt80Δ* RTGs. The x-axis represents the \log_{10} of the size of the LOH. (B) Size distribution of interstitial (grey) and terminal (black) LOH events for the intraspecies (light brown) and interspecies (dark brown) RTGs. The x-axis represents the \log_{10} of the size of the LOH. (C) Left panels: number of LOHs versus the relative distance from the centromere. For each background, the number of LOH events spanning 1000 bins of width 0.001 is reported as a black line. The plots represent aggregate analysis for all chromosomes except for events lying on chromosome II that were filtered out since positions of LOH in this chromosome were constrained by the presence of the *LYS2/URA3* system (see text). The smoothing of the data (red line) serves as an eye guide. Right panels: bar plot of the number of breakpoints in a 80 kbp window around all the centromeres (excluding chromosome II) for the intraspecies RTGs with 5 kbp bin size. Red bars represent the whole-genome median. The values exceeding the y-axis upper limit are annotated on top of the corresponding bar.

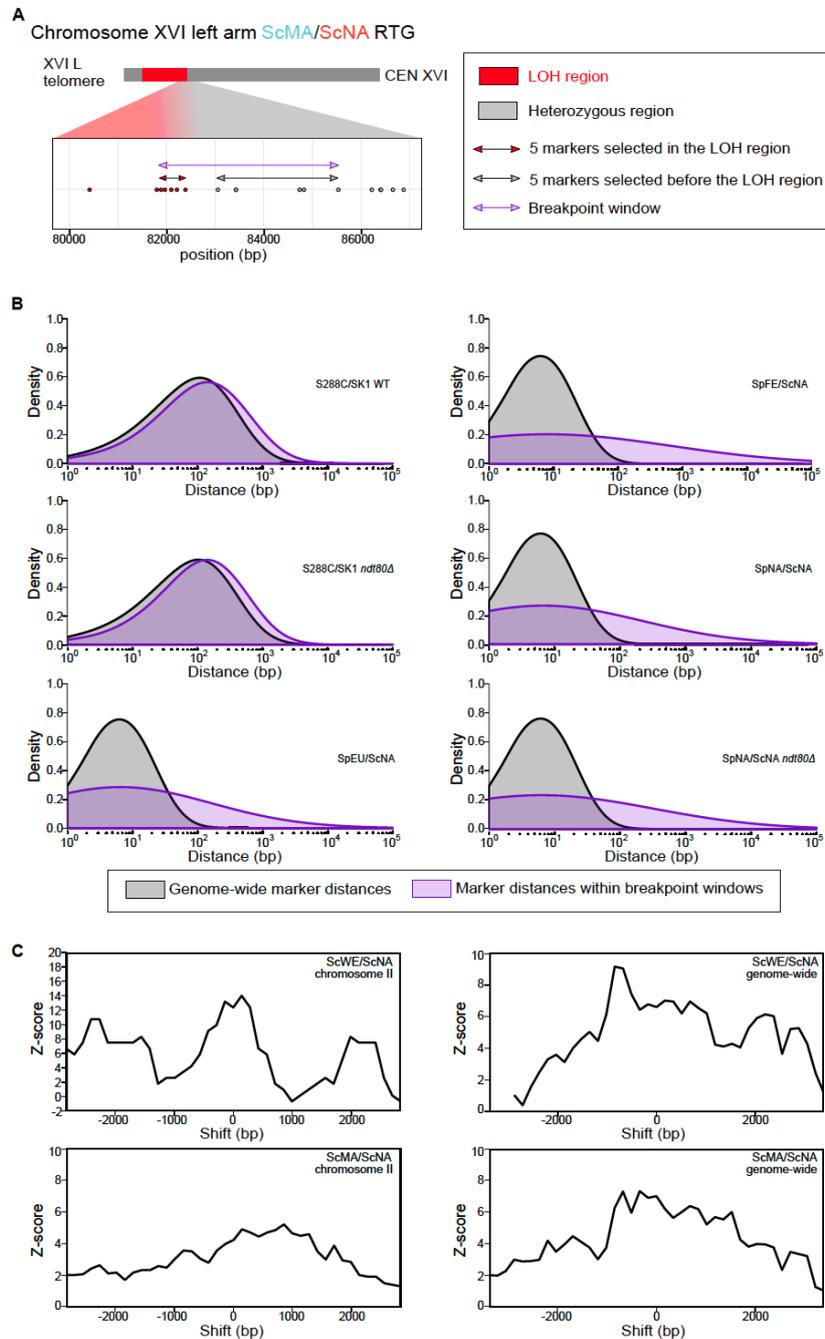
6.1 Supplementary Materials



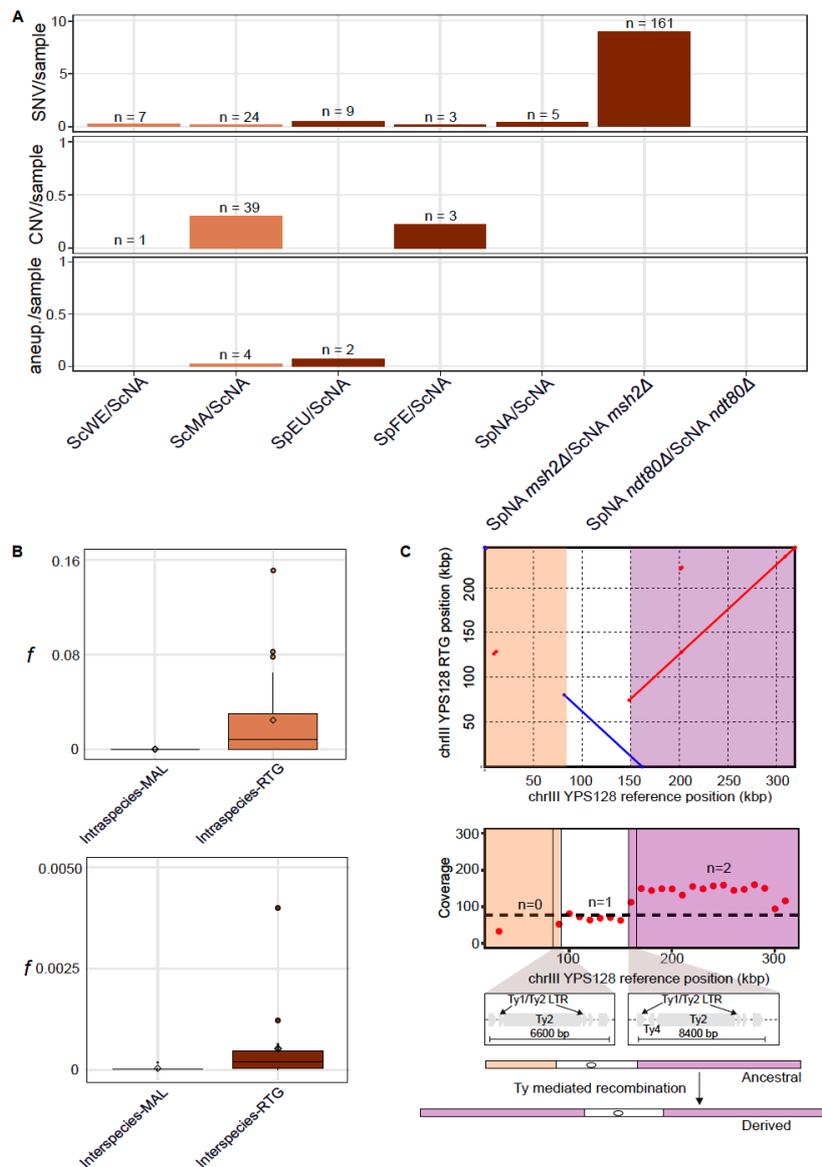
Supplementary Figure 2. Overview of LOH length and heterozygous/homozygous markers in RTG clones. (A) Boxplots of S288C/SK1 and S288C/SK1 *ndt80Δ* LOHs (left > 10 kbp and right < 10 kbp). The *p*-value refers to two-tailed Wilcoxon rank-sum test. The same test performed only on the mother cells of the *ndt80Δ* and WT populations confirmed this result (one sided Wilcoxon rank-sum test, p -value= 2.34×10^{-8}) (B) Boxplots of the LOH (left > 10 kbp and right < 10 kbp) for the intraspecies and interspecies hybrids colored according to figure 2. (C) RTG clones from intraspecies hybrids display high heterogeneity in the genome fraction in LOH (upper panels). We did not observe any LOH bias towards one of the two hybrid's subgenomes for intraspecies hybrids (Welch's t-test ScWE/ScNA, p -value = 0.8, Welch's t-test ScMA/ScNA, p -value = 0.74). Interspecies hybrids show a lower magnitude of genome in LOH (lower panels).



Supplementary Figure 3. Sporulation dynamics across the strain panel. (A) Meiotic progression measured by DAPI staining as the combined percentage of cells with 2 nuclei (MI) and 4 nuclei (MII). None of the samples had 2 or 4 nuclei at T0 (not reported) and T6. **(B)** Sporulation efficiency measured as the combined percentage of tetrads and dyads observed on the total number of counted cells.

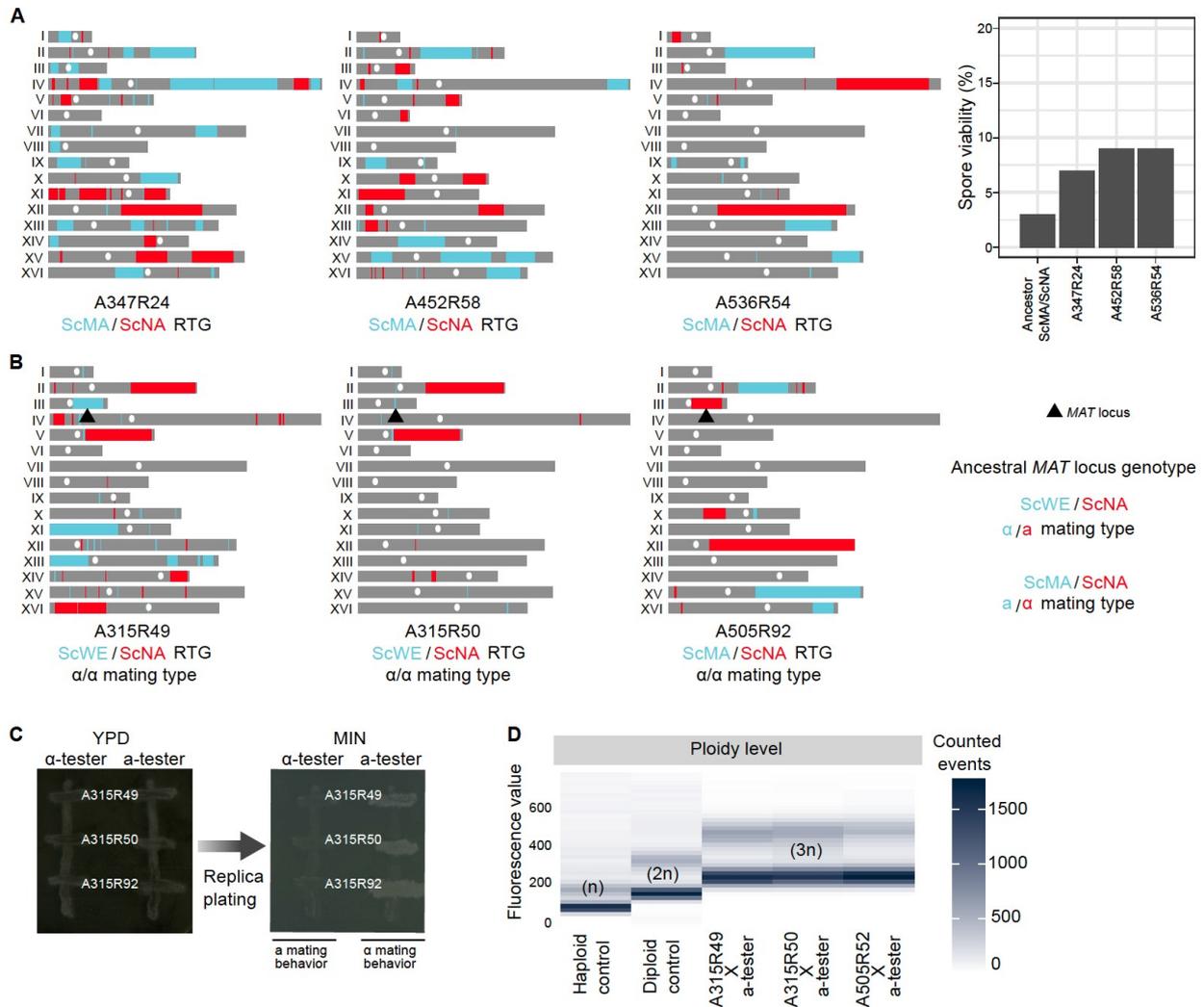


Supplementary Figure 4. LOH breakpoints are enriched in low heterozygosity regions and nearby meiotic recombination hotspots. (A) Zoom-in of a LOH breakpoint window that highlights which markers were selected to evaluate the local heterozygosity (see Figure 3 and Figure S4B). LOH breakpoint windows comprise the 5 heterozygous markers and the 5 homozygous markers closer to the breakpoint. **(B)** Distribution of marker distances genome-wide (grey) and in LOH breakpoint windows (purple) across other sequenced hybrids. **(C)** Z-score of the statistical test for LOH breakpoints and recombination hotspots association on chromosome II (left panel) and genome-wide (right panel) for the ScWE/ScNA and ScMA/ScNA evolved samples. The Z-score is plotted as a function of the shift (in bp) of the recombination hotspots regions.

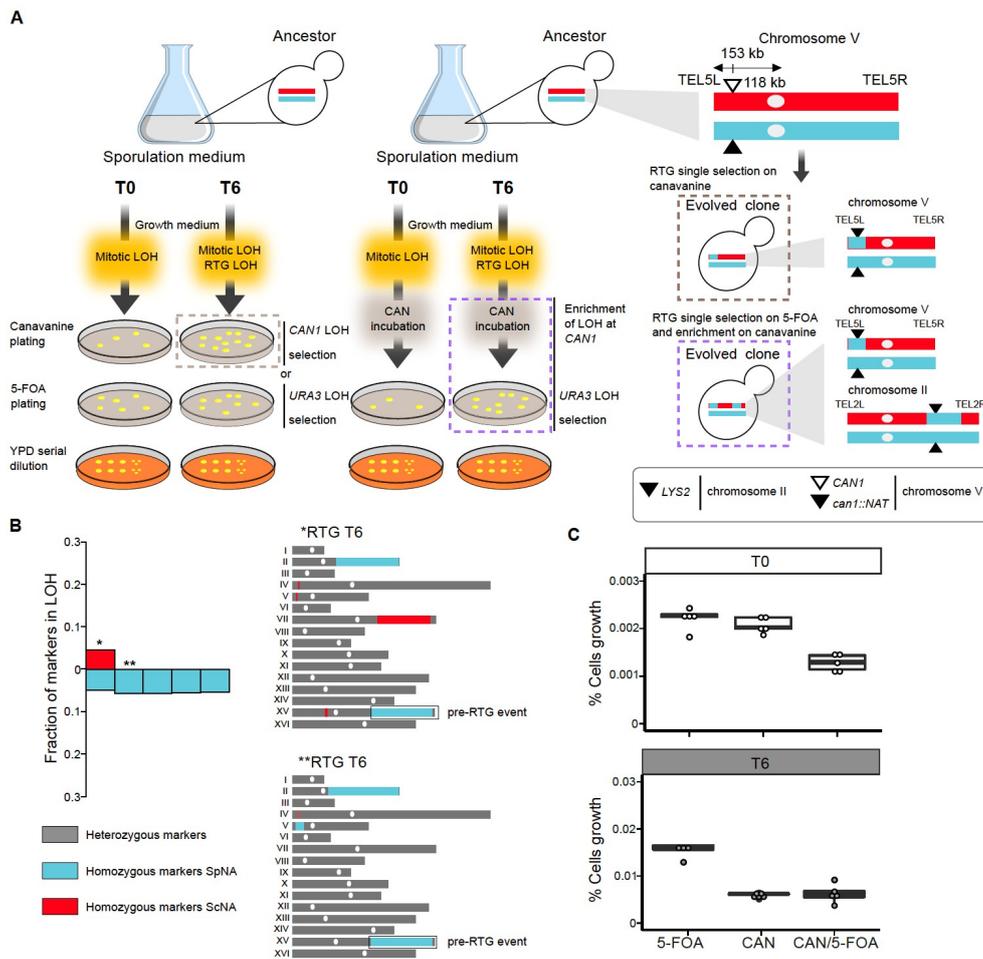


Supplementary Figure 5. Mutational landscape upon RTG. (A) Bar plots report the number of single nucleotide variants (SNVs), copy number variations (CNVs) and aneuploidies per sample. Genomes of evolved RTG clones are remarkably stable with no significant increase of aneuploidies, single nucleotide variants (SNVs) and copy number variations (CNVs). The increased SNVs in the SpNA/ScNA *msh2Δ* hybrid is expected given its mutator phenotype. Massive CNVs in the ScMA/ScNA samples arise as a result of recombination between non-collinear chromosome arms. (B) Top panel: LOH genomic impact on intraspecies ScWE/ScNA hybrids evolved through mutation accumulation lines (MAL) and RTGs; f is the fraction of genome in LOH per genome per bottleneck. Events lying on chromosome II of RTG samples were filtered out. Bottom panel: LOH genomic impact for interspecies SpEU/ScWE hybrids evolved through MAL and the SpEU/ScNA RTGs. (C) Top panel: dot plot of chromosome III from Nanopore long-read sequencing and *de novo* assembly of an RTG clone which underwent a complex rearrangement resulting in loss of the distal part of the left arm, inversion of the region encompassing the centromere and duplication of part of the right arm. The Illumina short-read coverage analysis support this rearranged structure, which was likely due to a Ty-mediated mechanism (bottom panel).

6.1 Supplementary Materials

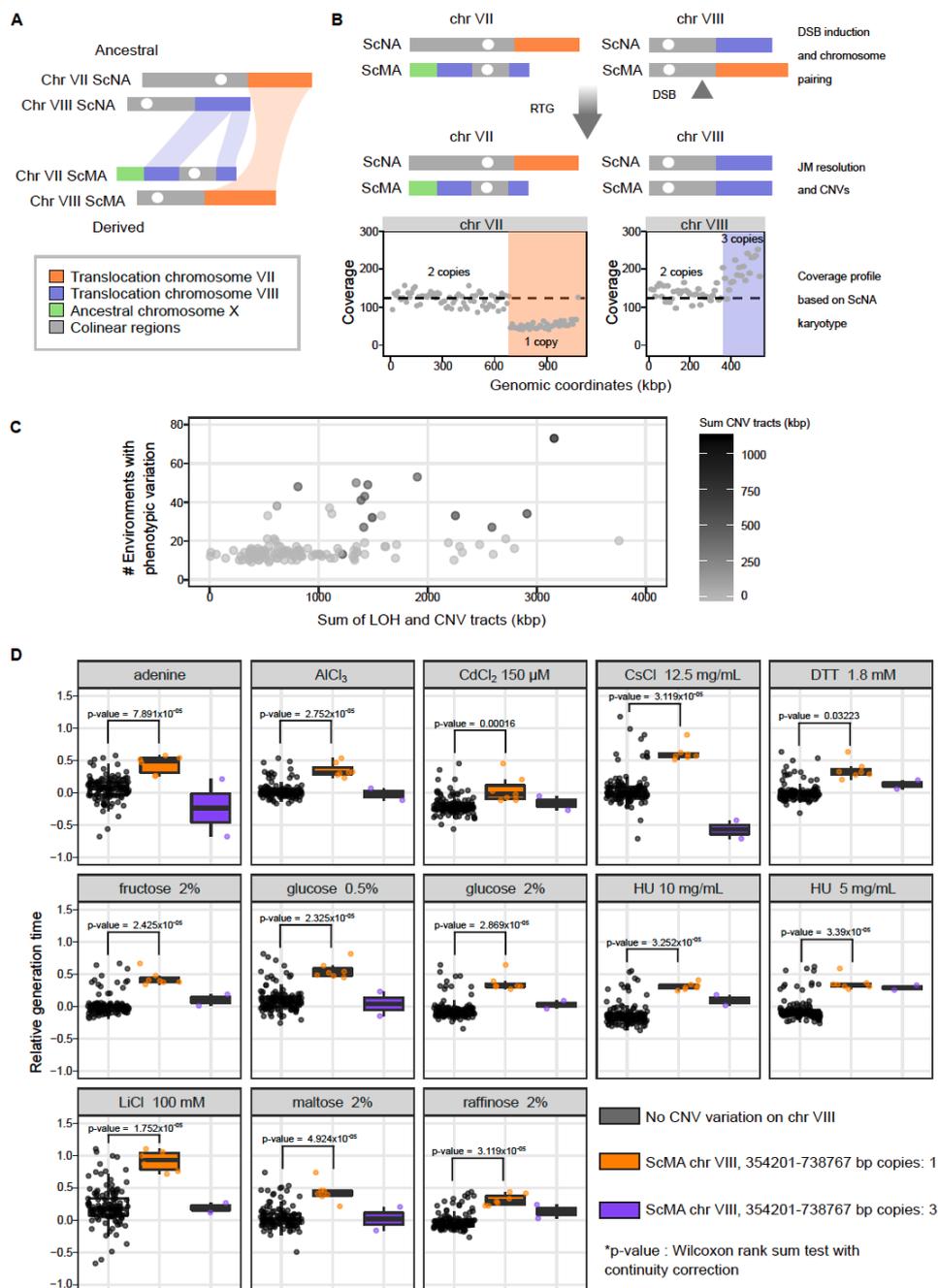


Supplementary Figure 6. RTG partially rescues hybrid fertility and can lead to polyploidization. (A) LOH landscapes of 3 selected ScMA/ScNA RTGs clones (left) and their respective spore viability compared to the ancestral hybrid (bar plot, right). (B) LOH landscape of two ScWE/ScNA RTGs and one ScMA/ScNA RTG with α/α mating type. (C) The mating-type test shows that RTG clones with recombination encompassing the *MAT* locus have haploid-like mating behavior and can generate polyploid strains if mated with a partner of the opposite mating-type. (D) The genome content of the mated strains has been validated by flow-cytometry.



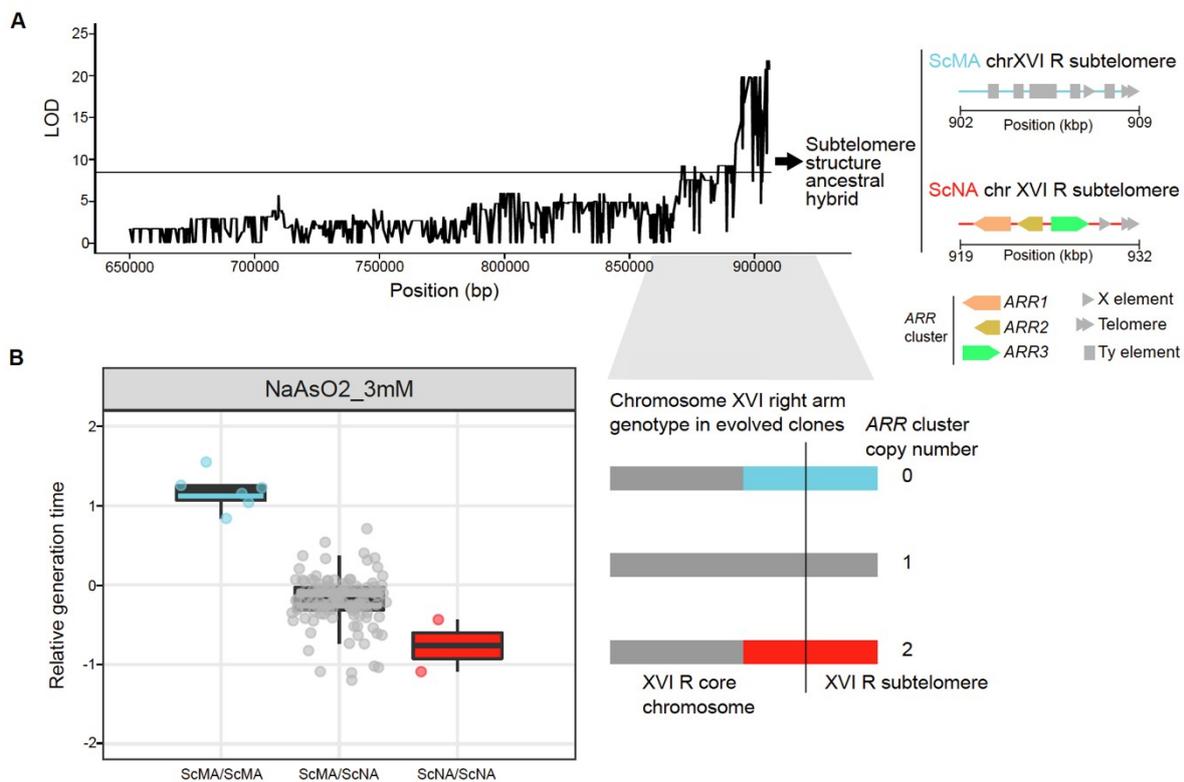
Supplementary Figure 7. Overview of the double LOH selection approach. (A) Left panel: RTG protocol used for the single selection approach performed either with 5-FOA or canavanine. Central panel: RTG protocol used for the enrichment of a second recombination event when cells are grown on canavanine before plating on 5-FOA. Right panel: sketch of the LOH recombination selected when plating on canavanine (brown box) and the event enriched upon canavanine incubation and plating on 5-FOA (purple box). The *CAN1* gene is deleted in the *S. paradoxus* subgenome so that the two selectable markers promote LOH in the same direction to avoid any genetic incompatibility effect by selecting two LOHs toward different subgenomes. (B) Fraction of markers in LOH in the sequenced RTG isolated with the double selection approach and LOH map of two RTGs sequenced. (C) Percentage of cells growing on 5-FOA or canavanine plates upon RTG. Three experiments are reported: single selection approach (5-FOA or canavanine) and double selection approach. T0 = no sporulation induction, T6 = 6 hours of sporulation induction. The incubation in canavanine does not kill the cells but allow to grow only those having resistance to the drug thus enriching cells harbouring LOH at *CAN1*. Once the cells are plated if the two events were completely independent the ratio of T6 compared to the T0 would have been expected to be the same and cells with only the LOH on chromosome II deleting *URA3* were expected to be retrieved.

6.1 Supplementary Materials



Supplementary Figure 8. Massive CNVs shape RTGs fitness. (A) Sketch of the non-colinear regions on chromosomes VII and VIII. The term “ancestral” refers to the ScNA karyotype whereas the term “derived” refers to the rearrangements in the ScMA karyotype. (B) Massive CNVs result from recombination of non-collinear chromosome arms of the subgenomes of a ScMA/ScNA hybrid. (C) Relationship between phenotypic variation and length of LOH and CNV events in the RTG samples; 2 samples were removed due to non-reliable CNV calls. (D) Boxplots of the relative generation time of RTGs with no CNV on chromosome VIII (black), RTG that have lost one copy of the arm of

chromosome VIII of the ScMA subgenome as a result of recombination (orange), and RTG that have gained one copy of the arm of chromosome VIII of the ScMA subgenome as a result of recombination (purple).



Supplementary Figure 9. Linkage analysis of arsenite resistance. (A) Plot of the LOD score along the right arm of chromosome XVI. The horizontal line indicates the statistical significance threshold. Right side show a zoom-in on the subtelomere structure of the two parental isolates used in the linkage mapping. The ScMA background lack the arsenite resistance cluster *ARR1-3*. (B) Phenotypic variation in arsenite across the ScMA/ScNA RTG samples. The data points in the boxplots are grouped based on the genotype of the last marker before the right subtelomere of chromosome XVI. The right panel shows the different number of *ARR* clusters according to the genotype of RTG clones.

Title: Unlocking the genetic potential of industrial sterile yeast hybrids by meiotic reversion

Authors:

Mozzachiodi S^{1,2}, Krogerus K³, Gibson B^{3,#}, Nicolas A⁴, Liti G¹

Affiliations:

¹Université Côte d'Azur, CNRS, INSERM, IRCAN, Nice, France.

²Meiogenix, 38, rue Servan, Paris 75011, France.

³VTT Technical Research Centre of Finland Ltd., Espoo, Finland.

⁴Institut Curie, Centre de Recherche, CNRS-UMR3244, PSL Research University, Paris 75005, France.

Notes :

[#]Current adress: Institute for Food Technology and Food Chemistry Department of Brewing and Beverage Technology, Technical University, 13353, Berlin, Germany

Abstract

Breeding and domestication have generated widely exploited crops, animals and microbes. Domesticated *Saccharomyces cerevisiae* yeast strains are largely sterile as a side-effect of domestication, and this prevent any genetic improvement strategy based on selective breeding. Here, we developed a novel yeast strain improvement approach based on the property of the budding yeasts to produce meiotic-like genetic recombination when cells encounter nutrients, interrupt meiosis and return-to-mitotic-growth (RTG). We applied the RTG framework to two unrelated and genetically distinct polyploid industrial *S. cerevisiae* strains, which suffer from nearly complete gametes unviability. We demonstrated that both strains were RTG-competent by engineering several deletion mutants and genetic systems through CRISPR-Cas9. We developed a non-GMO strategy based on natural colony phenotypes to screen for recombined RTG clones. Whole genome sequencing of the evolved clones proved RTG-induced recombination in both polyploid strains. The library of RTG evolved strains were extensively phenotyped for industrially relevant traits, demonstrating cases of phenotypic improvements and matching of the evolved RTG clones with industrial standards. We propose the RTG-framework as a fully non-GMO workflow to rapidly improve industrial yeasts that can be easily brought to the market.

Introduction

Humans have selected and improved domesticated organisms for centuries, leaving profound signatures in their genomes and lifestyles (1). Breeding has been among the first biotechnology advances that humankind has exploited to generate hybrids with improved performances and it is still widely used (2). Nowadays, with the advent of the new genetic engineering techniques, breeders and scientists can even act directly on the core biology of the organism introducing, editing or removing specific genes (3). However, the exploitation of genetic modified organisms (GMOs) in the food sector is still controversial and restricted in many countries.

Humans unwittingly domesticated yeasts of the genus *Saccharomyces* since the earliest fermentations of food and beverages were carried out (4, 5, 6), and novel fermentation processes selected new yeast strains and hybrids, which some of them are still in use today (7). Attempts to cross industrial yeasts to obtain new breeds are often inefficient because of the sterility of industrial strains, a typical signature of domestication on the yeast lifestyle (6, 8). This domestication-syndrome might have derived from the lack of selection on sexual reproduction, random genetic drift and adaptation to specific fermentation niches. Thus, other approaches to improve sterile industrial strains have been proposed as industrial yeasts are often selected based on historical reasons rather than on industrial

performances (9). Consistently, recent experiments showed that *de novo* hybrids were superior compared to common commercial strains (10, 11, 12).

Recently, we showed that aborting meiosis in lab-made sterile diploid hybrids and returning them to mitotic growth, a process called return-to-growth (RTG) generates genetic diversity and improved fitness in the evolved samples (Mozzachiodi, Tattini et al) (13). RTG relies only on the early meiotic phase where the enzyme Spo11p induces double-stranded breaks (DSBs) in the genome, leading to the formation of recombined crossover or noncrossover molecules (14). Uncommitted meiotic cells exposed to a rich-nutrient environment perform RTG by interrupting the expression of meiotic genes (15), bud, and resolve the meiotic recombinant molecules generating a mother and a daughter cells in which recombinant chromatids segregate. Moreover, beyond the generation of disperse genome-wide LOH tracts, RTG genomes are highly stable (Mozzachiodi, Tattini et al). Given that RTG induces recombination without a complete sexual reproduction, which often is defective in industrial strains, RTG might represent a powerful approach to unlock the genetic diversity of industrial strains. However, the underlying causes driving the extreme sterility observed in industrial strains are unclear. Industrial yeast genomes are highly complex and display hallmarks of systemic genomic instability. Their higher ploidy coupled with aneuploids, structural variation and the accumulation of loss of function mutations in the absence of sex can all contribute toward their sterility. The RTG paradigm has never been explored in such complex genomic scenario and whether it can induce recombination without triggering systemic genomic instability remain an open question. Furthermore, the selection of RTG recombinant clones has been mostly carried out using selective genetic markers (Mozzachiodi, Tattini et al), which involve GMO strategies (13). Thus, novel selection approaches are essential to transfer the RTG paradigm to industrial strains and solve the long-standing technical limitation of industrial strains sterility. Here, we developed an entire workflow to demonstrate the applicability of RTG to improve industrial yeasts and proposed an efficient strategy for selecting the RTG recombined clones. We generated two libraries of evolved samples harbouring broad phenotypic deviations from the ancestral hybrid with some of them showing improved industrial traits.

Results

Genomic portrait and sterility of industrial polyploid hybrids

Domesticated strains derived from wild yeast ancestors have accumulated signatures of domestication by random genetic drift and selection (**Fig 1a**). We selected two unrelated *S. cerevisiae* domesticated hybrids, hereafter called OS1364 and OS1431 (**Fig 1a**), to probe if the RTG framework could generate strains harbouring genetic and phenotypic diversity that ultimately result in improved

industrial strains (**Fig 1b**). OS1364 was isolated from a cassava factory in Brazil and belongs to the mosaic beer clade, whereas OS1431 was isolated from Ale fermentation in England and belongs to the Ale clade (15) (**Table S1**). We characterized the genome of the strains by short and long read sequencing and found multiple hallmarks of domestication in both hybrids, although they do not share recent evolutionary history as underlined by their distinct genetic ancestry. Both strains are polyploids with OS1364 being triploid (3n, with chromosome III aneuploid) and the OS1431 tetraploid (4n) (**Fig S1a-b**). Moreover, we detected in OS1364 one region harbouring non-reference genetic material partially derived from *Zygosaccharomyces bailii* inserted in the right arm of two chromosomes XIII (**Fig S1c**) and another region with non-reference material at the end of two chromosomes XIII (**Fig S1c**).

Both genomes harboured a considerable number of heterozygous positions ($n=40431$ OS1364, $n=39376$ OS1431) (**Fig 1d**) a common characteristic in strains that rarely go through sexual reproduction. However, the assembly of long-read failed to discriminate between the different haplotypes thus we could not phase these heterozygous positions. We detected a large region of loss-of-heterozygosity (LOH) on chromosome XII downstream the rDNA locus in both strains (**Fig S1d**) consistent with recent population genomic studies showing that this region is an LOH hotspot (5, 17). We also found several heterozygous missense variants ($n=10747$ OS1364, $n=13033$ OS1431, **Fig S1e**) and copy number variants (CNVs) (**Fig S1b**), the latter resulting in small amplifications or deletions similar to those previously observed (5). This diversity might reflect the evolutionary histories of the different haplotypes before or after the hybridization event that has formed the extant hybrids, in particular at the level of subtelomeres which are known to be highly dynamic (18).

We next investigated the reproductive capacity of these polyploid strains and observed a defective sexual reproductions (**Fig 1d**). Specifically, both strains showed slow and asynchronous meiotic progression and generated nearly completely unviable gametes. The high heterozygosity and lack of an effective sexual reproduction led us to wonder whether the strains accumulated deleterious mutations, which might affect the viability of their gametes and partially explain their sterility. We detected in the genomes many highly deleterious mutations ($n=137$ OS1364, $n=267$ OS1431) of which 9 (OS1364) and 19 (OS1431) were homozygous and affecting essential genes (19) however, it is difficult to understand their potential impact in driving the sterility (**Fig 1e, Table S2 and Table S3**).

Overall, the strains characterised here are typical examples of domesticated *S. cerevisiae* with complex polyploid genomes and represent ideal test cases for the RTG framework to probe the limit imposed by genomic complexity and extreme sterility.

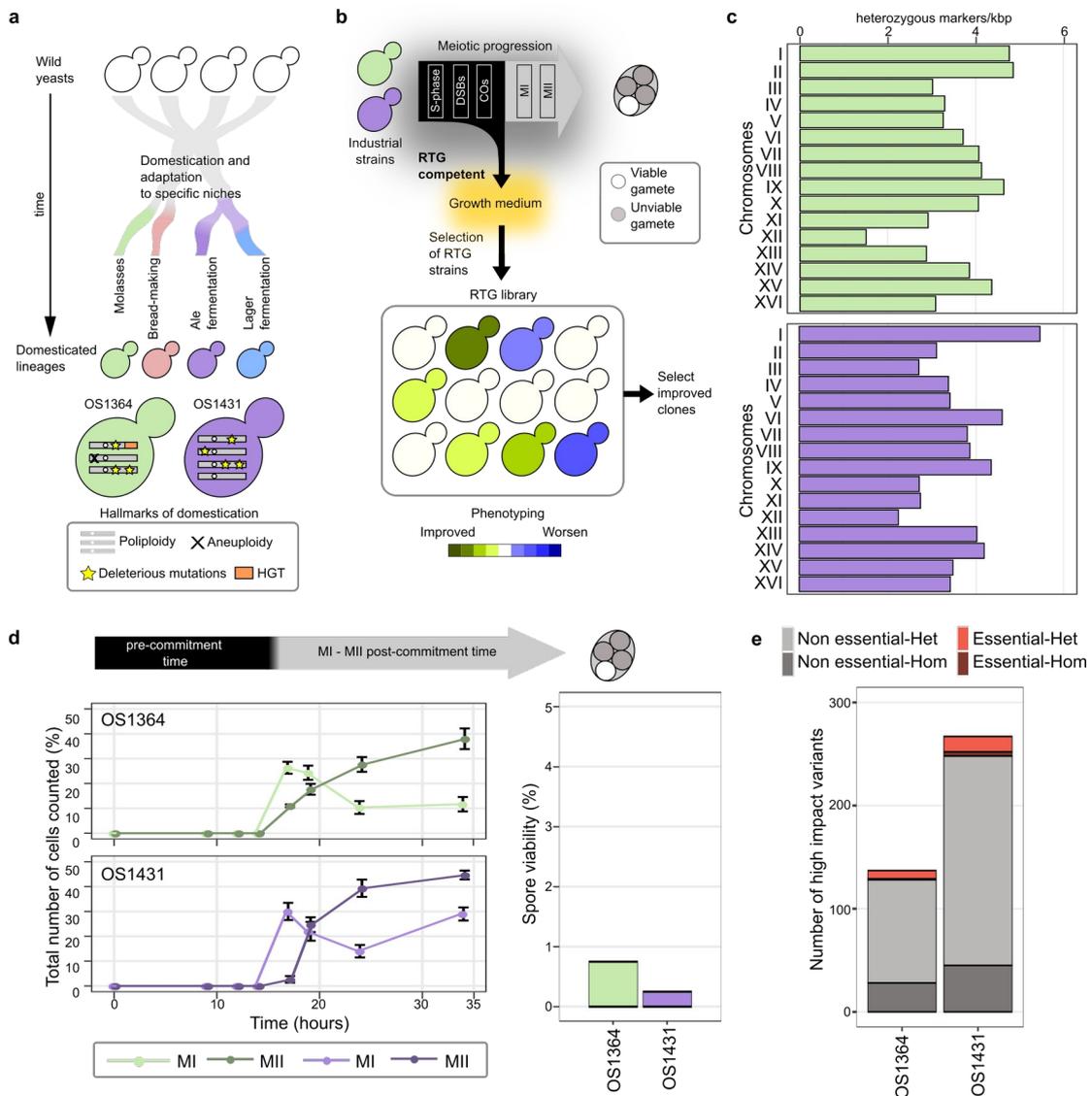


Fig 1 Genome complexity and sterility of industrial *S. cerevisiae* | (a) The process of domestication has produced strains highly adapted to specific human made niches. Genomic complexity and hallmarks of domestications are detected in both hybrids used in this study. (b) Example of the RTG framework applied to the polyploid strains of this study. The strategy relies on identifying if the industrial strains are RTG competent and then collecting several RTG samples in an RTG library that can be sequenced and phenotyped using high-throughput approaches in order to identify clones with improved desired industrial traits. (c) Level of heterozygosity (number of heterozygous markers/kbp) across each chromosome. (d) Meiotic progression measured as the percentage of cells that have passed the first (MI) and second (MII) meiotic division on the total cells counted. On the right, bar plot representing the spore viability of the two domesticated strains. (e) Bar plot representing high impact variants detected affecting essential (red) or non-essential (grey) genes. The variants are also divided into homozygous (Hom) if present in all haplotypes, and heterozygous (Het) if not present in all haplotypes.

Sterile polyploid hybrids are RTG competent

First, to probe if the two hybrids were RTG competent, we engineered a genetic system that we have broadly applied in RTG experiments (Mozzachiodi, Tattini et al) to measure LOH rate at a heteroallelic *LYS2/URA3* locus on chromosome II (hereafter referred to as *LYS2/URA3* regardless of the ploidy of the strain). To engineer this system in the polyploid strains, we first deleted all the copies of the gene *URA3* by CRISPR/Cas9 mediated multi-deletion (20), and then we inserted one *URA3* copy on chromosome II by classical lithium acetate transformation (**Fig 2A**). We validated the genotype by PCR and growth on selective media (**Fig S2, Table S4**). We evolved OS1364^{*LYS2/URA3*} and OS1431^{*LYS2/URA3*} following the RTG framework that was optimized for these strains based on their meiotic progression time course data (**Fig 2b**). We detected an increase in LOH rate of 10-fold in OS1364^{*LYS2/URA3*} and 3-fold in OS1431^{*LYS2/URA3*} when we induced RTG after 14 hours of sporulation compared to the basal level of LOH measured in the unsporulated culture (T0) (**Fig 2b, Table S5**). In contrast, the increase was non-significant at an early time point of the meiotic progression consistent with that recombination at the *LYS2/URA3* locus is driven by meiotic recombination and not genome instability (**Fig 2b, Table S5**). To further prove that RTG induced the increase of recombination at the *LYS2/URA3* locus, we deleted in OS1364^{*LYS2/URA3*} all the copies of *SPO11*, which is essential for inducing DSBs in meiosis (**Fig S2b**) and evolved this mutant through RTG. We did not detect any significant increase between the T0 and the respective T14 in the OS1364^{*LYS2/URA3*} *spo11Δ* (**Fig S2c, Table S5**) supporting that RTG caused the increased recombination.

We next performed whole-genome sequencing of the parental, control and evolved clones ($n=14$ OS1364, $n=4$ OS1431, **Table S1**) isolated on 5-FOA plates to evaluate the genome-wide impact of RTG. Our analysis revealed variable levels of recombination in the isolated RTGs as found in diploid hybrid RTGs (Mozzachiodi, Tattini et al) (**Fig 2c, Fig S2d**). RTG clones derived from OS1364^{*LYS2/URA3*} had up to 26% of the genome in which we detected recombination between heterozygous markers, with an average of 10% across samples. The value detected for OS1431^{*LYS2/URA3*} was 12.7% with an average of 6%. We also detected an LOH event generated upon the *URA3* deletion on chromosome V likely produced by the CRISPR/Cas9 genome editing (21), which was shared between all the RTGs and clones and therefore was excluded from the analysis. Moreover, we did not detect any chromosome loss accounting for 5-FOA resistant cells, showing that despite these strains are polyploids, LOH at the *LYS2/URA3* locus still arises more frequently than aneuploidy. We did not detect any aneuploidy genome-wide in all the sequenced RTGs derived from OS1364^{*LYS2/URA3*} supporting that overall genome content is preserved during RTG.

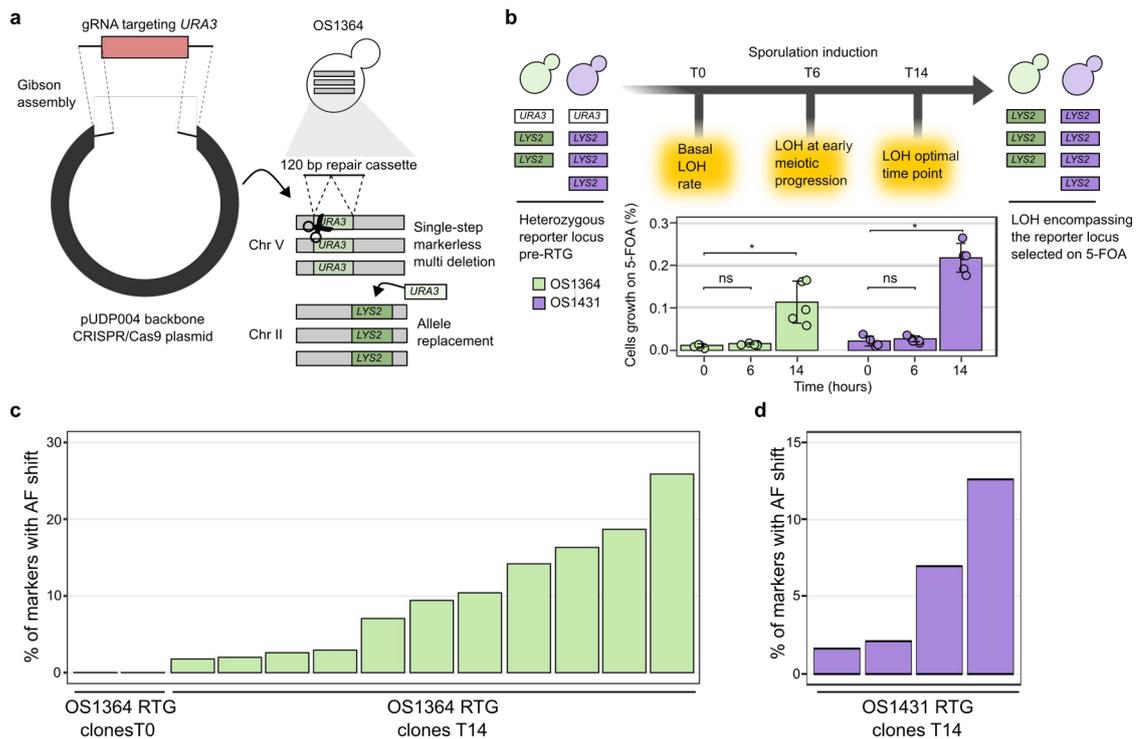


Fig 2 RTG induced recombination in polyploid sterile hybrids | (a) A CRISPR-based approach enabled the engineering of the *URA3*-loss genetic system in the polyploid strains. **(b)** RTG approach used to measure recombination rate at the heterozygotic locus *LYS2/URA3* (top panel). Bar-plots representing the average percentage of cells growing on 5-FOA at different time points: no sporulation induction (T0), 6 hours of sporulation induction (T6) and 14 hours of sporulation induction (T14) (bottom panel). The increase of cells growing on 5-FOA at T14 compared to T0 was significant for both samples (p -value < 0.05, Wilcoxon ranked-sum test one-sided) whereas the increase at T6 was not significant compared to the T0 (p -value > 0.05, Wilcoxon ranked-sum test one-sided). **(c)** Bar plot representing the percentage of markers in which we detected an AF shift for the RTG samples derived from OS1364^{*LYS2/URA3*} (pale green). **(d)** Bar plot representing the percentage of markers in which we detected an AF shift for the RTG samples of OS1431^{*LYS2/URA3*} (pale purple).

The engineered OS1431^{*LYS2/URA3*} strain harboured an aneuploidy on chromosome XIII and a CNV on chromosome XII that likely raised during CRISPR/Cas9 editing since they were absent in the parental strain, while they were inherited by all the RTG evolved clones (**Fig S3**). Besides that, we found only one additional aneuploidy and two CNVs in the RTG derived from OS1431^{*LYS2/URA3*} (**Fig S3**).

Altogether, our results demonstrate that domesticated polyploid hybrids are RTG competent despite their low sporulation efficiency and extreme sterility showing the RTG potential to generate genetic diversity in industrial strains.

Assisted selection of recombined RTG clones by natural colony phenotypes

The RTG selection based on *URA3*-loss requires genome editing and produces GMOs that have marketing constraints in the food industry. To overcome this limitation, we devised an alternative selection method based on the natural variability in colony phenotypes such as morphology or colour. Colony phenotypes are highly complex traits, influenced by both genetic and environmental factors (**Fig 3a**). Therefore, we explored if the genetic recombination induced by RTG generates variability in the genetic determinants regulating phenotypic traits that can be easily detected. We used a cheap YPD based media with different concentrations of dextrose (0.5% and 1%), a major environmental factor known to regulate colony morphology (22, 23) (**Fig 3a**). Thus, we applied the RTG protocol to the wild-type OS1364 and OS1431, plated the cells on YPD media with varying dextrose concentrations and inspected the plates daily (**Fig 3a Material and Methods**). We observed three classes of sectored colony phenotypes in the OS1364 (**Fig 3b, Fig S4, Table S5**) with frequency of 0.75 % and one class in the OS1431 with frequency of 0.5 % across all environment tested upon RTG (**Fig 3b, Table S5**). In contrast, we did not observe any of these phenotypes in control plates where cells were plated without inducing meiosis. Moreover, when we plated the RTG cells in which we allowed the first budding after RTG, we did not retrieve sectored colonies but only whole colonies with morphology variation. Thus, we hypothesized that the sectored colonies might arise upon budding of recombined mother-daughter (M-D) RTGs that has not completed yet the first division after RTG induction. An alternative explanation is that the sectored RTG colonies derived from residual sporulation and spore germination, although this was unlikely given the near to zero spore viability observed. To completely exclude that scenario, we deleted the gene *NDT80* by CRISPR/Cas9 multi-deletion in both hybrids thus generating mutants that cannot complete sporulation (**Fig S5a**). Then, we evolved the OS1364^{*ndt80Δ*} and OS1431^{*ndt80Δ*} mutants through the same RTG protocol used for the wild-type hybrids. We detected similar sectored phenotypes in the RTG plates of the *ndt80Δ* strains that cannot complete the first meiotic division (**Fig S5b**) thus excluding that residual sporulation contributed to the sectored colonies formation.

Then, we performed whole-genome sequencing to further probe whether sectored colonies were M-D RTGs, and we also sequenced colonies having a uniform variable morphology compared to the WT isolated from RTG plates and T0 controls (**Table S1**). Whole-genome sequencing of isolated clones and RTG M-D pairs revealed recombination in all the putative M-D RTG having a sectored phenotype by detecting AF shifts in the unphased heterozygous markers (**Fig 3c-d**). The fraction of the genome in which we detected recombination between heterozygous markers was highly variable in both strains (10.83±8.6% genome with AF shift in OS1364 WT-RTG, 4.8±2.4% genome with AF shift in OS1431 WT-RTG, average ± SD) mirroring the results obtained from the *URA3*-loss assay.

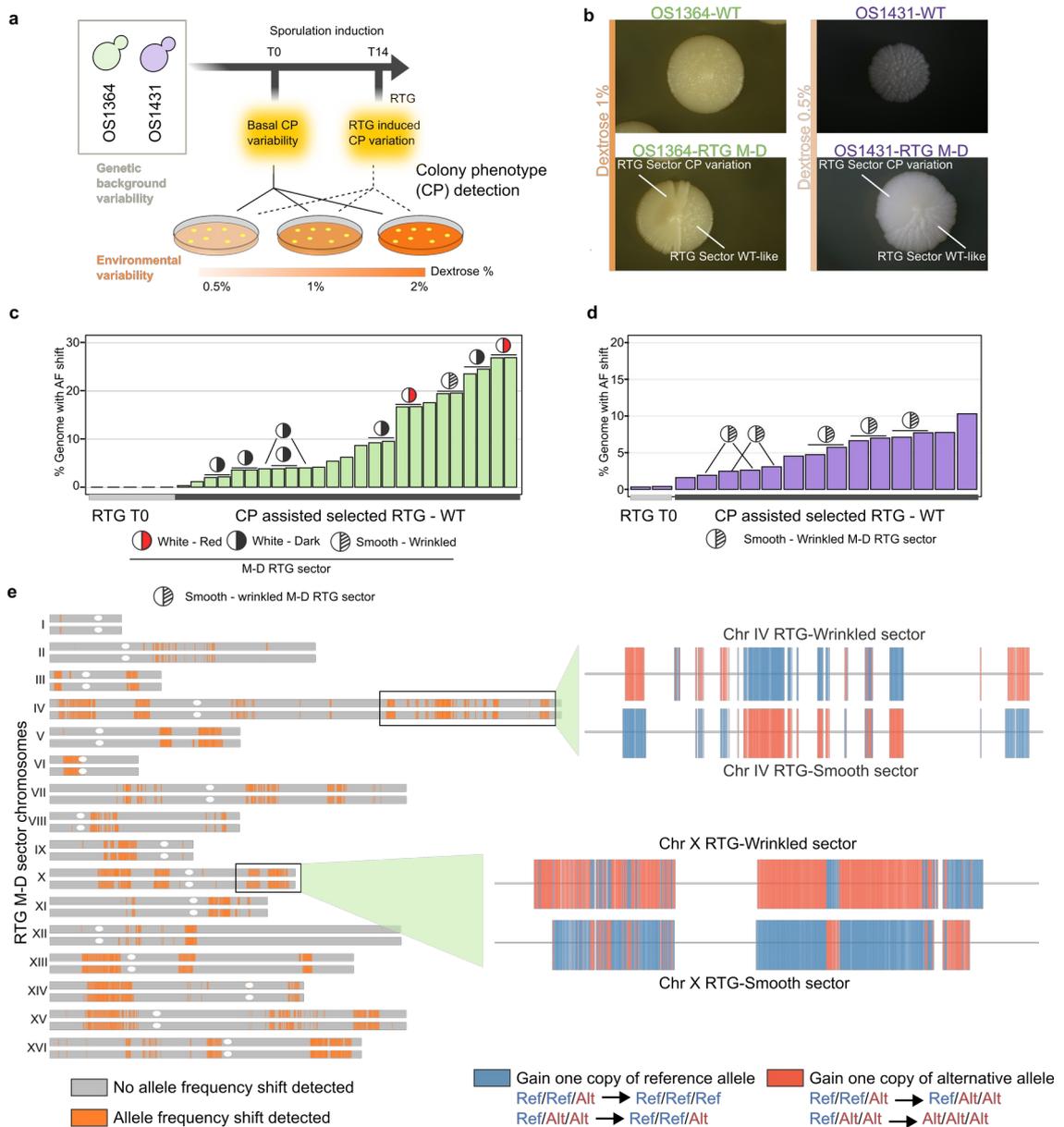


Fig 3 Colony phenotype variability revealed mother-daughter RTG pairs | (a) Varying dextrose concentrations were used to unveil hidden colony phenotypic variation upon RTG. **(b)** Wild-type (WT) colony phenotypes (top) compared to the sectored phenotypes emerging in RTG plates (bottom), with one sector resembling the WT and the other sector having a different phenotype. On the left is indicated the concentration of dextrose in the media. **(c)** Percentage of markers with allele frequency (AF) shift in the non-GMO samples in OS1364. The mother-daughter pairs for which both sectors were sequenced are indicated, single samples represent samples taken from RTG plates in which we allowed complete budding or sectors in which only one strain was sequenced. **(d)** As in panel **c** for the OS1431 background. **(e)** Karyotype of an RTG M-D pair (top chromosome: sm244/ bottom chromosome: sm245, derived from OS1364). Grey regions indicate the heterozygous markers without AF shifts, whereas orange regions contains heterozygous markers with AF shifts underlying LOH events. Zoom-in of two recombined regions are reported and colour coded to represent the genotype configuration of the heterozygous markers in the three homolog chromosomes.

Moreover, we provided two additional proofs that the sectored colonies were RTG M-D pairs. First, the AF shift for heterozygous markers was often reciprocal in the wild-type and *ndt80Δ* M-D pairs (**Fig 3e**, **Fig S6**, **Table S12**) as found in M-D pairs of diploid lab strains (13). Some of these regions were also shared between RTG M-D sectors with the same colony phenotype (**Fig S7a**) supporting the scenario that the colony phenotypes observed had a genetic determinant regulating it. Second, we observed in one M-D pair derived from OS1364 a complementary gain and loss of one chromosome (**Fig S7b**) compatible with chromosome missegregation between M-D during RTG. We exploited the recombination generated by RTG in OS1364 M-D RTGs to produce phased local haplotypes showing a potential application of RTG in polyploid yeasts (**Fig S7c**).

Overall, we showed that highly recombined RTGs M-D pairs can be selected in two unrelated industrial strains by exploiting natural phenotypic variation and we proved that these phenotypes arise as a result of RTG induced recombination.

RTG improves industrial fitness in polyploid yeasts

We next probed the potential of the RTG to improve industrial phenotypes. First, we characterised the fermentation performances of the parental OS1364 and OS1431 and compared it to the reference commercial strain WLP001 (**Table S1**). We confirmed that our two selected backgrounds are competitive for industrial fermentation, with OS1364 outperforming WLP001 and OS1431 for fast fermentation (**Table S10**) thus eventual improved RTG would have directly an industrial applicability. However, at the end of the 2-liter fermentation, both OS1364 and OS1431 had a massive decline in cell viability ranging from 25% to 50% compared to 8% of the commercial strain (**Table S11**), which is not a desirable trait when the same stock of yeasts is used in sequential fermentations. Therefore, we evaluated the resistance of the evolved non-GMO RTGs in several conditions mirroring industrial fermentations to identify samples with improved post-fermentation viability or displaying other traits of industrial interest. First, we performed a phenotypic screening in osmotic and alcoholic stressful conditions similar to those encountered during fermentation, and found that RTG samples had broad phenotypic variability with either a worsened, unchanged or improved phenotype compared to the parental strains (**Fig 4a-b**, **Table S6**). This result is consistent with the LOHs induced by RTG arising randomly in the genome and not as a by-product of the selective pressure. Moreover, some M-D RTG pairs showed complementary growth-rate variation (**Fig 4c**) that can be explained by complementary LOHs segregating weaker and stronger alleles in the recombined M-D RTG pairs. In the RTG library of OS1364, growth rate variation in ethanol and maltose appeared to be moderately correlated underscoring possible recombination in pleiotropic genes regulating both traits (**Fig S8a**). In contrast, we did not observe a similar correlation in OS1431

RTG variants but only moderate correlations between growth rate and yield in maltose and sorbitol (**Fig S8b**).

Then, we selected the best performing RTG samples derived from OS1364 ($n=12$), taking into account also the recombination profiles of a set of genes linked to the fermentative phenotype (**Table S7 and S8**), and all the M-D RTG pairs from OS1431 ($n=10$). We inoculated the selected RTGs in a high-gravity wort (30° Plato), and carried on a flask-scale fermentation for 13 days evaluating mass loss and alcohol produced at regular intervals for each pool (**Table S9**). Some samples showed increased fermentation kinetics (**Fig 4d**) as well as a superior alcohol production (**Fig S8c**) compared to the parental strains. We also noticed that in some M-D RTG derived from the Ale strain, the fermentation performances were complementary, in line with the complementary variation in growth rate observed in the previous phenotypic screening (**Fig S8d, Table S9**). Thus, we hypothesized that RTGs showing improved fermentation performances had improved resistance to the fermentation stress and potentially, higher post-fermentation cell-viability. Therefore, we selected two RTG variants among the best performers in the previous flask-scale fermentation, to carry on a 2-liter scale fermentation in high-gravity wort (20° Plato) and compared their performances to those of the parental strains previously characterized. We found that the OS1364 RTGs performed at least equally well to the respective parental strains, and the OS1431 RTGs were slightly better in fermentation performances (**Fig S9a, Table S10**) but did not have increased post-fermentation viability. In contrast, one OS1364 RTG had increased post-fermentation cell viability (**Fig 4d, Table S11**) compared to its parental strain, reaching a level similar to WLP001. Moreover, we wondered if the variable fraction of genome shaped by recombination (5 - 19 %) in the four RTGs selected has generated any trade-off in two unselected traits, the aroma profile and the sugar consumption, that are also relevant in the beer fermentation. The four RTG samples selected did not show any undesired variations for both phenotypes with the exception of a slight increase of acetaldehyde in sm408 although that was negligible (Threshold = 10 mg/mL, sm408 = 10.5 mg/mL, **Fig 4f, Table S11**).

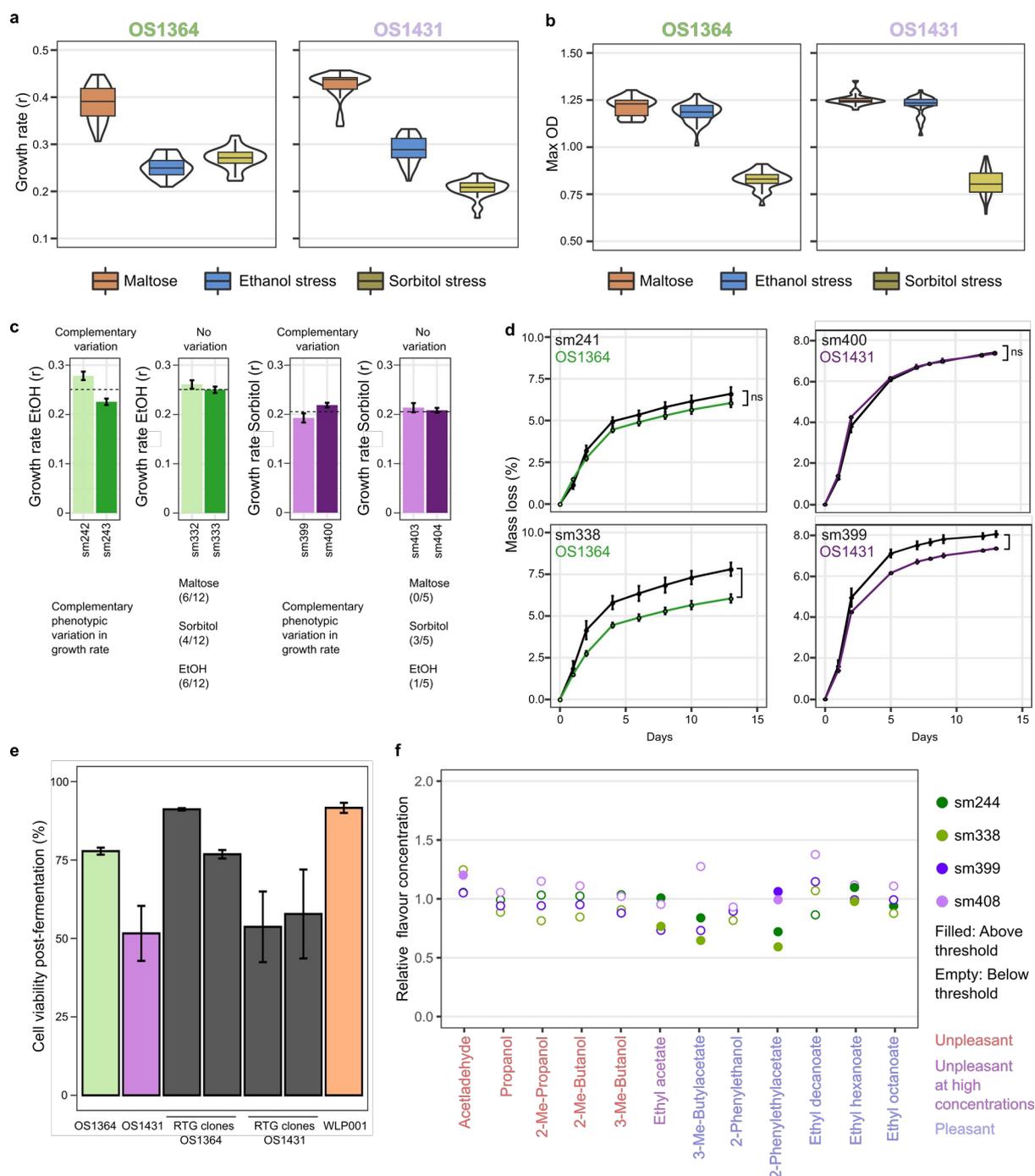


Fig 4 Fitness variability in industrial RTG variants | (a) Boxplots representing the variability of growth rate in the RTGs derived from OS1364 and OS1431. (b) Boxplots representing the variability of maximal optical density (OD) in the RTGs derived from OS1364 and OS1431. (c) Phenotypic diversification in sectored M-D pairs of OS1364 (green) and OS1431 (purple). The first bar plot represents an example where one sector of a M-D pair has a worsened phenotype but the other has an improvement compared to the average. The second bar plot shows an example where this variation is not detected. A similar example is reported on the left for OS1431. The dotted line represents the average phenotype in the library. On the bottom is reported the number of M-D pairs showing a complementary phenotypic variation. (d) Mass loss curves during the flask scale fermentation experiment of an RTG without variation (top) and an improved RTG (bottom) for OS1364 (green)

and OS1431 (purple). **(e)** Post fermentation viability measured after a 2L-fermentation in high gravity wort for the two parents, the respective RTG samples and a commercial strain. One RTG (sm244) derived from OS1364 showed increase viability. **(f)** Variability in the aroma profile across the four RTG samples in which the 2L scale fermentation was carried out.

In contrast, one RTG derived from OS1364 had a lower production of ethyl acetate (**Fig 4f, Fig S9c, Table S11**), that is compatible with recombination encompassing the gene *ATF2* involved in regulating this trait (**Table S8**). Moreover, the RTG derived from OS1364 showed diversified production of esters which may lead to a further differentiation of the sensory profile of the beer (**Fig 4f**).

Overall, our data demonstrate that RTG recombination in sterile polyploid strains can unlock phenotypic improvement in traits of industrial relevance (9), such as microbial stability or sensory quality of the product.

Discussion

In this work, we showed that RTG is an efficient approach to generate genetic and phenotypic diversity in two fundamentally different industrial sterile yeasts. Current approaches to improve industrial yeasts largely depends on designed targeted genetic modifications, facing market limitation and societal mistrust (9). The RTG approach coupled with the selection based on natural phenotypes does generate non-GMO yeast strains that can be unrestrictedly introduced to the market. Compared to other non-GMOs approaches such as serial transfer (24, 25), RTG induces random modifications as it does not select for a specific trait except for the genetic loci regulating the natural phenotype selected. Nevertheless, RTG has several benefits over the previous improvement strategies. We showed that the RTG process does not trigger genome instability in polyploid strains similar to previous results obtained in diploid hybrids (Mozzachiodi, Tattini et al.). This is in contrast with direct selection where the abundant number of generations often results in ploidy and chromosome copy number variation (14). Furthermore, trade-offs in unselected traits have been described as an undesired outcomes in adaptive evolution experiments (26, 27, 28), although some solutions have been proposed (29). We also observed RTG variants with impaired performances. However, we showed that often the RTG M-D pairs had worsened and improved fitness in the paired samples. Moreover, this result has another implication as it can aid in understanding the genetic architecture of complex industrial traits using large RTG libraries. Indeed, multiple deleterious trade-offs can be accumulated in RTG samples when the fraction of the genome that recombines is large. Nevertheless, one of the most recombined RTG did not show phenotypic decay in the phenotypic screenings performed; on the contrary, was more fit to the harsh fermentation conditions and had increased post-fermentative viability.

The RTG libraries produced are phenotypic agnostic and can harbour improved phenotypes useful for multiple applications as there is no selective pressure in RTG. The variability of “colony-associated-phenotypes” is an important parameter of the approach we devised for selecting M-D RTG recombinants, which might be limited by natural traits. However, a simultaneous screening of several environments could provide an effective solution to unveil variability in colony phenotypes. For instance, there are media which can trigger a morphological variation linked to relevant industrial traits such as the production of hydrogen sulfide detected in BiGGY agar plates (30). We envision that this approach can be easily automatized to quickly screen hundreds of colonies (31) in a very short time. Moreover, RTG is not a stand-alone approach as it can be integrated with other yeast improvement strategies. For instance, evolved mutants collected at the end of an adaptive evolution experiment could rescue generated trade-offs by evolving through RTG or the other way around RTG variants could be evolved through adaptive evolution to be further optimized. Furthermore, RTG can reshuffle also designed hybrid genomes as the only requirement is that the hybrid can enter meiosis and progress until prophase, even if with a low efficiency.

In conclusion, we propose that the RTG technology represents a novel avenue to induce genetic and phenotypic variability in industrial sterile yeasts generating RTG variants which can easily reach every market and can also enhance the understanding of complex genetic traits in industrial strains.

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S.M. contribution: supervised the project; performed the experiments and data analysis, wrote the draft paper.

Supplementary Materials

Materials & Methods

Detection of deleterious, missense mutations and pre-existing LOHs

Short reads of the parental strains were obtained from the 1011 yeast project (1) and mapped against the SGD reference using bwa-mem algorithm. Single nucleotide variants were called using Freebayes (v1.3.1-19) with appropriate ploidy set with the argument “-p” and quality selected to be higher than 20. The vcf generated was used to annotate impactful mutations using the Variant Effect Predictor (VEP) suite (2) and the impactful mutations used in the analysis were selected among those with a predicted “HIGH” impact. Variants in the subtelomeres or predicted as frameshift were filtered out. The list of essential genes used for the analysis was obtained from (3). The missense mutations were obtained from the same analysis. Pre-existing LOHs in the strains genome were identified as regions of 50 kbp in which 10 or less heterozygous markers were found. The plots were generated using ggplot2 and in-house R scripts.

Long read sequencing and HGT identification

Yeast cells were grown overnight in liquid YPD media (1% yeast extract, 2% peptone, 2% glucose). Genomic DNA was extracted using Qiagen Genomic-Tips 100/G according to the manufacturer's instructions. The MINION sequencing library was prepared using the SQK-LSK108 sequencing kit according to the manufacturer's protocol. The library was loaded onto a FLO-MIN106 flow cell and sequencing was run for 72 hours. We performed long read basecalling and scaffolding using the pipeline LRSDAY (4) however the canu assembler mostly merged the different haplotypes and thus was not possible to produce long read phased haplotypes. The dotplots were generated using mummerplot (5). The annotated non-reference regions were extracted from the fasta of the assembled genome and blasted using the application blast from NCBI <https://www.ncbi.nlm.nih.gov/> with “blastn” against the database of ascomycetes.

Sporulation monitoring and assessing spore viability

Yeast strains stored in glycerol stock at -80°C were patched in YPD (1% Yeast extract , 2% Peptone, 2% Dextrose) plates and incubated at 30°C for 24 hours. From the patch a streak for single was done and incubated at 30°C for 48 hours, then single colonies were taken and incubated in 10 mL of

liquid YPD and incubated at 30°C in a shaking incubator at 220 rpm overnight. The following day single colonies were taken for inoculating different tubes containing 10 mL of SPS (1% Peptone, 1 %, Potassium acetate, 0.5% Yeast extract, 0.17% Yeast nitrogen base, 0.5% Ammonium sulfate, 1.02%

Potassium biphthalate) as pre-sporulation and kept for 24 hours at 30°C with shaking at 220 rpm. The single tubes were then centrifuged and washed three times with sterile water and the cells were then resuspended in 25 mL of KAc 2% at a final OD of 1 in 250 mL erlenmeyer flasks and incubated at 23°C with shaking set at 220 rpm. Spores were withdrawn from the sporulated cultures and incubated between 30-60 minutes in 100 uL of zymolyase solution in order to perform spore dissection. At least 400 spores per sample were dissected on YPD plates which were incubated at 30°C for 4 days. Spore viability was assessed as the number of spores forming colonies growing at 4 days.

Plasmid engineering and genome editing with CRISPR/Cas9

The multi-deletions of *URA3*, *NDT80* and *SPO11* was engineered by using CRISPR/Cas9 genome engineering. The plasmid harbouring Cas9 was obtained from Addgene pUDP004 (6) and linearized with BsaI. The resistance to acetamide was replaced with the resistance cassette to Kanamycin. The gRNA with the necessary nucleotide for self-cleavage was designed on UGENE (7) and ordered as a synthetic oligo from Eurofins Genomics (TM). The synthetic oligo was cloned within the backbone using the Gibson assembly kit (NEB, Gibson Assembly®) and the ligation reaction was carried out for 1 hours at 50°C. Then, the assembled plasmid was transformed into DH5-alpha competent bacteria by heat shock and the bacteria were incubated in 3 mL of LB broth for 1 hour to induce the synthesis of the antibiotics and then plated on plates containing 100 µg/uL of ampicillin. The following day cells were screened by polymerase chain reaction (PCR) using primers to validate the correct assembly of the construct and selected bacterial colonies were inoculated in LB broth with 100 µg/uL of ampicillin for extracting the plasmid. The cells were harvested from the overnight incubation and the plasmid was extracted using the QIAprep Spin Miniprep Kit following the manufacturers instructions.

The 120 bp cassettes used for the deletion of *URA3*, *NDT80* and *SPO11* were designed to be flanking 60 bp upstream and downstream the candidate gene and ordered as a unique synthetic oligo at Eurofins. The forward and reverse cassettes were mixed at equimolar ratio heated at 95 °C for 15 minutes and then cooled down at room temperature to be ready for the transformation. The

7.1 Supplementary Materials

samples were transformed following the protocol from (6) and between 1-15 ug of cassette together with at least 200 ng of CRISPR/Cas9 plasmid were transformed. Cells were then plated on selective media containing kanamycin and incubated at 30°C for 3 - 7 days. Candidates transformed clones were validated by PCR using primers designed outside the deleted genes in order to evaluate the complete deletion of all the copies. Validated clones were streaked for single on YPD, grown for 2 days at 30°C, monitored for plasmid loss by plating again the single colonies in the selective media and good colonies were patched on YPD and stored at -80 °C in 75% glycerol tubes.

RTG selection by *URA3*-loss assay

URA3 knock-out OS1364 and OS1431 were transformed with the classical lithium acetate protocol for replacing with a *URA3* cassette the *LYS2* gene on chromosome II. The correct insertion was validated by checking for restored prototrophy on synthetic media lacking uracil and by PCR and positive clones were stored at -80 °C in 75% glycerol tubes. For performing the *URA3*-loss assay, cells from the frozen stocks were patched on YPD plates and incubated at 30°C for 2 days. After the patch was grown cells were streaked on plates lacking uracil and incubated at 30°C for 2 days. Single colonies were picked and sporulation was induced following the protocol described above for the wild-type strains. Cells from the sporulation cultures were taken at different time points washed with YPD three times and incubated in YPD for 12 hours at 30°C without shaking. Dilutions were spotted from the YPD liquid culture onto YPD plates, and an appropriate dilution for each strain was plated on 5-FOA plates. The plates were incubated at 30°C for 2 days and colonies formed on 5-FOA plates were counted for all the time points.

RTG selection by natural phenotypes

Sporulation of wild-type hybrids was induced as described in “Industrial yeast sporulation monitoring” for a time window compatible with RTG. Cells were withdrawn from the sporulation media before the appearance of MI cells measured by DAPI staining to do not plate committed meiotic cells. At the given time point cells were shifted from sporulation media to liquid YPD (1% yeast extract, 2% peptone, 2% glucose) and kept incubated between 2 - 3 hours to induce RTG but not budding or kept longer until complete budding and then were plated on modified YPD media (YPD0.5 : 1% Yeast Extract, 2% Peptone, 0.5% Dextrose. YPD1 : 1% Yeast Extract, 2% Peptone, 1% Dextrose.) and the plates were incubated at 30 °C and monitored daily for colony formation and variation in the morphology. Mother-daughter RTG pairs were selected from a single colony having

two sectors with different morphology of which one resembled the wild-type phenotype whereas the other was divergent. Cells from each sector were taken with a wooden stick and streak on the respective modified YPD to avoid any contamination from cells deriving from the other sector and incubated at 30°C for 2 days. Then a single colony was taken, patched on YPD and incubated at 30 °C for 2 days and finally stored in 75% glycerol tubes at -80 °C. Pictures of the sectoring colonies on the plates were taken with a stereomicroscope Discovery v.8 Zeiss. The scale bar was added to the pictures using the software ImageJ converting the known size of the colonies to pixel and then added using the option “Analyze/Tools”.

Genome analysis of parental strains and LOH detection in RTG samples

Short reads of the sequenced samples were mapped to the reference genome S288C generated by long-reads sequencing (8) using the bwa-mem algorithm. Mapped reads were then processed with SAMtools and the optical duplicates of the sequencing were removed using “samtools rmdup”, the deduplicated BAM files were indexed and coverage was extracted using “samtools index and depth”. Coverage along the chromosomes was plotted using in-house R script in which sliding windows of non overlapping 10000 bp were used to calculate a local average coverage which was normalized with the median coverage and the \log_2 profile was plotted to identify aneuploidies and large CNVs. For detecting smaller CNVs the median coverage was calculated again as before for sliding windows of non overlapping 1000 bp. From these windows, regions showing a CNV compared to the chromosome copy number were collected and matched with the previously detected CNVs in larger windows and used for LOH filtering. Furthermore, CNVs were tagged as subtelomeric if they were contained in the left or right subtelomere for each chromosome and only those not contained in subtelomeric, tagged as core, were used to filter LOH regions as subtelomeres are filtered during LOH identification. CNVs in RTG samples were analyzed with the same approach and plot of coverage profile genome-wide were manually inspected to detect artifacts due to “smiley pattern” (9) or to lower coverage of only small chromosomes. Following, vcf were generated by mapping short-reads of the parental strains to the S288C reference generated with long-reads using Freebayes (v1.3.1-19) with the argument “freebayes -p” to set variant calling with the appropriate ploidy. The parental vcf were then filtered to include only “SNP” markers with a quality higher than 20 and a depth higher than 10 using bcftools command “TYPE=snp, QUAL > 20, DP > 10”. The filtered parental vcf was then indexed with bgzip and tabix. To identify LOHs in the evolved RTG we used a list of markers generated by taking into account all the heterozygous

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positions between the reference strain and the parental genomes identified as described above. The calling of variants on the evolved RTG and control clones was done using freebayes with the argument `-p` for setting the appropriate ploidy “`-@`” to call variants at the previously identified heterozygous positions and additional settings for minimum depth, quality and avoid calling complex variants “`-m 30, -q 20 -i -X -u`”. The vcf generated were filtered with bcftools taking only “SNP” variants. As a further control the parental and samples “.vcf” files were intersected using bedtools “`-intersect`” and variants with shared positions were extracted from the evolved samples vcf. Following, using an in house R script the alternative and reference allele frequencies of each marker in the evolved samples were compared with the parental ones and when an allele frequency shift was detected in either direction the marker was tagged as laying in a recombined region. Moreover, the genotype of the marker tagged as being in an LOH region was compared with the known genotype of the marker present in the ancestral strain and those not matching the expected allele were filtered out. Given the complexity of polyploid analysis we decided to use a stringent threshold for filtering LOH regions in order to avoid calling false negative LOHs. LOH regions of at least 10 consecutive recombined markers were selected as candidate true positives. Among those, LOH events sharing more than 70% of their length between at least 70% of the samples were filtered out and considered false positives. The RTG selected by the *URA3*-loss assay were not filtered on chromosome II where we inserted the selection marker, but that chromosome was not counted for calculating the percentage of genome covered by LOH. Moreover, LOHs present in the parental samples engineered with the *LYS2/URA3* system or in which *NDT80* was deleted were removed from the respective derived samples. Plot of the LOH events were done using an in-house R script implemented with ggplot2 (v 3.6.1) which takes into account the genotype shift of each marker called in the LOH blocks.

Inferring the parental haplotypes from the mother-daughter RTG pairs

One M-D RTG pair derived from OS1364 was used as a proof of concept to phase a region of recombination on the left arm of chromosome IX which is likely resulted from a cross-over between two copies of chromosome IX. The genotype of each heterozygous marker was inferred based on the allele frequency shift (AF) in the M-D pair. In the recombination event when an AF shift toward the alternative allele was detected, that is the RTG has gained on more alternative alleles the genotype of the reference allele was assigned as the ancestral for the haplotype. The same approach was followed when an AF shift toward the reference allele was detected. The reconstructed two

haplotypes were validated on a second RTG M-D pair having an event spanning the same region. Then, the information of this reconstructed haplotypes was used to infer the third haplotype based on the allele dosage, that is the number of “Ref” or “Alt” copies estimated for each heterozygous marker.

Microplate cultivations

The osmotic stress and ethanol tolerance was assessed with microcultures in media containing 25% (w/v) sorbitol and 8% (v/v) ethanol, respectively. The microcultures were carried out in 100-well honeycomb microtiter plates at 25 °C (with continuous shaking), and their growth dynamics were monitored with a Bioscreen C MBR incubator and plate reader (Oy Growth Curves Ab, Finland). The wells of the microtiter plates were filled with 300 µL of YPM medium (1% yeast extract, 2% peptone, 1% maltose) supplemented with sorbitol (25%) and ethanol (8% v/v). Control cultivations in media without sorbitol or ethanol were also carried out. Precultures of the strains were started in 20 mL YPM medium and incubated at 25 °C with shaking at 120 rpm overnight. We measured the optical density at 600 nm, and pre-cultures were diluted to a final OD₆₀₀ value of 3. The microcultures were started by inoculating the microtiter plates with 10 µL of cell suspension per well (for an initial OD₆₀₀ value of 0.1) and placing the plates in the Bioscreen C MBR. The optical density of the microcultures at 600 nm was automatically read every 30 min. Four replicates were performed for each strain in each medium. Growth curves for the microcultures were modelled based on the OD₆₀₀ values over time using the ‘GrowthCurver’-package for R (10).

Flask-scale very high gravity wort fermentations

50 mL-scale fermentations were carried out in 100 mL Schott bottles capped with glycerol-filled airlocks. Yeast strains were grown overnight in 25 mL YPM medium at 25 °C. The pre-cultured yeast was then inoculated into 50 mL of 32 °P wort made from malt extract (Senson Oy, Finland) at a rate of 7.5 g fresh yeast L⁻¹. Fermentations were carried out in duplicate at 20 °C for 15 days. Fermentations were monitored by mass lost as CO₂. The alcohol content of the final beer was measured with an Anton Paar density meter DMA 5000 M with AlcoLyzer beer ME and pH ME modules (Anton Paar GmbH, Austria).

2-L scale high-gravity high gravity wort fermentations

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Strains were characterized in fermentations performed in a 20 °P high gravity wort at 20°C. Yeast was propagated essentially as described previously (11), with the use of a “generation 0” fermentation prior to the actual experimental fermentations. The experimental fermentations were carried out in triplicate, in 3-liter cylindroconical stainless steel fermenting vessels, containing 2 liters of wort medium. The 20 °P wort (98 g of maltose, 34.7 g of maltotriose, 24 g of glucose, and 6.1 g of fructose per liter) was produced at the VTT Pilot Brewery from barley malt and malt extract (Senson Oy, Finland). Yeast was inoculated at a rate of 5 g fresh yeast L⁻¹ (corresponding to approximately 15 × 10⁶ viable cells · ml⁻¹). The wort was oxygenated to 10 mg · liter⁻¹ prior to pitching (oxygen indicator model 26073 and sensor 21158; Orbisphere Laboratories, Switzerland). The fermentations were carried out at 20°C until the alcohol level stabilized, or for a maximum of 15 days. Wort samples were drawn regularly from the fermentation vessels aseptically and placed directly on ice, after which the yeast was separated from the fermenting wort by centrifugation (9,000 × g, 10 min, 1°C). Samples for yeast-derived flavor compound analysis were drawn from the beer when fermentations were ended.

Chemical analysis of fermentable sugars

The concentrations of fermentable sugars (maltose and maltotriose) were measured by HPLC using a Waters 2695 separation module and Waters system interphase module liquid chromatograph coupled with a Waters 2414 differential refractometer (Waters Co., Milford, MA, USA). A Rezex RFQ-Fast Acid H⁺ (8%) LC column (100 × 7.8 mm; Phenomenex, USA) was equilibrated with 5 mM H₂SO₄ (Titrisol, Merck, Germany) in water at 80°C, and samples were eluted with 5 mM H₂SO₄ in water at a 0.8 ml · min⁻¹ flow rate. The alcohol level (% vol/vol) of samples was determined from the centrifuged and degassed fermentation samples using an Anton Paar density meter DMA 5000 M with Alcolyzer beer ME and pH ME modules (Anton Paar GmbH, Austria). Yeast-derived higher alcohols and esters were determined by headspace gas chromatography with flame ionization detector (HS-GC-FID) analysis. Four-milliliter samples were filtered (0.45 μm pore size) and incubated at 60°C for 30 min, and then 1 ml of gas phase was injected (split mode, 225°C, split flow of 30 ml · min⁻¹) into a gas chromatograph equipped with an FID detector and headspace autosampler (Agilent 7890 series; Palo Alto, CA, USA). Analytes were separated on a HP-5 capillary column (50 m by 320 μm by 1.05 μm column; Agilent, USA). The carrier gas was helium (constant flow of 1.4 ml · min⁻¹). The temperature program was 50°C for 3 min, 10°C ·

min⁻¹ to 100°C, 5°C · min⁻¹ to 140°C, 15°C · min⁻¹ to 260°C, and then isothermal for 1 min. Compounds were identified by comparison with authentic standards and were quantified using standard curves. 1-Butanol was used as an internal standard.

Chemical analysis of aroma compounds in fermented beers

Yeast-derived higher alcohols and esters were determined by headspace gas chromatography with flame ionization detector (HS-GC-FID) analysis. Four-milliliter samples were filtered (0.45 µm pore size) and incubated at 60°C for 30 min, and then 1 ml of gas phase was injected (split mode, 225°C, split flow of 30 ml · min⁻¹) into a gas chromatograph equipped with an FID detector and headspace autosampler (Agilent 7890 series; Palo Alto, CA, USA). Analytes were separated on a HP-5 capillary column (50 m by 320 µm by 1.05 µm column; Agilent, USA). The carrier gas was helium (constant flow of 1.4 ml · min⁻¹). The temperature program was 50°C for 3 min, 10°C · min⁻¹ to 100°C, 5°C · min⁻¹ to 140°C, 15°C · min⁻¹ to 260°C, and then isothermal for 1 min. Compounds were identified by comparison with authentic standards and were quantified using standard curves. 1-Butanol was used as an internal standard.

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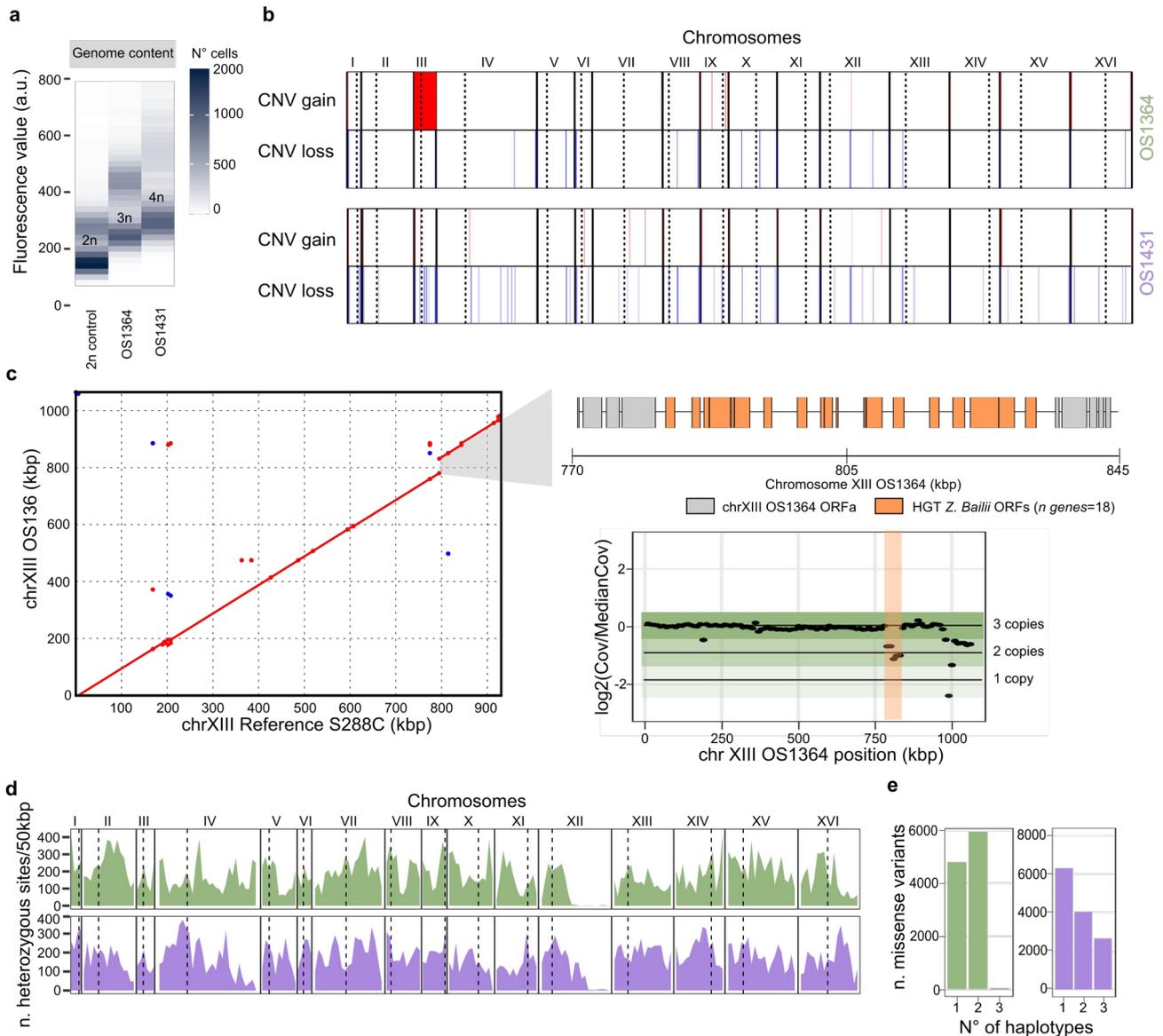
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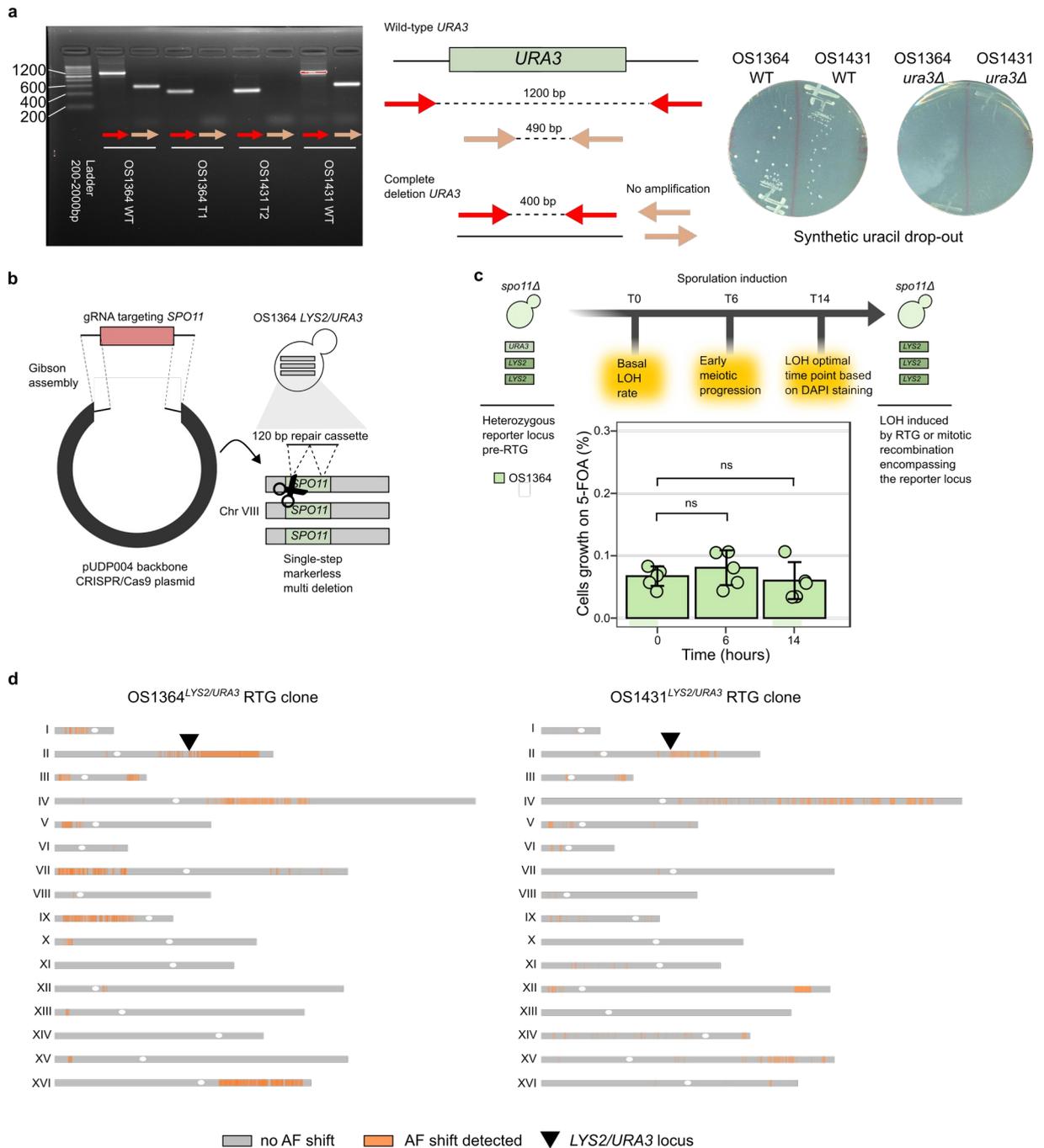
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SFig 1 Genome characterization of OS1364 and OS1431 | (a) Genome content estimated as the intensity of fluorescence (y-axis) per each industrial hybrid compared to a diploid control strain (b) On the left: dotplot of the alignment of OS1364 chromosome XIII (y-axis) against chromosome XIII of S288C (x-axis). On the right: highlighted the region present on the HGT derived from *Z. bailii* with on the bottom highlighted in orange the copy number of this region compared to the copy number of the chromosome. (c) Distribution of heterozygous markers genome-wide calculated on a window of 50 kbp. The dotted lines represent centromeres. (d) Barplots representing the number of missense variants (y-axis) based on the number of haplotype on which they are found. Missense variants found on 3 haplotypes in OS1364 are related to chromosome III. (e) Copy number variations (CNVs) detected in the two genomes with chromosome III CNVs manually merged.

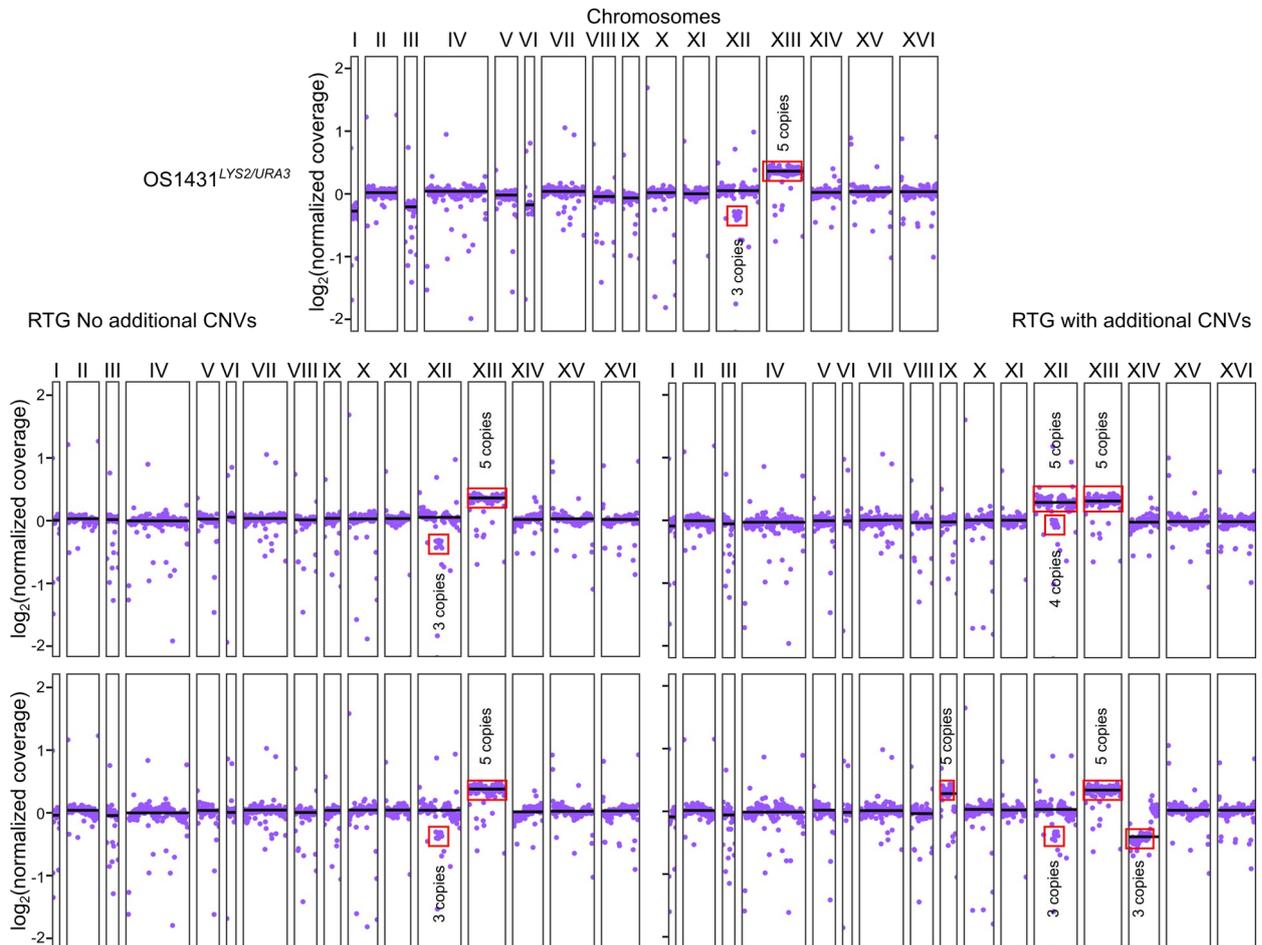


SFig 2 *URA3*-loss assay engineering, testing and outcome in polyploid hybrids | (a) Validation by PCR of the deletion of *URA3* on its native location on chromosome V with reported the example of the validation for both OS1364 and OS1431. The deletion of the gene by homologous-recombination with repair cassette flanking the gene produces a deletion which can be visualized by PCR as a band of reduced size using primers binding outside the gene (red) and by the absence of amplification of the gene with internal primers (brown). (b) The same strategy used for deleting *URA3* was used for deleting *SPO11* in OS1364 (c) *URA3*-loss assay performed with the mutant strain of OS1364^{*LYS2/URA3*, *spo11Δ*}. We did not detected increase in LOH rate neither at T6 (Wilcoxon

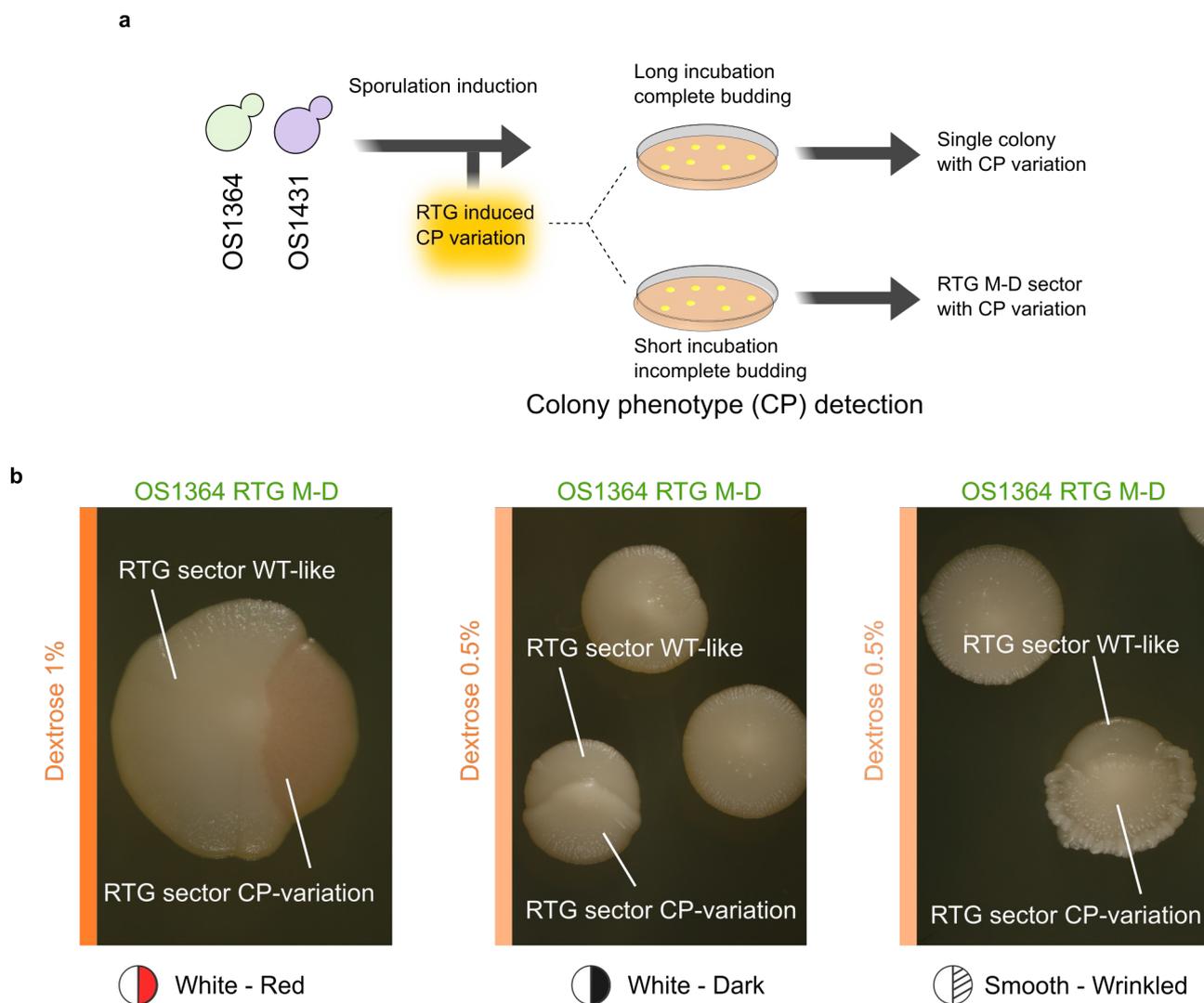
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rank-sum test, one tailed, p -value > 0.05) not at T14 (Wilcoxon rank-sum test, one tailed, p -value > 0.05) compared to the initial LOH rate **(d)** LOH map of two RTGs selected on 5-FOA in which are highlighted identified LOH regions with highlighted the region bearing the selection marker.

a

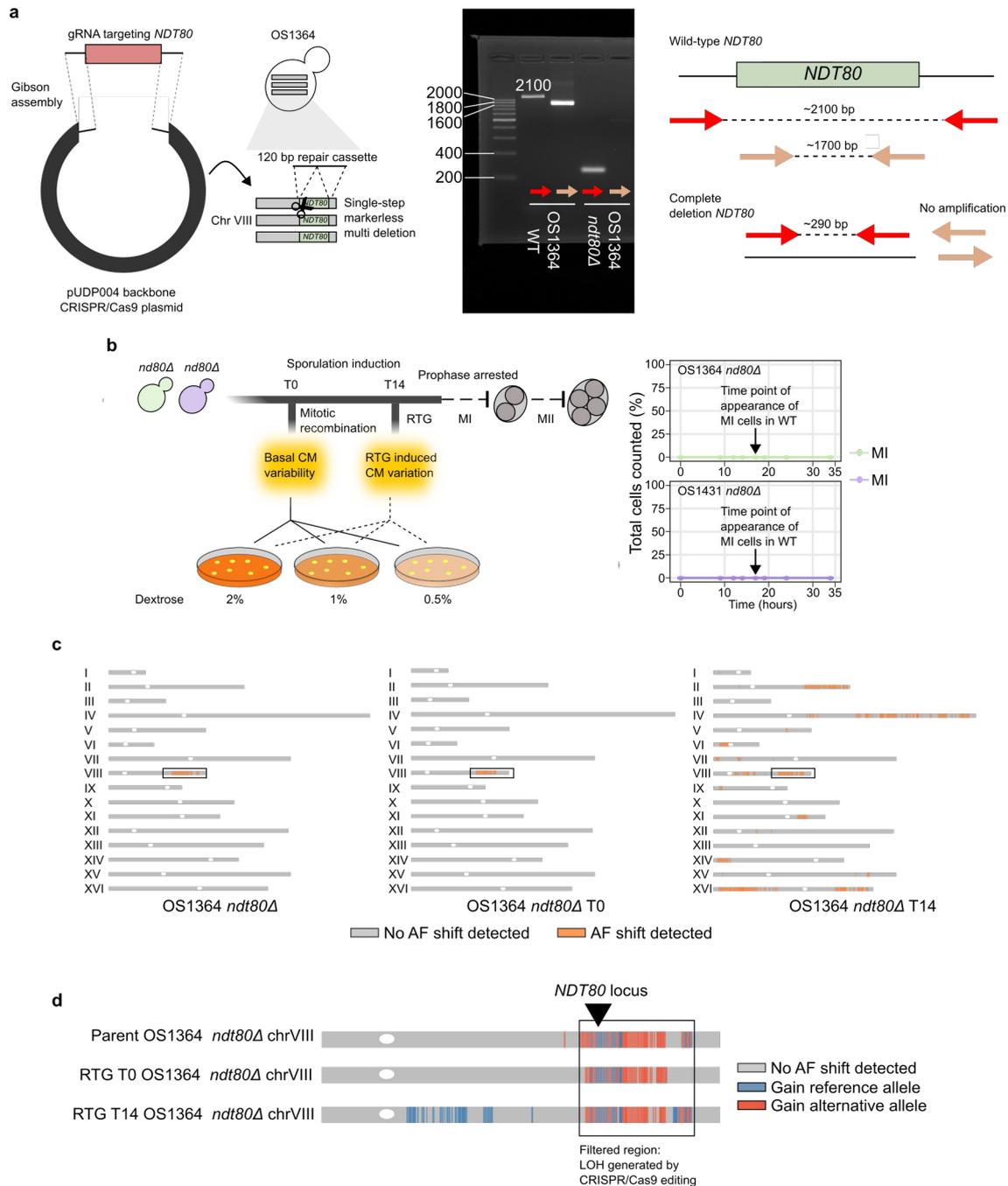


SFig 3 Genome stability induced by CRISPR/Cas9 in RTG derived from OS1431^{LYS2/URA3} | (a) Genome wide coverage plots of OS1431^{LYS2/URA3} and derived RTGs. On the top is reported the coverage profile of the engineered OS1431^{LYS2/URA3} with highlighted the existing copy number variations or aneuploidies which were not present in the initial strains. On the bottom: RTG derived samples selected on 5-FOA without additional CNVs (left) or with additional aneuploidies (right). The black lines represent the median coverage of each chromosome.



SFig 4 Identification of RTG M-D sector phenotypes | **(a)** Different plating times allowed to retrieve RTG M-D sectors. If cells are kept in rich media to induce RTG and complete budding the M-D RTG cells will bud and can be found as a single colony on the plate. However, if cells are left in rich media and plated before complete budding, RTG M-D can be retrieved as a colony with two sectors having diverged phenotypes. **(b)** Example of the three phenotypic classes of RTG M-D used for isolating RTG candidates in WT and *ndt80Δ* genetic backgrounds.

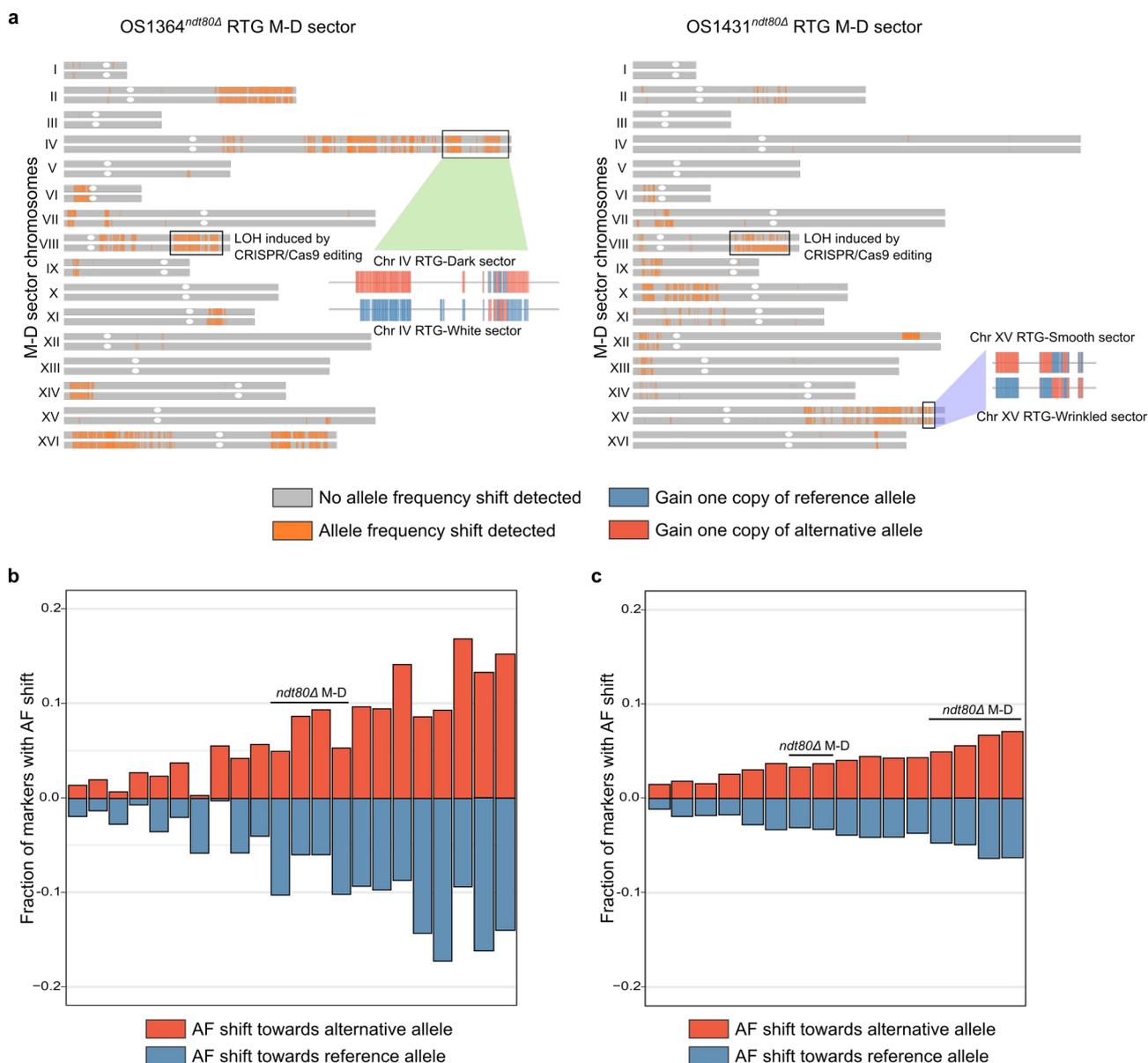
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SFig 5 Genome engineering to generate *NDT80* mutants in OS1364 and OS1431 backgrounds

| **(a)** Genome engineering to remove *NDT80* followed the same strategy used for removing *URA3* reported on Sfig.2. A validation example is reported for OS1364 following the same rationale used for *URA3* and *SPO11* deletions. **(b)** (Left) The *ndt80Δ* strains were evolved following the same RTG protocol used for the WT strains. (Right) The block in prophase was monitored by DAPI staining that confirmed absence of cells passing the first meiotic division (MI) even at later time points. **(c)** Three maps of recombination events are reported for *ndt80Δ* RTG of OS1364 with highlighted the LOH encompassing the *ndt80* locus on chromosome VIII. **(d)** Zoom-in on the

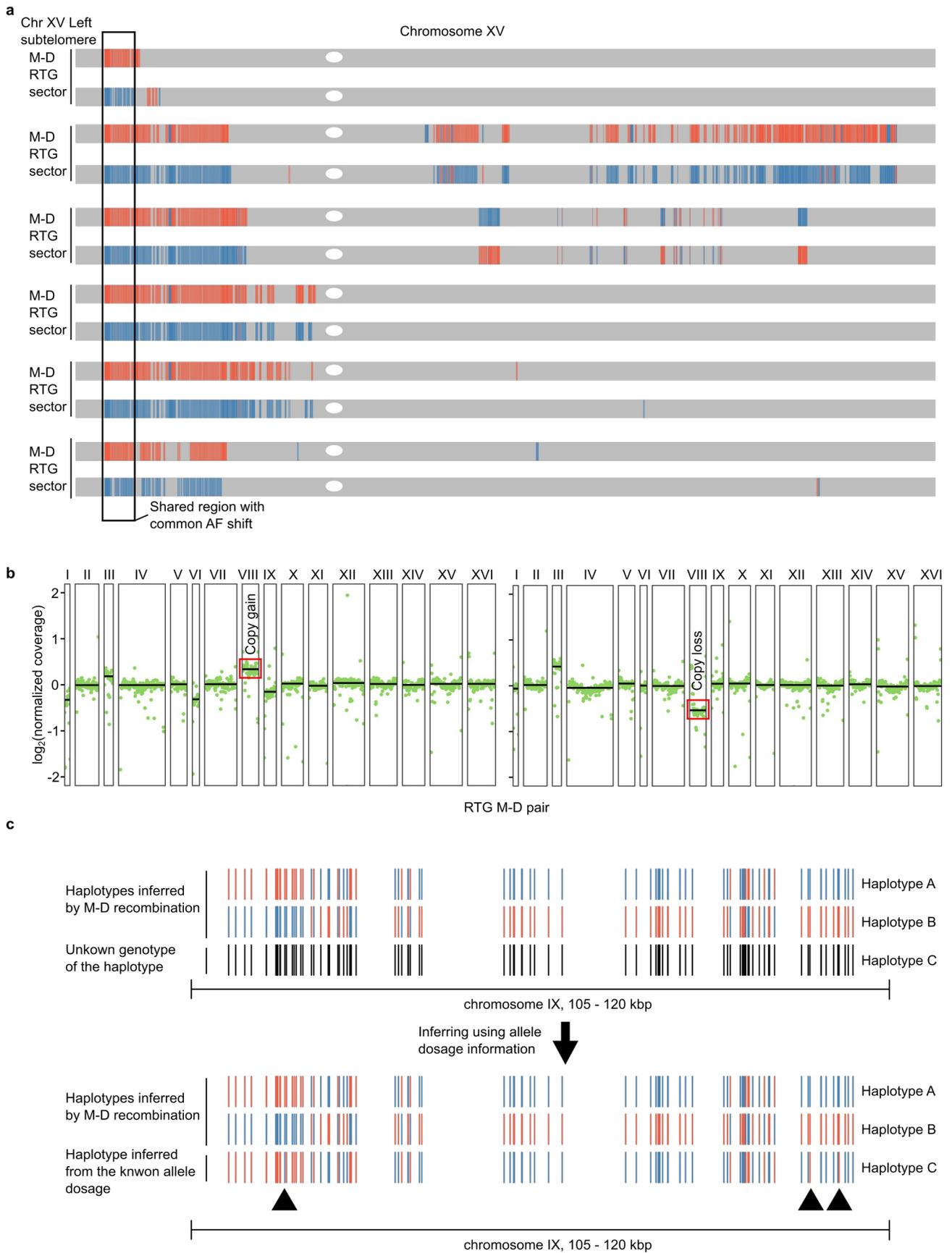
chromosome VIII recombination events of the samples reported above which share the common LOH generated upon genome-editing and present in all three samples.



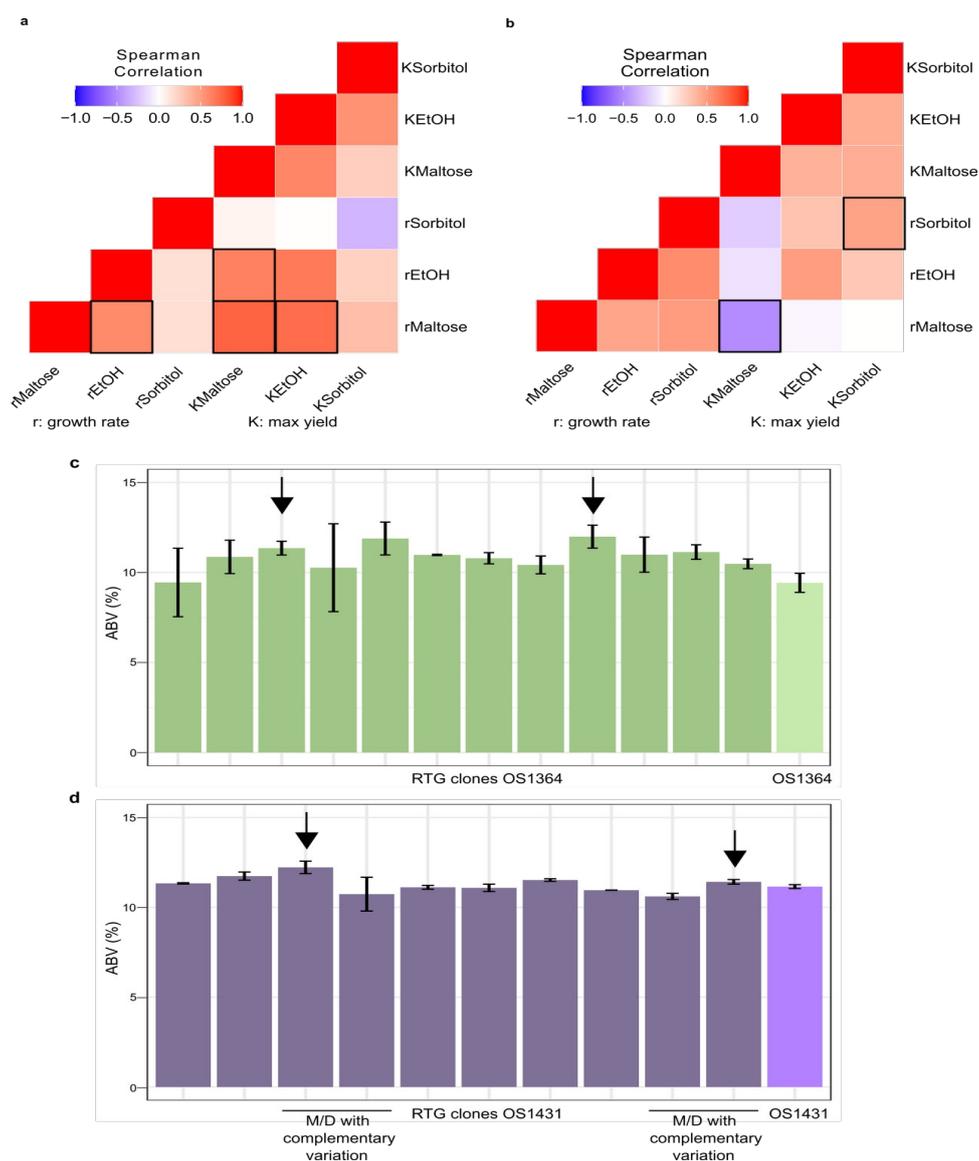
SFig 6 Recombination profile in RTG M-D sectors derived from WT and *ndt80Δ* strains | (a) LOH map of two M-D *ndt80Δ* RTG sectors derived from OS1364 and OS1431 with a zoom-in on two recombination events where the direction of allele frequency (AF) shift is reported for both RTG sectors. (b) Fraction of markers in which we detected an AF shift toward an increase of alternative allele (red) or parental allele (blue) as a result of recombination in OS1364 M-D RTG sectors. Each M-D RTG sector pair is reported sequentially from right to left. Each bar represents one RTG sample of the pair. We removed from the count the region in which recombination was induced by CRISPR-Cas9. (c) Fraction of markers in which we detected an AF shift toward an

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increase of alternative allele (red) or parental allele (blue) as a result of recombination in OS1431 M-D RTG sectors. Each M-D RTG sector pair is reported sequentially from right to left. Each bar represents one RTG sample of the pair. We removed from the count the region in which recombination was induced by CRISPR-Cas9.

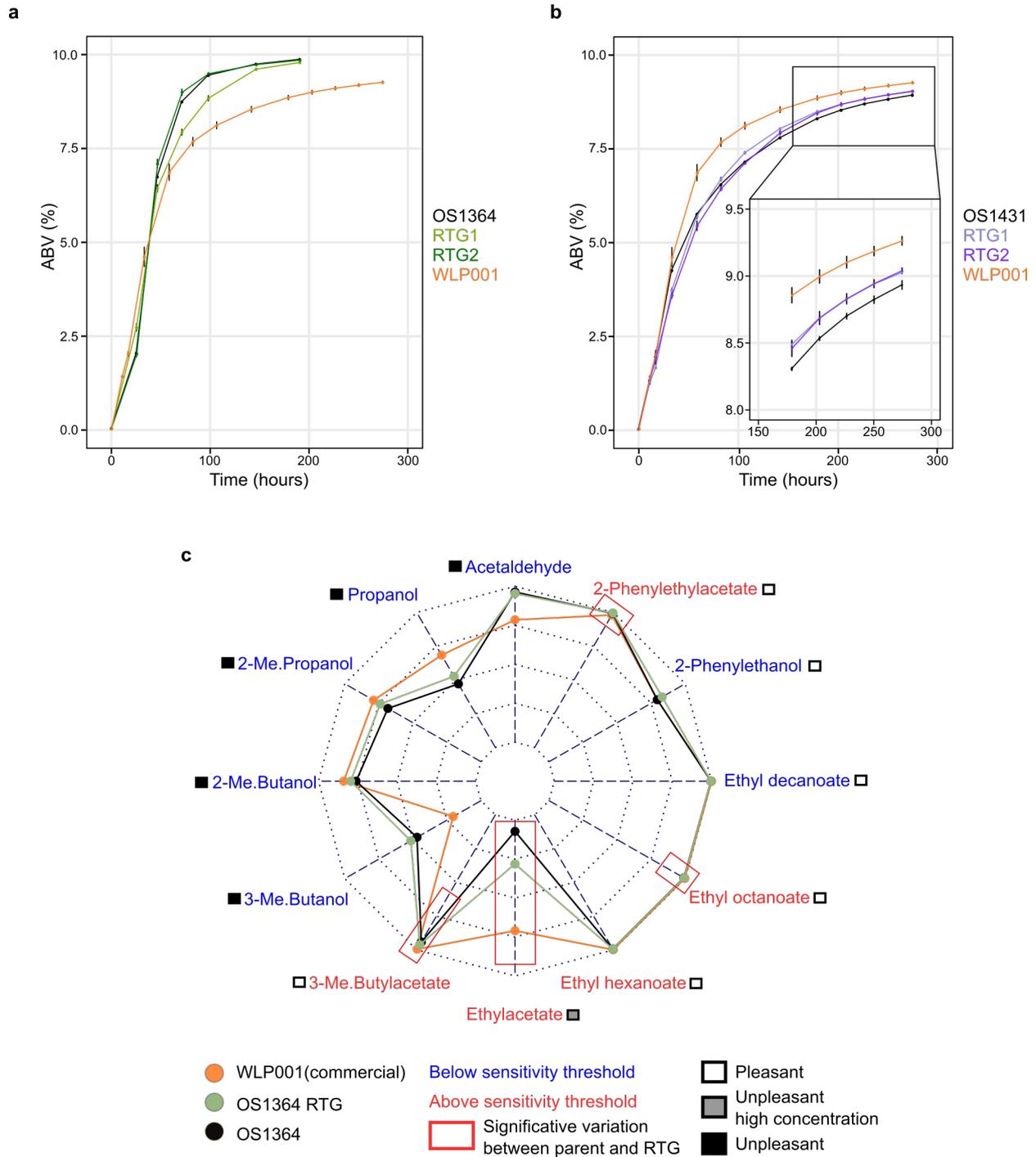


SFig 7 Analysis of RTG sector M-D pair recombinations | **(a)** Chromosome XV recombination pattern reported as in other figures where the colors represent a shift of the genotype of the heterozygous position toward the reference (blue) or alternative (red) allele. The rectangle highlight a shared region of recombination in five distinct RTG sector M-D pairs with the same sectoring phenotype (dark-white). **(b)** Genome wide coverage profiles with highlighted complementary gain (left) and loss (right) of chromosome VIII in a mother daughter RTG-sector isolated from OS1364, each plot represents one of the two samples. The black lines represent the median coverage of each chromosome. **(c)** Example of local haplotype reconstruction based on the recombination profile of chromosome VII of an RTG sector M-D pair (top), which is used to infer the third haplotype using the information of allele dosage for each heterozygous marker (bottom). The color code is the same used above, red represent a gain of an alternative allele whereas blue represents gain of a reference allele.



SFig 8 Phenotypic correlation and diversification analyses | **(a)** Spearman correlation between phenotypic performances in stressor or maltose conditions measured in OS1364 RTGs derived from the WT OS1364. The significant associations were calculated using Bonferroni-correction for multiple correlations and are enclosed in a black square. **(b)** Spearman correlation between phenotypic performances in stressor or maltose conditions measured in OS1431 RTGs derived from the WT OS1431. The significant associations upon Bonferroni correction for multiple comparisons are enclosed in a black square. **(c)** Bar plots representing alcohol by volume (ABV%) produced at the end of the flask-scale fermentation experiment in OS1364 RTG clones. The black arrows point to the samples selected for the 2L scale fermentation. The standard deviation is calculated from two replicates **(d)** Bar plots representing Alcohol by volume (ABV%) produced at the end of the flask-scale fermentation experiment in OS1431 RTG clones. The black arrows point to the samples selected for the 2L scale fermentation. The standard deviation is calculated from two replicates

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SFig 9 2L fermentation kinetics and sensory quality of the end product | (a) Fermentation profile of two RTGs (RTG1 = sm244, and RTG2 = sm338) derived from OS1364. On the y-axis is reported the alcohol by volume (ABV%) measured at different time points. WLP001 is the reference commercial strain. (b) Fermentation profile of two RTGs (RTG1 = sm399, and RTG2 = sm408) derived from OS1431. On the y-axis is reported the alcohol by volume (ABV%) measured at different time points. WLP001 is the reference commercial strain. (c) Web chart representing the aroma profile of the beer fermented by sm338. Significant variations that cannot be spotted by eye

are due to low sensory threshold (mg/L) compared to other flavours. The data relative to them are reported in Table S11. Aromas below (blue) or above (red) sensory threshold are color coded. The red squares highlight significant differences which are above the sensory threshold.

8. Conclusions and future perspectives

In this work we have shown that return-to-growth (RTGs) is an unconventional sexual cycle which molds hybrid genomes to different extents and offers an alternative pathway of evolution to sterile *Saccharomyces* yeasts. Moreover, we have shown that different strains derived from both wild and industrial environments can perform RTG supporting the hypothesis that this process is evolutionary conserved in other *Saccharomyces* strains and at least in *S. paradoxus* concluding that its impact into genome evolution might have been overlooked so far.

8.1 RTG in sterile diploid hybrids: an alternative route to genome recombination and phenotypic diversification

The first question we wanted to address was to what extent RTG can bypass common sterility barriers in the *Saccharomyces* genus. Here, by reanalysing RTG samples derived from a sterile strain lacking a key meiotic gene we confirmed evidence of RTG recombination. Thus, strains with defective middle and late meiotic genes may still recombine their genomes through RTG although they cannot complete meiosis. Indeed in a recent population genomic study, lineages with a defective *NDT80* gene or more late meiotic genes have been described (De Chiara et al. 2020). Moreover, we further expanded the paradigm of RTG to other hybrid genetic backgrounds with variable degrees of sterility whose parents were isolated from different ecological niches, suggesting that capacity to perform RTG is common across diverse *S. cerevisiae* isolates. As expected, the ease and efficiency with which strains performed RTG was linked with their meiotic progressions. Fast sporulator genetic backgrounds were more prone to enter RTG early, whereas other strains failed to do so. Then, we showed that a sterile intraspecies hybrid, with long stretches of non-structural homology between its subgenomes, can also evolve through RTG. Surprisingly, the level of recombination that we detected was similar to those found in an intraspecies hybrid with colinear subgenomes. However, recombination between non structural matching regions produced a massive copy-number-variation due to the loss of one chromosomal arm derived from one parental subgenome, which was replaced by the chromosomal arm of the respective homolog. Generally, clones bearing these rearrangements were unfit in several environments, however in few environments, the chromosomal arm CNV was beneficial supporting the scenario that increased gene dosage of specific genes might be helpful. Interestingly, we found that the signature of RTG

8. Conclusions and future perspectives

recombination is highly consistent to previous published meiotic recombination and Spo11p hotspots (Mancera et al. 2008, Pan et al. 2011) in both the intraspecies hybrids used in this work. Furthermore, the LOH breakpoints were more frequent in regions with low sequence diversity. On the same line, RTG recombination was less efficient in more diverged subgenomes, and local islands of sequence homology were driving the formation of LOH. As in meiosis (Hunter et al. 1996), we found that the mismatch machinery repair induced an antirecombination barrier that decreased the LOH formation during RTG. Finally, we showed that RTG could create a genetic understanding of complex traits in a sterile intraspecies hybrid. In this case, the main obstacle to the mapping of the quantitative-trait-loci (QTL) was the degree of recombination detected in the RTG library. The level of recombination also affected the degree of resolution of the QTLs. Nevertheless, it provided a novel way to study complex traits in sterile lineages, where the classical linkage analysis using haploid segregants is not feasible.

Further questions stem from this work. Hybrids between species of the *Saccharomyces* genus do not limit to *S. cerevisiae* and *S. paradoxus*, in fact, hybrids between different populations of *S. paradoxus*, between *S. eubayanus* and *S. uvarum*, or triple hybrids have been found (Morales and Dujon 2012). Is it possible that these diverse hybrids also recombine (or have recombined) through RTG? Indeed our work showed that the antirecombination is still active during RTG; thus, it is likely that diverged hybrids would recombine with less efficiency. However, sequential RTG cycles could have a synergistic effect as shown in a previous work made on a S288C/SK1 hybrid (Laureau et al. 2016) where, LOH formed in the first RTG cycle mediated further recombination and homogenization of the genome. This scenario remains possible and still unexplored.

8.2 RTG in sterile industrial hybrids: a strategy to generate GMO-free variants with improved industrial traits.

The second part of my work mainly aimed at translating the RTG paradigm to a framework that could be used for improving sterile industrial strains. This second part of the work has posed several challenges. First, since we wanted to have proof that inducing and then aborting meiosis in industrial hybrids was promoting RTG. To do so, we characterized the meiotic progression of these hybrids and identified the best condition to promote a fair degree of meiotic progression, which was then used for the following RTG experiments. Then, thanks to the recent advantage in genome engineering of industrial strains we engineered the *LYS2/URA3* system developed in the first

project using the CRISPR/Cas9 technology (Gorter de Vries et al. 2017). This genetic engineering method has been essential for generating several mutants in polyploid backgrounds that would have required otherwise many rounds of genome engineering. However, we found that despite all the mutants reported the desired genetic modification, in some cases, the engineering generated an LOH at the desired locus. This LOH likely formed during the process of cut and repair induced by Cas9, a possible outcome already reported in diploid hybrids (Gorter De Vries et al. 2019). Moreover, in one mutant engineered with CRISPR/Cas9, we even retrieved an additional aneuploidy, and an extensive copy number variation, that were not present in the ancestral strain. The recent articles published in the literature, as well as the data reported here, suggest that although genome engineering is becoming definitely feasible also in complex industrial strains, it might generate undesired off-target effects. Once we proved that the polyploid hybrids were RTG competent, we devised a new selection system which would enable to select GMO-free RTG variants in genetic backgrounds with a very low sporulation efficiency. This aspect of the work has been particularly challenging yet also rewarding and I end up developing a method of selection based on variation in colony phenotypes across different environments. In the cases reported in this thesis, variations in the concentration of the environmental glucose have been sufficient to capture colony phenotypic variation induced by RTG even when they were representing less than 1% of the population.

The genome analysis of polyploid recombining genomes represents still a challenge. Thanks to the isolation of M-D RTG recombinants and the isolation of selected recombinants using the *LYS2/URA3* system, I could validate the recombination pattern detected through bioinformatic analyses. In addition, long-read analyses of these complex genomes also proved to be difficult and despite having accomplished long-read sequencing of the genomes of the strains I used, phasing algorithms still fails in producing phased haplotypes due to the high complexity of industrial genomes. Interestingly, I phased one region of the triploid hybrid genome by using the gene dosage information and the recombination profile detected upon RTG recombination. This approach might be extended to other regions of the genome and could be informative for retaping the evolutionary history of the strain. However, this strategy is at the moment unfeasible in the tetraploid hybrid as the recombination profile generated is complicated by the number of haplotypes recombining and the possible scenario of chromosome segregation upon RTG. It is likely that when only a cross-over between two pairs of homologous is formed and resolved upon RTG, this will results in a clear signal also in the M-D pairs, whereas the recombination profile would be noisier in more

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complex scenarios.. Interestingly, we found in our RTG isolates a good level of genome stability as we did not detect many CNVs and aneuploidies in line with similar results obtained in diploid hybrids, which is also crucial as aneuploidy and CNVs can have massive detrimental effects on the fitness of the strain. Another goal we pursued was to show that RTG recombination also produces phenotypic variability and eventually trait improvement in sterile industrial strains. As RTG induces a genome-shuffling, it does not select specific phenotypic improvements unless they are somehow linked to the colony phenotype selected, thus RTG strains might have either an improved, worsened or unchanged phenotype. The chance of having isolated M-D RTG pairs has revealed fundamental because often the phenotypic variability was complementary. Indeed there were also variations to this pattern, likely depending on the complex nature of the traits screened. Moreover, even if we screened a small library, we were able to identify several strains in which RTG promoted a phenotypic improvement. The isolation of improved strains has always been critical in any improvement program (Steensels et al. 2014), and in this regard, RTG was not different. However, the selection of RTG M-D sectors is a powerful solution to this problem, and it is can be easily scaled-up to isolate many recombinants.

Although we replied to many questions, and we produced diverse evidence of RTG recombination in polyploid industrial strains, new questions and scenarios arise. Industrial strains that can recombine through RTG need at least to progress until the meiotic prophase, thus, strains that cannot enter in meiosis for loss-of-function of early meiotic genes cannot engage in RTG. Moreover, would be of great interest to develop alternative selection systems that enrich for RTG bearing specific LOH that ultimately impact a desired phenotype. This would be an important improvement, as it might help to isolate strains tailored for specific applications. This approach might also be helpful to isolate RTG candidates when natural phenotypes are not available.

8.3 RTG in natural environments and in other yeasts

One very fascinating implication of RTG, still untested, is directly testing its role in genome evolution in wild and domesticated environment. We did show that wild and domestic strains perform RTG, but that does not necessarily imply that these strains do so also in their natural habitats. Whether yeast hybrids occasionally perform RTG when they are on the bark of a tree, waiting to be used in the next fermentation batch or spending their time in the gut of insects is unknown. Intriguingly, signatures compatible with RTG recombination have been found even in

yeast hybrids evolved in lab conditions without a specific protocol tailored to trigger RTG (Dutta et al. 2017). Moreover, even yeast species that diverged for millions of years from the *Saccharomyces* genus produced LOH patterns compatible with RTG recombination without a specific protocol to trigger RTG (Brion et al. 2017). Therefore, it is possible that an RTG-like mechanism is also conserved in other yeast species where it promotes genome evolution in a more cryptic way than the classical sexual reproduction would. An example of abortive meiosis is reported also in yeasts of the CTG-clade (Mallet et al. 2012) which partly resembles the RTG process. On one hand, since RTG has been frequently overlooked in *S. cerevisiae*, where it was described for the first time, it is very likely that the same process has been neglected in other microbes that are less studied than *S. cerevisiae*. On the other hand, would be probably naive to think that across different taxa and genera the same process has been maintained and conserved without variations. It is possible that RTG might be just a drift of the sexual reproduction in facultative sexual species, where meiosis can be altered by environmental fluctuations and thus RTG would have evolved as an alternative pathway. Otherwise, RTG might have co-evolved along the evolution of sexual reproduction. However, at the moment both scenarios are just speculation and more evidence to understand how broad is the RTG phenomenon are needed.

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9. Annexe

Title: Accurate Tracking of the Mutational Landscape of Diploid Hybrid Genomes

Authors: Lorenzo Tattini, Nicolò Tellini, Simone Mozzachiodi, Melania D'Angiolo, Sophie Loeillet, Alain Nicolas, Gianni Liti

Abstract

Mutations, recombinations, and genome duplications may promote genetic diversity and trigger evolutionary processes. However, quantifying these events in diploid hybrid genomes is challenging. Here, we present an integrated experimental and computational workflow to accurately track the mutational landscape of yeast diploid hybrids (MuLoYDH) in terms of single-nucleotide variants, small insertions/deletions, copy-number variants, aneuploidies, and loss-of-heterozygosity. Pairs of haploid *Saccharomyces* parents were combined to generate ancestor hybrids with phased genomes and varying levels of heterozygosity. These diploids were evolved under different laboratory protocols, in particular mutation accumulation experiments. Variant simulations enabled the efficient integration of competitive and standard mapping of short reads, depending on local levels of heterozygosity. Experimental validations proved the high accuracy and resolution of our computational approach. Finally, applying MuLoYDH to four different diploids revealed striking genetic background effects. Homozygous *Saccharomyces cerevisiae* showed a ~4-fold higher mutation rate compared with its closely related species *S. paradoxus*. Intraspecies hybrids unveiled that a substantial fraction of the genome (~250 bp per generation) was shaped by loss-of-heterozygosity, a process strongly inhibited in interspecies hybrids by high levels of sequence divergence between homologous chromosomes. In contrast, interspecies hybrids exhibited higher single-nucleotide mutation rates compared with intraspecies hybrids. MuLoYDH provided an unprecedented quantitative insight into the evolutionary processes that mold diploid yeast genomes and can be generalized to other genetic systems.

S.M contribution. tested computational methods and performed experimental validations.

Title: An Out-of-Patagonia migration explains the worldwide diversity and distribution of *Saccharomyces eubayanus* lineages

Authors: Roberto F. Nespolo, Carlos A. Villarroel, Christian I. Oporto, Sebastián M. Tapia, Franco Vega-Macaya, Kamila Urbina, Matteo De Chiara, Simone Mozzachiodi, Ekaterina Mikhalev, Dawn Thompson, Luis F. Larrondo, Pablo Saenz-Agudelo, Gianni Liti, Francisco A. Cubillos.

Abstract

Population-level sampling and whole-genome sequences of different individuals allow one to identify signatures of hybridization, gene flow and potential molecular mechanisms of environmental responses. Here, we report the isolation of 160 *Saccharomyces eubayanus* strains, the cryotolerant ancestor of lager yeast, from ten sampling sites in Patagonia along 2,000 km of *Nothofagus* forests. Frequency of *S. eubayanus* isolates was higher towards southern and colder regions, demonstrating the cryotolerant nature of the species. We sequenced the genome of 82 strains and, together with 23 available genomes, performed a comprehensive phylogenetic analysis. Our results revealed the presence of five different lineages together with dozens of admixed strains. Various analytical methods reveal evidence of gene flow and historical admixture between lineages from Patagonia and Holarctic regions, suggesting the co-occurrence of these ancestral populations. Analysis of the genetic contribution to the admixed genomes revealed a Patagonian genetic origin of the admixed strains, even for those located in the North Hemisphere. Overall, the Patagonian lineages, particularly the southern populations, showed a greater global genetic diversity compared to Holarctic and Chinese lineages, in agreement with a higher abundance in Patagonia. Thus, our results are consistent with a likely colonization of the species from peripheral glacial refugia from South Patagonia. Furthermore, fermentative capacity and maltose consumption resulted negatively correlated with latitude, indicating better fermentative performance in northern populations. Our genome analysis, together with previous reports in the sister species *S. uvarum* suggests that a *S. eubayanus* ancestor was adapted to the harsh environmental conditions of Patagonia, a region that provides the ecological conditions for the diversification of these ancestral lineages.

S.M. contribution: Performed genome content measurements of the *S. erubayanus* isolates and data analysis.

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Writing this section is challenging as the number of people I would like to thank for all the help and support they gave me through these years are many.

First, I would like to really thank Gianni for letting me joining this adventure in his lab despite my initial self-doubts and insecurity. Along these years, he taught me to be a better scientist trying to bring out all the possible attitude I have for this complicated job with extreme patience, kindness and dedication. I learned that being a good scientist is a very complex trait. I am not still sure how much my genes can contribute to that, and I am sure I still have a lot to do before actually calling myself a "good scientist". Nevertheless, I want to acknowledge that Gianni has created an environment that allowed me to be a better version of myself in these years, both as a young yeast geneticist and as a person. He has been supportive when things were not going in the right direction and when things started to turn for the good one, pushing me when needed but always in a very positive way, which I believe distinguish true leaders from others. Thanks a lot for everything!

Then, I want to sincerely thank my two PhD fellows Melania and Ben, who have been with me through all these years. Melania has been an important person in all this adventure; she helped me through many bureaucracy and lab issues with extreme altruism and motivating me when bad events were really turning this PhD journey upside down. She supported me when I arrived disoriented and penniless in Nice, and she never refused her help if needed. We grow together during this trip. Instead, Ben has been my lead in the lab when I arrived. He is probably one of the most human "human being" (Sorry for citing you talking about me) I have ever met. I will be forever grateful for your help along the way, and I look forward to going back with you and all the crew to the Caraibes once the COVID pandemic will be over.

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Then there are the team's senior post-docs and past members: Lorenzo, Matteo, Jing, Jia-Xing, Sylvester and Agur. I cannot thank you all in detail because the list would become too long, but briefly, I would like to thank Lorenzo for introducing me to the power of coding in R and halted me from the temptation of using Perl. More generally, together with Matteo and Jia-Xing, thanks for having introduced me to the bioinformatics world. I will miss the dinner at the Chinese restaurant with Jing and Jia-Xing; you have been both very kind and friendly teammate, one day when the COVID pandemic will be over, I will come to visit you over in China. Finally, I thank Sylvester for his help in the wet lab experiments and his teachings about beer and wine, good luck with your future in Denmark.

Last but not least, for the team, I want to thank Sakshi, Nick and Chiara (the added member), the younger generation of PhD students. Now you are the next on the PhD defence list (Chiara first); I am sure you will do great! I also want to thank my students Neza and Angelika that in one way or the other taught me how to be a better supervisor.

Then, I would like to thank all the people from Meiogenix and Alain Nicolas' lab. Thanks, Alain, Luc, Giacomo, Alexandre and Sophie. It has been nice to work with you and develop novel ideas

together, and thank you for having granted me an extension of the contract during this last year which has been very helpful to finish all the experiments despite the pandemic.

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I also want to thank the CIFRE program and the ANRT for supporting this PhD fellowship, giving young students the possibility to explore the research from a basic and applied perspective.

Thanks Jacqueline, for your help and the pleasant English conversation we had before and during the early part of my PhD. I am sure now we will have a bit more time to catch up.

Thanks to all my friends in Italy, who have been supportive, particularly during the last year when the pandemic really challenged me and my determination; I hope there will be the possibility for you to come over more often before I finally leave this beautiful place.

Finally, I want to thank my twin brothers Angelo and Luca, my mother (Mi) Patrizia. They have patiently listened to my complaints along this adventure, and we have kept going on despite all the adversity we encountered. Although he is no longer with us, I also want to keep a line for my twin brother Franco whom I have no memory of, but somehow I felt with me along the way. Thanks, Angelo, for all the naturalistic-evolutionistic conversations we had along the way, which made exploring this new environment inspiring. Now, I understand the meaning of the experiments we made with the grasshoppers in the field when we were kids. I am sure that I would have never managed to achieve this result without my family's support. Thanks.

Curriculum Vitae

Mozzachiodi Simone

Nationality: Italian

Date of birth: 28/08/1992

Personal phone number: 0768490036, Professional phone number: +33 (0)4 93 37 77 28,

email: simone.mozzachiodi@gmail.com

twitter: @SimoMozzachiodi

Affiliations: Population genomics and complex traits (IRCAN) – 28 Avenue de Valombrose, 06107, Nice

Meiogenix, 27 rue du Chemin Vert, 75011, Paris

WORK EXPERIENCE

- March 2017 to date **IRCAN: Institute for Research on Cancer and Aging Nice, France. Meiogenix, Paris:** PhD fellow via CIFRE scholarship. Teams: Population genomics and complex traits (CNRS UMR 7284 - INSERM U1081), Meiogenix. Thesis directors: Dr Gianni Liti
- November 2015 - October 2016 **Department of Genetics at the University of Pisa, Pisa, Italy and Department of Science University of Roma Tre university :** Internship for Master's Thesis. Team: Bioinformatics and computational unit. Thesis director: Dr Roberto Marangoni co-director: Dr Pasquale Stano.
- May - September 2014 **Department of Genetics at the University of Pisa, Pisa, Italy:** Internship for Bachelor's Thesis. Team: Genetic unit at the department of Genetics. Thesis director: Dr Federica Gemignani.

ACADEMIC EDUCATION

- 2017 – current **Doctor of Philosophy in Life Sciences and Health.** Université Cote d'Azur, France.
- 2014 – 2016 **Master of Science in Molecular and Cellular Biology.** Cum Laude. University of Pisa, Italy.
- 2011 - 2014 **Bachelor of Science in Molecular Biology.** Cum Laude. University of Pisa, Italy.

AWARDS

- December 2016 Awarded a PhD fellowship from the CIFRE program.
- May 2016 Awarded a travel grant to attend the congress “BITS2016”.

JOB-RELATED SKILLS

| | |
|-------------------------------------|--|
| Molecular biology techniques | Electrophoresis, PCR, cloning, DNA/RNA extraction and purification, sequencing library preparation, q-PCR, phenotyping, flow cytometry, CRISPR/Cas9 genome editing, Oxford Nanopore sequencing, yeast wet-lab protocols. |
| Next generation sequencing analysis | Short and long reads mapping and assembly, coverage and CNV analysis, SNP detection and genotyping, genome annotation, linkage analysis, algorithmics and programming, phylogenetics. |
| Operative systems | Windows, Linux/Unix. |
| Programming languages | R (advanced), Bash (intermediate). |

PUBLICATIONS

Publications in peer-reviewed journals

Mozzachiodi S, Kristoffer K, Llored A, Gibson B, Nicolas A, Liti G. RTG induces genomic reshuffling and phenotypic improvement in sterile industrial hybrids. (**In preparation**)

Mozzachiodi S, Tattini L, Llored A, Irizar A, Skofljanc N, D'Angiolo M, De Chiara M, Barré B, Yue JX, Lutazi A, Loeillet S, Laureau R, Marsit S, Stenberg S, Baulande S, Persson K, Legras JL, Dequin S, Warringer J, Nicolas A, Liti G. Aborting meiosis overcomes hybrid sterility. (**Submitted**).

Nespolo F. R, Villaoruel A. C, Oporto I. C, Tapia M. S, Vega-Macaya F, Urbina K, De Chiara M, **Mozzachiodi S**, Mikhalev E, Thompson D, Larrondo F. L, Saenz-Agudelo P, Liti G, Cubillos A. F. An Out-of-Patagonia migration explains the worldwide diversity and distribution of *Saccharomyces eubayanus* lineages *Plos Genetics* (**2020**).

Tattini L, Tellini N, **Mozzachiodi S**, D'Angiolo M, Loeillet S, Nicolas A, Liti G. Accurate tracking of the mutational landscape of diploid hybrid genomes reveals genetic background effects. *Molecular Biology and Evolution* (**2019**).

COMMUNICATIONS

Conference presentations

Mozzachiodi S, Tattini L, Llored A, Barre B, Loeillet S, Nicolas A, Liti G. Overcoming genome complexity barriers in hybrids with meiotic-reversion. Journées de l'Ecole Doctorale de Nice. Nice (**2019**).

Posters

Mozzachiodi S, Tattini L, Llored A, Irizar A, Škofljanc N, D'Angiolo M, De Chiara M, Barré P.B, Yue JX, Lutazi A, Loeillet S, Laureau R, Marsit S, Stenberg S, Persson K, Legras JL, Dequin S, Warringer J, Nicolas A and Liti G. Overcoming sterility in yeast hybrids. EMBL: Molecular Mechanisms in Evolution and Ecology. Virtual (**2020**).

Mozzachiodi S, Tattini L, Llored A, Nicolas A, Liti G. Genome evolution in hybrid yeasts through meiotic reversion. Saint-Felix de Guixols, Spain (2019)

Mozzachiodi S, Tattini L, Llored A, Barre B, Loeillet S, Nicolas A, Liti G. Overcoming genome complexity barriers in hybrids with meiotic-reversion. Journées de l'Ecole Doctorale de Nice. Nice (2018).

Nicolas A, Loeillet S, Llored A, Marsit S, Laureau R, Tattini L, D'Angiolo M, Bedrat A, Serero A, Legras JL, **Mozzachiodi S**, Baulande S, Dequin S, Liti G. Recombination of yeast hybrids through meiotic reversion. EMBO Conference on Meiosis. Hvar (2017).

Mozzachiodi S, Cardelli L, Stano P, Altamura E, Mavelli F, Marangoni R. Environmental variables, modular Synthetic Biology and Systems Biology: a case study. 13th Annual Meeting of the Bioinformatics Italian Society. Salerno (2016).

RESEARCH INTERESTS

Genome evolution, Next Generation Sequencing (NGS), Genetic engineering, Microbial biotechnology, Evolutionary genomics, Speciation, Hybrid sterility, Domestication and artificial breeding, Linkage analysis, Population genomics, Single molecule real time sequencing (Pac Bio), MinION Nanopore sequencing.

TEACHING and MENTORING

2019 - 2020 March Supervision of Škofljanc Neža (Erasmus student)

2020 January - 2020 May Supervision of Angelika Zieminski (Master student)

LANGUAGE SKILLS

Italian (First Language), English (C1), French (A2), Korean (notions).

SOFT SKILLS & HOBBIES

Teamwork, Creative thinking, Conflict resolution, Networking,
Time management

Piano player (2002 – current), Diploma “Allievi Piano,

Accademia Bianchi”

