Spontaneous whole-genome duplication restores fertility in interspecific hybrids

Guillaume Charron, Souhir Marsit, Mathieu Hénault, Hélène Martin & Christian R. Landry

Interspecies hybrids often show some advantages over parents but also frequently suffer from reduced fertility, which can sometimes be overcome through sexual reproduction that sorts out genetic incompatibilities. Sex is however inefficient due to the low viability or fertility of hybrid offspring and thus limits their evolutionary potential. Mitotic cell division could be an alternative to fertility recovery in species such as fungi that can also propagate asexually. Here, to test this, we evolve in parallel and under relaxed selection more than 600 diploid yeast inter-specific hybrids that span from 100,000 to 15 M years of divergence. We find that hybrids can recover fertility spontaneously and rapidly through whole-genome duplication. These events occur in both hybrids between young and well-established species. Our results show that the instability of ploidy in hybrid is an accessible path to spontaneous fertility recovery.
nterspecific hybridization is common in animals, plants, and microorganisms, and is a potentially frequent source of genetic diversity over short time scales. However, hybrid lineages often suffer from poor fertility that reflects reproductive isolation between parental lineages. The poor fertility of hybrids can prevent their maintenance as independent populations, thus hindering their long-term survival and the process of speciation. Different molecular mechanisms underlie hybrid infertility, including genetic incompatibilities (nuclear and cytoplasmic) and changes in genome architecture (ploidy number or chromosome rearrangements). If the hybrids are to establish as an independent species, they need to recover from this low initial fertility. In obligatory sexual species, fertility restoration can be achieved by crosses among hybrid individuals or backcrosses with either parental species, allowing the purge of incompatibilities through recombination. Because of this gene flow, this process most often leads to the formation of introgressed species rather than hybrid species. In this context, the formation of hybrid species may necessitate other means of isolation from both parental species, which may include geographic or ecological isolation, which can prevent their maintenance as independent populations, thus allowing the purge of incompatibilities through recombination.

Using yeast as an experimental model system, we show that hybrids between closely and distantly related species can recover fertility spontaneously by whole-genome duplication and this, without the need for natural selection. Although rare, these events have large effects and bring fertility to the levels seen in parental species. Polyploidy is most common in plants but has also been observed in many animals and fungi, making this mechanism of fertility recovery accessible to many species.

Results

Hybrid survival through serial bottlenecks. We investigated the evolution of fertility in parallel experimental yeast hybrid populations during mitotic evolution. Using strong population bottlenecks to minimize the efficiency of natural selection allowed for the random accumulation of genetic and chromosomal changes, providing an estimate of the neutral rate of evolution. We examined whether fertility would increase or decrease with time and, if so, whether it would occur through gradual or punctuated changes (Fig. 1a). We considered hybridization over different levels of parental divergence from intra-population to interspecific crosses. These crosses represent up to 15 M years of divergence, which is sufficient to achieve almost complete (99%) postzygotic reproductive isolation in budding yeast. We used a collection of North American natural yeast isolates representing three lineages of the wild species Saccharomyces paradoxus and two wild isolates of its bona-fide sister species, S. cerevisiae. We examined whether fertility would increase or decrease with time and, if so, whether it would occur through gradual or punctuated changes (Fig. 1a). We considered hybridization over different levels of parental divergence from intra-population to interspecific crosses. These crosses represent up to 15 M years of divergence, which is sufficient to achieve almost complete (99%) postzygotic reproductive isolation in budding yeast. We used a collection of North American natural yeast isolates representing three lineages of the wild species Saccharomyces paradoxus and two wild isolates of its bona-fide sister species, S. cerevisiae. We used a collection of North American natural yeast isolates representing three lineages of the wild species Saccharomyces paradoxus and two wild isolates of its bona-fide sister species, S. cerevisiae (Supplementary Table 1). The S. paradoxus lineages (SpA, SpB, and SpC) are incipient species that exhibit up to 4% of genetic divergence (SpA–SpB) and up to 60% of reduction in hybrid fertility compared to within-lineage crosses. These species and populations occur in partially overlapping geographical ranges, even for the most distant pair, S. paradoxus and S. cerevisiae, making hybridization possible in a natural context. Including this sister species extends nucleotide divergence between parental strains up to 15%. We mated two SpB strains to two other SpB strains and to two strains of the diverged lineages and species, producing four different types of crosses in duplicates that we classified in terms of divergence: Very Low (VLSpB) = SpB × SpB; Low (Ldiv) = SpB × SpC; Moderate (Mdiv) = SpB × SpA, and High (Hdiv) = SpB × S. cerevisiae (Supplementary Table 2 and 3). Ninety-six diploid hybrid lines from independent mating events were generated for all but the VLSpB crosses, for which 48 hybrids were generated, for a total of 672 independent lines (96 lines × 3 types of crosses × 2 pairs of strains + 2 × 48 VLSpB crosses) (Fig. 1b). We randomly selected and streaked colonies on plates every 3 days for 35 passages with an estimated number of mitotic generations of 22 per passage (Fig. 1c, Supplementary Fig. 1).

Not all lines could be propagated through the entire experiment. After 770 mitotic generations, 77.9% of the lines (524 out of the 672 initial lines) were still propagated using standard conditions. About 50% (n = 72) of the extinct lines were lost within the first 250 mitotic generations. This suggests that the loss of these lines is mostly due to genomic instability that arises rapidly after hybridization rather than spontaneous mutations, which would happen at a much slower pace. The Ldiv, Hdiv, and one of the Mdiv crosses (M1) had a significantly lower proportion of surviving lines (averages of 69.3% for Ldiv, 70.3% for Hdiv, and value of 79.2% for M1, Fig. 1d, Supplementary Fig. 2) compared to the VLSpB and M2 (average of 88% for VLSpB and value of 91.7% for M2, Fig. 1d, P < 0.01, Log-rank test, Fig. 1d, Supplementary Table 4). This suggests that hybrids from divergent parents may suffer from exacerbated genomic instability that lead to the rapid collapse of populations when faced with serial bottlenecks. The cause of line extinction remains to be investigated in detail, but the data suggest that it may be because of the frequent segregation of highly deleterious variants generated by genome instability. Indeed, the within-species control crosses of VLSpB, which are expected to be stable because of their low heterozygosity and thus represent a measure of experimental noise, show little line extinction. In general, the extent of line loss correlates with genetic divergence of the parental strains, with the exception of the Mdiv crosses, which shows elevated line extinction. The Ldiv crosses is also the one that shows the greater level of ploidy instability (see the section ‘Ploidy evolves following hybridization’), suggesting that these hybrids are generally less stable. Therefore, these results suggest that yeast hybrids, even when maintained by mitotic division only, segregate very unstable clones at high frequency. This is also supported by the fact that the rate of loss decreases with time, which we would expect if unstable genotypes are eliminated through replication. However, replicating multiple colonies from the last available glycerol stocks of some extinct Ldiv and Hdiv lineages over four passages (80 generations) could not recapitulate the high extinction rate we observed during the experimental evolution. This may indicate that the pre-cultures preceding the glycerol stock constitute a selection step favoring the most genetically stable individuals within the colony, even if they are in minority.
Fraction of lines that lost their sporulation capacity at $T_{\text{ini}}, T_{\text{mid}}$, and $T_{\text{end}}$. The number of strains tested per cross type is indicated over the corresponding bars.

HSD) and $M_{\text{div}}$ crosses (averages of 36.4% and 18.1%, $P < 0.01$, Tukey HSD). These differences are probably due to strain specific genetic variation or even genomic architecture leading to variable levels of postzygotic isolation$^{19}$. Unexpectedly, spore viability could not be assessed for all the lines at $T_{\text{end}}$ because 17% ($n = 37$) of the tested lines lost their ability to enter sporulation. We found that the probability of successful sporulation at the end points considered is negatively correlated with parental divergence (Fig. 1c, $r = -0.76$, $P < 0.01$, logistic regression). The loss of sporulation ability in yeast is multifactorial$^{22}$ but we hypothesized that it could be caused by mitochondrial malfunctions. Indeed, functional aerobic respiration is necessary for sporulation and thus requires the maintenance of functional mitochondrial DNA (mtDNA)$^{23}$. The genotyping of two mitochondrial loci revealed a strong association between the loss of ability to sporulate and the absence of at least one mitochondrial marker (Fisher’s exact test, odds ratio $> 77$, $P = 1.43 \times 10^{-3}$, Supplementary Fig. 4C). This observation suggests that mtDNA instability could contribute to reproductive isolation among closely related yeast populations by leading to sterility, as shown for more distant species$^{24}$, and that this effect increases with genetic distance. There are also rare cases in which lines lost their sporulation ability while both mitochondrial markers were detected (Supplementary Fig. 4), suggesting that the loss of mtDNA integrity is not the only cause of sporulation inability. As the Saccharomyces Genome Database (SGD) reports over 200 genes which, when knocked out in $S$. paradoxus (SGD) reports over 200 genes which, when knocked out in $S$. cerevisiae (H$_{\text{div}}$), each type of cross involves two biological replicates, i.e., involving independent strains, and each individual cross was performed independently to represent independent hybridization events. The 672 hybrids were evolved in conditions of weak selection to examine the neutral spontaneous changes of fertility. Mitotic propagation was performed through repeated bottlenecks of single cells. Fertility was measured by estimating spore viability after meiosis at $T_{\text{ini}}, T_{\text{mid}}$, and $T_{\text{end}}$. 37) of the tested lines lost their ability to enter sporulation. We found that the probability of successful sporulation at the end points considered is negatively correlated with parental divergence (Fig. 1c, $r = -0.76$, $P < 0.01$, logistic regression). The loss of sporulation ability in yeast is multifactorial$^{22}$ but we hypothesized that it could be caused by mitochondrial malfunctions. Indeed, functional aerobic respiration is necessary for sporulation and thus requires the maintenance of functional mitochondrial DNA (mtDNA)$^{23}$. The genotyping of two mitochondrial loci revealed a strong association between the loss of ability to sporulate and the absence of at least one mitochondrial marker (Fisher’s exact test, odds ratio $> 77$, $P = 1.43 \times 10^{-3}$, Supplementary Fig. 4C). This observation suggests that mtDNA instability could contribute to reproductive isolation among closely related yeast populations by leading to sterility, as shown for more distant species$^{24}$, and that this effect increases with genetic distance. There are also rare cases in which lines lost their sporulation ability while both mitochondrial markers were detected (Supplementary Fig. 4), suggesting that the loss of mtDNA integrity is not the only cause of sporulation inability. As the Saccharomyces Genome Database (SGD) reports over 200 genes which, when knocked out in $S$. cerevisiae (H$_{\text{div}}$), each type of cross involves two biological replicates, i.e., involving independent strains, and each individual cross was performed independently to represent independent hybridization events. The 672 hybrids were evolved in conditions of weak selection to examine the neutral spontaneous changes of fertility. Mitotic propagation was performed through repeated bottlenecks of single cells. Fertility was measured by estimating spore viability after meiosis at $T_{\text{ini}}, T_{\text{mid}}$, and $T_{\text{end}}$.
sporulation ability is significantly associated with a change in red pigment saturation for colonies growing on the standard media used for the evolution experiment (Supplementary Fig. 5B, logistic regression, \( P = 0.029 \)). Although the increase of saturation with mtDNA loss was contrary to the phenotype expected from ADE2 deletants, this pigmentation change seems to be related with mitochondrial function, as it is well correlated with the ability to grow on glycerol (Supplementary Fig. 5B, Pearson’s \( r = 0.547, P = 1.336 \times 10^{-26} \)). These results suggest that the failure of mtDNA maintenance or function plays a major role in causing rapid and irreversible sterility in experimental yeast hybrids through the loss of sporulation ability.

**Fertility evolves through time.** To investigate whether the spore survival component of fertility improved over the experiment, we calculated a fertility recovery score (FRS) as the difference in spore viability between \( T_{\text{end}} \) and \( T_{\text{ini}} \) (Fig. 2a, b). As a point of comparison for fertility restoration with sexual reproduction, we performed 12 meiotic generations of intra-tetrad crosses (ITC) in randomly chosen diploid \( L_{\text{div}} \) lines and calculated FRS, all of which were positive (Fig. 2c, Supplementary Fig. 6). This is in stark contrast with mitotic lines, in which we found no statistically significant bias in FRS values as the distributions were unimodal and centered around 0 (Fig. 2b, Supplementary Fig. 7), showing that spore viability is as likely to increase as it is to decrease. To make sure that low spore viability was not due to the intrinsic inability of strains to produce viable spores, due to dominant mutations for instance, we performed autodiploidization on a random set of 16 haploid spores from the \( L_{\text{div}} \) and \( M_{\text{div}} \) crosses and this, at the three timepoints. In most cases, fertility was restored to more than 85% upon selfing (Fig. 2c), showing that infertility mostly derives from the presence of two divergent genomes in the same cell.

Although no general trend towards the recovery of spore viability was observed during the experiment, 23 individual lines presented statistically significant differences in their fraction of viable spores between \( T_{\text{ini}} \) and \( T_{\text{end}} \) (Fisher’s exact test, FDR corrected, Supplementary Table 6). Eleven of those 23 lines (5 \( VL_{\text{div}} \), 5 \( L_{\text{div}} \), and 1 \( M_{\text{div}} \)) showed a decreased fertility and the remaining 12 (7 \( L_{\text{div}} \), 4 \( M_{\text{div}} \), and 1 \( H_{\text{div}} \)) showed improvement. Among the lines that showed improvement, seven (4 \( L_{\text{div}} \), 2 \( M_{\text{div}} \), and 1 \( H_{\text{div}} \) ) displayed spectacular recovery in spore viability within 352 mitotic generations: their FRS values were close to that of ITC lines (Fig. 2b), and their spore viability was similar to what is typically observed for their non-hybrid diploid parents. One line presented a low FRS value, which is explained by the fact that its fertility returned to its initial value by \( T_{\text{end}} \) (Fig. 2b). As infertility of Saccharomyces hybrids is mainly due to anti-recombination caused by the mismatch repair machinery acting on homeologous chromosome pairs, leading to chromosome missegregation, there could be at least three main explanations for these sudden increases in fertility. All of these mechanisms rely on the loss of heterozygosity across the genome and re-establishment of correct chromosome pairing during meiosis.

The first potential mechanism would be an endoreduplication event, i.e., spontaneous chromosome doubling following a failed cell division during mitosis. Such an event would lead to the production of identical homologues that would restore correct chromosome segregation. The second mechanism would be damage to a copy of the MAT locus that would convert the diploid hybrid into behaving as a gamete. Two such diploid gametes could then mate, generating a fertile tetraploid hybrid. This path to fertility recovery was recently observed in hybrid species of the Zygosaccharomyces genus, so it is in principle an accessible path to fertility recovery. However, this would need rare events to co-occur in the same colony and to produce two diploid gametes of opposite mating type. The last potential mechanism would be that strains could have sporulated during the experiment and spores of opposite mating types could have mated. This would be the equivalent of our ITC lines where sometimes a single cross between two spores can bring fertility back to high values (Supplementary Fig. 6). This third option is very unlikely because sporulation happens under very specific environmental conditions, principally nitrogen starvation. The frequency of streaking to fresh media during the experiment would prevent such depletion to happen. In addition, this scenario would often lead to spores that are aneuploid, making fertility recovery unlikely even after mating. All these mechanisms would prevent such depletion to happen. In addition, this scenario would often lead to spores that are aneuploid, making fertility recovery unlikely even after mating.
would generate strains with increased spore viability, but the lines are expected to show a change from diploidy to tetraploidy in the first two scenarios, allowing to differentiate these mechanisms. Mitotic loss of heterozygosity could also be involved but would not be expected to lead to such dramatic recovery of fertility33. We tested these potential mechanisms by measuring the total cellular DNA content of the lines to infer ploidy, genome-wide genotyping and whole-genome sequencing of some of the strains.

**Ploidy evolves following hybridization.** We measured ploidy in the 214 randomly selected lines at the three timepoints Tini, Tmid, and Tend using DNA staining and flow cytometry. Surprisingly, these analyses revealed that some lines already deviate from diploidy after hybridization. While almost all the hybrids are diploid, both independent Ldiv crosses show frequent triploidy (average at Tini of 54% triploid lines) (Fig. 3a, b, Supplementary Fig. 12). It appears that this triploidization is a major driver of low initial spore viability in the Ldiv hybrids (both crosses), with an average reduction of 45% compared to diploids (20.7% compared to 37.6% considering all timepoints, Supplementary Fig. 8). This triploidy could either be a consequence of aneuploidies that led to an overall DNA content equivalent to triploidy, or as a consequence of whole-genome duplication of one of the parental genomes. We examined the genotype of hybrids using genotyping-by-sequencing (GBS) and found that at Tini, all Ldiv triploid hybrids were composed of two copies of the SpC genome and one copy of the SpB genome (Fig. 3c), suggesting that the change in ploidy predates mating. This would be possible if some SpC haploid cells were in fact diploid. We indeed observed a small fraction of diploid clones in both parental SpC haploid stocks (Supplementary Fig. 9). These clones were identified pseudo-haploids, i.e., diploid but competent for mating (Supplementary Fig. 10). Triploidy appears to be frequent in the Ldiv cross and was observed in the two biological replicate matings performed with independent strains of SpC and SpB (Fig. 1). It however does not seem to occur in all crosses between these two species (Supplementary Figs. 11 and 12) and is variable among replicates (Supplementary Table 7) suggesting some stochastic effects within and between strains. The proportion of triploids among hybrids may depend on the initial proportion of diploid parents (pseudo-haploid) in the preculture used for the crosses, which itself is stochastic (Supplementary Fig. 9). The frequency of SpC diploid parents may also depend on whether it appears early or late in the cell culture. The absence of triploids in some other Ldiv crosses indicates that diploidization in parental strains is background-dependent. Phenotypic, genomic, and karyotypic diversity have been observed among strains of the SpB and SpC lineages17-19. These results suggest that some SpC haploid strains may be prone to spontaneous genome doubling, the origin of which will need more investigations. For instance, variation that affect key genes involved in cell cycle regulatory pathways could be on the origin of this process.

![Fig. 3](https://example.com/fig3.png)
consistent with the hypothesis above based on spore viability recovery by polyploidization, the 7 lines that displayed significant spectacu
lar fertility recovery and one line that showed significant fertility improvement, but of lower magnitude, during evolution became tetraploid (Fig. 4). This is also true even for hybrids between S. paradoxus and S. cerevisiae, which were initially completely sterile (Fig. 4). One of the two tetraploid M1 lines (M1_40) returned to diploidy at T_end but this is more likely to be due to segregating ploidy and colony heterogeneity at T_mid rather than return to diploidy (for more details see Supplementary Note 2). GBS analysis revealed that tetraploid hybrids have equal copies of parental genomes, since allele frequencies across 171 markers among the 16 chromosomes are around 50% (Fig. 3c, Supplementary Figs. 13 and 14).

As stated above, whole-genome doubling could occur either by endoreduplication, which is a consequence of cytokinesis failure\(^3^4\) or by means of damage to one copy of the MAT locus in the hybrid\(^3^6,3^1\) (Supplementary Fig. 15). This damage to the MAT locus could cause hybrid cells to behave as a haploid, switch mating type and hence autotetraploidize. In this experiment, mating type switching may not occur using the standard process because the necessary HO gene was deleted. The main way by which autotetraploidization could occur by mating in our study is to have two hybrids with damage to the opposite MAT loci that are in the same colony and are close enough to mate with each other (Supplementary Fig. 15), which is a very unlikely event. To investigate this, we sequenced the genome of the 8 tetraploid lines (5 L_div, 2 M_div, and 1 H_div) at T_in, T_mid, and T_end. We indeed found that the frequency of parental alleles across the genomes are roughly 50%, showing that the strains are not aneuploids that would have DNA content equivalent to tetraploidy (Fig. 3d, Supplementary Figs. 16 and 17). One exception is observed for M1_40 line that show allele frequency corresponding to a triploid state at T_end (Fig. 3d, Supplementary Fig. 16) while GBS data show a tetraploid state at T_mid and a diploid state at T_end (Supplementary Fig. 14). A different colony was isolated each time from these timepoints. This is again consistent with segregating ploidy and colony heterogeneity, which are probably due to extreme genomic instability in this particular line (for more details see supplementary Note 2). Next, we investigated the total or partial chromosome loss or loss of heterozygosity (LOH) of the MAT locus region (Supplementary Fig. 15). We identified only two tetraploids with aneuploidy on chromosome III containing the MAT locus (Supplementary Figs. 18, 19, and 20). However, these aneuploides affect only one copy of the mating type (Supplementary Note 1 and Supplementary Fig. 21). We thus find no evidence of damage to the MAT locus of tetraploid hybrids that could have caused mating between diploid hybrids. Thus, these results suggest that endoreduplication is the most likely mechanism of whole-genome doubling. However, we cannot exclude that damage to the MAT loci occurring by the loss of chromosome III could be undetectable because this chromosome could have been regained following tetraploid formation. Chromosome III was indeed found to be the most unstable chromosome among S. cerevisiae diploids, triploids, and S. cerevisiae-S. bayanus hybrids\(^3^5\). Furthermore, transient dynamics of aneuploidy were repeatedly observed in yeast laboratory evolution experiments under stressful conditions\(^3^6,3^7\). Such successive aneuploides could happen in our hybrids lines because aneuploidy is prevalent (Fig. 3c, Supplementary Fig. 18) and tetraploids are notorious for showing genome instability\(^3^8\).

**Discussion**

We measured the rate of recovery from reproductive isolation in yeast hybrids using experimental evolution. We propagated parallel hybrids with serial bottlenecks to measure the neutral rate of recovery. We eliminated the potential confounding effect that natural selection on growth rate could have on genome dynamics and consequently on components of fertility.

One of the most striking changes to fertility we observed is a complete loss of the ability to sporulate within thirty generations, effectively reducing fertility to 0. While decrease in sporulation efficiency was observed in similar experiments performed on homozygous strains\(^3^9,4^0\), such complete loss of the ability to sporulate in strains that initially were able to sporulate at high efficiencies was, to our knowledge, not reported before. Our results show that a greater genetic distance between the founding parents led to higher probability of losing the ability to sporulate, suggesting that genome instability or genetic incompatibilities could cause this decrease of fertility. This, added to the results indicating that those strains also lost some or all of their mitochondrial DNA and show growth defects on non fermentable sources, indicate that genetic incompatibilities or instabilities...
involving the mitochondrial genome may be responsible for these fertility losses.

We find that reproductive isolation as assessed by spore viability does not have a global directional fate during the mitotic propagation of hybrid lines. The different scenarios presented in Fig. 1a were almost all observed. Overall, spore viability of hybrids evolved like a neutrally evolving quantitative trait, with incremental gains and losses, with no overall particular direction. However, we did observe spectacular punctuated improvements in spore viability for some lineages. From the 23 lines that had statistically significant differences between the initial hybridization and the end of the experiment, fertility decreased for 11 of them. Reductions in spore viability was observed in all crosses except the Hhxq crosses, which were already completely infertile at the beginning of the evolution and could not be reduced further. One explanation for such decrease includes the accumulation of genomic rearrangements, which would lead to incorrect segregation of chromosomes during meiosis to various degrees depending on the extent of the rearrangements. The segregation of recessive lethal alleles following a de novo mutation could also be implicated and would lead to strains exhibiting halved fertilities, which were rarely observed, but more complex patterns of fertility decrease are also possible given the potential for genetic interactions in these heterogeneous genetic backgrounds.

Punctual and almost complete restoration of fertility was observed at low frequency in all but the lowest parental divergent crosses. We show that these lines experienced a genome duplication, most likely caused by the doubling of all chromosomes. It was shown before that artificially induced chromosome doubling in fertile hybrids between S. paradoxus and S. cerevisiae hybrids could restore fertility. Our results show that this happens spontaneously, without the need for natural selection and between species that can naturally hybridize. Finally, a more gradual form of statistically significant recovery was also observed in about six lines, which were not subjected to genome duplication. For four of those lines, fertility almost doubled compared to their ancestral lines. The other two have improvement of three and almost five times their initial fertilities. As mitotic recombination often leads to gene conversion events spanning tens of thousands of base pairs, accumulation of a large number of mitotic loss of heterozygosity events might allow such recoveries. More in-depth genomic analyses will therefore be needed to understand the basis of these recoveries. This would suggest that the contribution of mitotic recombination to the recovery of fertility in hybrid lineages could be a slow process compared to meiotic recombination and whole-genome duplication.

It is important to note that our results also show that there is some variation for fertility and ploidy in all the colonies that were replicated and frozen, (2) among lines within a cross, and (3) between biological replicates of the same divergence category. This variation could be due to the interactions of the parental genetic backgrounds, generating instability in the hybrid lines, instability that starts as early as in the zygotes after the initial mating. Therefore, taking into account the initial genetic background of experimentally evolved hybrids and considering multiple independent hybridization events is crucial for the understanding of their possible evolutionary fates.

Sexual reproduction is thought to be extremely rare in yeast and thus may not always explain successful hybridization and introgression in the wild. The slow rate of improvement of spore viability by mitotic growth alone, without change in ploidy, could therefore contribute to improved fertility because mitotic proliferation is much more frequent than sexual reproduction. This could be even more likely for instance if natural selection could accelerate the loss of heterozygosity, although this remains to be tested. Our data show that hybrids can recover fertility by becoming allopolyploids in less than 400 cell divisions, which could represent less than 250 days in nature. In principle, fertility recovery could therefore happen before sexual reproduction occurs. The rate of fertility restoration by genome doubling observed in our experiments might be an underestimate of the rate that would occur in yeast hybrids that contain the HO gene. In the event of damage to one of the mating type loci, the wild-type HO would allow the mating type switch and self mating in hybrids, leading to a duplicated genome. How frequently whole-genome duplication occurs in nature remains to be examined but it could contribute to important events. For instance, it was shown recently that the whole-genome duplication in an ancestor of the Saccharomyces genus originated from interspecies hybridization. The success of such an event would have been unlikely if the F1 hybrids were completely sterile and if sexual reproduction would have been needed to recover fertility. Our results suggest that whole-genome duplication could have happened spontaneously and neutrally, thus restoring fertility at the same time. Fertility recovery without sex is likely to apply to multicellular eukaryotes as well because somatic chromosome doubling in diploid tissues or zygotides can lead to the emergence of polyploids, which may display both restored fertility and reproductive isolation with parental species. While polyploidy has been shown to be prevalent in plants, it is not common in animals. However, the animal lineages containing stable polyploids species often have access to asexual reproductive strategies such as parthenogenesis, which improve tolerance to polyploidy and could enable hybrid fertility restoration without sex. As ploidy changes contribute to restore fertility in partially fertile hybrids, we also find that ploidy instability generates triploids in some of our crosses, which contributes to poor fertility. Ploidy changes are therefore a double-edged sword, causing both reproductive isolation and fertility recovery.

Methods

Strain construction. We used the ade2-Δ marker to help with visual identification of respiration deficient colonies, a strategy used in past mutation accumulation experiments. As described in the main text, this marker did not faithfully indicate insufficient respiration of the strain backgrounds that we used. The heterothallic S. paradoxus strains were generated previously (Supplementary Table 1). The ADE2 and HO loci of the two wild S. cerevisiae strains were deleted following the method described by Güldener et al. (Supplementary Table 1). The ADE2 locus of the S. paradoxus strains were replaced by homologous recombination with resistance cassette following the same procedure as for HO in S. paradoxus. Oligonucleotides with overhangs (Supplementary Table 8) specific to each lineage were used to generate the deletion cassettes from pFA-hphNT13 to prevent recombination with the cassettes already present at the HO locus (KANMX and NATMX cassettes).

Experimental crosses. Two crosses were made for each of the divergence levels (l1, M1, H1, V1 and L2, M2, H2, V2, L2) (Supplementary Table 3). All incubation steps were performed at room temperature (RT). Haploids to be crossed were precultured overnight in 5 mL of YPD (1% yeast extract, 2% tryptone, and 2% D-glucose). Pre-cultures were then diluted at ODIonos of 1.0 in 500 µL aliquots. The aliquots from two strains to be crossed were mixed together and 5 µL were used to inoculate 200 µL fresh YPD medium in 96 replicates so all strains would derive from independent mating events and would be truly independent hybrids. Cells were given 6 h to mate after which 5 µL of the mating cultures were spotted on a diploid selection medium (YPD, 100 µg/mL G418, 10 µg/mL Nourseothricin). From each of the 96 spots, one colony was picked as a founding line for the evolution experiment, resulting in 96 independent lines for each of the six interlineage crosses (48 lines for the two intra-lineage crosses).

Evolution experiment. Each of the independent lines (single colonies) were streaked on one third of a YPD agar plate. To facilitate the detection of potential lines mixing during the experiment, each Petri was streaked with three different crosses (series L1, M1, H1 and L2, M2, H2). Crosses V1 and V2 were streaked on two different sets of Petri dishes, with three lineages per Petri. The 192 plates were split into three sets of 64 (lines 1–64 and lineages 65–96). Plates were incubated at room temperature for 3 days after which a new single colony was streaked as a progenitor for the new generation. Each set was rotated between three
Estimation of generation time. To evaluate the generation time on plates and thus estimate the total number of mitotic divisions during the experiment, three lines from each cross were randomly selected. Strains for $T_{1}$ and $T_{end}$ were thawed (48 total strains tested), streaked onYPD solid medium and let grow for 3 days. Strains were then replicated on freshYPD solid medium following the same protocol as for the evolution experiment. After 3 days of incubation, the colony closest to the predefined mark was extracted from the media using a sterile scalpel. The agar block with the colony was put in a sterile 1 mL Eppendorf tube and the colony was resuspended in 500 µL of sterile water. Optical density at 600nm (OD$_{600}$) of the resuspensions was estimated using a TECAN Infinite 200 plate reader (TECAN, Männedorf, Switzerland). These resuspensions were diluted in 200 µL of sterile water to obtain OD$_{600}$ values of about 0.05 (cells µL$^{-1}$). The dilutions were then analyzed with a Guava® easyCyte HT (Millipore Sigma, Burlington, USA) flow cytometer to estimate actual cell numbers. The estimated number of cells µL$^{-1}$ were used to calculate the initial number of cells in the volume used in the dilution and then in the initial 500 µL. The log of this number represents the number of cell doublings during the colony growth for one passage of the experiment assuming the colony was formed from a single cell (Supplementary Fig. 5, Supplementary Data 1).

Sporulation protocol. Strains were thawed and 2 µL of the stocks were spotted on a freshYPD medium and incubated for 3 days. A small number of cells was used to inoculate 4 mL of freshYPD media and incubated for another day. From these cultures, a new 4 mL culture was inoculated at 0.6 OD$_{600}$ in freshYPD and grown for 3 h. Cultures were then centrifuged at 250 × g and the YPD was replaced with 4 mL of YEPA medium (1% yeast extract, 2% tryptone, and 2% potassium acetate). Cultures were incubated for 24 h after which they were centrifuged again at 250 × g washed once with sterile deionized water and put into 4 mL of SB medium (0.3% potassium acetate 0.02% α-Raffinose). After 3–5 days of incubation, the strains were disassembled in Charron et al. with a SporePlay®-dissection microscope (Singer Instruments, Somerset, UK) on YPD plates and incubated for 5 days. Pictures of the plates were taken after the incubation and fertility was determined as the number of spores formed a colony visible to the naked eye after 5 days.

Mitochondrial DNA genotyping. Two mitochondrial loci were genotyped for presence or absence of PCR. Total DNA extractions were performed using the method described by Looke et al. The two PCR assays target loci in the RNL and ATP6 mitochondrial genes, respectively, as described in Leducq et al. Multiplex PCR with both primer pairs (Supplementary Table 8) was performed with the following cycle: 40 times the following cycle: 3 min at 94 °C; 30 s at 512 nm). The distributions of the green fluorescence values were processed to find the two main density peaks, which correspond to the two cell populations, respectively, in

Fluorescence microscopy. The SYTOX™ green dye was loaded into the cultures (see Note 2) and imaged using a Guava® easyCyte HT flow cytometer. Images were analyzed with the Guava® easyCyte HT software (version 3.6.1, Millipore Corp., USA) to determine the fraction of cells with more than one SYTOX™ green fluorescence signal. The number of cells was multiplied by two to estimate the number of cells per colony. The results were compared with the number of colonies on the plates and the results were correlated with the colony size analysis.

Flow cytometry. Measurement of the cell DNA content was performed using flow cytometry with the SYTOX™ green staining assay (ThermoFisher, Waltham, USA). Cells were first thawed from glycerol stocks on solidYPD in 96-well plates (room temperature, 3 days) including controls. The parental strain Sp8 (MHS604) was used as control on both its haploid and diploid (wild strain) state. LiquidYPD cultures of 1 ml in 96-deepwell (2mL) plates were inoculated and incubated for 24 h at room temperature. Cells were subsequently prepared as described in Gerstein et al., cells were first fixed in 70% ethanol for at least 1 h at room temperature. RNAs were eliminated from fixed cells using 0.25 mg ml$^{-1}$ RNase A during an overnight incubation at 37 °C. Cells were subsequently washed twice using sodium citrate (50mM, pH7) and stained with a final SYTOX™ green concentration of 0.6 µM for 30 min. Fluorescence was measured using a FACSAria flow cytometer (BD Biosciences, San Jose, CA, USA). The compensation was adjusted to be around a cell concentration less than 500 cells µL$^{-1}$. Five thousand cells for each sample were analysed on a Guava® easyCyte HT flow cytometer using a sample tray for 96-well microplates. Cells were excited with the blue laser at 488 nm and fluorescence was collected with a green fluorescence detection channel (peak at 512 nm). The distributions of the green fluorescence values were processed to find the two main density peaks, which correspond to the two cell populations, respectively, in G1 and G2 phases. The data were analysed using R version 3.4.1.

Mating type DNA genotyping. The MAT locus was genotyped for the presence of MATa, MATa or both copies by PCR in the stock of the haploid Sp parental strains (LL11_004 and LL11_009). Genomic DNA was extracted following standard protocols (Qiagen DNeasy Blood and Tissue Kit). Genomic DNA was amplified from five isolated colonies from the Sp duplicate plates and from the same stocks, two triploid
Genotyping by sequencing. We performed genotyping-by-sequencing (GBS) to investigate the genomic composition of the triploid and tetraploid hybrids. We sampled 1199 reads in total: 672 diploids and 16 triploids from the Lm/Mp crosses at Tend, all eight tetraploids and as controls two diploids from each cross Lm/Mp, Mmp, and Hmp at Tmmp, and Tmp and as all parental strains. DNA was extracted from overnight cultures issued from one isolated colony following standard protocols (QIAGEN DNAeasy, Hilden, Germany). As controls, we prepared artificial hybrid genomes by mixing DNA of parental strains with different proportion from each 0.50, 0.60, 0.60.33, or 0.33,0.60. DNA was quantified using Accuquant® Ultrapure sensitivity dsDNA Quantiﬁcation kit (Biotium, Fremont, USA) in a Spark® microplate reader (TECAN, Männedorf, Switzerland). DNA concentration was normalized to 10 ng µl

Whole-genome sequencing. We performed whole-genome sequencing to investigate the genomic composition of the eight tetraploid hybrids at Tmmp, and Tmmp for all crosses at Tmmp, as well as the six corresponding haploid parental strains. Genomic DNA was extracted from overnight cultures issued from one isolated colony following standard protocols (QIAGEN DNAeasy, Hilden, Germany). Libraries were prepared with the Illumina Nextera kit (Illumina, San Diego, USA) following the manufacturer’s protocol and modiﬁcations from by Baym and colleagues65. Pooled libraries were sequenced in paired end, 150bp mode on different lanes of HiSeqX (Illumina, San Diego, USA) at the Genome Quebec Innovation Center (Montreal, Canada). The 22 genomes were sequenced with an average genome-wide coverage of 90x. Raw sequences are accessible at NCBI (bio project ID PRJNAS15073).

Statistical analyses. Survival curves were produced and analysed using the R packages survival68 and survminer69. The analysis of the correlation between sporulation and genetic divergence was performed using the glm R function to perform a logistic regression with the formula: Sporulation Tend~ Genetic Divergence. Statistical analyses and figure creation for fertility data were done using custom scripts from Supplementary Data 1 in Python version 3.3.9. Figure generation and analyses for mtDNA loss and sporulation capacity were performed using custom scripts in Python (version 3.6.3).

ade2-3 colony coloration phenotype. Although we used the ade2-Δ marker as a visual aid to track loss of mitochondrial DNA, we still passed strains that seem to have lost mitochondrial DNA. During the experiment some colonies from all crosses suddenly turned bright or light orange. This pale coloration was correlated with the absence of sporulation in the strains. The only thing that changed during the evolution experiment is the yeast extract (EMD millipore, Burlington, USA), for which the lot of number changed. Further testing suggests that the pink/red coloration of the ade2-Δ mutants is media dependent. On one of the yeast extract lot used, white colonies appear red and show slower growth while on the other, most colonies are white and show normal growth. The slower growth is common to all ade2-Δ strains (Supplementary Fig. 22).

Received: 19 February 2019 Accepted: 19 August 2019

Published online: 11 September 2019

References
1. Mallet, J. Hybridization as an invasion of the genome. Trends Ecol. Evol. 20, 239–237 (2005).
2. Stuekenbrock, E. H. The role of hybridization in the evolution and emergence of new fungal plant pathogens. Phytopathology 106, 104–112 (2016).
3. Grant, P. R. & Grant, B. R. Phenotypic and genetic effects of hybridization in Darwin’s finches. Evolution 48, 297–316 (1994).
4. Arnold, M. L. & Martin, N. H. Adaptation by introgression. J. Biol. 8, 82 (2009).
5. Lewontin, R. C. & Birch, L. C. Hybridization as a source of variation for adaptation to new environments. Evolution 20, 315–336 (1966).
6. Maheshwari, S. & Barbash, D. A. The genetics of hybrid incompatibilities. Annu Rev. Genet 45, 331–355 (2011).
7. Rieseberg, L. H. Chromosomal rearrangements and speciation. Trends Ecol. Evol. 16, 351–358 (2001).
8. Marsden-Jones, E. M. The genetics of Geum intermedium wild. Haud ehrh., And its back-crosses. J. Genet. 33, 377–395 (1930).
9. Mallet, J. Hybrid speciation. Nature 446, 279–283 (2007).
10. Anderson, E. & Stebbins, G. L. Jr. Hybridization as an evolutionary stimulus. Evolution 8, 378–388 (1954).
11. Hiltu, K. W. Polyploidy and the evolution of domesticated plants. Am. J. Bot. 80, 1494–1499 (1993).
12. Otto, S. P. & Whitton, J. Polyploid incidence and evolution. Annu Rev. Genet. 40, 401–437 (2000).
13. Albertson, W. & Marullo, P. Polyploidy in fungi: evolution after whole-genome duplication. Proc. Biol. Sci. 279, 2497–2509 (2012).
14. Liti, G., Barton, D. B. & Lewis, E. J. Sequence diversity, reproductive isolation and species concepts in Saccharomyces. Genetics 174, 839–850 (2006).
15. Hittinger, C. T. Saccharomyces diversity and evolution: a budding model genus. Trends Genet 29, 309–317 (2013).
16. Kuehne, H. A., Murphy, H. A., Francis, C. A. & Snigheid, P. D. Allopatric divergence, secondary contact, and genetic isolation in wild yeast populations. Curr. Biol. 17, 407–411 (2007).
17. Leducq, J. B. et al. Speciation driven by hybridization and chromosomal plasticity in a wild yeast. Nat. Microbiol 1, 15003 (2016).
18. Eberlein, C. et al. Hybridization is a recurrent evolutionary stimulus in wild yeast speciation. Nat. Commun. 10, 923 (2019).
19. Charron, G., Leducq, J. B. & Landry, C. R. Chromosomal variation segregates within incipient species and correlates with reproductive isolation. Mol. Ecol. 23, 4362–4372 (2014).
20. Lynch, M. et al. A genome-wide view of the spectrum of spontaneous mutations in yeast. Proc. Natl Acad. Sci. USA 105, 9272–9277 (2008).
21. Nishant, K. T. et al. The baker’s yeast diploid genome is remarkably stable in vegetative growth and meiosis. PLoS Genet 6, e1001109 (2010).
22. Neiman, A. M. Sporulation in the budding yeast Saccharomyces cerevisiae. Genetics 189, 737–765 (2011).
23. Kienzi, M. T., Tingle, M. A. & Halvorson, H. O. Sporulation of Saccharomyces cerevisiae in the absence of a functional mitochondrial genome. J. Bacteriol. 117, 80–88 (1974).
24. Chou, J. Y., Hung, Y. S., Lin, K. H., Lee, H. Y. & Leu, J. Y. Multiple molecular mechanisms cause reproductive isolation between three yeast species. PLoS Biol. 8, e1000432 (2010).
25. Cherry, J. M. et al. Saccharomyces Genome Database: the genomics resource of budding yeast. Nucleic Acids Res. 40, D700–D706 (2012).
26. Josephs, S. B. & Hall, D. W. Spontaneous mutations in diploid Saccharomyces cerevisiae: more beneficial than expected. Genetics 168, 1817–1825 (2000).
27. Zhu, Y. O., Siegal, M. L., Hall, D. W. & Petrov, D. A. Precise estimates of mutation rate and spectrum in yeast. Proc. Natl Acad. Sci. USA 111, E2310–E2318 (2014).
28. Guldener, U., Timmusk, M., Louis, E. J. & Borst, R. H. A role for the mismatch repair system during incipient speciation in Saccharomyces. J. Evol. Biol. 16, 429–437 (2003).
29. Rogers, D. W., McConnell, E., Ono, J. & Greig, D. Spore-autonomous meiotic mechanisms. Proc. Natl Acad. Sci. USA 109, 20110–20115 (2012).
30. Zeyl, C., Curtin, C., Karnap, K. & Beauchamp, E. Antagonism between sexual sterility and hybrid sterility in Saccharomyces. Proc. Biol. Sci. 279, 1157–1159 (2012).
31. Harari, Y., Ram, Y. V. & Kupiec, M. Frequent ploidy changes in growing yeast cultures. Curr. Genet. 64, 1001–1004 (2018).
32. Zhu, Y. O., Siegal, M. L., Hall, D. W. & Petrov, D. A. Precise estimates of mutation rate and spectrum in yeast. Proc. Natl Acad. Sci. USA 111, E2310–E2318 (2014).
33. Ortíz-Merino, R. et al. Evolutionary restoration of fertility in an interspecies hybrid yeast, by whole-genome duplication after a failed mating-type switch. PLoS Biol. 15, e2002128 (2017).
34. Janke, C. et al. A versatile toolbox for PCR-based tagging of yeast genes: new methods. Methods 9, 357–359 (2012).
35. Grant, V. Plant speciation. Annu. Rev. Ecol. Syst. 35, 455–481 (2004).
36. Lee, P. S. et al. A ne-structure map of spontaneous mitotic crossovers in the yeast Saccharomyces cerevisiae. Mol. Biol. Evol. 27, 1553–1565 (2010).
37. Guldener, U., Heck, S., Fielder, T., Beinhauer, J. & Hegemann, J. H. A new gene collection and data entry system for the budding yeast Saccharomyces cerevisiae. Nucleic Acids Res. 24, 2519–2524 (1996).
38. Janke, C. et al. A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. Yeast 21, 947–962 (2004).
39. Yona, A. H. et al. Yeast meiotic chromosome mis-segregation as the principal cause of hybrid sterility in yeast. PLoS Biol. 16, e2003568 (2018).
40. Zeyl, C., Curtin, C., Karnap, K. & Beauchamp, E. Antagonism between sexual sterility and hybrid sterility in Saccharomyces. Proc. Biol. Sci. 279, 1157–1159 (2012).
41. Lee, P. S. et al. A ne-structure map of spontaneous mitotic crossovers in the yeast Saccharomyces cerevisiae. Mol. Biol. Evol. 27, 1553–1565 (2010).
42. Tsai, I. J., Bensasson, D., Burt, A. & Koufopanou, V. Population genomics of yeasts: the principal cause of hybrid sterility in yeast. PLoS ONE 10, e0128036 (2015).
43. Kellis, M., Patterson, N., Endrizzi, M., Birren, B. & Lander, E. S. Sequencing and comparison of yeast species to identify genes and regulatory elements. Nature 423, 241–254 (2003).
44. Mable, B. K. M. N. J. P. D. A. A versatile toolbox for PCR-based tagging of yeast genes: new methods. Methods 9, 357–359 (2012).
45. Mable, B. K. M. N. J. P. D. A. A versatile toolbox for PCR-based tagging of yeast genes: new methods. Methods 9, 357–359 (2012).
46. Grant, V. Plant speciation. Annu. Rev. Ecol. Syst. 35, 455–481 (2004).
47. Lee, P. S. et al. A ne-structure map of spontaneous mitotic crossovers in the yeast Saccharomyces cerevisiae. Mol. Biol. Evol. 27, 1553–1565 (2010).
48. Gerstein, A. C., Chun, H.-J. E., Grant, A. & Otto, S. P. Genomic convergence toward diploidy in Saccharomyces cerevisiae. PLoS Genet. 2, e1-45 (2006).
49. Janke, C. et al. A versatile toolbox for PCR-based tagging of yeast genes: new methods. Methods 9, 357–359 (2012).
50. Guldener, U., Heck, S., Fielder, T., Beinhauer, J. & Hegemann, J. H. A new gene collection and data entry system for the budding yeast Saccharomyces cerevisiae. Nucleic Acids Res. 24, 2519–2524 (1996).
51. Catchen, J., Hohenlohe, P. A., Basham, S., Amores, A. & Cresko, W. A. Stacks: an analysis tool set for population genomics. Mol. Ecol. 22, 3124–3140 (2013).
52. Li, H. et al. The sequence alignment/map format and SAMtools. Bioinformatics 25, 2078–2079 (2009).
53. Li, H. et al. Inexpensive multiplexed library preparation for megabase-sized genomes. PLoS ONE 10, e0128036 (2015).
54. Li, H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv preprint. https://arxiv.org/abs/1303.3997 (2013).
55. Kellis, M., Patterson, N., Endrizzi, M., Birren, B. & Lander, E. S. Sequencing and comparison of yeast species to identify genes and regulatory elements. Nature 423, 241–254 (2003).
56. Kasambabara, A. K. M. S. Survival: Drawing Survival Curves using ggplot2'. https://cran-r-project.org/web/packages/survminer/index.html (2018).
57.Acknowledgements
We thank the members of the Landry lab for discussions and J. Hallin, A. Dubé, C. Eberlein, A. Fijarczyk, N. Aubin-Horth, J. Anderson, L. Kohn, S. Otto, C. Mérot, and A.M. Dion-Coté for useful comments on the manuscript. We thank C. Mérot for help with the library construction protocols and A. Fijarczyk for help with genome data analysis. This work was supported by grants from the NSERC Discovery and Canada Research Chair to C.R.L., FRQNT scholarship to G.C. and M.H., NSERC Alexander Graham-Bell scholarship to G.C. and FRQs post-doctoral fellowship to S.M.

Author contributions
Conceptualization: C.R.L., G.C., M.H., and S.M.; Data curation: G.C., S.M. and M.H.; Funding acquisition: C.R.L.; Experimental work, formal analysis, and interpretation: G.C., S.M. and M.H.; Writing—original draft: G.C. and M.H.; Writing—review and editing: all authors.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-12041-8.

Competing interests: The authors declare no competing interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Peer review information Nature Communications thanks Aleeza Gerstein, Rike Stelken and the other, anonymous, reviewer for their contribution to the peer review of this work. Peer reviewer reports are available online.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Downloaded from https://doi.org/10.1038/s41467-019-12041-8