Evolutionary trajectories are contingent on mitonuclear interactions

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Abstract

Critical mitochondrial functions, including cellular respiration, rely on frequently

interacting components expressed from both the mitochondrial and nuclear genomes. The

fitness of eukaryotic organisms depends on a tight collaboration between both genomes.

In the face of an elevated rate of evolution in the mitochondrial genome, current models

predict that maintenance of mitonuclear compatibility relies on compensatory evolution

of the nuclear genome. Mitonuclear interactions would therefore exert a strong influence

on evolutionary trajectories. One prediction from this model is that the same nuclear

genomes but evolving with different mitochondrial haplotypes would follow distinct

molecular paths towards higher fitness peaks. To test this prediction, we submitted 1344

populations derived from seven mitonuclear genotypes of *Saccharomyces cerevisiae* to

more than 300 generations of experimental evolution in conditions that either select for a

mitochondrial function, or that do not strictly require this organelle for survival.

Performing high-throughput phenotyping and whole-genome sequencing on

independently evolved individuals isolated from endpoint populations, we identified

numerous examples of gene-level evolutionary convergence among populations with the

same mitonuclear background. We recapitulated a subset of prominent loss-of-function

alleles in the ancestral backgrounds. This confirmed a generalized pattern of mitonuclear-

specific and highly epistatic fitness effects. Together, these results demonstrate how

mitonuclear interactions can dictate evolutionary divergence of populations with identical

starting nuclear genotypes.

Keywords: mitonuclear evolution, mitochondria, evolutionary convergence,
experimental evolution

Introduction

According to the endosymbiotic hypothesis, mitochondria derive from an ancestral bacterial symbiont that gradually specialized in its current cellular functions (Dyall et al. 2004; E.S. Fritsch et al. 2014). While most mitochondrial proteins are encoded in the nucleus, the vestigial genome of the organelle retains key genes, thought to favor responsiveness to changing conditions (Allen et al. 2003; Lane and Martin 2010; Lane 2011). Gene products encoded in both genomes are thus required for mitochondrial function. The mitochondrion is now the site of cellular respiration, and as such, plays a central role in energy production (Malina et al. 2018). Other important metabolic processes involve the mitochondrion, including the biosynthesis of heme (Ferreira 1995), iron-sulfur clusters (Read et al. 2021) and steroids (Jordá and Puig 2020). Mitochondria are also involved in nutrient sensing, the regulation of redox homeostasis, and several signaling pathways (Malina et al. 2018).

Components encoded in the nuclear and mitochondrial genomes interact extensively, sometimes encountered together as part of mitochondrial protein complexes (Malina et al. 2018). Cytochrome *c* oxidase (Cooper et al. 1991) and the cytochrome *bc1* complex (Hunte et al. 2003), components of the respiratory electron transport chain, are assembled from proteins encoded by both genomes. In yeast, the mitogenome encodes for mitochondrial ribosomal RNAs and one ribosomal polypeptide, while most protein components are found in the nuclear genome (Desai et al. 2017). In humans, the nucleus-encoded protein LRPPRC is required for cytochrome *c* maturation via interaction with mitochondrial mRNAs, and mutations that alter this function are associated with severe disease (Xu et al. 2012). These examples illustrate the critical importance of mitonuclear interactions, and how their disruption may severely reduce fitness.

Yet, because they are distinct and segregated to separate cell compartments, molecular evolution in the nuclear and mitochondrial genomes is partially uncoupled. Mutation rate tends to be higher in mtDNA (Fritsch et al., 2014; Lynch et al., 2008), especially in organisms with uniparental inheritance and lacking a dedicated mitochondrial mismatch

repair system (Mason and Lightowlers 2003; Havird and Sloan 2016). In many
organisms, uniparental inheritance also leads to smaller effective population size and the
virtual lack of recombination (Lynch et al. 2006; Hagström et al. 2014). More generally,
mitochondria evolve in a regulated microenvironment – the cytoplasm with its nuclear
supply of mitochondrial proteins – that differs from the extracellular milieu affecting the
entire cell. Illustrating this peculiar aspect of mitochondrial evolution is the suppressivity
of ρ^- mitogenomes in yeast. In these cells, despite a strongly deleterious effect on
organismal fitness, copies of the mitochondrial genome deleted at loci essential for
respiration outcompete healthy copies thanks to their higher replication origin density (de
Zamaroczy et al. 1981; Mangin et al. 1983; Bernardi 2005). In summary, because of
unique characteristics and selection pressures, the rate of evolution tends to be higher in
the mitochondrial genome than in the nuclear genome, with a critical influence on genetic
divergence, speciation, reproductive strategies, and morphology (Hill 2015).

A model has emerged in which rapid and essentially non-adaptive changes to the
mitochondrial genome exert a constant selective pressure on the nuclear genome to
accumulate compensatory mutations (Dowling 2014). Rapid changes in mtDNA would
drive the nucleus towards increasing divergence, the evolution of Dobzhansky-Muller
incompatibilities favoring speciation via post-zygotic reproductive isolation (Freel et al.
2015). Such incompatibilities are expected to arise following hybridization between
allopatric populations, whose mitonuclear genotypes have evolved independently under
different environmental conditions. As extensively described in the copepod *Tigriopus*
californicus, hybrids may display fitness loss and reproductive incompatibility when
initially separated populations come into contact, generating mismatched mitonuclear
genotypes (Burton and Barreto 2012; Barreto et al. 2018; Burton 2022). A growing body
of evidence accumulates in favor of this model. Early studies showed that species
divergence is associated with a loss of mitonuclear compatibility (Niki et al. 1989;
Osuský et al. 1997; Dey et al. 2000a; McKenzie and Trounce 2000; Špírek et al. 2000;
Yamaoka et al. 2000; Kenyon and Moraes 2002). More recent reports provide examples
of reproductive isolation and hybrid fragility associated with mitonuclear
incompatibilities (Lee et al. 2008; Chou et al. 2010; Paliwal et al. 2014; Ma et al. 2016;

Haddad et al. 2018). The power of mitochondrial markers and genome structures as
phylogenetic predictors further supports a driving role for the evolution of mtDNA in
divergence and speciation (Robba et al. 2006; Hendrich et al. 2010; Wolters et al. 2015).
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Current understanding about the cell and molecular biology of the mitochondrion owes
much to methods and approaches pioneered in the yeast *S. cerevisiae* (Rutter and Hughes
2015). Their capacity to proliferate in absence of functional mitochondria enables explicit
experimental selection for respiratory function, via the use of fermentable and non-
fermentable carbon sources. This facilitates cytoduction, in which mitochondrial
genomes are exchanged between individuals of the same or different species, generating
mitonuclear hybrids (Zakharov and Yarovoy 1977; Barrientos et al. 1998). Cytoduction
has been applied to the study of evolutionary questions in mitochondrial biology, for
example investigating the compatibility of mitochondrial genomes between closely
related yeast species. Coherent with a role for mitochondria in defining the interspecific
barrier, these studies showed a rapid decline in the efficiency of oxidative
phosphorylation as species divergence increases between the nuclear and mitochondrial
parents of mitonuclear hybrids (Barrientos et al. 1998; Dey et al. 2000a; Dey et al. 2000b;
McKenzie and Trounce 2000; Yamaoka et al. 2000; Kenyon and Moraes 2002; Lee et al.
2008; Ma et al. 2016). Cytoduction has also helped identify key genes and molecular
functions involved in the mitonuclear incompatibilities that arise between yeast species
(Spirek et al. 2015). Large collections of yeast mitonuclear hybrids have recently helped
explore the genetic space of mitonuclear interactions, revealing the impact of mitonuclear
interactions on fitness, and the complex third-order relationships that exist between
environmental conditions, the nuclear genome, and the mitochondrial genome (Paliwal et
al. 2014; Nguyen et al. 2020).
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Standing models thus predict that mitonuclear interactions must influence the path of
evolution. Available evidence from wild individuals and populations supports this model,
yet direct experimental testing of this hypothesis in naturally evolving populations is
challenging. Experimental evolution in microbes is ideally suited to address this problem,
because it can be replicated, enabling the observation of repeatable patterns among

identically but independently evolved individuals and populations. Indeed, one of the

fundamental questions that underlie much of the experimental evolution literature

concerns the predictability of evolution. Given identical genetic and environmental

circumstances, will independently evolving populations take the same evolutionary path?

Will phenotypes be identical, will the same alleles be selected, or at least will the same

genes and functional systems be affected? Repeatable outcomes have been reported in

terms of both phenotype and genotype (Wichman et al. 1999; Cooper et al. 2003;

Mcdonald et al. 2009; Herron and Doebeli 2013; Lang et al. 2013). Available evidence

does not decisively argue for predictability (Bull and Molineux 2008; Łuksza and Lässig

2014), yet the controlled context of experimental evolution seems to lead to evolutionary

convergence at the functional complex and gene levels (Tenaillon et al. 2012). Another

important advantage of experimental evolution in microbes is the conservation of

evolving populations at different steps of the evolutionary process. Evolved individuals

can thus be compared experimentally to their ancestors. Mutations selected by evolution

can be reconstituted in ancestral backgrounds and assessed for their impact on fitness

using marker-assisted competition assays (Breslow et al. 2008; Lang et al. 2013),

allowing the precise dissection of the genetic architecture of evolved mutants. Building

on these methodological foundations, experimental evolution studies have demonstrated

that the path to adaptation is contingent on the genetic makeup of the evolving population

and the characteristics of its environment (Blount et al. 2008; Meyer et al. 2012).

Therefore, analysis of evolutionary convergence in replicated experimental evolution

setups appears as a mean to assess the impact of specific environmental and genetic

factors, such as mitonuclear interactions, on evolutionary trajectories and outcomes.

In this study, we leverage a previously reported collection of yeast mitonuclear hybrids

(Paliwal et al. 2014; Nguyen et al. 2020) to investigate the evolutionary impact of

mitonuclear interactions. We performed experimental evolution on 1344 replicate

populations of mitonuclear hybrids of *S. cerevisiae*, varying the intensity of selection on

mitochondrial function in an experiment spanning more than three hundred generations.

Evolving mitonuclear hybrids provided accelerated mimics of the evolutionary dynamics

encountered in organisms with rapidly diverging mitogenomes. The level of replication

provided us with a wealth of identically yet independently evolved individuals. We

predicted that high-throughput growth assays and whole-genome sequencing on these

individuals would allow us to detect patterns of evolution influenced by mitonuclear

interactions, at the phenotypic or genetic levels.

Results

Negative epistasis in mitonuclear yeast hybrids.

Our founding intent was to study the influence of mitonuclear interactions on the path of

evolution. We therefore screened a collection of mitonuclear hybrids of *S. cerevisiae* for

evidence of such mitonuclear interactions. This collection contains 225 strains of yeast

generated by cytoduction in four replicates and representing all possible combinations of

nuclear and mitochondrial genomes from 16 strains of *S. cerevisiae* (Paliwal et al. 2014;

Wolters et al. 2018). We recorded growth curves for all strains of the collection in a

condition – glycerol as the sole carbon source – that requires mitochondrial activity to

sustain the growth of facultative anaerobic yeast. As observed previously (Nguyen et al.,

2020; Paliwal et al., 2014; Wolters et al., 2018), the screen showed the predominant

effect of the nuclear genome on fitness but revealed a modulating influence from the

mitochondrial genome (**Fig S1**A). Some pairs of nuclear and mitochondrial parents

displayed a significant depression in fitness upon cytoduction, indicative of negative

epistasis between mismatched nuclear and mitochondrial genomes. Mitonuclear hybrids

formed between strain DBVPG6044 (D) and strains 273614N (N) and Y12 (Y) were

submitted to further scrutiny, confirming the loss of fitness incurred by cytoduction in

these backgrounds (**Fig S1**BC). Following a two-letter nomenclature, indicating the

nuclear background first and the mitochondrial background second, genotypes of interest

were: NN, ND, DN, DD, DY, YD, and YY (**Fig 1**A). Assays on mitochondrial enzymes

in these strains and their mitonuclear hybrids further suggested perturbation of metabolic

activities associated with the mitochondrion when organellar genomes were swapped

(**Fig S2**). We thus found evidence of negative interactions between nuclear and

mitochondrial genomes in mitonuclear hybrids of strains N, D and Y, enabling the study

of how the mitochondrial environment can influence the evolution of the nuclear genome.

Experimental evolution of yeast mitonuclear hybrids leads to fitness increases in both

fermentable and non-fermentable media.

We evolved seven mitonuclear backgrounds derived from strains N, D and Y. Four

biological replicates of each mitonuclear background were used to found 24 replicate

evolution experiments, amounting to a total of 96 evolving populations. This experiment

was performed in both fermentable and non-fermentable media, hence in conditions that

either do not explicitly require or that absolutely necessitate respiratory function,

respectively. We cultured 1344 independently evolving populations by daily serial

passage for 58 days, amounting to approximately 300 generations (**Fig 1**B). Frozen

stocks were prepared at the beginning and end of the experiment, as well as every seventh

passage. While a subset of 230 populations went extinct or were terminated because of

bacterial contamination, 1114 populations survived to the end. A single individual was

isolated by streaking for single colonies from stocks of each of the surviving populations

We next measured the extent of adaptation in evolved individuals by recording growth on

both fermentable and non-fermentable media, in an automated and highly replicated

manner (see Methods). As expected, evolution generally led to increases in fitness, but

the magnitude of the median fitness gains was dependent on the fitness of the founding

strains (**Fig 1**CD). Best illustrating this trend are strains carrying the D nuclear genome

for which comparingly low initial fitness in fermentable media represented potential for

large gains (**Fig 1**C). In comparison, mean fitness gains in fermentable medium are

modest (Y nucleus) if not null (N nucleus) with other nuclear backgrounds. Interestingly,

in fermentable medium, a similar endpoint fitness, close to the value of some of the

ancestors (NN, YY) is reached in all backgrounds, suggesting that evolved individuals

are, on average, approaching fitness optimum. Evolution in non-fermentable medium also

generally led to gains in fitness. Also similar between conditions is the lower apparent

evolvability of strains endowed with the N nuclear genome, associated with the lowest

median fitness among evolved individuals. Yet, variation between ancestral backgrounds

is not as pronounced in non-fermentable medium, leading to less variation in the

magnitude of fitness gains. Endpoint fitness also appears more variable in this

environment. Of note, in non-fermentable medium, the fittest of the ancestral

backgrounds (YY) also displays one of the largest median gains in fitness, suggesting

individuals evolved in this environment are still largely far from fitness optimum.

Fitness of all evolved individuals were assayed simultaneously in their home

environment, as well as their non-native environment. Gains achieved in one carbon

source often realized in the other. Indeed, in most mitonuclear backgrounds, individuals

evolved in fermentable and non-fermentable medium display similar fitness in both

carbon sources (**Fig S3**). Strikingly, in non-fermentable conditions, strains evolved in this

medium are frequently matched or outperformed by those evolved in fermentable

medium (**Fig S3**BD). Similarly, in fermentable medium, while non-fermentable-evolved

individuals tend to lag behind their fermentable-evolved counterparts, they still

demonstrate significant fitness gains over ancestral levels (**Fig S3**AC). These

observations suggest that much of the gain in fitness is related to aspects common to both

environments, either in terms of composition or culture conditions.

In a complementary analysis, we tested the impact of nuclear, mitochondrial and

mitonuclear backgrounds on fitness in ancestral and evolved individuals, with special

attention to carbon source effects. We hypothesized that strains evolved in non-

fermentable medium, and thus explicitly selected for respiratory function, would reveal

large fitness effect differences between mitochondrial backgrounds compared to strains

evolved in fermentable medium (**Fig S4**A). We therefore performed a series of two-way

ANOVAs on fitness proxies in fermentable or non-fermentable media, partitioning data

according to nuclear and mitochondrial backgrounds (**Fig S4**B). This analysis confirms

the strong differences in fitness between backgrounds in the ancestral strains, and how

they tend to resorb with evolution. Neither nuclear, mitochondrial nor mitonuclear

backgrounds are associated with difference in fitness among strains evolved in

fermentable medium, indicating full resorption of ancestral divergences in growth.

However, individuals evolved in non-fermentable conditions display differences in

fitness associated with nuclear and mitonuclear backgrounds, albeit no significant

mitochondrial effects are detected. In both conditions, populations evolved for a

comparable number of generations and accumulated a similar number of mutations (**Fig**

2B). Our results thus suggest that mitochondrion-specific selective pressures favor

phenotype-level divergence associated with mitonuclear interactions. More generally, our

assessment of fitness in evolved individuals strongly indicates that the magnitude of gains

is primarily contingent on the nuclear background of ancestral strains, with influence

from the mitochondrial background exacerbated in presence of explicit selection for

respiratory function.

Sequencing of evolved genomes reveals patterns of evolutionary convergence.

To gain insight in the molecular mechanisms involved in the adaptation of evolved

isolates, we sequenced each of their genomes, along with that of their ancestors, aligning

to published sequences of ancestral nuclear and mitochondrial backgrounds. Our partially

redundant dataset comprised 1505 non-empty sequencing libraries and provided genomic

data for 1114 evolved isolates and 28 ancestral strains. With a 31x median depth of

coverage, most libraries were suitable for the confident calling of mutations, from the

whole-chromosome to single-nucleotide scales (**Data S1**). In contrast with published

methods that rely on median depth at stably covered loci *COX1* or *COX3* (Puddu et al.,

2019), and acknowledging the complex and highly branched topology of *S. cerevisiae*

mtDNA (Bendich 1996; Bendich 2007), we abstained from claims about mitogenome

copy number, and used the fraction of sequence bases mapped to the mitochondrial

genome as a proxy for the relative abundance of mtDNA per cell in ancestral and evolved

strains (**Fig 2**A, **Data S2**). We observed changes in mtDNA abundance in mitonuclear

hybrids, corroborating other observations (**Fig S2**) indicating a disruption of

mitochondrial homeostasis upon cytoduction. We also observed a trend towards decrease

of mtDNA abundance among evolved isolates. Lower abundance of mtDNA in evolved

strains is likely not the result of widespread petite genotypes, since most isolates display

fitness in non-fermentable media that is equal or superior to their non-evolved ancestors

(**Fig S3**BD). All these observations seem specific to each mitonuclear background. For

example, large disruptions in mtDNA abundance are observed in cybrids ND, and to a

lesser extent DY, while the DN and YD backgrounds appear much less affected.

Similarly, extent of the contraction in mtDNA abundance is dependent on both

mitonuclear background and evolution conditions. Magnitude of the decrease is largest in

either fermentable or non-fermentable media, depending on the mitonuclear background.

Because environmental demands on respiratory metabolism affect mtDNA copy

numbers, our observations about mtDNA abundance are chiefly relevant to the

fermentable conditions in which we propagated the yeast used for gDNA extraction

(Galeota-Sprung et al., 2022).

We detected copy number increases for several chromosomes, prominently including

chromosomes XII, IV and IX (**Fig S5**). We detected aneuploidies at chromosome XII in

46 independently evolved strains: the vast majority bore the N nuclear genome, and most

carried the matching mitochondrial genome. We observed the same pattern for

aneuploidies of chromosome IV. In a similar manner, we most often found extra copies

of chromosome IX in mitonuclear hybrid derivatives of strain D (**Fig 2**B). Along with

changes in mtDNA abundance, these repeated large-scale genomic changes suggested a

pattern of convergent evolution influenced by the identity and interaction of nuclear and

mitochondrial genomes.

We next identified smaller scale genetic changes. Following stringent bioinformatics

pipelines, we called 272 copy number variants (CNVs), ranging in size from 184 bp to

1091 bp, and 3710 single nucleotide variants (SNVs) (**Data S3**). Neither CNVs nor SNVs

were distributed randomly among mitonuclear backgrounds and carbon sources (chi^2 test

p-values (3.37×10^{-22}) and (7.23×10^{-84}), respectively). This fact may appear most

obvious observing that only nine CNV calls were made in nuclear derivatives of strain D.

In stark contrast, ND derivatives evolved in fermentable medium and YD derivatives

evolved in non-fermentable medium accumulated 92 and 86 CNVs, respectively (**Fig

2**B). Interestingly, one may note that CNVs are overwhelmingly called in backgrounds

ND and YD, both derivatives of mitochondrial swaps. In the same way, SNVs are

unevenly distributed, yet the large number of calls ensures that more than a hundred

mutations were detected per mitonuclear background in each carbon source. Together,

these small-scale changes provided further hints that both mitonuclear interactions and

the environment inflected adaptive trajectories in our mitonuclear hybrids.

To determine if patterns of specificity were observed at the gene level, we examined

annotations associated with CNV and SNV calls (**Data S4**). As for aneuploidies, gene-

level annotation indicated evolutionary convergence associated with several loci. Indeed,

hundreds of genes are associated with more than one mutation among evolved clones,

and 43 display ten or more. Along with aneuploidy at chromosome XII, nuclear genes

RSN1, *HPF1*, *MNN4*, *AMN1* and mitochondrial gene *COX1* are the annotations most

affected by changes, with tens to more than a hundred calls per locus mapping to these

areas of the genome. Examining mutation counts associated with these loci in each

mitonuclear background and carbon source (e.g. **Fig S6**), we detected several examples

of apparent specificity. Our survey of genetic changes in evolved individuals, at all scales

examined, thus provided decisive evidence for evolutionary convergence within a given

mitonuclear background, and strongly suggested that adaptive changes followed a pattern

of specificity to both mitonuclear background and environmental conditions.

Mutations are associated with changes in fitness in evolved individuals.

Quantitative fitness data and a list of genetic changes are available for 957 evolved

individuals, along with fitness data in the 28 ancestral individuals. Similarly, we have

1110 individuals with both mtDNA abundance data and associated mutations. We

therefore tested the statistical association between mutant annotations and available

phenotype measurements. For each annotation, we tested whether individuals bearing the

associated mutations had phenotype significantly different from their ancestral strains.

We also tested the probability that phenotypic changes of a given magnitude, or more

extreme, could be obtained by chance from a random set of mutants of the same size. A

significant result to both tests identified annotations associated with phenotypic changes

(see Materials and Methods for details). Using this approach, we identified mutant

annotations significantly associated with changes in phenotype (**Fig 3**). Comparing

fitness in individuals that carry mutations at these loci with evolved and ancestral strains

wild type at the same loci may indicate mitonuclear background-specific effects (**Fig S7**).

Candidate fitness modifiers were most prominently identified at loci displaying strong

signals of evolutionary convergence, likely benefitting from the increased statistical

power afforded by a larger number of data points. While we did not obtain a definitive

list of evolutionary driver mutations using this analysis, we gained additional hints

concerning the contribution of certain genetic changes to adaptation in our evolution

experiment.

Mutations at several loci display mitonuclear and environmental specificity and thus

evolutionary contingencies.

We accumulated several lines of evidence suggesting that genetic changes selected in

evolved yeast populations displayed specificity towards either mitonuclear background,

carbon source, or both. To test this hypothesis, for all annotations, we performed chi^2-

tests of independence on contingency tables partitioning all mutations according to their

association with the annotation, and according to either mitonuclear background or

carbon source. We also computed mutual information from the same contingency tables.

Both a significant p-value from the test and significantly above average mutual

information were required to identify specific annotations (**Fig 4**). Similar computations

were performed, partitioning mutation counts according to nuclear and mitochondrial

backgrounds alone, or conjunctions of background with carbon source (**Fig S8**). These

analyses revealed loci or genomic regions biased towards mutation in a mitonuclear

background- and carbon source-specific manner. For example, out of 50 aneuploidies

detected at chromosome XII, 38 were found in background NN, and they appear to have

been selected almost exclusively in non-fermentable medium. Similarly, 94% of the 88

CNVs at gene *HPF1* were selected in background ND evolving in fermentable medium

(**Fig S6**). Background and carbon source specific mutations both represented

approximately 15% of all identified mutations.

To further investigate patterns of mitonuclear and environmental specificity, we

performed pairwise comparisons on the identity and counts of mutations in all

mitonuclear backgrounds and both carbon sources. For any given pair, we compared the

divergence in identity and counts of mutations between fermentable and non-fermentable

media (**Fig S4**C). This revealed that evolution in non-fermentable conditions was

associated with a significantly stronger divergence in the D and Y nuclear backgrounds.

This would suggest that selection for respiratory function favors mitonuclear background-
driven evolutionary divergence. Yet, the N nuclear background is associated with the
reverse tendency, regardless of the mitonuclear backgrounds to which it is compared,
including comparison of the NN and ND backgrounds. This suggests that the
evolutionary path adopted in presence of the N nuclear background tends to differ more
strongly from that of the other backgrounds in fermentable medium than non-fermentable
conditions. Taken together, our analysis of mutational specificity provided additional
evidence for mitonuclear contingency, whereby the interaction of nuclear and
mitochondrial genomes, as well as an environment that explicitly selects for respiratory
function both had a decisive impact on the adaptive trajectories favored by natural
selection.
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Reconstituting loss-of-function mutations in ancestral backgrounds leads to mitonuclear
background-specific fitness effects
Using patterns of convergent evolution, phenotypic association and both mitonuclear and
carbon source specificity, we identified a core set of annotations potentially involved in
adaptation or otherwise implicated in the evolution of our mitonuclear hybrids. Mapping
of mutations on these annotated features and prediction of their mutational effects
revealed widespread stop codons alongside nonsense mutations at conserved positions
(e.g., **Fig S9**). We therefore hypothesized that loss-of-function at these and other loci was
a widespread adaptive mechanism selected by our experiment, as observed for other
experimental populations evolved in constant environments (Kvitek and Sherlock 2013).
To mimic the effect of loss-of-function alleles, we generated a replicated set of knockouts
in fluorescent derivatives of each of the seven ancestral backgrounds at eight of the most
prominent loci identified in our dataset. We competed these knockouts against their wild
type counterparts in the conditions of the evolution experiment. From these competitions,
we estimated the fitness effect of loss-of-function alleles at these loci in all mitonuclear
backgrounds and both fermentable and non-fermentable media (**Fig 5**). Loss-of-function
at these loci led in most cases to significant gains in fitness, validating their roles in
adaptive evolution. The magnitude of the changes in fitness associated with each knock-
out differed significantly between mitonuclear backgrounds (ANOVA p-value < 0.01).

Some loci even showed opposite effects between different mitonuclear backgrounds,

further underlining the strongly epistatic nature of their impact on fitness. Furthermore,

examining mitonuclear backgrounds in a pairwise manner, we found that significant

differences in fitness effect were widespread, including between strains that shared the

same nuclear genome but differed only in terms of their mitochondrial genome (Tukey

HSD test p-value < 0.01). This provided additional support for a decisive role of

mitonuclear interactions in shaping and inflecting the path of adaptive evolution.

Competition assays provided additional support for evolution contingent on mitonuclear

interactions. Yet, for most tested loci, differential fitness effect patterns did not match

predictions derived from our analysis of evolutionary convergence (**Fig 5**, **Fig S10**).

Indeed, contrary to expectation, apart from *RIM20* knockouts growing in fermentable

medium, rank correlation was poor between estimated fitness effects and mutation

frequency in evolved mutants (Spearman correlation p-value on the slope > 0.05). In

other words, frequent mutation at a locus does not reliably predict strong gains in fitness

in knockouts of that locus. In contrast, in fermentable medium, predictions of locus

preference based on mutation frequency matched fitness effect-based predictions of

preference, for half of our mitonuclear backgrounds (**Fig 5, Fig S10**).

We explored possible explanations for the partial disconnect between convergence- and

fitness effect-based predictions. The experiment was based on the simplifying assumption

that complete loss-of-function is the primary and sole mechanism underlying the fitness

effect of mutation at all examined loci. Different or concurring mechanisms are likely

involved and may explain some of the discrepancy between fitness data and our analysis

of evolutionary convergence. Such mechanisms may include protein truncation, loss of

regulatory interactions, and partial reductions in activity, to name but a few. Yet, beside

the raw fitness effect of mutations in the ancestral strains, other mechanisms not related

to mitonuclear interactions may account for the observed patterns of mitonuclear and

carbon source specificity. Fitness of the ancestral strains (**Fig 1**CD**, Fig S3**) differs

markedly between mitochondrial backgrounds. If the fittest backgrounds are indeed

approaching fitness optimum, as hypothesized above, and given patterns of diminishing

returns epistasis common in experimental evolution (Chou et al. 2011; Wiser et al. 2013;

Couce and Tenaillon 2015), the magnitude of fitness gains accessible to different

mitonuclear pairs likely differed, constraining evolutionary paths to adaptation.

Considering that initial fitness itself is influenced by mitonuclear interactions, this effect

may prove difficult to deconvolute from other similarly influenced mechanisms.

However, we did identify loci uniquely or preferentially mutated in mitonuclear

backgrounds that otherwise display comparable initial fitness to closely related

backgrounds (**Fig S6**). More generally, because our competition assays establish that the

effect of loss-of-function alleles at the tested loci is epistatic in a mitonuclear

background-specific manner, we counted the number of mutation calls in evolved

individuals. We found that the median number of mutations detected per evolved

individual is two. This number increases to three, four and even 23 in strains mutant at

MNN4, *AMN1* and *SUC2*, respectively (**Data S3**). Hence, in evolved individuals, loss-of-

function alleles normally interact with other mutant loci, contrary to our simplified

experiment, performed in the ancestral backgrounds. This aspect appears critical when

considering mutational order: selection for mutation at any given locus is contingent on

the identity of mutations most likely to fix earlier during evolution (Weinreich et al.

2006). This itself may be influenced by mitonuclear interactions and illustrates the

difficulty of predicting patterns of mutational specificity and evolutionary convergence

based solely on fitness effects measured in the ancestral strains.

We also found that the mutational bias observed in backgrounds NN and YY differed

significantly, albeit slightly, from that of the entire dataset (**Fig S11**). This would support

the hypothesis that patterns of background-dependent evolutionary convergence may, at

least in part, be dictated by differential access to beneficial mutations rather than

mitonuclear interactions. Yet, because the nuclear genome, by virtue of its much larger

size, chiefly determines mutational bias, this hypothesis does not account for differences

in mutational specificity observed between backgrounds that carry the same nuclear

genome (as illustrated in **Fig S6**). Moreover, fitness effects inferred from association tests

in evolved mutants also correlated poorly with both convergent evolution signals and

competition data (**Fig S10**). Together, these results indicated that different mitonuclear

backgrounds may have access to different mutational spectra, in presence of multiple and
complex epistatic networks. Mitonuclear interactions are a critical component of these
epistatic networks and appear to exert a decisive impact on the path of evolutionary
adaptation in *S. cerevisiae*.
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496 **Discussion**
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The current model of mitonuclear evolution proposes that the mitochondrial genome,
through interactions with nuclear-encoded genetic material, exerts an influence on
evolutionary divergence of populations and eventually on the process of speciation. An
elevated rate of evolution observed in the mitogenome can exert a pressure for
compensatory evolution of the nuclear genome, leading to negative interactions between
mitonuclear pairs that have evolved independently (Dowling 2014). Evidence of
mitonuclear incompatibility between both closely related species (Niki et al. 1989;
Osuský et al. 1997; Dey et al. 2000a; McKenzie and Trounce 2000; Špírek et al. 2000;
Yamaoka et al. 2000; Kenyon and Moraes 2002; Lee et al. 2008; Chou et al. 2010; Ma et
al. 2016) and geographically isolated populations of the same species (Paliwal et al.
2014; Wolters et al. 2015; Barreto et al. 2018; Wolters et al. 2018; Nguyen et al. 2020)
corroborates this model. Yet, direct experimental evidence demonstrating the impact of
mitonuclear interactions on evolutionary trajectories and outcomes was still lacking. We
designed an experimental evolution study aimed at filling this gap. We mimicked the
potential pressure exerted by the mitochondrial genome on the nuclear genome by
swapping mitochondrial genotypes among yeast strains. We propagated these strains in a
highly replicated manner for several hundred generations, in conditions that either
explicitly select for a mitochondrial function or relax the selective pressure exerted on
this organelle. If the mitochondrial genome exerts a pressure on the nuclear genome,
independent populations of the same nuclear genotypes but with distinct mitochondrial
haplotypes should converge on different adaptive mutations.
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Mitonuclear interactions and their impact on evolutionary trajectories

The outcomes of our evolution experiment and the analyses herein support our prediction
that specific mitonuclear interactions led to distinct evolutionary trajectories. Our
phenotypic data suggests a weak to moderate influence on fitness attributed to
mitonuclear interactions. Such effects depended on conditions, the evolution regimen,
and fitness proxies retained for analysis. Fitness in the evolved individuals that we
isolated is on average comparable albeit not identical among the mitonuclear
backgrounds under study. Genomic scrutiny in those individuals provides more
compelling evidence for the impact of mitonuclear interactions on evolutionary
outcomes. Thanks to our highly replicated dataset, we were able to detect signals of
evolutionary convergence at the whole-chromosome and gene levels, as observed in
previous evolution experiments (Tenaillon et al. 2012). This allowed us to identify
features of the yeast genome specifically or preferentially targeted by selection in a
mitonuclear background-specific manner. This included changes that were specific to one
single mitonuclear background, to the exclusion of closely related ones. We similarly
detected environment-specific patterns of selection. Interestingly, we also showed that
divergence between mitonuclear backgrounds, both in terms of phenotype and identity of
mutations, is modulated by selection on mitochondrial function. Furthermore,
recapitulating loss-of-function alleles at convergent loci in the ancestral strains of our
experiment, we demonstrated that their effect on fitness, while generally favourable,
varied markedly as a function of mitonuclear background and carbon source. Taken
together, this evidence therefore upholds standing models of mitonuclear evolution,
whereas specific interactions between the nuclear and mitochondrial genomes lead to
divergent evolutionary trajectories.

Screening our collection of mitonuclear hybrids, we observed that the nuclear genome
exerted a larger impact on fitness than did the mitochondrial genome. Highly replicated
assessment of fitness in the ancestral strains confirmed this observation but indicated a
modulating influence for the mitochondrial genome. Our data further indicated that the
mitochondrial genome exerted much of its impact on fitness via mitonuclear interactions
rather than on its own account. Further supporting this view, we show evidence that the
disruption of co-evolved mitonuclear pairs via cytoduction has a strong impact on

mitochondrial homeostasis. Namely, increases in mtDNA content and changes in the

baseline activity of mitochondrial enzymes appear specifically associated with artificial

mitonuclear pairs. These founding contingencies in the experimental evolution represent,

on their own, strong predictors of the divergent evolutionary trajectories predicted from

different mitonuclear backgrounds. Indeed, an organism's specific evolutionary history -

historically contingent factors - creates a unique ensemble of constraints on the set of

alleles likely favored by selection. In other words, it potentiates specific evolutionary

pathways, and examples of this are reported in the experimental evolution literature

(Blount et al. 2008).

Evolved individuals displayed greater phenotypic uniformity than their unevolved

ancestors. Less adapted mitonuclear backgrounds readily caught up to fitter ones. This is

coherent with the rapid and reproducible gains in fitness generally observed in the early

stages of experimental evolution (Wiser et al. 2013) and with patterns of diminishing

returns epistasis expected in fitter individuals (Chou et al. 2011; Couce and Tenaillon

2015). Evolved individuals display phenotypic uniformity in the environment in which

they evolved, but also in conditions to which they were not explicitly adapted. This could

be a sign of positive pleiotropy, meaning that many of the mutations selected in one

environment tend to be adaptive in the other. More prosaically, we note that the two

environments differ solely by the identity of the available carbon source. Hence, we posit

that both environments are more alike than different, and alleles selected in either will

tend to be adaptive in both by virtue of the same mechanisms. Despite this, we did

observe several environment-specific mutant annotations. We also observed slightly

lower fitness in fermentable medium for individuals evolved in non-fermentable medium.

Therefore, environment-specific adaptive strategies still arose, which may steer the path

of evolution towards more strongly distinct pathways over a longer evolutionary period.

Mitonuclear cooperation posits a complex network of genetic and physical interactions.

Compatible with this framework, patterns of mitonuclear-specific evolutionary

convergence from our study suggests selection affected by high-order epistasis.

Recapitulating some of the selected mutations as loss-of-function alleles further argued

for that view, as their effect in the ancestral backgrounds was strongly dependent on the

mitonuclear background. Furthermore, the locus preferences we could infer from the

fitness of these reconstituted mutants did not match predictions from patterns of

evolutionary convergence. Since we know that each evolved individual carries more than

one mutation, we can reasonably posit that the effect of mutation at any locus in our

mutants, and the probability for their selection during evolution, is affected by other

mutations found in the same cells, especially those that arose earlier. The factor that

unifies all these considerations is the founding difference in mitonuclear background.

Limitations of the study

Our evolution experiment lasted approximately 300 generations. While published

experimental evolution studies of a few hundred generations are common, this remains

short on the evolutionary time scale. For comparison, this would correspond to roughly

7500 years of human evolution. While most studies cannot realistically last for tens of

thousands of generations, a few thousand generations are commonplace (Lenski 2017).

The typical workaround for short-term experimental evolution is increased replication

(Mcdonald 2019). With more than 1300 starting populations, our experiment certainly

meets the high replication standard, yet studies with similar levels of replication have

reported lengths of up to a thousand generations (Lang et al. 2011; Lang et al. 2013).

Despite this limitation, we were able to identify numerous and clear patterns of

evolutionary convergence. In addition, we could assign both mitonuclear and carbon

source specificity to many mutant loci. Furthermore, it is uncertain that prolonged

evolution would have increased the statistical power of our study. While it may have

exacerbated patterns of specificity at some loci, over long periods, the accumulation of

fixation events coupled to clonal interference and negative epistasis could have reduced

the diversity of detectable patterns. However, it could also have enabled the detection of

patterns that could only emerge after the fixation of earlier mutations. In short, by

affording time for divergence, a longer evolution may have helped observe more sharply

defined evolutionary trajectories for each mitonuclear background.

By evolving yeast in two environments that differed solely by the identity of the available
carbon source, we hoped to test for the effect of explicitly selecting for a mitochondrial
function, while minimizing confounding factors. In retrospect, comparing outcomes of
evolution in two highly similar environments may have led to excessively similar
outcomes, especially at the phenotypic level. Variation of an additional factor, such as
nitrogen source, temperature, or even carbon source concentration, may have helped
further differentiate populations evolving in distinct conditions, and better identify effects
specific to mitochondrial function. Nevertheless, our experimental setup proved sufficient
to identify carbon source-specific mutational patterns and compare patterns of divergence
associated with different selective pressures.

The seven mitonuclear backgrounds that we submitted to experimental evolution were
derived from just three original strains. We screened our collection of cytoductants for
mitonuclear mismatches with readily detectable phenotypic differences, maximizing our
chances of observing bona fide examples of mitonuclear interactions. This may have
unduly restricted the breadth of observable patterns of mitonuclear interaction and
evolution, biasing our results towards specific evolutionary outcomes. An alternative
would have been to propagate a much wider diversity of mitonuclear backgrounds,
potentially evolving our entire collection. This agnostic approach would have limited
potential artefactual biases induced by the experimental design, at the expense of per
background replication.

Most of the evidence presented in this study relies directly or indirectly on the results of
sequencing. We did phenotype each of our evolved isolates. We also attempted
recapitulation of some of the loss-of-function alleles identified by sequencing, generating
corresponding knockouts in ancestral backgrounds. Yet, these experiments either
imperfectly mimicked the conditions of evolution, were limited in scope or relied on
simplifying assumptions. Indeed, systematic assessment of growth kinetics in evolved
isolates was performed on solid medium for ease of replication and automation, whereas
evolution was performed in liquid to accelerate evolution. Medium composition may
essentially be the same in both systems, but our own evidence indicates notable

differences in growth between them (as illustrated by comparing growth of ancestral
strains in **FigS1**BC and **Fig1**CD). Thus, phenotypic insight from growth on solid may not
accurately reflect growth in the conditions of evolution. In contrast, high-sensitivity
competition assays on recapitulated loss-of-function alleles assessed fitness effects in the
exact conditions used for evolution. Yet, those assays concerned but a limited number of
loci. For technical reasons, mutations to the mitochondrial genomes and aneuploidies had
to be excluded from these tests. More generally, our restricted choice of loci may not
reflect most mutations, biasing our view of fitness effects. Potentially more impactful is
our simplifying assumption that mutations at the chosen loci were pure loss-of-function
alleles, which we chose to recapitulate using whole gene knockouts. Careful
reconstitution of the exact mutations identified by sequencing, while less scalable, may
have provided a more accurate view of fitness effects. Finally, knockouts were prepared
in ancestral backgrounds, ignoring epistatic effects incurred by other mutations
accumulated in evolved individuals. We thus acknowledge that our phenotypic data
represents an approximation, potentially complemented by more targeted future studies.
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Future directions
The model of mitonuclear evolution tested in this study predicts that evolutionary
divergence is driven by the high rate of evolution experienced by the mitochondrial
genome. In turn, the nuclear genome is believed to accumulate compensatory mutations
aimed at maintaining compatibility. Data presented in this study support this model by
showing the influence of specific mitonuclear interactions on the evolutionary trajectories
and range of selected mutations observed in adapting mitonuclear hybrids. We also
showed evidence of mitonuclear incompatibility and metabolic disruption in the
mitonuclear hybrid ancestors of the experiment. In addition, we provided evidence for the
resolution of the incompatibilities. However, this study shows no evidence about the site
of compensatory evolution in either the nuclear or mitochondrial genomes. Strains
evolved during our experiment may yet enable such demonstrations. Using cytoduction,
we may generate novel mitonuclear hybrids by introducing an ancestral mtDNA or
nuclear genome into the evolved strains. From a combination of growth, competition and
enzymatic assays, along other cellular properties, we should identify which of the nuclear

or mitochondrial genome is responsible for compensation. As an example, crossing
evolved ND individuals with their ancestor, we could determine if evolved N nuclear
genomes converged towards D, or if rather the D mitochondrial genome converged
towards N. This experiment would provide an additional test for the standing model of
mitonuclear evolution, potentially providing experimental support.

Our sequencing efforts have identified several mutant loci, with potential relevance to
mitochondrial biology and mitonuclear evolution (see **Text S1**). Detailed scrutiny of the
cell and molecular biology of adaptive mutations at these loci should provide insight into
their underlying mechanisms, shedding light not only on paths of mitonuclear
compensation, but also fundamental cellular processes. For example, aneuploidies at
chromosome IV have been associated with increased gene dosage at *TSA2* in response to
oxidative stress. We could therefore study the impact of overexpression of this gene in
various mitonuclear backgrounds, testing for differential effects. Similarly, artificial
expansion of the rDNA locus, as in (Kwan et al. 2016), may help investigate the adaptive
influence of chromosome XII copy number on mitonuclear homeostasis. Long read
sequencing at *HPF1* and *MNN4* loci in individuals mutant would provide an accurate
map of the associated CNVs. This would enable their reconstitution in various
backgrounds. Along with already available *RIM20* knockouts, expression of altered
HPF1 and *MNN4* constructs could help to study cell wall mannoproteins. We could
further study their role in yeast buoyancy, in addition to their impact on respiration,
oxidative stress and chronological lifespan.

Summary and conclusion
In this study, we used experimental evolution of yeast mitonuclear hybrids and whole-
genome sequencing of the resulting individuals to uncover patterns of evolutionary
convergence specific to unique mitonuclear backgrounds. We also confirmed that
phenotypic outcomes of evolution are primarily contingent on the nuclear background,
with a minor, modulating influence for mitonuclear interactions. We further showed that
mitonuclear interactions exert an influence on the molecular mechanisms of adaptation,
potentially constraining and potentiating long term evolution. To our knowledge, this

study represents the first direct experimental demonstration of mitonuclear interactions
inflecting the evolutionary trajectories of adapting populations. In addition to the support
provided to standing evolutionary theory, the strains generated in this study and the
accumulated data provide material for further studies of mitonuclear evolution and the
cell biology of yeast.

Materials and Methods

Culture media. Below are the recipes for the media used in this study. Medium
ingredients were purchased from BioShop (Burlington, ON, Canada). Experimental
evolution, with its starter cultures, as well as growth and competition assays were all
performed in media assembled from ingredients of the same production lot (see below).

Yeast peptone dextrose (YPD). 10 g/L yeast extract, 20 g/L tryptone, 20 g/L glucose.
2XYPD is assembled by doubling the concentration of all components in YPD.

Yeast peptone glycerol (YPG). 10 g/L yeast extract, 20 g/L tryptone, 20 g/L glycerol.

Yeast peptone ethanol glycerol (YPEG). 10 g/L yeast extract, 20 g/L tryptone, 30 g/L
ethanol, 20 g/L glycerol.

Enhanced YPD (eYPD). 15 g/L yeast extract, 40 g/L tryptone, 40 g/L glucose, 5 g/L malt
extract, 2.5 g/L glycerol, 0.4 g/L L-cysteine hydrochloride, 0.83 g/L K_2HPO_4, 3 g/L Tris
pH 6.0.

Amino acid dropout without uracil and without histidine. The following compounds were
mixed in powder form: 0.5 g L-adenine sulfate dihydrate, 2 g L-arginine hydrochloride, 2
g L-aspartic acid, 2 g L-glutamate monosodium salt, 10 g L-leucine, 2 g L-lysine
monohydrochloride, 2 g L-methionine, 2 g L-phenylalanine, 2 g L-serine, 2 g L-
threonine, 2 g L-tryptophan, 2 g L-tyrosine, 2 g L-valine.

Enhanced synthetic complete (eSC). 3.48 g/L yeast nitrogen base without ammonium
sulfate and without amino acids, 2 g/L monosodium glutamate, 40 g/L glucose, 2.5 g/L
glycerol, 0.4 g/L L-cysteine hydrochloride, 2.4 g/L amino acid dropout without uracil and
without histidine, 0.83 g/L K_2HPO_4, 3 g/L Tris pH 6.0.

Solid medium was made by adding 1.5%-2% agar to the above recipes.

Evolution plates. Experimental evolution and related experiments were performed in

standardized microtiter culture vessels, assembled as follows. Flat-bottom polypropylene

microtiter plates, in 96 well format (cat. nb. 951040005, Eppendorf, Hamburg, Germany)

served as reusable, autoclavable culture vessels. A clean 2.5 mm diameter glass bead

(Biospec, Bartlesville, OK, USA) was placed in each well. Wells were filled with 240 µL

media, then sealed with an adhesive foil seal (VWR, Wayne PA, USA). Plates were next

covered with clean polypropylene lids held with autoclave tape. Plates were sterilized

with a 20 min liquid autoclave cycle at 121°C. Plates were left at room temperature to

cool and dry. Plates were then wiped of any residual water, placed in airtight plastic bags,

and stored at 4°C until use. Before use, plates were equilibrated at room temperature and

centrifuged at 2000 rpm for 2 min. Immediately before inoculation, lids were removed,

and foils seals discarded. Following inoculation, plates were sealed with breathable

adhesive membranes (VWR, Wayne PA, USA), and covered with polypropylene lids

held with masking tape at all four corners. For re-use, plates were emptied, collecting and

rinsing beads. Beads were sanitized by a 1 hr+ incubation in warm 10% bleach, rinsed

thoroughly with tap water, and dried at 56°C in a Pasteur oven. Plates were washed in

soapy water, rinsed thoroughly with tap water, and left inverted at room temperature until

fully dry.

Growth curves on collection of yeast cybrids. A collection of yeast cybrids was

previously constituted, as described in (Paliwal et al., 2014; Wolters et al., 2018). It

contains all possible combinations of nuclear and mitochondrial genomes from a set of

fifteen strains of *S. cerevisiae*, in four biological replicates. Each strain in the collection

was assayed for growth in non-fermentable YPG medium as follows. All manipulations

were performed following standard sterile technique. Five microliters from thawed

glycerol stocks of the collection were transferred to YPD evolution plates and incubated

for 48 hours at 30°C, 65% relative humidity (RH), with shaking at 165 rpm in a

microplate incubator shaker (Multitron, Infors HT, Basel, Switzerland). With the help of

a liquid handling robot (Freedom Evo, Tecan, Männedorf, Switzerland), 5 µL from these

prolonged cultures were transferred to the wells of a flat bottom polystyrene microtiter

plate (Greiner, Kremsmünster, Austria), each well containing 235 µL of YPG and a 2.5

mm diameter glass bead. This second plate was covered with a transparent lid, with sides

wrapped in three layers of parafilm (Bemis, Sheboygan Falls, WI, USA). Plate was

placed in a Tecan Infinite M Nano microplate reader and incubated for 48 hours at 30°C,

recording A_{595} at 20 min intervals. Each 20 min interval consisted of 13 min orbital

shaking (6 mm diameter), 5 min absorbance measurement, and 2 min resting time. Raw

growth curve data was fitted to a modified Gompertz model, as in (Zwietering et al.,

1990). Area under the fitted curve was calculated using the trapezoidal rule.

Preparation of whole extracts from strains of *S. cerevisiae* for enzyme assays.

Precultures were inoculated by transferring 5 µL from thawed glycerol stocks to 5 mL

2XYPD medium. Cultures were incubated with shaking at 30°C for 24 hrs. Half a

milliliter from precultures was used to inoculate 5 mL YPG, and this new culture was

incubated with shaking at 30°C for 24 hrs. This second culture was diluted in 45 mL of

pre-warmed YPG medium. This subculture was incubated at 30°C with shaking until it

reached A_{600}=0.5-1.0. Cells were pelleted by centrifugation, then suspended in water.

Cells were pelleted once again by centrifugation, washed in 25 mM potassium phosphate

pH 8.0, then frozen as pellets at -80°C. After overnight freezing, pellets were thawed on

ice, and suspended in 0.5 mL of freshly prepared, ice-cold 25 mM Tris-HCl pH 7.5, 1

mM EDTA, 100 mM NaCl + cOmplete Mini, EDTA-free Protease Inhibitor Cocktail

(Roche, Basel, Switzerland). Suspension was bead beaten at 4°C for 10 cycles of 2 min

beating, 2 min rest on ice. Lysate was frozen at -80°C.

Enzyme assays on whole cell extracts from *S. cerevisiae*. Enzyme activities were

determined spectrophotometrically using a Mithras LB940 microplate reader (Berthold

technologies, Germany) and data analyzed with MikroWin 2010 software (Labsis

Laborsysteme, Germany). Enzymatic capacities were expressed as mU·mg proteins^{-1}

(U·mg proteins^{-1} in the case of CAT), where U refers to 1 µmol of substrate transformed

to product per minute. Chemicals were purchased from Sigma-Aldrich (Oakville,

Ontario, Canada). Enzymatic assays were performed at 30°C in the following conditions:

Malate dehydrogenase (MDH, EC 1.1.1.37). MDH activity was measured at 340 nm for 4

min, following the oxidation of NADH ($\varepsilon_{340} = 6.22$ mM^{-1}·cm^{-1}). The medium consisted

of 100 mM potassium phosphate, 0.2 mM NADH and 0.5 mM oxaloacetate (OAA), pH

7.5. The background activity in absence of sample was subtracted from the main results

(Bergmeyer, 1983).

Mitochondrial complex I + III (ETS, EC 7.1.1.2 and 7.1.1.8). ETS activity was measured

at 490 nm using NADH as electron donor, following the reduction of p-

iodonitrotetrazolium violet (INT, $\varepsilon_{490} = 15.9$ mM^{-1}·cm^{-1}) for 6 min. The medium

consisted of 50 mM potassium phosphate, 0.85 mM NADH, 2 mM INT and 0.03% (v/v)

triton X-100, pH 7.5. The specificity of the reaction was verified in absence of NADH

and the residual activity was subtracted from the main results (Hunter-Manseau et al.,

2019).

Cytochrome c oxidase (CCO, EC 7.1.1.9). CCO activity was measured at 550 nm

following the oxidation of cytochrome c (cyt c, $\varepsilon_{550} = 18.5$ mM^{-1}·cm^{-1}) for 6 min. Cyt *c*

was reduced through the addition of 4.5 mM dithionite. The medium consisted of 50 mM

potassium phosphate, 50 µM cyt c, 1 mM ADP and 0.03% (v/v) triton X-100, pH 7.0.

The specificity of the reaction was verified in presence of 40 mM sodium azide (CCO-

inhibitor), and the residual activity was subtracted from the main results (Lemaire and

Dujardin, 2008; Spinazzi et al., 2012; Hunter-Manseau et al., 2019).

ATP-synthase (ATPase, EC 7.1.2.2). ATPase activity (ATP hydrolysis) was measured at

340 nm following the oxidation of NADH ($\varepsilon_{340} = 6.22$ mM^{-1}·cm^{-1}) for 4 min. The

medium consisted of 250 mM sucrose, 20 mM HEPES, 5 mM MgSO$_4$, 0.35 mM NADH,

2.5 mM phosphoenolpyruvate (PEP), 2.5 mM ATP, 5 µM antimycin A, 4 units · mL^{-1}

lactate dehydrogenase (LDH) and 4 units · mL^{-1} pyruvate kinase (PK), pH 8.0. The

specificity of the reaction was verified in presence of 25 µM oligomycin (ATPase-

inhibitor), and the residual activity was subtracted from the main results (Barrientos et al.,

2009; Haraux and Lombes, 2019; Rodriguez et al., 2020).

Catalase (CAT, EC 1.11.1.6). CAT activity was measured at 240 nm following the
disappearance of H_2O_2 (ε_{240} = 43.6 $M^{-1} \cdot cm^{-1}$) for 1 min. The medium was composed of
100 mM potassium phosphate, 0.1% (v/v) triton X-100 and 60 mM H_2O_2, pH 7.5. The
background activity in absence of sample was subtracted from the main results (Page et
al., 2009; Pichaud et al., 2010; Orr and Sohal, 1992; Bettinazzi et al., 2021; Christen et
al., 2020).

Protein content. Protein concentration (mg·mL^{-1}) was determined with the BCA assay
(Smith et al., 1985).

Preparation of starter cultures for experimental evolution. All procedures were
performed under laminar flow within a sterile enclosure. This protocol was performed
identically for strains of each of the following mitonuclear backgrounds: 273614N^{273614N}
(NN), 273614N^{DBVPG6044} (ND), DBVPG6044^{273614N} (DN), DBVPG6044^{DBVPG6044} (DD),
DBVPG6044^{Y12} (DY), Y12^{DBVPG6044} (YD), Y12^{Y12} (YY). Streaks from each of the four
biological replicates from each of the mitonuclear backgrounds were prepared on YPEG
agar, and incubated at 30°C for 48 hrs. From each streak, 24 colonies were picked at
random with sterile micropipette tips and inoculated into YPD evolution plates. Hence,
96 cultures were launched from each of the seven mitonuclear backgrounds submitted to
experimental evolution. Plates were incubated overnight at 30°C, 65% RH with shaking
at 200 rpm. Glycerol stocks were prepared by mixing 100 µL from the starter cultures
with 100 µL 75% glycerol in the wells of conical bottom polypropylene plates (Greiner,
Kremsmünster, Austria). Remainder of the cultures were used immediately to launch
experimental evolution.

Experimental evolution on mitonuclear hybrids of *S. cerevisiae*. Experimental
evolution was performed on 96 replicates from each of the seven mitonuclear
backgrounds by propagating them by daily serial passage in both fermentable (YPD) and
non-fermentable (YPG) medium for 58 days. All procedures were performed under
laminar flow within a sterile enclosure. Starter cultures were used to inoculate a first
batch of experimental evolution cultures. With the help of a liquid handling robot

(Freedom Evo 150, Tecan, Männedorf, Switzerland), 5 µL from each starter culture were

transferred to YPD evolution plates, and in parallel to YPG evolution plates. Tips used

for transfer were washed with sterile water and kept for further transfer of the same

cultures. Plates were incubated for 24 hrs at 30°C, 65% RH with shaking at 200 rpm.

Evolving populations were then transferred to new evolution plates for 58 consecutive

days. On day 2 and every 7 days afterwards until day 58, glycerol stocks were prepared

by mixing 100 µL from the evolving populations with 100 µL 75% glycerol in the wells

of conical bottom polypropylene plates.

Isolation of independently evolved individuals. Glycerol stocks from day 58 of the

evolution experiment were thawed at room temperature. Ten microliters from each stock

were transferred to the surface of a YPD agar petri dish. The resulting drop from the

glycerol stock was streaked for single colonies. Petri dishes were incubated for 48 hrs at

30°C. From each Petri dish, a single colony was picked at random using a sterile tip and

placed into a YPG evolution plate. Plates were incubated for 24 hrs at 30°C, 65% RH

with shaking at 200 rpm. Glycerol stocks were prepared by mixing 100 µL from the

starter cultures with 100 µL 75% glycerol in the wells of conical bottom polypropylene

plates (Greiner, Kremsmünster, Austria).

Assessment of ploidy in select strains. Ploidy was determined for all ancestral strains

and a subset among evolved individuals, following a method inspired by (Charron et al.

2019). Thawed glycerol stocks of the strains were streaked for single colonies on YPD

agar. Single colonies were picked and inoculated into 1 mL YPD, in a well of a

polypropylene DWP. DWPs were incubated for 24 hrs at room temperature. Cells were

washed with 2 x 1 mL water, then fixed for 1 hr in 70% ethanol. Ethanol was removed by

washing cells twice with 1 mL water. Cells were next incubated overnight at 37°C in 1

mL of freshly prepared 0.25 mg/mL RNase A. Cells were washed twice with 1 mL of 50

mM sodium citrate pH 7.0. A small volume (25 μL) of the washed cell suspension was

diluted in 225 μL of 0.667 μM SYTOX green (Thermo Fisher Scientific) and incubated

in the dark at room temperature for 1 hr. Cell density in the stained sample was adjusted

to approximately 500 cells/μL, and analyzed by flow cytometry, recording 5000 events

per sample. Mean green fluorescence was measured for cells in G1 phase. All

manipulations were performed in triplicate.

Assessing the growth of evolved individuals on fermentable and non-fermentable

solid media. High-throughput assessment of growth in evolved individuals and their

ancestral strains was performed by recording the growth of colonies on YPD and YPG

agar medium. Replication and array of samples in preparation for growth curves were

performed with robotically manipulated pin tools (BM5-SC1, S&P Robotics, Toronto

ON, Canada). Thawed glycerol stocks were replicated from 96-well plates to omnitrays

(Nunc, Rochester, NY, USA) in 96-array format on YPD agar medium. Replicas were

incubated at 30°C until large, well-defined colonies were visible. Replicas were then

cherry-picked and re-arrayed to 384-array format on YPD agar, randomizing positions.

Following outgrowth, plates were combined into 1,536-array format on YPD agar

omnitrays. Evolved individuals of a given mitonuclear background, evolved in both

fermentable and non-fermentable conditions, were printed on a single 1,536 array. Each

evolved individual was printed as six replicates. Hence, one 1,536 array was prepared per

mitonuclear background for a total of seven arrays, whereas nine replicates of each of the

ancestral strains were printed onto all seven arrays. To avoid border effects, top and

bottom two rows of the arrays were printed with a filler strain, as were the two extreme

left and right columns, and these were excluded from analysis. Next, these randomized

arrays in 1,536 format were outgrown, then replicated on YPD and YPG agar. The

replicas were incubated at 30°C for 48 hrs (YPD) or 72 hrs (YPG) in a spImager custom

robotic platform (S&P Robotics), recording photographs of the plates at 2-hour intervals.

The outgrown replicas were replicated to fresh plates for two additional rounds of

growth. Data from the second and third rounds was retained for analysis.

Analysis and quantitation of growth on solid medium. Colony sizes from pictures

were measured with R package Gitter (Wagih and Parts, 2014). Growth curves were

drawn for each colony, and fitted to a modified Gompertz model, as described above,

extracting estimates of the maximum specific growth rate (μ) and carrying capacity (A).

Ancestral controls were used to estimate plate-specific effects on growth parameters and

to normalize estimates across arrays. Specifically, the effect of a given plate on a growth

parameter was calculated by dividing each individual ancestral estimate by the geometric

mean of the ancestral estimates over all plates in each carbon source, then extracting the

median of that ratio over all ancestral estimates from the same plate. The normalization

factor thus obtained was used to multiply all estimates from a plate. True value for

growth parameters of any given strain was estimated using the median (if $n \geq 25$) or

geometric mean (if $n < 25$) over all replicates, with confidence intervals obtained by

bootstrapping. A series of two-way ANOVAs with interaction was performed on growth

data with nuclear and mitochondrial backgrounds as factors, Separate ANOVAs were

conducted for each combination of fitness proxy, evolution regimen, and carbon source.

Preparation of total DNA from evolved individuals and ancestral strains. Total DNA

from each individual evolved strain and all ancestral strains was extracted and purified as

starting material for the preparation of next-generation sequencing libraries. A custom

protocol using the DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands) was

applied in 96-well format. Each strain was inoculated from glycerol stock in 4 x 1 mL

YPD medium, in a polypropylene 96-well deep well plate (DWP). Each DWP was

covered with a breathable adhesive membrane and incubated for 24 hours at 30°C with

shaking at 200 rpm. Cells were harvested by centrifugation for 10 min at 5000 x g and

suspended in 250 µL water. Suspensions of each strain were pooled into a single well,

and centrifuged once more for 10 min at 5000 x g. Pellets were suspended in 600 µL of 1

M sorbitol, 100 mM sodium EDTA, 14 mM β-mercaptoethanol, supplemented with 6 U

zymolyase (Bioshop, Burlington ON, Canada), then transferred to collection microtubes

provided with the DNeasy kit, and incubated at 30°C for at least 30 min. Resulting

spheroplasts were pelleted by centrifugation for 10 min at 5000 x g, suspended in 180 μl

Buffer ATL + 20 μl proteinase K solution, and mixed thoroughly. Lysis suspensions were

incubated at 56°C for at least 30 min, mixing occasionally to disperse the samples.

Samples were shaken vigorously for 15 s, then centrifuged briefly to collect all liquid at

the bottom of tubes. Samples were supplemented with 4 μL of 100 mg/mL RNase A,

mixed vigorously, and incubated at room temperature for at least 5 min. A 50:50 mix of

buffer AL and ethanol was prepared fresh, and 400 μL were dispensed to each tube,

mixing once again by vortexing. Lysates (max 900 µL) were applied to DNeasy 96

purification columns. Columns were centrifuged for 10 min at 3800 x g. Columns were

washed with 500 µL buffer AW1, centrifuging for 5 min at 3800 x g. Columns were

further washed with 500 µL buffer AW2, centrifuging for 15 min at 3800 x g. Columns

were next placed on a clean rack of elution microtubes and 100 µL of buffer AE were

added to each sample. Samples were incubated for at least 1 min at room temperature,

then centrifuged for 2 min at 3800 x g. Each eluate was next supplemented with 4 µL of

10 mg/mL RNase A, agitated vigorously, and incubated at room temperature for 15 min.

The RNase-treated eluates were further purified by mixing 50 µL from each sample with

20 µL beads and incubating at room temperature for 5 min. After the bead mixture was

applied to a magnet for 60s, liquid was removed from the beads, which were washed

twice with 200 µL of 80% ethanol. Beads were suspended in 40 µL of 10 mM Tris-HCl

pH 8.0 and incubated for 5 min at room temperature. Eluate was separated from beads

using a magnet, as described above. Sample quality was evaluated by agarose gel

electrophoresis. Purity and concentration of DNA was assessed by measuring absorbance

at 260 nm and 280 nm using a NanoDrop spectrophotometer (ThermoFisher Scientific,

Waltham MA, USA). Concentration of DNA in the sample was further estimated using

the AccuClear Ultra High Sensitivity dsDNA Quantitation Kit (Biotium, Fremont CA,

USA) following manufacturer's instructions.

Preparation of next-generation sequencing libraries. Sequencing libraries were

prepared using the Tagment DNA Enzyme and Buffer Large Kit (Illumina, San Diego

CA, USA), the KAPA HiFi HotStart ReadyMix 2X (Roche, Basel, Switzerland) and

custom DNA barcodes. Genomic DNA samples were diluted with water to a target

concentration of 2.5 ng/µL or less. Tagmentation reactions were assembled by mixing

1.25 µL tagment DNA buffer, 0.25 µL tagment DNA enzyme and 1 µL gDNA dilution.

Reactions were incubated at 55°C for 5 min. Custom DNA barcodes were added by PCR

as follows. Tagmented DNA was mixed with 3.76 µL 2X KAPA ReadyMix, and 0.625

µL   both   primers.   Primers   had   sequences

5'AATGATACGGCGACCACCGAGATCTACAC-(8-nucleotide custom i5 barcode)-

TCGTCGGCAGCGTC3' and 5'CAAGCAGAAGACGGCATACGAGAT-(8-nucleotide

custom i7 barcode)-GTCTCGTGGGCTCGG3'. A collection of 32 custom i5 barcodes

and 48 custom i7 barcodes was used, enabling the preparation of 1,536 unique barcode

combinations. PCR was performed by cycling as follows: 72°C for 3 min, 98°C for 2:45

min, then 8 cycles of 98°C for 15s, 62°C for 30s, 72°C for 3 min, followed by a 1 min

final extension at 72°C. Library preparation was completed by reconditioning PCR.

Barcoding reactions were mixed with 8.5 μL 2X KAPA ReadyMix, 0.5 μL of 10 μM

primer P1 (5'AATGATACGGCGACCACCGA3') and 0.5 μL of 10 μM primer P2

(5'CAAGCAGAAGACGGCATACGA3'). Reactions were then cycled as follows: 95°C

for 5 min, then 4 cycles of 98°C for 20s, 62°C for 20s, 72°C for 2 min, followed by a 2

min final extension at 72°C. Final PCR products were diluted with 14 μL PCR grade

water, then bead purified as described above, except that only 18 μL beads were added to

the PCR reactions. Library concentration was estimated using the AccuClear Ultra High

Sensitivity dsDNA Quantitation Kit following manufacturer's instructions. A subset of

the libraries was inspected with a BioAnalyzer 2100 using a High Sensitivity DNA chip

(Agilent Technologies, Santa Clara CA, USA) for insert size and monodispersity.

Equimolar amounts of all libraries were pooled and sequenced on an Illumina

NovaSeq6000 S4 PE150 sequencing lane at the Génome Québec Expertise and Service

Center (Montréal QC, Canada).

1001

Pre-treatment of sequencing results and alignment to reference genomes. Quality

control was performed individually on each sequencing library using FastQC (Andrews,

2010), and results were aggregated using MultiQC (Ewels et al., 2016). Sequencing

adapters were trimmed with Trimmomatic (Bolger et al., 2014). Overlapping read pairs

were merged using BBmerge (Bushnell et al., 2017). Single read files from the BBmerge

output were concatenated into single files. Primary read mapping to reference genomes

was performed using bwa mem (Li and Durbin, 2009). Libraries were mapped to the

published genomes of their associated ancestral strains. The *S. cerevisiae* 273614N

reference genome sequence was obtained from the diArk database (Hammesfahr et al.,

2011) as published in (Liti et al., 2009). Reference genome sequences for *S. cerevisiae*

strains DBVPG6044 and Y12 were obtained from NCBI BioProject PRJEB7245,

BioSamples SAMEA2757762 and SAMEA2757763, respectively, as published in (Yue

et al., 2017). Chimeric reference genomes were assembled for cybrid strains by manually

replacing the mitochondrial genome contig published for the nuclear parent's genome

with that published for the mitochondrial parent. Alignment was performed separately for

merged and paired reads. Hence, BAM outputs from bwa mem were merged and then

sorted with samtools (Li et al., 2009). Following the addition of read groups and

deduplication with picard (Broad Institute, 2016), indel realignment was performed using

GATK3 (McKenna et al., 2010; van der Auwera and O'Connor, 2020). Alignment and

depth metrics were collected using picard and samtools, respectively. Whole-genome

pileups were extracted for each sequencing library with samtools.

1023

Identification of aneuploidies from sequencing data. Aneuploidies were called from

pileup. Telomeric and subtelomeric regions were excluded from this analysis by masking

25 kb at both ends of each chromosome. High quality depth of coverage was computed at

each position by counting reads with both base and mapping quality above 30 on the

phred scale. Finally, any position found with average depth of coverage in all biological

replicates of the ancestral strains above 5 times the median depth over the whole genome

was also excluded from analysis. Next, the ratio between the mean depth of the

chromosome and that of the rest of the genome was calculated. If this ratio was between

0.75 and 1.25, a p-value for the mean coverage of the chromosome was computed,

assuming normal distribution of depth of coverage across the genome, with mean and

standard deviation computed over the rest of the genome. If this p-value was smaller than

the length of the chromosome divided by the length of the whole genome, an aneuploidy

was suspected. Aneuploidy candidates were confirmed visually by inspecting heatmaps

of depth of coverage along the length of chromosomes.

1038

Primary identification of copy number variants (CNVs) from sequencing data.

Primary CNV calling was performed essentially according to (Yoon et al., 2009).

Analysis was performed from filtered pileups, as described above for aneuploidies. Each

chromosome was divided in 100 bp windows, extracting mean depth of coverage for each

window. Average and standard deviation on window means were computed across

chromosomes, enabling computation of a z-score and p-value for each window. Next,

whole chromosomes were scanned with rolling intervals of various window sizes, starting

with 2 windows in length. If the maximum p-value for windows within an interval fell

below a threshold, determined by both window and chromosome length, the interval was

marked as a candidate CNV. Increasing interval sizes were used until a threshold

determined by both interval and chromosome length was reached. Overlapping CNV

candidates were merged. Any candidate with median depth between 0.75x and 1.25x the

median chromosome depth was excluded. A p-value was computed for the mean depth of

each candidate, based on mean and standard deviation over windows. If this p-value was

greater than 10^{-6}, the candidate was excluded. Finally, if the same area had mean depth of

coverage between 0.75x and 1.25x the chromosome-wide median depth in the ancestral

strains, the candidate CNV was also excluded.

1056

Resolution of artefactual CNV calls caused by misalignment at homologous loci.

Reference genome sequence at CNV coordinates were blasted against the whole

reference genome sequence, identifying loci with high homology to CNV candidates.

Blast hits with an E-value of less than 1.0, length above 150 bp and percent identity with

the CNV locus above 80% were retained for further scrutiny. Sequences at the CNV call

and homologous hit loci were aligned with MUSCLE (Edgar, 2004), identifying locus

specific positions. A system of linear equations was built from the sums of each base

calls at each locus specific positions in the alignment. Multiple linear regression was then

used to identify the most likely depth of coverage, with 95% confidence interval, at each

homologous locus. If the inferred depth was between 0.75x and 1.25x the chromosome

median depth, and the confidence interval on depth at the homologous locus did not

overlap with the confidence interval for depth over the whole chromosome, a CNV call

was asserted. Otherwise, or if locus specific information was too scarce to lift ambiguity

caused by homology, CNV call was not retained. Applying this methodology, several

CNVs were rejected, others were retained, while a subgroup was reassigned to more

likely loci.

1073

1074   **SNV calling.** Primary SNV calling was performed using two parallel methods, starting

1075   with GATK HaplotypeCaller. SNV calls were combined into a single GVCF file using

1076　GATK CombineGVCF. Initial filtering was performed with GATK FilterVariants and

1077　SelectVariants. Additional filtering was done with vcftools. A list of SNVs was produced

1078　in csv format using bcftools.

1079

In parallel, custom SNV calling was attempted directly from pileup. The following

regions of the genome were filtered out of pileups in preparation for SNV calling: high

coverage regions in the ancestral strains, subtelomeric regions, positions with mean base

and mapping quality below 30, positions with depth of coverage below 10, and positions

with N or otherwise missing sequence in the reference. A baseline error model was built,

identifying the number of each base call expected at each position, considering the

reference base and mean base quality at that position, and the average error rates

observed in the data. A chi^2 test for goodness of fit was performed, comparing the

expected base counts predicted by the error model with the observed base counts. An E-

value was obtained by multiplying this p-value with the number of positions in the

genome. Positions with an E-value < 1.0 were retained as potential SNVs, while any

position with non-reference base call count less than 25% of total depth was rejected.

The intersection between the results of both primary SNV calling pipelines was kept as

the overall primary list of SNVs. SNV calls were additionally filtered for strand, base

quality and mapping quality biases. A few libraries were observed to display SNVs that

overlapped strongly with those from libraries that were found in close proximity on

library preparation plates. Those SNVs were flagged as potential cross-contamination

artifacts and removed from further scrutiny. Furthermore, SNVs found in two or more

libraries that shared a barcode were tagged as potential index hopping artifacts and

removed from SNV calls. Moreover, the same substitutions arising repeatedly at the exact

same position were deemed unlikely: any SNV encountered in four libraries or more was

rejected. SNVs expected to arise from alignment of reads to the wrong reference genome

(e.g. DBVPG6044 reads mistakenly aligned to the Y12 reference genome) were also

removed. Next, using Fisher's exact test, SNV calls were identified that were more

compatible with the homozygous non-mutant genotype than the heterozygous or

homozygous mutant scenarios, and thus rejected. Finally, libraries that presented an

unusually high number of SNVs (more than 300 SNVs and more than 2 standard

deviations above average number of SNVs per library) were rejected. A heterozygous

genotype was called for many SNVs, despite uniformly haploid starting populations. A

small sample of evolved individuals were thus assessed for ploidy, as described above,

showing diploidization in approximately half sampled isolates (**Fig S12**).

1111

Estimating the fraction of mtDNA per cell. The fraction of sequence bases mapped to

the mitochondrial genome was used as a proxy of the abundance of mitochondrial DNA

in sequenced strains. This was computed from pileup, summing the depth of coverage

with base quality greater than 20. This sum across the mitochondrial genome divided by

the same sum over the whole genome provided an estimate of the fraction of

mitochondrial DNA in each cell.

1118

1119 **Annotation of CNV and SNV calls.** Annotations of the S288c reference genome (Engel

1120 et al., 2022) were used for annotating SNV and CNV calls in evolved isolates. Sequences

1121 from mutation-carrying loci were blasted against the S288c reference genome to identify

1122 corresponding coordinates. Annotations corresponding to these locations were identified

1123 with Yeastmine (Balakrishnan et al., 2012).

1124

Analysis of association between phenotypic changes and mutant loci. Quantitative

phenotypic data, including fitness proxies (carrying capacity and growth rate) in both

fermentable and non-fermentable media, as well as the fraction of mtDNA per cell, are

available for each evolved mutant. A list of annotated mutations is also associated with

each mutant, enabling tests of the statistical association between mutant loci and

phenotypic changes. For each mutant locus and each phenotype in each carbon source,

the association was tested as follows. A t-test was performed to determine if the fitness of

individuals that display mutations at the locus of interest differed significantly from

fitness of their ancestral strains, yielding a p-value. In parallel, using the same data, effect

size of mutation at the locus was evaluated by calculating Cohen's *d* corrected for sample

size, with associated standard error. P-value of obtaining a given effect size at least as

extreme by chance was computed empirically by drawing 10 000 random samples of the

same size among mutants of the same mitonuclear background and calculating an effect

size for each. These calculations were performed separately for each mitonuclear

background since ancestral strains displayed widely differing phenotypes. P-values

obtained for each mitonuclear background were combined using Stouffer's method, with

the square root of sample sizes used as weights. Significance thresholds were corrected

for false discovery rate applying the method of Benjamini and Hochberg on all loci as

described in (Kuo, 2017). Loci with both p-values below threshold were deemed

significantly associated with quantitative changes in phenotype.

1145

Specificity of mutant loci for carbon sources and nuclear and mitochondrial

backgrounds. The same strategy was applied to test the specificity of mutant loci for

carbon source, nuclear background, mitochondrial background, and all combinations

thereof. For each locus, a contingency table was assembled reporting counts of mutations

within and outside the locus in each category. This table was submitted to a chi^2 test of

independence, yielding a p-value. Mutual information (MI, reported as dits) was

calculated from the same contingency table. Significance thresholds on chi^2 p-values

were corrected for false discovery rate on all loci. Loci with chi^2 p-value below threshold

and mutual information two standard deviations above mean MI were deemed non-

randomly distributed between categories, and thus displaying specificity.

1156

Generation of fluorescent knock-out mutants at putative loss-of-function loci. First,

GFP- and mCherry-tagged derivatives of all ancestral strains were generated from three

biological replicates. Parental strains all carried the *MATa ho::hphMX ura3::KanMX*

genotype. They were transformed with both the GFP and mCherry selection cassettes,

which shared the same general structure. The expression of both fluorescent proteins was

placed under the control of the *S. cerevisiae TEF2* promoter and *ENO1* terminator.

Fluorescent protein expression cassettes were fused to the *natNT2* selection cassette. This

full-length cassette was flanked on both extremities with 40 bp homology to the *HIS3*

locus and transformed into the ancestral strains using the lithium acetate method.

Transformants were selected on eYPD agar medium (as described in *Culture media*

subsection above) supplemented with 100 μg/mL hygromycin, 100 μg/mL geneticin and

75 μg/mL nourseothricin. Integration of the fluorescent protein expression cassettes at the

HIS3 locus was confirmed by growth in presence of nourseothricin, loss of histidine

prototrophy, detection of a fluorescent signal by flow cytometry and successful PCR

amplification at the 5' and 3' junctions between the *HIS3* locus and heterologous

sequences. Knockouts of each of the following genes were prepared in all fluorescent

derivatives of the ancestral strains: *AMN1*, *GPB2*, *IRA2*, *MNN4*, *RIM20*, *SIR3*, *SIR4*, and

SUC2. Open reading frames at each of these loci were deleted by transformation with the

Kluyveromyces lactis URA3 expression cassettes amplified from plasmid pUG72

(Gueldener et al., 2002) and flanked in 5' and 3' with 40 bp homology to the promoters

and terminators of the target genes. Transformants were selected on eSC agar devoid of

uracil. Knockouts were scored for uracil prototrophy; resistance to hygromycin, geneticin

and nourseothricin; detection of a fluorescent signal by flow cytometry and successful

PCR amplification at the 5' and 3' junctions between the target locus and heterologous

deletion cassette.

1182

Effect of the environment on the mitochondrion-driven response to selection.

Mutational range for a given mitonuclear genotype was defined as the vector of mutation

counts associated with each annotation. Mutational ranges were compared for all pairwise

combinations of mitonuclear genotypes by means of a chi^2-test of independence, with a

p-value < 0.05 indicating a significant difference in mutational range. Euclidean distance

between significantly different ranges was computed to quantitate the extent of their

divergence. Difference in distance between fermentable and non-fermentable evolved

mutational ranges was calculated to determine which condition, if any, was associated

with the strongest divergence. A 95% confidence interval was computed for differences

in mutational range divergence by bootstrapping, resampling individual mutations. If this

confidence interval did not overlap 0, then carbon source was deemed as affecting the

extent of divergence between mitonuclear backgrounds.

1195

1196   **Competition assays between knockouts and their ancestral strains.** Changes in fitness

1197   incurred by deletion at loci of interest were quantified by performing competition assays

1198   in fermentable and non-fermentable media, using a method inspired by (Breslow et al.,

1199   2008). Knockouts at each of the eight loci of interest, in the mitonuclear background

under study, as three biological replicates, labeled with both GFP and mCherry were arrayed as glycerol stocks in 96-well format. Matching arrays of the fluorescently labeled ancestors were also prepared. Precultures in 96-well format of both the ancestral and knock-out strains were inoculated by transferring 5 μL from thawed glycerol stock arrays to YPD evolution plates. These preculture plates were incubated for 24 hrs at 30°C, 65% RH with shaking at 200 rpm. Outgrown precultures of the knockouts and matching ancestral strains were mixed by transferring 5 μL from both to YPD and YPG evolution plates. Control competitions of the mCherry and GFP labeled ancestral strains were also prepared, to account for differences in fitness caused by the fluorescent labels. For three consecutive days following mixing of the strains, competition cultures were propagated and transferred as described above for experimental evolution. On each day, outgrown cultures were diluted 1:50 in non-sterile water. Two thousand cells from each dilution were analyzed by flow cytometry, counting mCherry and GFP positive cells. Flow cytometry measurements were performed with the help of a Guava easyCyte HT (Luminex, Austin TX, USA) or Accuri C6 (BD, Franklin Lakes NJ, USA) instrument. All microbiological procedures were performed under laminar flow within a sterile enclosure, except for endpoint measurement by flow cytometry. All pipetting was performed with the help of a liquid handling robot (Freedom Evo, Tecan, Männedorf, Switzerland). Triplicate competitions were performed on both fluorescent derivatives of all biological replicates in both carbon sources.

Analysis of competition data. The log_2 ratio of mCherry count over GFP count was calculated for each time point in each competing culture. The log_2 ratio at time zero was subtracted from time points of a given competition so that samples may share a common y-intercept. Linear regression was performed on all data points derived from the same biological replicate, fitting to the following model:

$$log_2 \frac{n_{mCherry}}{n_{GFP}}$$

where $n_{mCherry}$ is the number of mCherry-labeled cells, n_{GFP} is the number of GFP-labeled cells, $\Delta\mu_{WTvWT}$ is the difference in growth rate between the mCherry and GFP labeled

ancestor, t is time, $\Delta\mu_{KO}$ is the difference in growth rate between the knock-out and its

ancestor, M is a variable with value 0 when a time point belongs to a ancestor vs ancestor

competition, and 1 when it belongs to a knock-out vs ancestor competition, and β_0 is a

constant. The fit thus provides an estimate for the change in growth rate associated with

knocking out the gene of interest in the current mitonuclear background. Standard error

(SE) on this coefficient estimate is used to test whether the coefficient is null, and to

compute a sum-of-squares error (SS) on the estimate. Estimates for three biological

replicates and two fluorescent labels thus provided six estimates for each locus in each

mitonuclear background. They were combined into a single estimate by calculating an

average weighted by sample size. P-values were combined using Stouffer's method,

weighted by sample size. Similarly, sum-of-square errors were combined by summing

them and adding the product of sample size with the square deviation of each estimate

from the overall mean estimate. Mean fitness effect estimates, and the combined sum-of-

squares were used to perform ANOVAs to test if fitness effect of a given locus in each

carbon source differed between mitochondrial backgrounds, with accompanying Tukey

HSD tests on pairwise differences between backgrounds. Similarly, ANOVAs were

performed to determine if fitness effects of loci differed on given mitonuclear

backgrounds. Spearman rank correlation was calculated between fitness effects estimated

by competition assays and frequency of mutations at the loci in each background, as

determined from sequencing data.

1251

1252 **Mutational bias.** We counted every possible single-nucleotide change among all SNV

1253 calls in our dataset, and assigned them to their corresponding mitonuclear background.

1254 Counts of for each mitonuclear background were compared to the dataset-wide counts

1255 applying a chi^2-test of independence, with a p-value threshold of 0.05.

1256

1257

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1259

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1267

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1283

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Figure 1. Adaptive evolution of mitonuclear hybrids. **(A)** *Yeast strains submitted to*
experimental evolution were derived from strains 273614N (NN), DBVPG6044 (DD) and
Y12 (YY). Cytoduction of NN and YY with DD yielded two cybrids each. (ND and DN, DY
and YD). We thus evolved seven ancestral mitonuclear backgrounds. **(B)** *Each of these*
backgrounds was evolved in fermentable (FF) and non-fermentable (NF) media, as 96
replicates. Evolving populations were propagated for approximately 300 generations by
daily robot-assisted serial passage. At the end of the experiment, a single independently
evolved individual was isolated from each population. Evolved individuals were assessed
for growth rate in fermentable **(C)** *and non-fermentable* **(D)** *media, showing significant*
gains in fitness in most cases. Asterisks indicate median fitness in evolved strains
significantly higher than ancestors (Mann-Whitney U-test p-value < 0.05).
1496
Figure 2. Whole-genome sequencing reveals mitonuclear background- and carbon
source-specific genomic changes in experimental evolution isolates. **(A)** *Fraction of*
sequenced bases mapped to the mitochondrial genome was used as a proxy for the
proportion of DNA belonging to the mitochondrial genome, revealing a mitonuclear
background-specific impact for both cytoduction and evolution. **(B)** *Changes in*
chromosome copy numbers (aneuploidies), and smaller, gene-scale copy number variants
(CNVs) we detected using depth of coverage information. Many single-nucleotide
variants (SNVs) were also called. All those changes were unevenly distributed among
mitonuclear backgrounds and carbon sources.
1506
Figure 3. Mutation at certain loci is associated with quantitative phenotype changes.
Mutant loci associated with quantitative changes in phenotype were identified by
performing two statistical tests. Scatter plots show on the x-axis the negative log p-value
that individual mutants at a given locus have phenotype identical to their parents, and on
the y-axis, the negative log p-value that a phenotypic effect of this size could be observed
from a set of random individuals of equal size. Dotted lines indicate significance
thresholds for p-values > 0.05, corrected for false-discovery rate. Color of the data
points indicates the mitonuclear background most often affected by mutation at the locus,
while shape indicates carbon source in which the locus was most often mutant. This
analysis was performed for carrying capacity **(A, B)** *and growth rate* **(C, D)***, in*
fermentable **(A, C)** *and non-fermentable* **(B, D)** *media, as well as for the fraction of*
mtDNA per cell **(E).**
1519
Figure 4. Mutations display convergence that is carbon source and mitonuclear specific.
Loci were considered specific for a certain carbon source **(A)** *or mitonuclear background*
(B) *if their distribution among categories differed significantly from that of all detected*
mutations, taken as a whole. Chi^2 tests were performed on contingency tables of all
mutations, classifying them as mapping or not to the locus, and according to either
carbon source or mitonuclear background. Mutual information was calculated from the
same contingency tables. Specificity thresholds, indicated by dotted lines, were set at a p-
value of 0.05, corrected for false-discovery rate, and at two standard deviations above
mean (~95th percentile) mutual information across all loci.
1529

Figure 5. Fitness effect for loss-of-function at select loci is dependent on mitonuclear
background and carbon source. *Gene knockouts were performed at the indicated loci in*
fluorescent-derivatives of all seven ancestral strains used in the evolution experiment,
mimicking loss-of-function alleles. These knockouts were competed against their
*deletion-free counterparts of the same mitonuclear genotype in fermentable (***top***) and*
*non-fermentable (***bottom***) media. This enabled estimation of the fitness effect of loss-of-*
function at these loci, reported in shades of red (positive effect) or blue (negative effect).
For each locus, effect of deletion was found to differ significantly between mitonuclear
genotypes (ANOVA p-value < 0.01). Similarly, loci had differing effects on any given
mitonuclear genotype (ANOVA p-value < 0.01). Fitness effect of each locus in across
mitonuclear genotypes did not correlate with frequency of mutation at the locus in each
genotype, with one exception indicated by an asterisk on the right-hand side (spearman
rho > 0.95). Asterisks at the top of heatmaps indicate genotypes that display rank order
correlation between locus fitness effect preference and frequency of mutation at the locus
(spearman rho > 0.7, p-value<0.05 that slope of regression =0).
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A

273614N

NN ND DN **DBVPG6044** DD DY YD **Y12** YY

B

NN
ND
DN
DD
DY
YD
YY

Fermentable   Non-fermentable   ~300 generations   Isolate 1 clone per evolved population

C

Fermentable (FF)

Growth rate (AU/hr)

ancestral
evolved

Mitonuclear background

NN ND DN DD DY YD YY

D

Non-fermentable (NF)

ancestral
evolved

Mitonuclear background

NN ND DN DD DY YD YY

Fitness gain - competition