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Title

Mitochondrial recombination reveals mito-mito epistasis in yeast

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Mito-Mito Epistasis in Yeast

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ABSTRACT

Genetic variation in mitochondrial DNA (mtDNA) provides adaptive potential although the underlying genetic architecture of fitness components within mtDNAs is not known. To dissect functional variation within mtDNAs, we first identified naturally occurring mtDNAs that conferred high or low fitness in *Saccharomyces cerevisiae* by comparing growth in strains containing identical nuclear genotypes but different mtDNAs. During respiratory growth under temperature and oxidative stress conditions, mitotype effects were largely independent of nuclear genotypes even in the presence of mitonuclear interactions. Recombinant mtDNAs were generated to determine fitness components within high and low fitness mtDNAs. Based on phenotypic distributions of isogenic strains containing recombinant mtDNAs, we found that multiple loci contributed to mitotype fitness differences. These mitochondrial loci interacted in epistatic, non-additive ways in certain environmental conditions. Mito-mito epistasis (i.e. non-additive interactions between mitochondrial loci) influenced fitness in progeny from 4 different crosses, suggesting that mito-mito epistasis is a widespread phenomenon in yeast and other systems with recombining mtDNAs. Furthermore, we found that interruption of coadapted mito-mito interactions produced recombinant mtDNAs with lower fitness. Our results demonstrate that mito-mito epistasis results in functional variation through mitochondrial recombination in fungi, providing modes for adaptive evolution and the generation of mito-mito incompatibilities.

ARTICLE SUMMARY

The genetic architecture underlying the mitochondrial contribution to adaptation is not understood. We generated *Saccharomyces cerevisiae* strains with recombinant mtDNAs and assessed how mitochondrial admixtures influenced growth. Phenotypic distributions indicated that multiple loci contribute to growth through genetic interactions between mitochondrial loci (mito-mito epistasis). Interruption of coadapted mito-mito interactions led to recombinant mtDNAs with low fitness. Evidence for mito-mito epistasis was observed between numerous divergent mitotypes suggesting this phenomenon is widespread in yeast. Given that mitochondrial recombination is frequent in fungi and has been observed across eukarya, mito-mito interactions are likely an important process in mtDNA evolution and adaptation.

INTRODUCTION

Mitochondrial DNA (mtDNA) haplotypes are frequently associated with environmental temperature gradients across eukarya, suggesting that mitochondrial performance plays an important role in adaptation (MISHMAR *et al.* 2003; LUCASSEN *et al.* 2006; CHATELAIN *et al.* 2011; SCOTT *et al.* 2011; LAGISZ *et al.* 2013; DINGLEY *et al.* 2014; MELO-FERREIRA *et al.* 2014; SILVA *et al.* 2014; CONSUEGRA *et al.* 2015; LI *et al.* 2016). To understand the adaptive potential of mtDNAs, it is necessary to dissect the genetic and environmental factors that influence the functional variation in mtDNAs. This is particularly challenging in systems where mtDNA inheritance is uniparental because the lack of recombination makes it difficult to differentiate between mitochondrial alleles that contribute to functional variation and those that are neutral.

Homologous recombination between mtDNAs should promote the reorganization of mitochondrial genes and increase the efficacy of selection on adaptive loci. Mitochondrial recombination is common in systems with biparental mtDNA inheritance, such as fungi and many plants (BARR *et al.* 2005; GUALBERTO AND NEWTON 2017). In *Saccharomyces* yeasts, the diverse and highly reticulated mtDNAs show signatures of recombination and horizontal gene transfer within and between species (PERIS *et al.* 2014; WOLTERS *et al.* 2015; WU *et al.* 2015; LEDUCQ *et al.* 2017; PERIS *et al.* 2017). In predominantly asexual fungi, mitochondrial recombination occurs more frequently than expected (BRANKOVICS *et al.* 2017). The machinery for homologous recombination is found in the mitochondria of mammals (DAHAL *et al.* 2017) and there is some evidence of mitochondrial recombination in mammals (PIGANEAU *et al.* 2004), other vertebrates (CIBOROWSKI *et al.* 2007; UJVARI *et al.* 2007; SAMMLER *et al.* 2011; WANG *et al.* 2015;

PARK *et al.* 2016) and invertebrates (LADOUKAKIS AND ZOUROS 2001; PASSAMONTI *et al.* 2003). Mitochondrial recombination occurs with enough frequency that it should play an important role in the evolution of mtDNAs, especially in fungi.

The effects of mitochondrial recombination on selection and adaptive potential are not understood. In *Saccharomyces* yeasts, mitochondrial recombination can occur in zygotes containing different mtDNAs but because heteroplasmic mtDNA states are not maintained, a single mtDNA haplotype (either parental or recombinant) becomes fixed after approximately 20 generations (BERGER AND YAFFE 2000). Hybrids of *S. cerevisiae* and *S. uvarum* contained different species-specific mtDNA genetic markers, depending on whether the hybrids were created in a laboratory (VERSPOHL *et al.* 2018) or isolated from industrial settings (MASNEUF *et al.* 1998; RAINIERI *et al.* 2008), suggesting that environmental conditions influence the selection for mitochondrial alleles or entire mitotypes. Supporting this, mitochondrial allele inheritance during hybridization of *S. cerevisiae* and *S. paradoxus* was altered by changing laboratory conditions during matings (HSU AND CHOU 2017).

Mitochondrial alleles that participate in mitonuclear interactions will also influence the adaptive success of recombinant mtDNAs. Mitonuclear incompatibilities occur between (SULO *et al.* 2003; CHOU *et al.* 2010; SPIREK *et al.* 2014) and within (PALIWAL *et al.* 2014; HOU *et al.* 2015) *Saccharomyces* species and species-specific compatible mitonuclear genetic combinations were universally maintained in rare, viable meiotic progeny from *S.cerevisiae/S.bayanus* hybrids (LEE *et al.* 2008). The extent of recombination in these hybrid studies is not known due to the limited number of mitochondrial markers followed. Laboratory-derived isogenic *S. paradoxus* hybrids

containing different recombinant mtDNAs were phenotypically variable, consistent with the presence of functionally distinct mitochondrial or mitonuclear alleles (LEDUCQ *et al.* 2017). Selection on such functional units could explain the existence of recombinant mtDNAs found in natural *S. paradoxus* hybrids (LEDUCQ *et al.* 2017; PERIS *et al.* 2017).

Mitochondrial genes encode for physically interacting subunits of respiratory complexes and so there is potential that mitochondrial genes coevolve through compensatory mutations or selection for adaptive mito-mito allele combinations in recombinant mtDNAs. In support of this, comparative analysis of mtDNA and *in silico* analysis of protein structures revealed coevolving amino acids within mitochondrial genes within primates (AZEVEDO *et al.* 2009). In *S. cerevisiae,* mitochondrial suppressor mutations that counteracted respiratory deficient mitochondrial mutations were characterized (FOX AND STAEMPFLI 1982; DI RAGO *et al.* 1995). These examples of functional interactions suggest that epistasis between mitochondrial alleles (mito-mito epistasis) could contribute to functional variation in mtDNAs.

In this work, we identified mitotypes that conferred strong adaptive potential by examining fitness in *S. cerevisiae* strains containing different combinations of mtDNA and nuclear genomes. Certain mitotypes provided growth advantages during respiratory growth under temperature and oxidative stress conditions, irrespective of nuclear backgrounds, indicating direct effects on fitness. To determine whether these growth advantages were due to one or more interacting mitochondrial loci, we generated and phenotyped strains containing recombinant mtDNAs. The effects of these mitochondrial alleles were non-additive and revealed that mito-mito epistasis contributes to phenotypic variation. Furthermore, interruption of coadapted mito-mito interactions produced

recombinant mtDNAs with lower fitness in media mimicking natural yeast environments, indicating that mito-mito epistasis may play an important role in hybridization and mtDNA evolution.

MATERIALS AND METHODS

Strains and Media

Strain names and genotypes are provided (Tables S1 and S2). The creation of strains containing synthetic combinations of nuclear and mitochondrial genotypes was previously described (PALIWAL *et al.* 2014). Basically, mtDNAs from mtDNA donor haploid strains were serially passaged through a strain containing a *kar1-1* mutation to mtDNA recipient haploid strains. The *kar1-1* mutation inhibits nuclear fusion and allows the formation of heterokaryotic zygotes containing mixed cytoplasmic components including mitochondria (ROSE AND FINK 1987). Haploid progeny containing mtDNA from the donor strain and the nuclear genotype of the recipient strain were identified by scoring auxotrophic markers.

Five different media were used (Table S3). These included rich, undefined, media containing fermentable (YPD) or nonfermentable (YPEG) carbon sources, minimal defined media containing fermentable (CSM) or nonfermentable (CSMEG) carbon sources, and a minimal media emulating oak tree exudate (SOE). Nonfermentable media require mitochondrial respiration for growth. The oxidative stress agent, menadione, was added to 20 µM when indicated (YPEGM). Agar (2%) was added to solid medium. Undefined media types contained yeast extract for which the

exact nutrient composition is not known while defined media contained precisely specified nutrient compositions.

Colony Arrays

Cells from the haploid mito-nuclear (9 n x 9 mt) and recombinant mtDNA (YJM975 x Y12) diploid strain collections were printed onto OmniTrays (Nunc) in randomized 1,536 colony arrays using a BM3-BC colony processing robot (S&P Robotics, Inc.). Arrays were first printed to YPD, and then to test media. Strains were acclimated to test media (YPD, CSM, SOE, YPEG with and without menadione, and CSMEG, Table S3) for three days at 30°, 35° and 37°, and then reprinted to the same media. Each strain in the mito-nuclear collection was printed in 15 replicates. Strains in the recombinant mtDNA collection, including two biological replicates for each parental mtDNA, were printed in 25 replicates. Photographs were taken at times 0 and 48 hours. Colony sizes were extracted from images using ImageJ 1.440 software (DISS *et al.* 2013). Size differences were determined using custom R scripts available at https://github.com/JFWolters/Wolters-Genetics-2018/.

A block design was used for additional phenotyping of diploid strains containing mitochondrial recombinants from all crosses. The phenotyping strategy was completed as before with the following modifications. Phenotyping was conducted on YPD, YPEG, and SOE media at 30° and 37°. The block design was similar to that used in (STROPE *et al.* 2015), modified such that each strain existed on the plate as a four row by eight column colony block. Colonies on the edges of blocks were excluded from analysis to remove neighbor effects. Images were taken over seven days. Image analysis was

completed using Gitter (WAGIH AND PARTS 2014). A custom R script pipeline using logistic regressions to fit growth curves and estimate growth rate is available at https://github.com/JFWolters/Robot-Image-Analysis-master.

Competition Assay

Haploid strains (*MAT***a**) containing the YJM975 nuclear genotype and Y12 or YJM975 mitotypes (strains SP15a1ρ26 and NCYC3954, respectively, Table S1) were grown overnight in liquid YPD media at 30°, mixed in equal volumes, spread as lawns onto solid YPEG media and grown for 2 days at 30° and 37°. The pooled cells were then retrieved from the solid plates in sterile water, diluted, plated for single colonies on YPEG media, and incubated at 37° for 4 days. Cells harboring the Y12 mitotype produced large colonies as compared to cells with the YJM975 mitotype. Numbers of large and small colonies were used to determine the proportion of each genotype. Significance was assessed using a test of equal proportions and *p*-values were assessed versus a stringent α after Bonferroni correction (3 tests, $\alpha = 0.017$).

Mitochondrial recombination

To produce mitochondrial recombinants, matings between congenic haploid strains containing congenic nuclear backgrounds (YJM975) and different mitotypes (YJM975 x Y12, YJM975 x YPS606, YJM975 x Y55, and YPS606 x Y55) were conducted on solid YPD media at 30° for 2 days. Diploids were selected by printing mated cell mixtures onto SD media and grown at 30° for 1 day. To fix mitotypes, diploid strains were streaked for single colonies twice on SD media with 2 days of growth at 30°. Each strain containing putative recombinant mtDNAs resulted from independent

matings. Two independent biological replicates of diploid parental control strains were created by mating congenic strains containing only one mitotype.

To look for evidence of mitochondrial recombination, restriction fragment length polymorphism (RFLP) assays were performed. Total DNA was isolated from 5 ml overnight cultures grown in YPD using a glass-bead cell disruption and phenol-chloroform extraction, as previously described (HOFFMAN AND WINSTON 1987). Three mtDNA loci were amplified using the primers and amplification conditions listed in Table S4. Amplified products were digested using *Hin*fI (*ATP6*), *Ase*1 (*COX2*), or *Hin*dIII (*COX3*) and separated on 2% agarose gels. Additionally, total mtDNA was isolated from 21 diploid strains according to (DEFONTAINE *et al.* 1991), including a DNase treatment (WOLTERS *et al.* 2015), and modified for smaller volumes. The total mtDNA was digested with *Eco*RV, and separated by electrophoresis on 0.8% agarose gels. Mitochondrial recombination was inferred through non-parental haplotype and total mtDNA RFLP patterns.

To compare colonies formed from single cells (Figure 5 inset), cells were streaked directly onto YPEG media and photographed following incubation at 37° for 4 days.

Statistics

All statistical analyses were performed in R (version 3.4.0). The differences in colony sizes from the arrays between 0 and 48 hours were used as a proxy for growth differences. Random effects ANOVAs (containing terms for nuclear genotype, mitotype, and nuclear x mitotype interaction, and when appropriate temperature and media) were performed using the lmer function of the lme4 package (BATES *et al.* 2014). Phenotypic

variances were determined from each model, and normalized by dividing the mean for a given condition. Direct effects of mitotypes were evaluated in each nuclear genotype by dividing the average growth of each strain by the average growth of all strains containing the identical nuclear genotype, and converting to percentages. To determine whether strains with potentially recombinant mtDNAs had phenotypes that differed from parental controls, individual ANOVAs were performed on each strain (fixed factor comparing Parent 1, Parent 2, and strain), followed by a post-hoc analysis using the TukeyHSD function to assess whether the recombinant strain was significantly different from both parental controls. A Bonferroni correction was applied to compare results under a standard (α=0.05) and stringent (α=0.0012) significance thresholds (Table S5).

Estimating Mito-Mito Epistasis

Epistasis between mitochondrial variants (mito-mito epistasis) was explicitly tested for these crosses using a variance partitioning approach developed for haploid organisms (SHAW *et al.* 1997). Recombinant mitotypes were nested within each parental cross and represented by multiple technical replicates. The variance attributed to parental crosses (σ^2_{PC}) and putative recombinants (σ^2_R) were estimated from a nested random effects model using the lmer function in the lme4 package (BATES *et al.* 2014). Additive by additive epistasis (i.e. mito-mito epistasis) was estimated as: $V_{AA} = 2 * (\sigma^2_R - \sigma^2_{PC})$. Parental cross, putative mitochondrial recombinant and technical replicates correspond to the terms “diploids”, “haploids” and “clones” as described by (SHAW *et al.* 1997).

The significance of V_{AA} was evaluated with several criteria. First, the significance of the putative recombinant term (nested within parental cross) was tested in the

random effects model via a hierarchical comparison of models with and without that term. Second, the significance of the difference between the putative recombinant variance (σ_R^2) and the parental cross variance (σ_{PC}^2) was tested by comparing the observed value to 1000 permutations of the data where individual data points were permuted (ANDERSON AND TER BRAAK 2003). Third, we determined whether 95% confidence intervals of V_{AA} were positive and did not overlap zero. Confidence intervals were calculated using a percentile bootstrap approach by resampling from within the technical replicates such that the overall structure of the putative recombinants and diploid crosses was maintained (QUINN AND KEOUGH 2002). V_{AA} was considered significant if and only if all three criteria were met.

Data Availability

All growth measures used for analyses are provided in Tables S6, S7 and S8. Strains are available upon request.

RESULTS

Respiratory growth is influenced by mtDNA haplotypes under stress conditions.

In *S. cerevisiae,* mitonuclear interactions can explain the majority of mitotype-related growth differences (PALIWAL *et al.* 2014). We previously observed that in yeast cells growing on fermentable sugars at high temperatures, mitotypes had direct effects on growth (PALIWAL *et al.* 2014). Here, we hypothesized that temperature stress would reveal mitotype-specific effects on nonfermentable media where oxidative phosphorylation is essential for energy production. To test this, we plated *S. cerevisiae* cells containing identical nuclear genotypes and two divergent mitotypes onto solid media containing fermentable and nonfermentable sugars and followed colony sizes as

a proxy for mitochondrial genotype fitness (Figure 1). Cells containing the nuclear genotype from a strain of European origin (YJM975) and its own mitotype or that from a divergent Sake strain (Y12) showed no differences in fitness when grown on fermentable or nonfermentable sugars at standard temperature (30°). However, at high temperature (37°) growth differences due to mitotypes were observed. On nonfermentable media, where growth requires mitochondrial respiration, cells containing the Y12 mitotype formed larger colonies than isonuclear cells containing the YJM975 mitotype while colony size differences on fermentable media were barely observable. To determine if these respiratory growth differences were due to direct effects of mitotypes or to mitonuclear interactions, the mitotypes were introduced into a second nuclear background. In the Y12 nuclear background, no differences in fitness between mitotypes were observed although cells containing the Y12 nuclear genotype grew better on nonfermentable media than those with the YJM975 genotype (Figure 1). The variable effect of these mitotypes is consistent with mitonuclear epistasis, however, nuclear alleles in the Y12 genotype with large effects on growth may have obscured independent effects of mitotypes.

We expanded our assay to identify mitochondrial genotype specific fitness differences between 81 strains containing unique combinations of 9 divergent nuclear genotypes and mtDNAs (9 nuclear x 9 mtDNA, Table S1). This large and systematic array of mitonuclear combinations provides a powerful platform to determine whether phenotypic differences are due to genetic differences in nuclear genomes, mitotypes, or mitonuclear interactions. Cells were grown on solid media under atmospheric oxygen levels using high-density colony arrays in 18 environmental conditions, including

fermentable and non-fermentable sugars at three temperatures, and fitness differences were analyzed by ANOVAs (Table 1). Strains bearing the SK1 nuclear genotype failed to transfer or form uniform colonies due to severe flocculation and were omitted from further analysis. In all media and temperature conditions, mitonuclear interactions and nuclear genotypes were highly significant contributors to fitness differences. Mitotypes showed significant independent fitness effects in each nonfermentable media at elevated temperature. In the presence of strong interaction terms, interpretation of main effects is difficult. Still, the main effect for mitotype showed increasing χ^2 statistics and decreasing *p*-values with increasing temperatures, suggesting that mitochondrial respiration was directly impacted by variation in mitotypes during temperature stress. Temperature-related mitotype effects were even stronger in media containing the exogenous oxidative stress agent menadione (YPEGM).

To more closely follow independent effects of mitotypes, we compared the respiratory growth of each strain with the average growth for all strains with the same nuclear genotype (Figure 2). The Y12 mitotype provided a respiratory growth advantage under temperature stress in the YJM975 nuclear background, and also in 6 of the 7 nuclear backgrounds tested. Four additional mitotypes showed relative growth increases at high temperatures in each of the respiratory and oxidative stress conditions tested, while two mitotypes decreased growth (Table 2). Two mitotypes (including YJM975) showed both increased or decreased growth, depending on the nuclear backgrounds, although these fitness differences are relatively small compared to growth averages.

Direct effects from mitotypes can also be observed in colony size variances. Overall phenotypic variances in colony sizes increased with increasing temperatures in defined fermentable and nonfermentable media (Figure 3A). This is consistent with previously observed responses to temperature in yeasts grown in liquid culture (PALIWAL *et al.* 2014) and on complex traits in *Drosophila* (BUBLIY *et al.* 2001). In contrast, phenotypic variances were generally higher in rich media whose exact nutrient components are not defined (YPD, YPEG, YPEGM) and decreased as cells were grown at higher temperatures. The contributions of the nuclear genome, mitotype, and mitonuclear interactions to total phenotypic variances were estimated using variance component analyses of the full ANOVA models (Figure 3A and Table S9). Mitotypes did not explain any proportion of these variances at lower temperatures, but as temperatures increased, the proportions of variance due to mitotypes increased during respiratory growth, particularly in the presence of an oxidative stress agent (YPEGM, Figure 3B). The variance component due to mitotypes exceeded that of mitonuclear interactions in this oxidative-stress environment (18.3% vs. 16.0% respectively, Table S9).

A competition experiment was performed to determine if the growth differences of strains grown in isolation on solid media corresponded to fitness advantages when grown in mixed cultures. Non-mating cells with isogenic nuclear genotypes and either a neutral (YJM975) or high fitness (Y12) mitotype were pooled in equal amounts and grown in direct competition on nonfermentable media (Figure 4). The proportion of mitotypes were approximately equal before (61.4%) and after competition (52.4%) at 30° (nonsignificantly different with $p = 0.03$ vs α of 0.017 after Bonferroni correction), but

after competitive growth at the elevated temperature, the proportion of cells containing the high fitness mtDNA increased substantially (95.6%, $p < 2x10^{-16}$). This result demonstrates that larger colony sizes due to high fitness mtDNAs reflect competitive advantages.

Taken together, these results show that genetic variation in mitochondrial haplotypes directly impacts mitochondrial fitness. Even in the presence of strong mito-nuclear interactions, certain mitotypes affect yeast growth in consistent patterns and can be considered as high and low fitness mtDNAs. These effects are largely contingent on stress conditions that tax mitochondrial functions.

Mitochondrial recombination reveals functional interactions between mitochondrial loci

In nature, biparental inheritance of yeast mtDNAs during matings could introduce different mtDNAs into the same cell. Mitochondrial recombination would result in allele reorganization, revealing novel phenotypes due to direct effects and epistasis that may promote adaptive evolution.

Single or multiple alleles within the mitochondrial genome could be contributing to fitness differences. To test this, we created isonuclear diploid strains containing recombinant mtDNAs by mating haploid strains with congenic nuclear genotypes (differing by mating type and a selectable marker) and different mitotypes (Table S2). A total of 41 independent matings were performed, followed by clonal propagation of unique diploid strains to fix mitotypes. Restriction analysis of whole mtDNAs and 3 amplified mitochondrial markers showed evidence of recombination in 41% of tested diploids (Table S10), though the low resolution of these assays likely underestimates

353 the number of diploids containing recombinant mtDNAs. If the high fitness mtDNA was
354 due to a single locus, then recombinant mtDNAs would have either the high or low
355 fitness allele and would be expected to generate phenotypes identical to a parental
356 mtDNA. We found that on rich nonfermentable media at high temperature (YPEG 37°),
357 colony sizes from strains with recombinant mtDNAs were sometimes in between either
358 control strain containing parental mitotypes (Figure 5A). Fitness differences between
359 strains with recombinant versus both parental mtDNAs were observed in 10 of the 18
360 environmental conditions examined (Table S5). These results indicate that multiple
361 mitochondrial loci underlie mitotype-specific phenotypic differences.
362 Multiple loci could contribute to phenotypes through additive or epistatic
363 interactions between mitochondrial alleles. For polygenic traits with additive alleles,
364 recombination would produce mtDNAs that generate phenotypes both above and below
365 those of the parental mtDNAs. If one parental mtDNA contained all positive alleles and
366 the other none, then progeny containing recombinant mtDNA would only generate
367 intermediate phenotypes. For epistatic loci, recombination would generate novel
368 interacting allele combinations that could present phenotypic distributions that resemble
369 additive alleles. However, if high fitness epistatic allele combinations were interrupted,
370 recombination could produce low fitness mtDNAs that result in lower growth than
371 parental mtDNAs.
372 The intermediate phenotypes observed in strains with recombinant mtDNAs on
373 respiratory media are consistent with a model with multiple loci with additive effects
374 (Figure 5A). However, when strains were grown at high temperature on a media
375 designed to emulate exudate from oak trees (SOE), a natural habitat for

376 *Saccharomyces*, the phenotypic distribution is consistent with negative epistasis (Figure
377 5B). In this condition, growth rates of strains with parental mtDNAs were similar to each
378 other, while 28 (of 41) strains containing recombinant mtDNAs grew worse than either
379 parent ($p < 0.05$ for independent ANOVAs comparing growth of each recombinant strain
380 to each parent; Table S5). It is likely that the interruption of one or more coadapted
381 mito-mito allele combination led to the observed negative epistasis in this ecologically
382 relevant media.

383 To determine if mito-mito epistasis was a general feature among mtDNAs, we
384 created additional recombinant mtDNAs between four different mitotypes (Table S2).
385 Growth rates were collected for isonuclear strains containing fixed recombinant mtDNAs
386 (mitotypes YJM975 x Y12, YJM975 x YPS606, YJM975 x Y55, and YPS606 x Y55). To
387 explicitly test for mito-mito epistasis, we used a variance partitioning approach
388 developed to test for epistasis in haploid genetic systems (SHAW *et al.* 1997).
389 Specifically, additive by additive (i.e. mito-mito) epistasis (V_{AA}) was estimated as twice
390 the difference between the variances attributed to the parental crosses (PC) and the
391 putative recombinants (R) i.e, $V_{AA} = 2 * (\sigma^2_R - \sigma^2_{PC})$ (see Methods). There was evidence
392 for mito-mito epistasis ($p < 0.001$) in each environmental condition (Table 3). These
393 analyses were repeated for balanced subsets of the data, such that each mitotype was
394 only represented exactly once or twice (using data from mitotype crosses YJM975 x
395 Y12 and YPS606 x Y55 or YJM975 x YPS606, YJM975 x Y55, and YPS606 x Y55,
396 respectively). In both balanced designs, V_{AA} estimates were significant and positive,
397 with one exception (double representation, YPD at 37°). The negative V_{AA} estimate in
398 this exception suggests a violation of test assumptions and could be generated if the

interacting loci were tightly linked. If mitochondrial recombination was not sufficient to interrupt linkage in these crosses, our power to observe mito-mito epistasis would be reduced. Still, we detected positive mito-mito epistasis in most cases.

In sum, mito-mito epistasis appears to be a common feature in yeast mtDNAs. Mito-mito epistasis estimates were highest for the ecologically relevant media (SOE) at high temperatures, and suggest that selection for interacting mitochondrial alleles has influenced mtDNA evolution.

DISCUSSION

The evolutionary significance of mitochondrial recombination is not well understood. In this work, we show that recombination can reveal interactions between mitochondrial loci that affect fitness. Our analyses were facilitated by using strains with isogenic nuclear genotypes and parental or recombinant mtDNAs in fixed environments, such that phenotypic variances could be directly attributed to mtDNAs. Because mtDNAs are homoplasmic in yeast, dominance effects (V_D) could be eliminated, and the remaining phenotypic distributions must be due to additive (V_A) and/or epistatic (V_I) components within mtDNAs. In most cases, recombinant mtDNAs created distributions that were consistent with additive effects. However, negative interactions between mitochondrial alleles best explains the lowered fitness values of nearly all strains with recombinant mtDNAs shown in Figure 5B. Epistasis can be difficult to detect because for most allele frequencies the additive (main) effects of interacting loci will be greater than the total epistatic variance unless the epistatic loci have strong deleterious interactions (MACKAY 2014). Still, statistically significant non-zero values of mito-mito epistasis (V_{AA}) were estimated when assessing recombinant mtDNAs from four distinct

parental mitotypes. Mito-mito epistasis is most likely a general feature underling fitness differences in yeast mtDNAs.

Given the large number of physical interactions between the products of mitochondrial genes, it is possible that multiple mito-mito interactions contribute to mtDNA fitness. In the simplest case where each parental mtDNA contains unique alleles for two interacting loci (eg. X_1Y_1 and X_2Y_2), offspring with recombinant mtDNA should, at most, form two distinct phenotypic classes (representing X_2Y_1 and X_1Y_2). While our sample sizes are too low to robustly describe distribution shapes, the phenotypic distribution in Figure 5B appears broad, consistent with the interruption of multiple different interacting genes. Large deletions within mtDNAs arise in clonally replicating cells (resulting in '*petites*' (EUPHRASSI 1949)) and so we omitted non-respiring diploid strains resulting from the crosses generating recombinant mtDNAs. Our observed numbers of *petites* varied between crosses (Table S11) and could reflect additional deleterious mito-mito interactions between certain mtDNAs that were not analyzed in this study.

We found that recombination between parental mtDNAs generated lower fitness mtDNAs using laboratory crossing schemes, showing that mito-mito incompatibilities exist within species. In nature, mito-mito incompatibilities generated via mitochondrial recombination may lead to reduced fitness in hybrids. In *S. paradoxus*, recombination between mtDNAs from diverging populations generated hybrid diploids with lower fitness than hybrids with parental mtDNAs (LEDUCQ *et al.* 2017). Given the heterozygous nuclear genotypes in these hybrids, dominant interactions between mitochondrial and nuclear alleles could not be ruled out entirely. However, it is likely that negative

interactions between mitochondrial loci could be contributing to incipient speciation events. In natural hybridization zones, *S. paradoxus* hybrids contain mtDNAs with specific recombination patterns of mtDNAs (LEDUCQ *et al.* 2017; PERIS *et al.* 2017), consistent with selection for mito-mito (and possibly higher order mito-mito-nuclear) interactions. Coevolved mito-mito interactions are likely to strengthen hybridization barriers already imposed by mitochondrial-nuclear incompatibilities in *Saccharomyces* yeasts (SULO *et al.* 2003; LEE *et al.* 2008; CHOU *et al.* 2010; SPIREK *et al.* 2014).

Strains with recombinant mtDNAs had different phenotypic distributions when grown in different environmental conditions. Thus, selection potential for direct mitotype effects (including mito-mito epistasis) versus mitonuclear interactions will be altered in different environments. In the 9 mitotype x 9 nuclear genotype strain collection, we found that direct effects of mitotypes were significant only when respiring cells were grown in temperature and oxidative stress conditions. This is perhaps a reflection of increased ATP demands (POSTMUS *et al.* 2011) and a mitochondrial role in stress responses at elevated temperatures in cells (KNORRE *et al.* 2016; MUNRO AND TREBERG 2017). In contrast, mitonuclear interactions were highly significant in each condition tested, as shown here and in (PALIWAL *et al.* 2014), including fermentable media where mitochondrial metabolism is downregulated due to glucose repression. It is possible that mitonuclear interactions play roles that are more related to cellular homeostasis rather than oxidative phosphorylation. In support of this, cosegregating mitochondrial and nuclear alleles in natural populations of fish mapped to genes with roles in mitochondrial protein regulation and translation but not to genes encoding subunits of oxidative phosphorylation complexes (BARIS *et al.* 2017).

While coevolved mitonuclear genome combinations generally show higher fitness than synthetic genome combinations, exceptions are routinely observed, including in yeast (Figure 1A and (PALIWAL *et al.* 2014)), copepods, (WILLETT AND BURTON 2003) and flies (RAND *et al.* 2006). Mitochondrial alleles with strong direct effects may help to explain these observations. Without recombination, selection for beneficial mitochondrial alleles may be impeded by linkage to other mitochondrial alleles causing mitonuclear incompatibilities. Recombination may promote adaptive evolution by separating beneficial alleles from linked deleterious alleles. Bi-parental inheritance of plastid DNA can lead to rescue of a cyto-nuclear incompatibility (BARNARD-KUBOW *et al.* 2017) suggesting the same is possible with mitochondrial recombination. Low levels of recombination in natural populations may weaken mitonuclear coevolution, especially if there is selection on direct effects that could promote admixture. However, strong mito-mito incompatibilities may reinforce mitonuclear incompatibilities in the presence of mitochondrial recombination.

Mitochondrial recombination has played a role in the evolution of mtDNAs in yeasts (WU *et al.* 2015; LEDUCQ *et al.* 2017; WANG *et al.* 2017). High rates of recombination have been reported in laboratory conditions (FRITSCH *et al.* 2014), although mitochondrial heteroplasmy does not necessarily occur in the diploid cells produced following matings (ZINN *et al.* 1987). We observed that approximately 40% of mated cells contained recombinant mtDNAs as identified through low resolution genotyping approaches. Additional mated strains had mtDNA genotypes that matched one parental mtDNA but phenotypes that matched the alternative mtDNA parental control strain, suggesting that many recombination events went undetected. A closer

491 analysis of mitochondrial recombination is necessary to develop mapping approaches
492 and to understand how functional variation is created in nature.

493 Mito-mito epistasis adds to the complexity of genetic interactions affecting life
494 history traits. In systems where mtDNA inheritance is uniparental and largely absent of
495 recombination, mildly deleterious mutations are frequently maintained (NEIMAN AND
496 TAYLOR 2009). Secondary mutations that compensate for an interrupted epistatic
497 interaction should be selected for and promote mito-mito coevolution. Selection for
498 interacting mitochondrial loci may also contribute to unexpected maintenance of mtDNA
499 heteroplasmy (YE *et al.* 2014). In recombining systems, mitochondrial recombination will
500 create new mito-mito allele combinations that could influence the removal of deleterious
501 mutations, hybrid fitness, and coevolutionary processes. Generation and
502 characterization of recombinant mtDNAs as done here and experimental evolution in
503 yeast offer approaches to test such evolutionary mechanisms in the future.
504

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FIGURE LEGENDS

Figure 1. Mitochondrial genotype affected respiratory growth at elevated temperature. *S. cerevisiae* strains containing different combinations of nuclear and mitochondrial genomes were grown overnight in liquid fermentable media, serially diluted and grown for 2 days on fermentable (YPD) or nonfermentable media (YPEG) media at 30º and 38º. Nonfermentable media requires mitochondrial respiration for growth. Nuclear and mitotype origins are indicated.

Figure 2. Fitness effects of mitotypes in nonfermentable media. Heat maps show the relative increase (red) or decrease (blue) in fitness by specific mitotypes. Relative fitness is shown for each nuclear background in media where direct effects of mitotypes were statistically significant. Mitotypes had observable direct effects in nonfermentable media (CSM, YPEG, YPEGM) at 37º. Percent changes in growth were determined by comparing the growth rate of each mitonuclear genome combination with the average growth of all strains with the same nuclear genotype. Average percent increases and decreases are provided in Table 2.

Figure 3. Temperature increases phenotypic variances due to mitotypes under stress conditions. (A) Total normalized phenotypic variances and individual variance component analyses including nuclear (blue), mitotype (red), and mitonuclear epistasis (orange) and residual (grey) are presented as stacked bar graphs. (B) The proportion of total phenotypic variance due to independent effects of mitotypes is plotted across increasing temperature for each media. Line colors match media as shown in (A).

Figure 4. High fitness mitotypes provide competitive advantage. Overnight cultures of yeast strains containing the YJM975 nuclear genotype and YJM975 (blue) or Y12 (red) mitotypes were mixed and competitively grown on solid respiratory media (YPEG_R). The proportion of cells containing each mitotype was determined before (t = 0) and after competition on YPEG media for two days by counting numbers of large (Y12) and small (YJM975) colonies that formed on YPEG media at 37°. Proportions shown are based on total numbers of colonies counted before (n = 223) and after competition at 30° (n = 391) and 37°(n = 205). Significance codes following Bonferonni correction: 'ns' $p > 0.05$; '*' $p < 0.05$; '**' $p < 0.005$; '***' $p < 5x10^{-6}$.

Figure 5. Mitochondrial recombination generates novel phenotypes. Diploid strains containing isogenic homozygous nuclear genotypes and parental (Y12 (red) or YJM975 (blue) or recombinant (green) mitotypes were spotted via robotic transfer onto solid media and colony sizes were quantified after 48 hours. Two biological replicates for each parental control diploid were included. Colony sizes distributions are plotted as histograms. (A) YPEG, 37°. The intermediate phenotypes of representative recombinant strains (R1 and R2) are shown in the insert. (B) SOE, 37°. See Figure S1 for phenotypes for additional media and temperatures.

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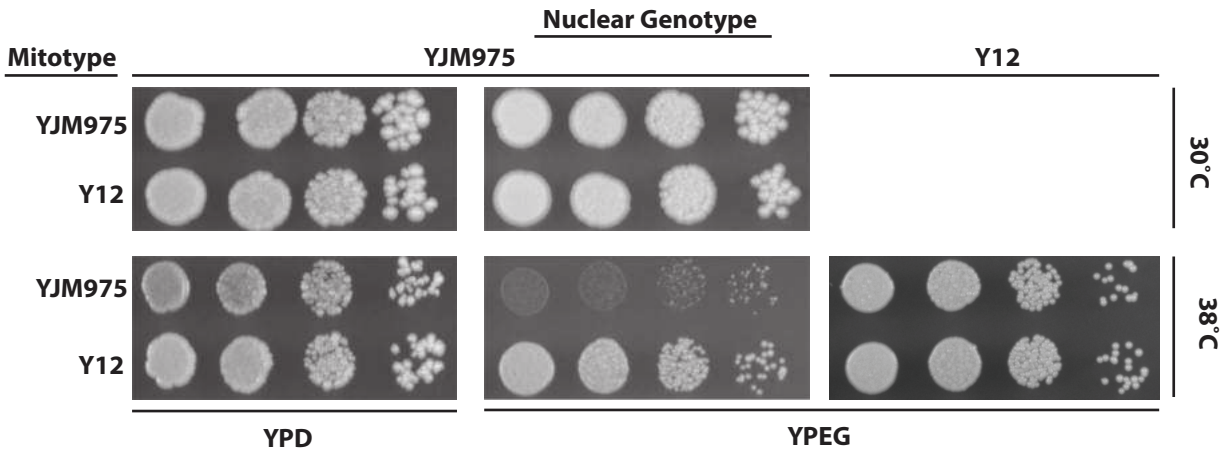
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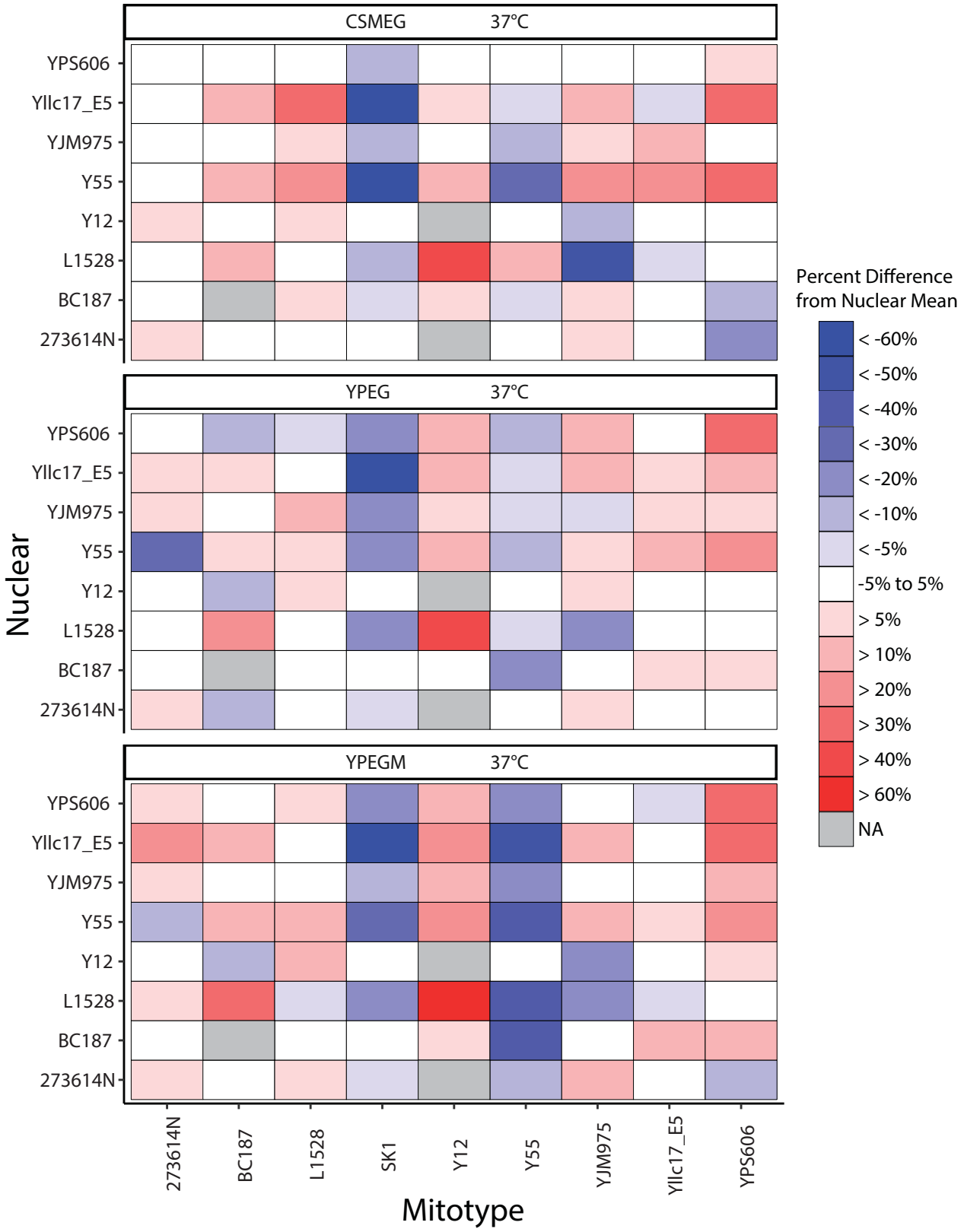
Y12
YJM975
Y12
YJM975
Y12
30°C
38°C
YPD
YPEG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YPEG 37°C
YPEGM 37°C
Nuclear
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YJM975
Y55
Y12
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BC187
273614N
Mitotype
273614N
BC187
L1528
SK1
Y12
Y55
YJM975
Yllc17_E5
YPS606
Percent Difference from Nuclear Mean
< -60%
< -50%
< -40%
< -30%
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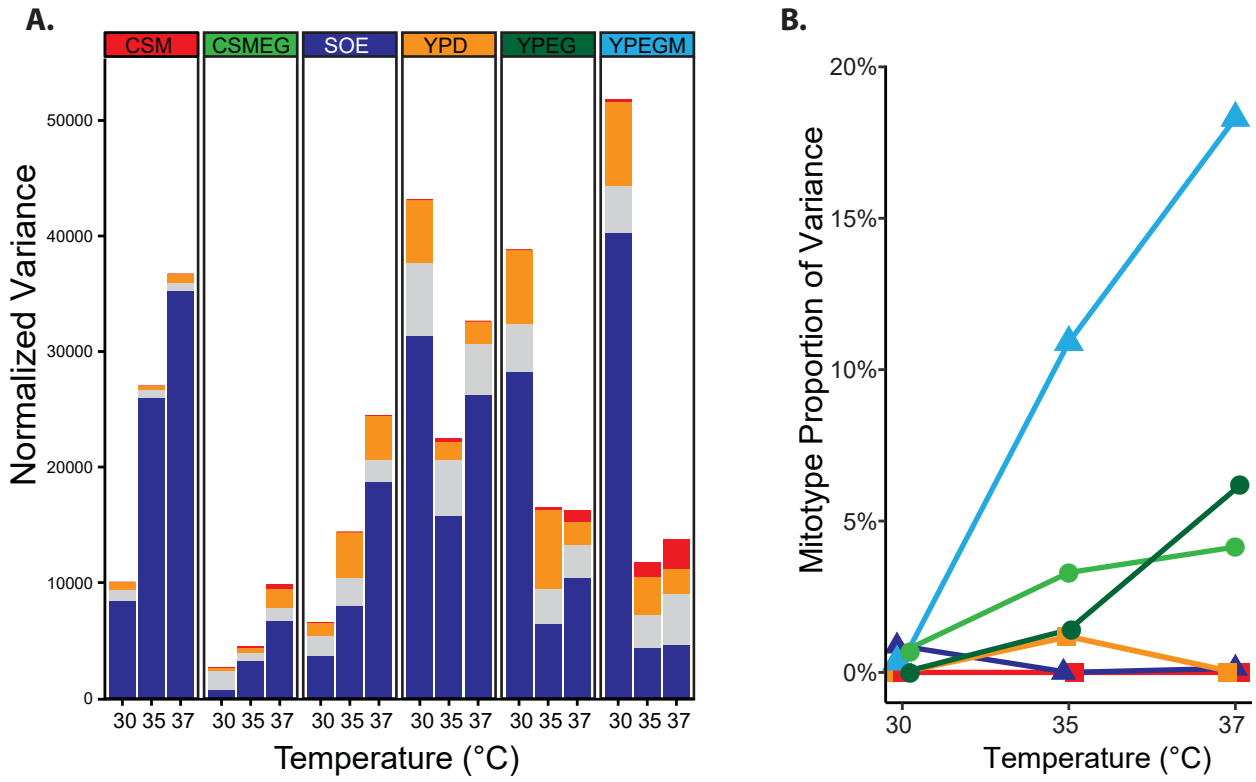
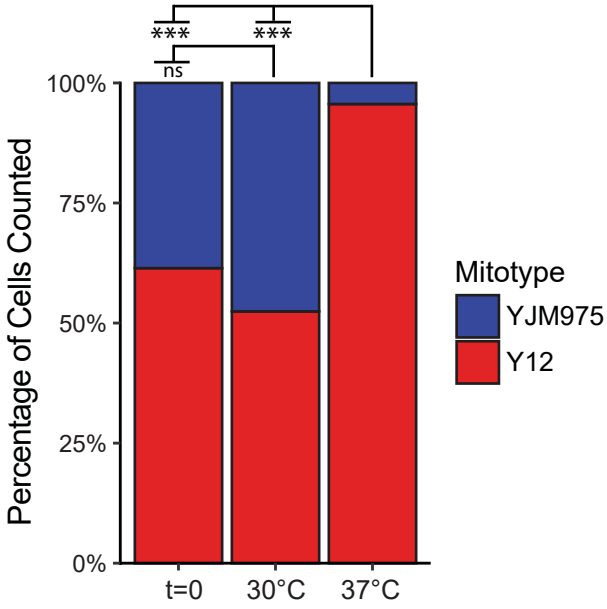
> 10%
> 20%
> 30%
> 40%
> 60%
NA


A.
CSM
CSMEG
SOE
YPD
YPEG
YPEGM
Normalized Variance
0
10000
20000
30000
40000
50000
30 35 37
Temperature (°C)
B.
Mitotype Proportion of Variance
0%
5%
10%
15%
20%
30
35
37
Temperature (°C)


***
***
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Percentage of Cells Counted
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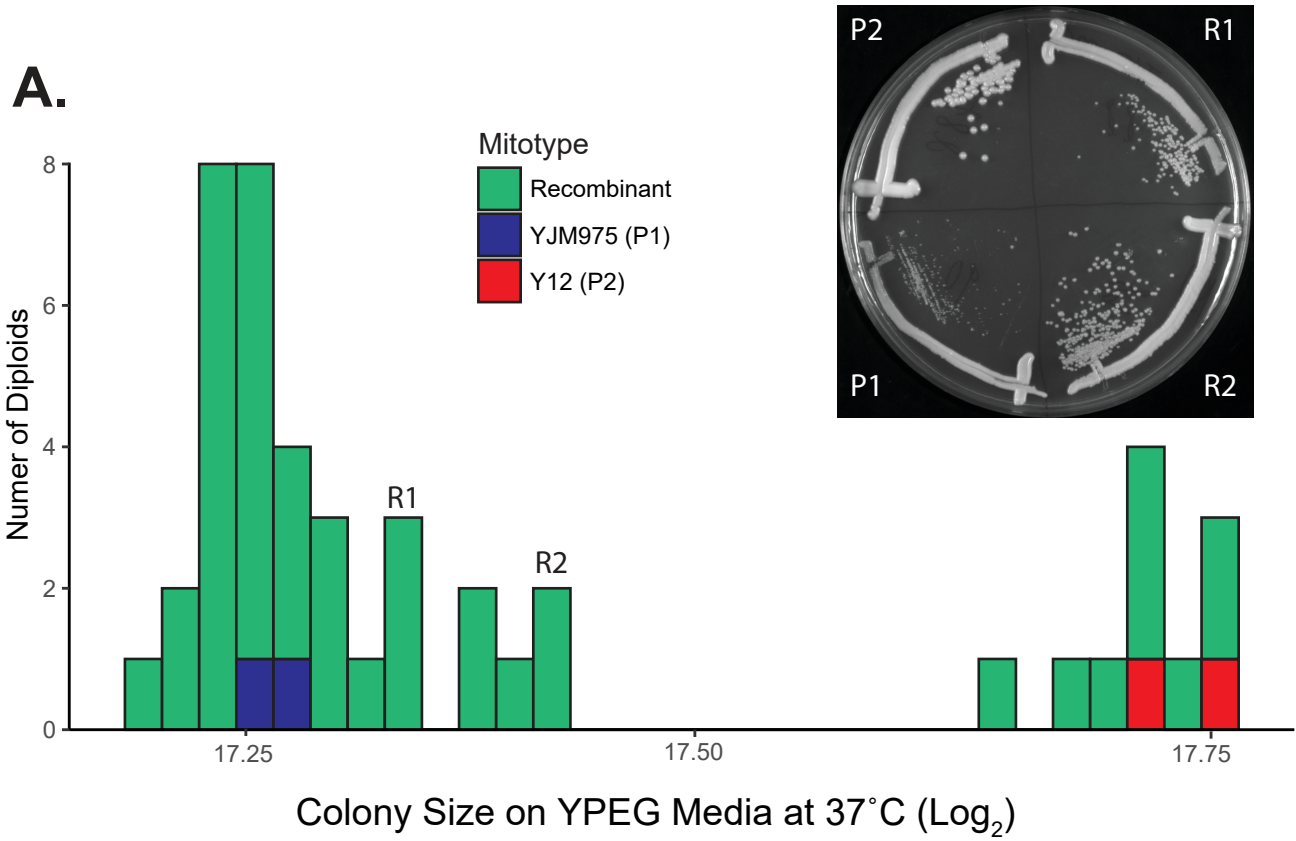
30°C
37°C
Mitotype
YJM975
Y12


A.
Mitotype
Recombinant
YJM975 (P1)
Y12 (P2)
P2
R1
P1
R2
Numer of Diploids
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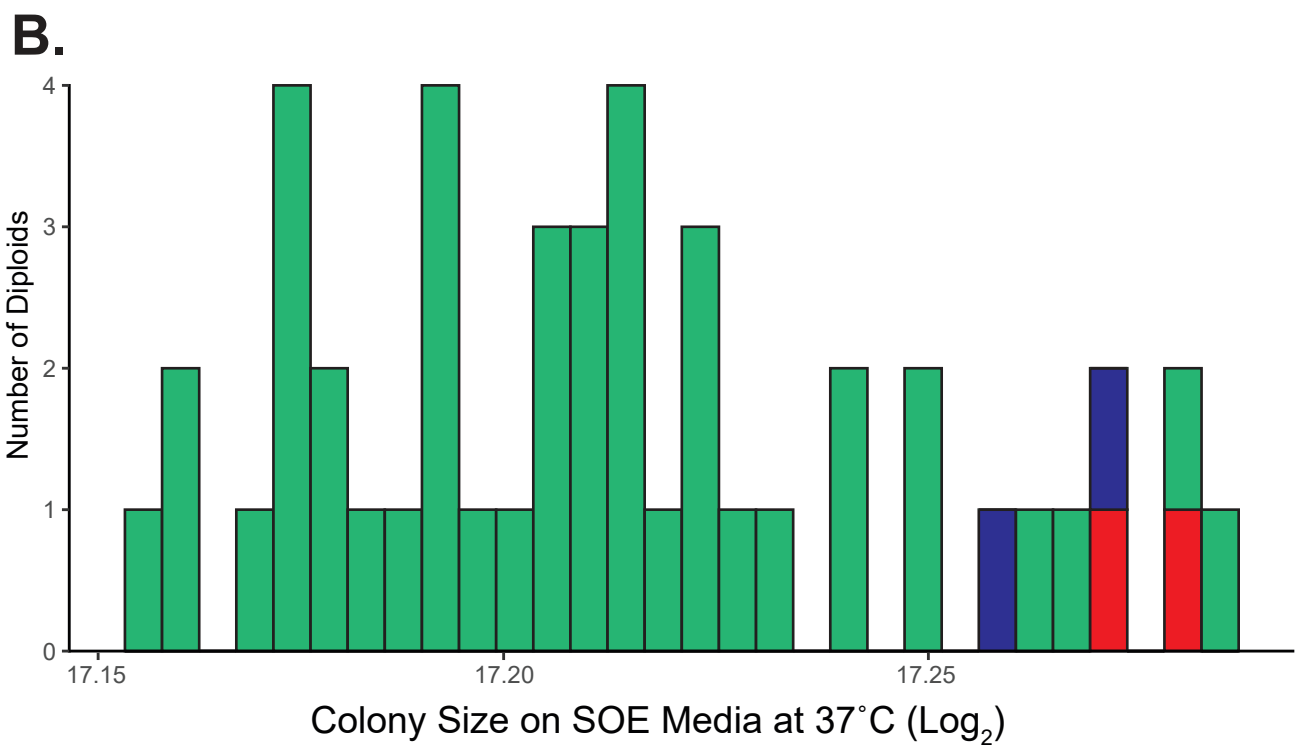
4
6
8
R1
R2
17.25
17.50
17.75
Colony Size on YPEG Media at 37˚C (Log2)


B.
Number of Diploids
0
1
2
3
4
17.15
17.20
17.25
Colony Size on SOE Media at 37˚C (Log2)

TABLES

Table 1: Nuclear, Mitotype, And Mitonuclear Effects On Growth

Condition		Nuclear			Mitotype			Mito-nuclear		
Media	°C	df	χ^2	*p*	df	χ^2	*p*	df	χ^2	*p*
CSMEG	30	5	42.87	***	5	0.27	ns	5	81.69	***
	35	5	100.62	***	5	7.58	* (0.0059)	5	261.39	***
	37	5	69.39	***	5	4.46	* (0.0346)	5	666.08	***
YPEG	30	5	79.04	***	5	0	ns	5	638.80	***
	35	5	23.76	***	5	0.12	ns	5	836.52	***
	37	5	78.33	***	5	10.06	** (0.0015)	5	318.40	***
YPEGM	30	5	89.38	***	5	0.08	ns	5	706.30	***
	35	5	32.09	***	5	7.76	* (0.0053)	5	484.19	***
	37	5	42.47	***	5	24.21	***(8.65×10^{-7})	5	219.67	***
CSM	30	5	127.82	***	5	0	ns	5	363.13	***
	35	5	222.92	***	5	0	ns	5	259.84	***
	37	5	204.09	***	5	0	ns	5	506.38	***
SOE	30	5	62.14	***	5	0.30	ns	5	298.82	***
	35	5	45.21	***	5	0	ns	5	659.51	***
	37	5	80.46	***	5	0.01	ns	5	799.73	***
YPD	30	5	87.81	***	5	0	ns	5	398.46	***
	35	5	110.05	***	5	2.37	ns	5	124.93	***
	37	5	130.99	***	5	0	ns	5	203.22	***

To determine the significance of each term, ANOVAs comparing the full model (nuclear + mitotype + (nuclear x mitotype)) with a model lacking the indicated term were evaluated. Factors were treated as random effects. Significance codes: 'ns' $p > 0.05$; '*' $p < 0.05$; '**' $p < 0.005$; '***' $p < 5 \times 10^{-6}$.

Table 2. Mitotype Effects On Growth

Mitotype	YPEG 37°	CSMEG 37°	YPEGM 37°	Average
Y12	15.5%	11.1%	23.8%	16.8%
BC187	1.0%	8.6%	8.1%	5.9%
YPS606	10.7%	4.2%	14.2%	9.7%
L1528	2.5%	11.2%	5.4%	6.4%
Yllc17_E5	4.3%	3.5%	1.2%	3.0%
SK1	-21.9%	-29.1%	-20.0%	-23.7%
Y55	-8.3%	-6.2%	-31.7%	-15.4%
YJM975	0.8%	-2.4%	0.3%	-0.4%
273614N	-0.6%	3.0%	5.7%	2.7%

The percent increase or decrease in fitness by each mitotype (as shown in Figure 2) were averaged across all nuclear genotypes. Average fitness differences across three stress conditions are provided in the final column.

Table 3. Mito-Mito Epistasis (V_{AA}) Estimates

Media		SOE		YPD		YPEG	
Temperature (°C)		30	37	30	37	30	37
All Crosses	V_{AA}	0.0051	0.0360	0.0011	0.0028	0.0019	0.0042
	CI Lower	0.0046	0.0354	0.0009	0.0026	0.0017	0.0041
	CI Upper	0.0078	0.0400	0.0014	0.0032	0.0022	0.0044
Single Representation	V_{AA}	0.0112	0.0351	0.0010	0.0030	0.0010	0.0037
	CI Lower	0.0109	0.0345	0.0008	0.0027	0.0008	0.0036
	CI Upper	0.0141	0.0394	0.0014	0.0033	0.00144	0.0039
Double Representation	V_{AA}	0.0060	0.0413	0.0034	-0.0030	0.0030	0.0037
	CI Lower	0.0045	0.0395	0.0033	-0.0033	0.0029	0.0035
	CI Upper	0.0091	0.0473	0.0037	-0.0027	0.0033	0.0038

Positive (nonzero) V_{AA} estimates indicate mito-mito epistasis. The overall magnitude of the V_{AA} values do not provide information regarding its relative contribution to genetic variance. All estimates were significantly different from zero based on permutation analysis (see Methods). Positive estimates were confirmed based on upper and lower confidence intervals (CI) that did not overlap zero estimated from bootstrapping analysis. Subsets of the data were analyzed for single representation (YJM975 x Y12 and YPS606 x Y55 crosses) or double representation (each mitotype represented twice, YJM975 x Y12 excluded).