Title: Mitochondrial DNA and temperature tolerance in lager yeasts

Authors: EmilyClare P. Baker[1,2], David Peris[1,3,4], Ryan V. Moriarty[1,3], Xueying C. Li[5,6,7,8], Justin C. Fay[5,6,7,8] Chris Todd Hittinger[1,2,3,*].

Affiliations:

[1]Laboratory of Genetics, Genome Center of Wisconsin, Wisconsin Energy Institute, J. F. Crow Institute for the Study of Evolution, University of Wisconsin-Madison, Madison, WI, USA.

[2]Microbiology Doctoral Training Program, University of Wisconsin-Madison, Madison, WI, USA.

[3]DOE Great Lakes Bioenergy Research Center, University of Wisconsin-Madison, Madison, WI, USA.

[4]Department of Food Biotechnology, Institute of Agrochemistry and Food Technology (IATA), CSIC, Paterna, Valencia, Spain.

[5]Molecular Genetics and Genomics Program, Washington University, St. Louis, MO, USA.

[6]Department of Genetics, Washington University, St. Louis, MO, USA.

[7]Center for Genome Sciences and System Biology, Washington University, St. Louis, MO, USA.

[8]Department of Biology, University of Rochester, Rochester, NY, USA.

*Correspondence to: Chris Todd Hittinger (cthittinger@wisc.edu)

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Abstract: A growing body of research suggests that the mitochondrial genome (mtDNA) is important for temperature adaptation. In the yeast genus *Saccharomyces*, species have diverged in temperature tolerance, driving their use in high or low temperature fermentations. Here we experimentally test the role of mtDNA in temperature tolerance in synthetic and industrial hybrids (*Saccharomyces cerevisiae* x *Saccharomyces eubayanus*, or *Saccharomyces pastorianus*), which cold-brew lager beer. We find that the relative temperature tolerances of hybrids correspond to the parent donating mtDNA, allowing us to modulate lager strain temperature preferences. The strong influence of mitotype on the temperature tolerance of otherwise identical hybrid strains provides support for the mitochondrial climactic adaptation hypothesis in yeasts and demonstrates how mitotype has influenced the world's most commonly fermented beverage.

One Sentence Summary: Mitochondrial genome origin affects the temperature tolerance of synthetic and industrial lager-brewing yeast hybrids.

Main Text:

Introduction

Temperature tolerance is a critical component of how species adapt to their environment. The *mitochondrial climatic adaptation* hypothesis (*1*) posits that functional variation between mitochondrial DNA (mtDNA) sequences (mitotypes) plays an important role in shaping the genetic adaptation of populations to the temperatures of their environments. Clines of mitotypes along temperature gradients and associations between mitotype and climate have been observed for numerous metazoan species, including humans (*1*, *2*). Experiments in invertebrates have demonstrated directly that different mitotypes can alter temperature tolerance (*3*, *4*), and mitotype has been associated with adaption to temperature in natural environments (*1*, *5*).

Recent work has suggested that mitotype can also play a role in temperature tolerance in the model budding yeast genus *Saccharomyces* (*6*–*8*). The eight known *Saccharomyces* species are broadly divided between cryotolerant and thermotolerant species (*9*–*11*). Thermotolerant strains (maximum growth temperature ≥36˚C) form a clade that includes the model organism *Saccharomyces cerevisiae* (*12*), while the rest of the genus is more cryotolerant. Most prior research has focused on thermotolerance or the function of mitochondria under heat stress (~37˚C), on mitotype differences within *S. cerevisiae* (*6*, *8*), or on interspecies differences between *S. cerevisiae* and its moderately thermotolerant sister species, *Saccharomyces paradoxus* (*13*). The genetic basis of cryotolerance in *Saccharomyces* has been difficult to determine using conventional crosses focused on the nuclear genome (*14*–*16*). Nonetheless, given how common mitochondrial adaption to cold conditions is among arctic metazoan species (*17*–*19*), mitotype could conceivably influence cryotolerance in *Saccharomyces*.

In a companion study, Li et al. found that the parent providing mtDNA in hybrids of *S. cerevisiae* and the cryotolerant species *Saccharomyces uvarum* had a large effect on temperature tolerance (*20*). Since *Saccharomyces eubayanus* is the sister species of *S. uvarum* but ~7% genetically divergent, we wondered whether the effect of mitotype would extend to industrial hybrids of *S. cerevisiae* x *S. eubayanus,* sometimes called *Saccharomyces pastorianus* syn. *S. carlsbergensis* (*21*). While *S. cerevisiae* is well known for its role in human-associated fermentations, it is generally not used to produce lager-style beers, which are brewed at colder temperatures than *S. cerevisiae* can tolerate. Instead, the world's most commonly fermented beverage is brewed using cryotolerant *S. cerevisiae* x *S. eubayanus* hybrids (*21*) that inherited their mtDNA from *S. eubayanus* (*22*, *23*). The recent discovery of non-hybrid strains of *S. eubayanus* (*21*) has sparked substantial interest in understanding the genetics of brewing-related traits to understand how lager strains were domesticated historically and to develop novel lager-brewing strains (*24*–*28*).

Temperature tolerance of *S. cerevisiae* and *S. eubayanus*

To establish the temperature tolerance of *S. cerevisiae* and *S. eubayanus,* relative growth scores were calculated at temperatures ranging from 4-37˚C. Two strains of *S. cerevisiae* (a laboratory strain and a strain used to brew ale-style beers) and two strains of *S. eubayanus* (a derivative of the taxonomic type strain from Patagonia (*21*) and a strain isolated from North Carolina that is closely related to the ancestor of lager yeasts (*29*)) were tested (Table S1 is a complete list of strains and genotypes). Strains were spotted onto plates containing either glucose (a fermentable carbon source) or glycerol (a non-fermentable carbon source that requires respiration to assimilate) and grown for several days (high temperatures) or up to two months (low temperatures).

S. eubayanus and *S. cerevisiae* had reciprocal temperature responses. *S. eubayanus* strains grew at all temperatures, except 37˚C, while *S. cerevisiae* strains began to decline in relative growth at 15˚C and were completely unable to grow at 4˚C (Fig. 1A-B, Fig. S1-4). Strain-specific differences were also apparent. The *S. cerevisiae*-laboratory strain (*Sc*) and the *S. eubayanus*-North Carolinian strain (*SeNC*) grew relatively weakly compared to conspecific strains. For *Sc*, relatively poor growth was likely driven by multiple auxotrophies and differences in growth rates between diploid and haploid yeast strains. The reason for *SeNC's* poor performance is unknown.

Fig. 1

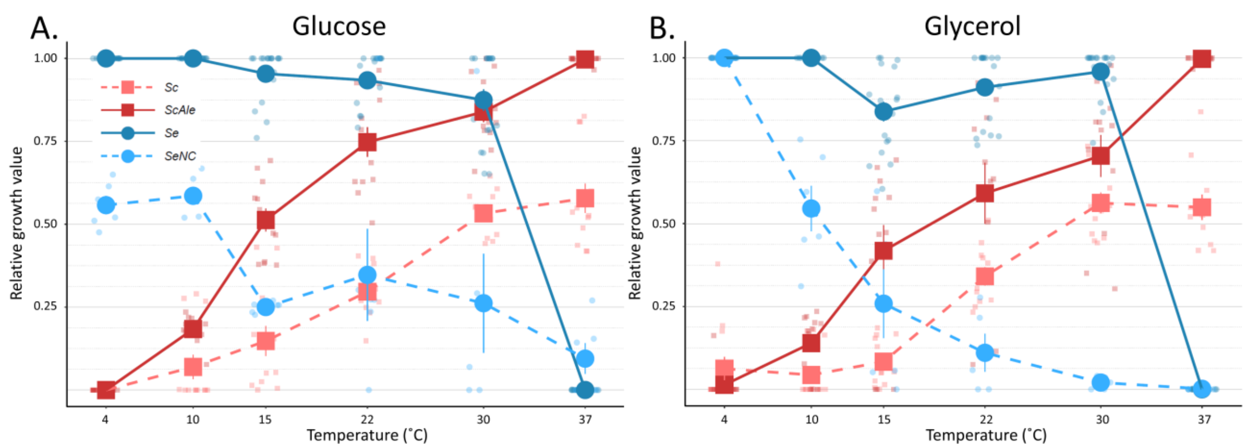

Fig. 1. Relative growth of *S. cerevisiae* and *S. eubayanus* strains. Relative growth scores of *S. cerevisiae* and *S. eubayanus* strains carrying their native mtDNA from 4-37˚C combined from all tests on A) glucose and B) glycerol. Strains are: *S. cerevisiae*-laboratory strain (*Sc*), *S. cerevisiae*-ale strain (*ScAle*), *S. eubayanus*-type strain (*Se*), and *S. eubayanus*-North Carolinian strain (*SeNC*). Error bars represent standard errors. Parents were not tested for significant differences.

Influence of mitotype in synthetic lager hybrids

To directly test the role of mtDNA in temperature tolerance, we constructed a panel of synthetic hybrids of *S. cerevisiae* x *S. eubayanus*, controlling the source of mtDNA using crosses between ρ^0 strains lacking mtDNA and ρ^+ strains retaining their native mtDNA (Fig. 2A). The generation of ρ^0 strains for crosses requires treating parent strains with ethidium bromide, a known mutagen. To control for possible variation in growth as a result of spurious nuclear mutations, we generated ρ^0 strains of each parent in triplicate and used each independently generated ρ^0 strain to make synthetic hybrids, which were all tested. We further verified, by ANOVA analysis of ρ^0 relative growth scores, that variation between ρ^0 replicates across temperatures was minimal (Fig. S5).

Synthetic hybrids tolerated an increased range of temperatures compared to their parents, regardless of mitotype (Fig. 2B-C, Fig. S1-4). These results support a strong role for the nuclear genome in temperature tolerance and indicate some level of codominance between alleles supporting thermotolerance and cryotolerance. Despite generally robust growth across temperatures, synthetic hybrids with different mitotypes displayed clear and consistent differences in relative growth. At higher temperatures, *S. cerevisiae* mitotypes permitted increased growth relative to *S. eubayanus* mitotypes, while the same was true for *S. eubayanus* mitotypes at lower temperatures. Relative growth was typically high for both mitotypes on glucose, but statistically significant differences were detected at 5 of 6 temperatures when data was considered in aggregate (Fig. 2B). On glycerol, the impact of mitotype was exaggerated (Fig. 2C), and the differences in growth were significant at all temperatures. Subtle background-specific effects were also observed, including a growth defect at 37˚C for the *ScAle* x *SeNC* hybrid carrying *ScAle* mtDNA (Fig. S1). Arrhenius plots approximated using the relative growth data displayed the same overall trends (Fig. S6-7).

Fig. 2

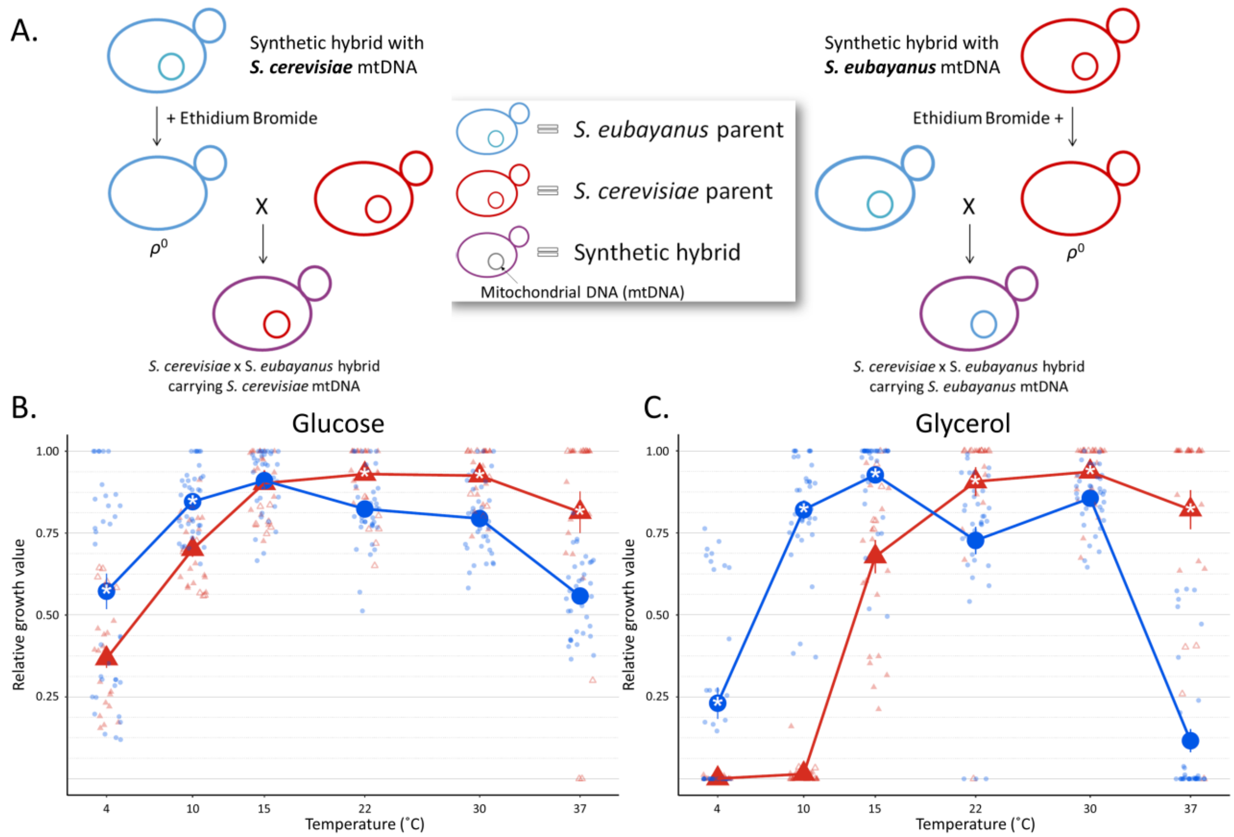

Fig. 2. Mitotype affects temperature tolerance in synthetic lager hybrids. A) Outline of the procedure to control the mitotype of synthetic *S. cerevisiae* x *S. eubayanus* hybrids. Yeast cells represent nuclear genomes, and inner circles represent mtDNA. Red indicates genetic material of *S. cerevisiae* origin, blue of *S. eubayanus* origin, and purple hybrid nuclear material. B) On glucose and C) glycerol, relative growth scores of *S. cerevisiae* x *S. eubayanus* synthetic hybrids with alternate mitotypes from 4-37˚C, combined across all experiments (tiny circles and triangles). Each hybrid of each mitotype is represented in the above graphs. Mean data for all synthetic hybrids carrying *S. eubayanus* mtDNA are represented by large blue circles, and mean data for all synthetic hybrids with *S. cerevisiae* mtDNA by large red triangles. Parent strains: *S. cerevisiae*-laboratory (*Sc*), *S. cerevisiae*-ale (*ScAle*), *S. eubayanus*-type (*Se*), and *S. eubayanus*-North Carolinian (*SeNC*). Synthetic hybrids: *Sc* x *Se*, *ScAle* x *Se*, *Sc* x *SeNC*, and *SeAle* x *SeNC*.

ScAle x *SeNC* and *Sc* x *SeNC* hybrids carrying *S. cerevisiae* mtDNA, for which only single biological replicates of the crosses were available (see below), are represented by open tiny triangles. Differences in relative growth between hybrids of different mitotypes with p-values of <0.05 were considered statistically significant and are indicated by an asterisk.

Because we encountered challenges forming hybrids with a *S. cerevisiae* x *SeNC* nuclear background and an *S. cerevisiae* mitotype, hybrids of *Sc* x *SeNC* with *Sc* mtDNA and *ScAle* x *SeNC* with *ScAle* mtDNA were both represented by single biological replicates. The behavior of these strains suggests that incompatibilities related to mitochondrial function may exist in these hybrids. To confirm that our results were not being driven by the unusual behavior of these hybrids, we also excluded these data and again compared the growth of synthetic hybrids with *S. cerevisiae* and *S. eubayanus* mtDNA (Fig. S8). Analyses on this restricted dataset had slightly less power, but they still suggested that the *S. eubayanus* mtDNA conferred vigorous growth at colder temperatures, while the *S. cerevisiae* mtDNA conferred vigorous growth at warmer temperatures.

The challenges obtaining *S. cerevisiae* x *SeNC* hybrids with *S. cerevisiae* mtDNA suggest that strain-specific dominant cytonuclear incompatibilities may exist between *S. cerevisiae* and *S. eubayanus*. Recessive cytonuclear incompatibilities are common both within and between *Saccharomyces* species (*7*, *8*), but dominant cytonuclear incompatibilities affecting hybrids could explain why *Saccharomyces* interspecies hybrids tend to lose more nuclear genetic material from the parental genome that did not contribute mtDNA (*30*, *31*). Another group recently described a separate strain-specific incompatibility between *S. cerevisiae* and *S. eubayanus* (*28*), and the companion manuscript of Li et al. also describes potential dominant interactions between hybrid genomes and mtDNA in crosses between *S. cerevisiae* and *S. uvarum* (*20*). More research is needed to better characterize this class of cytonuclear incompatibilities.

Influence of mitotype in industrial lager cybrids

To test if mtDNA still plays a role in temperature tolerance in industrial lager-brewing hybrids that have been evolving to lagering conditions for many generations, we replaced the native lager mtDNA of *S. eubayanus* origin (*23*) with *S. cerevisiae* mtDNA from *Sc* and *ScAle*, creating lager cybrids (Fig. 3A). Consistent with results for synthetic hybrids, lager cybrids carrying *S. cerevisiae* mtDNA had greater growth at higher temperatures and decreased growth at colder temperatures, especially on glycerol (Fig. 3B-C, Fig. S9). On glucose, strain-specific differences between lager cybrids were particularly apparent. At 30˚C and below, lager cybrids carrying *ScAle* mtDNA grew significantly less than the parental lager strain with its native mtDNA (from the lager *S. eubayanus* parent) (Fig. 3B, Fig. S9A, B), while there was no difference in growth between the parental lager strain and cybrids carrying *Sc* mtDNA, except at temperature extremes (4˚C and 33.5˚C) (Fig. 3B, Fig. S9A, B). On glycerol, both lager cybrids grew significantly less than the industrial strain at 15˚C and below, while they grew significantly more at 22˚C and 30˚C (Fig. 3C, Fig. S9A, C), displaying a shift from lager-brewing toward ale-brewing temperatures. Approximate Arrhenius growth plots revealed similar trends (Fig. S10). These results show that the strong effect of mtDNA on temperature tolerance seen in synthetic hybrids extends to industrial lager strains under at least some conditions.

Fig. 3

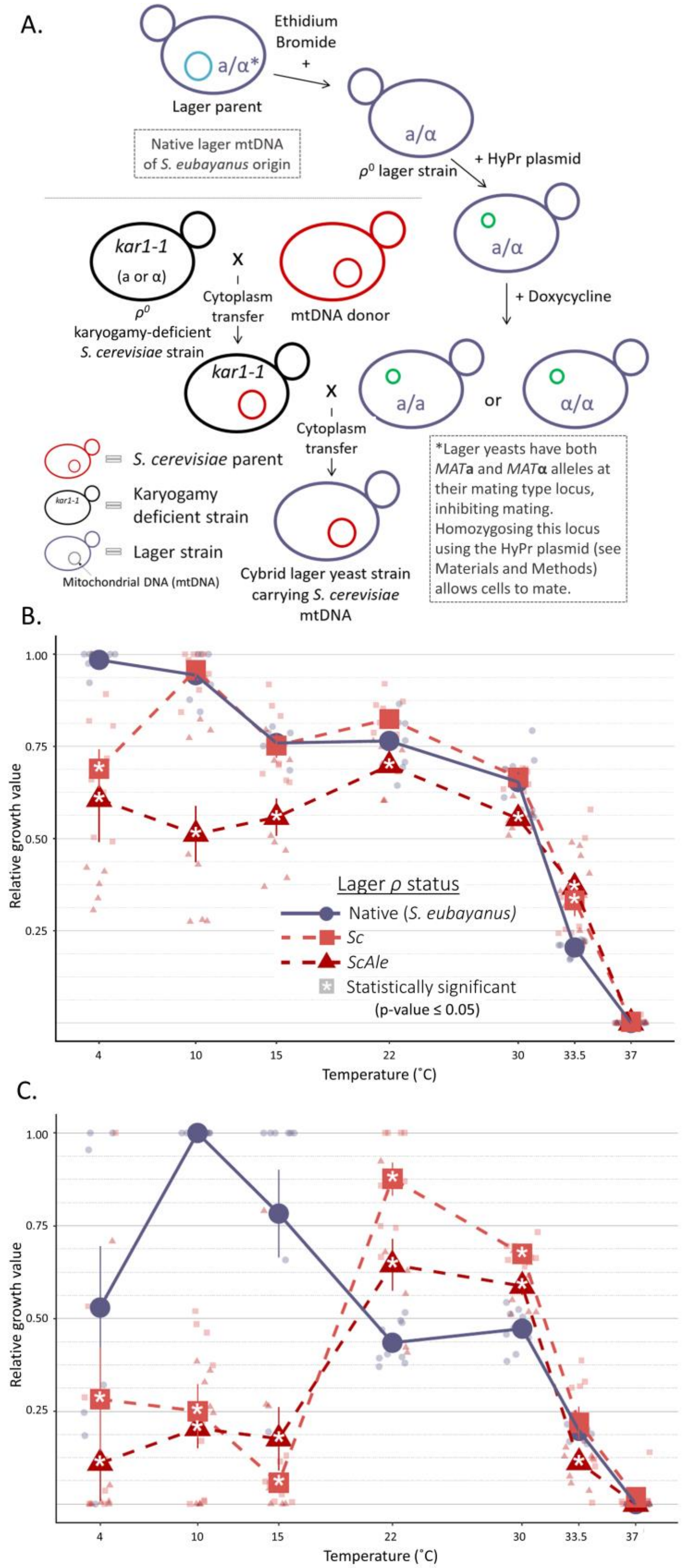
A.
Ethidium Bromide +
a/α*
Lager parent
Native lager mtDNA of S. eubayanus origin
a/α
ρ0 lager strain
+ HyPr plasmid
a/α
+ Doxycycline
kar1-1
(a or α)
x
Cytoplasm transfer
mtDNA donor
ρ0 karyogamy-deficient S. cerevisiae strain
kar1-1
x
Cytoplasm transfer
a/a
or
α/α
*Lager yeasts have both MATa and MATα alleles at their mating type locus, inhibiting mating. Homozygosing this locus using the HyPr plasmid (see Materials and Methods) allows cells to mate.
S. cerevisiae parent
Karyogamy deficient strain
Lager strain
Mitochondrial DNA (mtDNA)
Cybrid lager yeast strain carrying S. cerevisiae mtDNA
B.
Relative growth value
Lager ρ status
Native (S. eubayanus)
Sc
ScAle
Statistically significant
(p-value ≤ 0.05)
Temperature (˚C)
C.
Relative growth value
Temperature (˚C)

Fig. 3. *S. cerevisiae* mtDNA increases the thermotolerance and decreases the cryotolerance of an industrial lager strain. A) Outline of crosses and strain engineering to produce lager cybrids. Yeast cells represent the nuclear genome, large inner circles represent mtDNA, and small green inner circles represent the HyPr plasmid (*32*). Lower case "**a**" and "**α**" indicate mating types. Karyogamy-deficient (*kar1-1*) strains can be of either mating type and are mated to the opposite mating type. Black indicates genetic material from the *S. cerevisiae* karyogamy-deficient strain; red, genetic material from a *S. cerevisiae* parent; blue, genetic material of *S. eubayanus* origin; and purple, a hybrid (i.e. lager) nuclear genome. B) On glucose and C) glycerol, growth of a lager strain with native mtDNA (inherited from *S. eubayanus* lager parent) and lager cybrids with *S. cerevisiae* mtDNA. Error bars represent standard errors, and asterisks indicate statistically significant differences in growth between the cybrid and lager with native mtDNA (p-value <0.05).

Origin of the mitotype of industrial lager yeasts

Compared with ale strains or new hybrids carrying *S. cerevisiae* mtDNA, the increased cold tolerance conferred to new interspecies hybrids carrying *S. eubayanus* mtDNA would have provided an immediate selective advantage at the lower temperatures at which lagers are brewed. It is likely that additional changes occurred that affected temperature tolerance during adaption to lagering conditions, much of which are likely attributable to changes within the nuclear genome. Even so, our data suggest that mitotype had a disproportionate impact on temperature tolerance, considering the limited number of genes encoded by mtDNA. Along with previous research suggesting hybrid lager yeasts acquired most of their aggressive fermentation traits from *S. cerevisiae* (*25*, *27*, *28*), our results suggest they acquired their cold tolerance from *S. eubayanus* in large part by retaining *S. eubayanus* mtDNA. Our results and methods provide a roadmap for constructing designer lager strains where temperature tolerance can be controlled for the first time (*24*–*28*). Shifting the temperature preference of synthetic or industrial lager strains to warmer fermentation temperatures could substantially reduce the cost of lager brewing by reducing production time and infrastructure requirements. The strain-specific differences observed further suggest that the *S. cerevisiae* parent, the *S. eubayanus* parent, and cytonuclear incompatibilities (*34*), should all be considered during strain construction. Along with the companion study of Li et al. (*20*), the identification of a role for mtDNA in temperature tolerance of these yeasts extends support for the *mitochondrial climatic adaptation* hypothesis (*1*) to fungi and suggests that the outsized role of mtDNA in controlling temperature tolerance may be general to eukaryotes.

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Supplementary Materials:

Materials and Methods

Figures S1-S10

Table S1

References (*35*-*55*)