Evolutionary genetics and dissection of brewing related traits in *Saccharomyces eubayanus* and its hybrids with *Saccharomyces cerevisiae*.

By

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ABSTRACT

Successful colonization of new environments requires that organisms evolve to cope with conditions they could not previously tolerate and to exploit resources they could not previously utilize. These changes in selective pressures leave an imprint on organisms from the level of the gene to the whole genome. Over time, some strains of *Saccharomyces* yeasts have successfully evolved to thrive in industrial fermentations. Here, they regularly contend with conditions and experience population sizes they would have rarely encountered in wild settings. Understanding the genetic basis for their success can provide greater insight into the molecular basis of adaptation across the tree of life. Because many of the strains of *Saccharomyces* found in fermentative environments are hybrids between distantly related species this system also provides the opportunity to explore not only how interactions between an organism and its external environment have shaped its genome, but also how interactions between two diverged genomes when brought together in single nucleus shape each other. The work reported in this thesis explores evolutionary genetics in the yeast *Saccharomyces eubayanus* and its hybrids with *Saccharomyces cerevisiae*. Taking advantage of the deep foundation of tools and knowledge from *S. cerevisiae* research, evolution and adaptation of *S. eubayanus* and its hybrids is explored at scales ranging from the whole genome to a single genetic locus and across a range of conditions. This work provides a deeper understanding of the mechanisms of adaptation and demonstrates the potential of *S. eubayanus* and its hybrids as a system for future genetic studies.

Chapter 1

Introduction

"There is no such thing as applied sciences…only applications of science"

- Louis Pasteur (Baxter 2001)

Overview of the *Saccharomyces* genus

The *Saccharomyces* genus is a genetically diverse clade of yeasts encompassing a wide range of genetic and phenotypic diversity. The genus includes well-known and thoroughly studied species, such as *Saccharomyces cerevisiae,* which has been a leading model organism for decades (Duina et al. 2014) and was the first eukaryote to have its entire genome sequenced (Goffeau et al. 1996), as well as newly described species such *Saccharomyces jurei,* which was first described in 2017 (Naseeb et al. 2017) and whose genome sequence, as of this writing, is still forthcoming. To date, eight phylogenetically and biologically distinct species have been characterized (Fig. 1), three of which were only identified within the last decade (Wang and Bai 2008; Libkind et al. 2011; Scannell et al. 2011; Naseeb et al. 2017). For most *Saccharomyces* species, genetically, geographically and environmentally specific populations have also been described (Kuehne et al. 2007; Liti et al. 2009; Schacherer et al. 2009; Almeida et al. 2014; Leducq et al. 2014; Peris et al. 2014; Strope et al. 2015; Barbosa et al. 2016; Gallone et al. 2016; Gonçalves et al. 2016; Peris & Langdon et al. 2016; Peter et al. 2018). Species divergences range from between ~7% nucleotide sequence divergence between sister species *S. uvarum* and *S. eubayanus* (Libkind et al. 2011) to ~25% nucleotide sequence divergence between *S. cerevisiae* and the early branching members of the *Saccharomyces bayanus* species complex (Kellis et al. 2003; Hittinger 2013). For perspective, these scales of nucleotide sequence divergence are similar to the divergence between humans and Old World monkeys and between humans and birds, respectively (Dujon 2006; Gibbs et al. 2007) (Fig. 1).

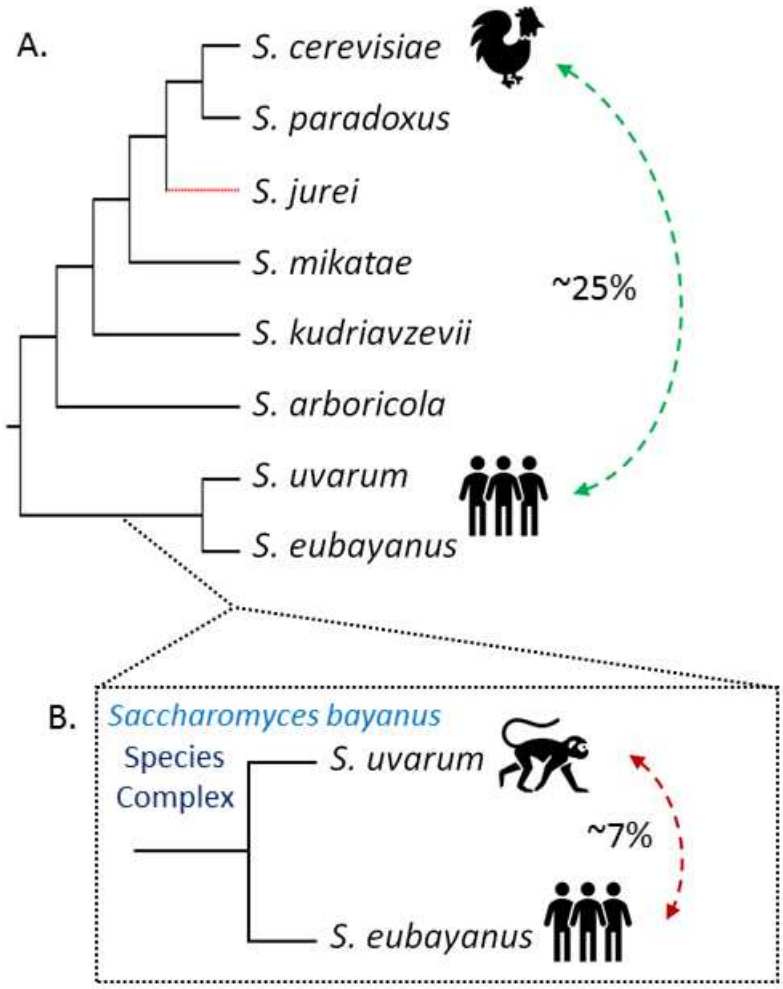

Figure 1. Phylogeny of the *Saccharomyces* genus. Percentages show approximate nucleotide divergences between the groups indicated by arrows and images represent vertebrate lineages with roughly equivalent divergences. A) Tree consisting of all known species of the *Saccharomyces* genus (adapted from (Naseeb et al. 2017)). Red line along the *S. jurei* branch indicates putative species placement. Nucleotide divergence between *S. cerevisiae* and the *Saccharomyces bayanus* species complex is shown and is roughly equivalent to human-bird divergence. B) Enlargement of the *S. bayanus* species complex. Nucleotide divergence between *S. uvarum* and *S. eubayanus* is shown and is close to the amount of divergence between humans and Old World monkeys.

Not only is *S. cerevisiae* a proven model system in a wide range of fields, the entire genus of *Saccharomyces* yeasts is an emerging model system (Hittinger 2013). The main reason for this is the decades of intensive genetic and molecular work carried out in *S. cerevisiae*. Many of the technical tools developed for *S. cerevisiae* are directly portable to other members of the genus, while the extensive molecular and genetic studies carried out in *S. cerevisiae* provide a foundation for comparative studies with other members of the genus. This rich technical foundation and the wide range of genetic diversity within and between species of the genus have made the *Saccharomyces* genus a compelling system in which to study a variety of topics including population genetics; molecular incompatibilities, especially cytonuclear; and for comparative studies in systems biology (Hittinger 2013).

***Saccharomyces* hybrids**

One of the characteristics of *Saccharomyces* yeasts that makes them such a facile system for so many comparative, molecular, and genetic studies is the ease with which even extremely divergent lineages form hybrids (Morales and Dujon 2012; Hittinger 2013). Despite the extensive amount of genetic divergence between different members of the genus *Saccharomyces*, all species tested to date can readily form F_1 hybrids (Fig. 2), though some strain and condition-specific incompatibilities do exist (Lee et al. 2008; Chou and Leu 2010; Mertens et al. 2015).

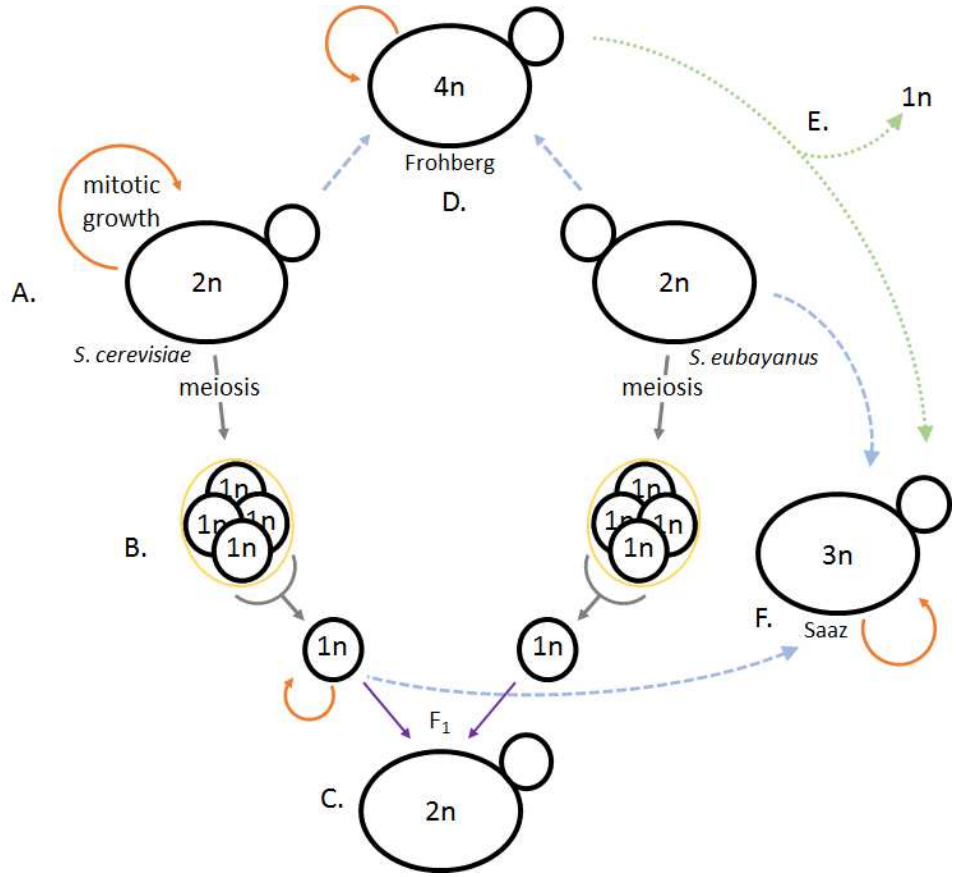

Figure 2. Reproduction and hybridization of *Saccharomyces* yeasts as illustrated by *S. cerevisiae* and *S. eubayanus.* Ploidy (n) is labeled within each cell. Orange arrows indicate that mitotic growth (asexual reproduction) can occur at the ploidy level presented. A) Most replication occurs mitotically in diploid (2n) pure species (Hittinger 2013). B) Occasional meioses result in the production of 1n ascospores (Hittinger 2013). C) Fusion between ascospores results in the production of a diploid F_1 hybrid. Most within species matings occur in this manner, and these are the most commonly performed mattings in laboratory settings D-F) Simplified schematic of rare alternative mating events that could have produced lager yeasts of the Frohberg and Saaz lineages. D) Fusion of diploid parents to produce a tetraploid hybrid strain giving rise to the Frohberg-type lager yeasts. E) Substantial loss of chromosomes from the hybrid tetraploid giving rise to the Saaz lineage. F) Alternative origin of the Saaz-type yeasts by the mating of a haploid *S. cerevisiae* spore with a diploid *S. eubayanus*.

By studying hybrids, both natural and synthetic (that is hybrids created using laboratory techniques), we can begin to elucidate the functional consequences of both neutral and positively selected changes that have shaped the parent genomes (Lee et al. 2008; Tirosh et al. 2009; Chou et al. 2010; Chou and Leu 2010; Tirosh and Barkai 2011; Li and Fay 2017). In addition, how two distinct genetic backgrounds have or can evolve in a common nucleus can provide insights into the molecular mechanisms underpinning the adaptation of hybrid genomes both to their external and genetic environments (Dunn and Sherlock 2008; Belloch et al. 2009; Peris et al. 2012; Dunn et al. 2013; Peris, Moriarty, et al. 2017).

While rare or unstable in nature, hybridization seems to be fairly common in industrial settings, and most hybrid *Saccharomyces* have been isolated from human-related fermentations (Morales and Dujon 2012; Peris et al. 2014; Barbosa et al. 2016; Peris, Arias, et al. 2017). Hybridization brings together diverse genetic strategies for dealing with unprecedented or uniquely intense stressors, which is probably why the stressful conditions of brewing environments often favor hybrid genomes over pure species (Peris, Moriarty, et al. 2017; Lopandic 2018). Combining genetic material from multiple species can be so favorable in human-related fermentations that strains with genetic contributions from up to four different species have been isolated from brewing environments (Almeida et al. 2014).

Of the hybrid *Saccharomyces* strains that are used in brewing, the most economically important are the *S. cerevisiae* x *S. eubayanus* hybrids that brew lager-style beers. With the discovery of pure species of *S. eubayanus* (Libkind et al. 2011), there is considerable interest in creating new *S. cerevisiae* x *S. eubayanus* hybrids for use in industrial brewing and in developing *S. eubayanus* as a brewing strain itself (Hebly et al. 2015; Krogerus et al. 2015; Mertens et al. 2015; Krogerus et al. 2016; Krogerus, Magalhães, et al. 2017; Krogerus, Seppänen-Laakso, et al. 2017; Hittinger et al. 2018; Nikulin et al. 2018). The study and development of brewing-related strains provides the opportunity to gain insights not simply into

what makes a good beer, but also address questions of basic scientific interest. Among these questions are: how do the genomes of hybrid organisms change over time, what are the molecular and genetic underpinning of adaptation of organisms to their environments, and what evolutionary paths are open to organisms to evolve novel functions? The work of this thesis aims to address these questions through the study of industrial lager hybrids, synthetic *S. cerevisiae* x *S. eubayanus* hybrids, and pure strains of *S. eubayanus*.

Lager brewing

While many different styles of beer exist, lager style beers constitute over 90% of the world beer market (Riese and Eßlinger 2009). There are several characteristics that distinguish lagers from other styles of beers. One is the "crisp and clean" flavor profile associated with the final product. The behavior of lager-brewing yeasts during the fermentation process itself is also important for differentiating lagers from other styles. Unlike the other major style of beer, ale, the yeasts that brew lagers have a tendency to drop to the bottom of fermentation vats rather than float at the top; this is why lagers are sometimes referred to as bottom-fermenting yeasts (Tenge 2009). What truly makes lager brewing unique though, is not bottom fermentation, but long fermentation times, as long as several months, at low temperatures, usually between 7 and 15˚C (Boulton and Quain 2001; Meussdoerffer 2009; Tenge 2009; Boulton 2013). In contrast, ales are brewed at relatively high temperatures, between 18 and 25˚C and fermentation can be accomplished over the course of several days (Boulton and Quain 2001).

Lager brewing originated around the 16th century in the region that now constitutes Bavaria and parts of the Czech Republic (Meussdoerffer 2009). The origin of lager brewing and thus lager-brewing yeasts is intimately associated with the famous Bavarian Reinheitsgebot, more commonly known in English as the German Beer Purity Law. While bottom fermenting beers are attested in the region prior to the

institution of the Reinheitsgebot, it was the particular stipulations of the law that ensured that lager brewing would come to predominate in the region. Specifically, it was a clause that prohibited brewing during the summer months that made slow fermentation at cool temperatures essential. This condition was probably added for both economic (controlling the price/supply of grain for bread) and quality reasons; beers brewed during hot summer months were more likely to be contaminated by other less desirable microbes (Unger 2004; Meussdoerffer 2009). Because new beer could not be made for several months of the year, brews that could be stored over the summer (the word lager in German relates to storage) were needed to ensure the continual supply of this staple commodity. Thus, the innovation of having beer started during the cold winter and early spring months and allowed to ferment very slowly in cool cellars or caves through the summer, ensuring that the beer would remain fresh throughout that time. Such conditions favored a different type of yeast from those used to brew ales: a cold-tolerant strain that consumed the sugars in wort relatively slowly. It was this style of beer that, with the advent of modern cooling technology, would come to dominate the world beer market.

The role of yeasts in the fermentation of beer would not come to light until the 19th century. Louis Pasteur and Robert Koch, despite their personal enmity (Baxter 2001), were jointly responsible for shedding a scientific light on the production of beer. Through insights into germ theory and active investigation of the brewing process, Pasteur was the first to establish the central role of yeasts in the fermentation of wort to beer (Baxter 2001; Meussdoerffer 2009), while methods developed by Koch were essential for the isolation and culturing of pure strains of yeast (Tenge 2009).

Influenced by the work of Pasteur and Koch, Emil Christian Hansen at the Carlsberg laboratories in Copenhagen began isolating pure cultures of brewing yeast for use by the Carlsberg brewery in the late 19th and early 20th century. It is not clear by whom, but eventually, two pure strains of lager-brewing

yeasts were isolated and disseminated throughout the lager-brewing industry. Some sources indicate that the two pure lager strains were isolated by the microbiologist Paul Lindner, famous for the discovery of *Schizosaccharomyces pombe*, but more likely it was Emil Christian Hansen and the Carlsberg brewery that were responsible for the isolation and spread of these strains (Martini and Kurtzman 1985; Martini and Martini 1987; Barnett and Lichtenthaler 2001; Tenge 2009; Gibson et al. 2013; Wendland 2014; Gibson and Liti 2015; Monerawela and Bond 2018). Regardless who was responsible, all modern lager-brewing yeasts apparently originate from just these two original pure cultures (Gibson et al. 2013).

In 1930 Paul Lindner described a "strong fermenting" yeast isolate obtained from a brewery in Frohberg Bavaria and a "weak fermenting" yeast from a brewery in Saaz, located in what is now the Czech Republic (Tenge 2009). Based on Lindner's descriptions, lager yeast isolated from breweries were classified as either Saaz-type or Frohberg-type based on their fermentative characteristics. Modern genomic research would later establish that these two designations correspond to the two genetically distinct lineages that gave rise to modern brewing yeasts (Dunn and Sherlock 2008).

A note on lager-brewing yeast nomenclature

After the spread of the original two pure cultures of lager yeast throughout the brewing industry, later researchers, unaware of the common origin of lager-brewing yeasts, isolated yeast from fermentations and characterized them as separate species. Because different strains and species of yeast are largely indistinguishable by the human eye and the physiological characteristics that were historically used to characterize yeasts can be polymorphic within a single species (Martini and Kurtzman 1985), species distinctions within *Saccharomyces* are difficult in the absence of some form of genomic data. As a result, prior to the development modern genomic techniques, a myriad of "species" of *Saccharomyces* existed, which are now classified as synonyms of the same species or as interspecies hybrids. Consequentially,

lager yeast hybrids are referred to by a number of different species designations, the most common being *Saccharomyces pastorianus* and *Saccharomyces carlsbergensis,* the original designations for the lager yeast strains isolated by Emily Christian Hansen (Martini and Martini 1987), and less frequently *Saccharomyces monacensis*, *Saccharomyces uvarum,* and *Saccharomyces bayanus,* despite all being *S. cerevisiae* x *S. eubayanus* hybrids belonging to only one of two lineages (Dunn and Sherlock 2008; Nguyen and Boekhout 2017). Also note that the designation *S. uvarum* is now used to refer to pure strains of the sister species of *S. eubayanus* (Fig. 1), while *S. bayanus* is a now invalid species designation that used to refer to either pure strains of *S. uvarum* or a complex set of *S. uvarum, S. eubayanus,* and *S. cerevisiae* hybrids (Nguyen and Gaillardin 2005; Nguyen and Boekhout 2017).

Despite being hybrids, lager yeasts are often referred to by the species designation *S. pastorianus*. To distinguish between strains belonging to the two different lineages of lager yeast, three nomenclature systems are currently in widespread use. The first uses Saaz and Frohberg to refer to the triploid and tetraploid lager lineages, respectively; the second refers to these same groups as Group 1 (I) and Group 2 (II); and the third system utilizes the species designations assigned to the type strains of the Saaz and Frohberg lineages, *Saccharomyces carlsbergensis* and *Saccharomyces pastorianus,* respectively (Dunn and Sherlock 2008; Wendland 2014; Gibson and Liti 2015; Magalhães et al. 2016). Nguyen and Boekhout have proposed a standardized method for naming all *Saccharomyces* hybrids, which combines the names of the parent species along with information on relative ploidy or percent genetic contribution from each parent (Nguyen and Boekhout 2017). Under this system the Saaz/Group I/*S. carlsbergensis* lager yeasts are *S. cerevisiae* (1n) x *S. eubayanus* (2n), while Frohberg/Group II/*S. pastorianus* lager yeasts are *S. cerevisiae* (2n) x *S. eubayanus* (2n). I consider the naming system proposed by Nguyen-Boekhout to be preferred, as names constructed using this system are the most intrinsically informative. However, the length of hybrid names generated by this approach makes consistent use of this system

throughout a manuscript somewhat impractical. In addition, the hybrids used in the lager-brewing industry are distinct in a number of ways from synthetic hybrids created in the lab, which are also discussed in this manuscript. This includes their history, genome content and architecture, and extensive evolution and adaption to industrial lager-brewing conditions. For this reason, I refer to the hybrids that have historically been used in the lager brewing industry as lager, or lager-brewing yeasts, unless otherwise noted, and new hybrids created in the lab as *S. cerevisiae* x *S. eubayanus* hybrids. When distinguishing between different lineages of lager brewing yeasts, Saaz and Frohberg are used to refer to the triploid and tetraploid lineages, respectively.

Genetic characterization of lager-brewing yeasts

Because of the difficulty distinguishing species in *Saccharomyces,* it was not until the 1980's, as new molecular and genetic tools became available, that the hybrid nature of lager-brewing yeasts became apparent. Chromosome transfer experiments (Nilsson-Tillgren et al. 1981) first showed that radical chromosomal differences existed between *S. cerevisiae* and the lager-brewing yeast *S. carlsbergensis.* Shortly thereafter, DNA hybridization assays by (Martini and Kurtzman 1985) indicated that, not only were *S. carlsbergensis* and *S. pastorianus* closely related to each other, but their sequence similarity to *S. cerevisiae* and *S. bayanus* suggested that they shared genetic material with both. Further work confirmed the coexistence of genetic material from two separate species within lager-brewing yeasts (Hansen et al. 1994; Hansen and Kielland-Brandt 1994; Tamai et al. 1998).

Differences in genome composition between different strains of lager yeasts were noted as early as 2001 (Casaregola et al. 2001). Shortly thereafter, LTR-retrotransposon data suggested that lager yeasts were comprised of two genetically distinct lineages (Liti et al. 2005). The existence of two distinct lineages of lager yeasts was confirmed by microarray analysis using as probes DNA from *S. cerevisiae*

and *S. uvarum* (a close relative of the as-yet-unknown 2nd parent of lager-brewing yeasts) (Dunn and Sherlock 2008). This study showed that, not only do the two lineages differ in the number and distribution of LTR-retrotransposons, but that they also contain large differences in their genetic architecture. The first group of lager yeasts are approximately triploid and contain roughly a haploid *S. cerevisiae* genome and a diploid genome of another species closely related to *S. uvarum*. The second group is approximately tetraploid and has maintained almost full diploid genomes of both parents. This study was also the first to note that the two lager lineages corresponded with previous fermentation-performance based distinctions made between Saaz (triploid group) and Frohberg (tetraploid group) type lager yeasts.

Variation between and within the lager-brewing yeast lineages

Two characteristics stand out as being particularly important in making the hybrids used in fermenting lager style beers especially well-suited to this use; the ability to utilize the maltotriose present in wort and the capacity to ferment even at low temperatures. However, the degree and even the presence of these characteristics has been found to be polymorphic both between and within the two lager yeast lineages. As noted above, Paul Lindner in the early 20th century was the first to classify Frohberg type strains as “strong fermenters”, meaning (relatively) rapid and complete utilization of brewing sugars, while Saaz type strains were “weak fermenters”, whose fermentations proceeded more slowly and might contain residual brewing sugars, particularly the sugar maltotriose (Tenge 2009; Gibson et al. 2013).

Modern studies have further refined our understanding of the physiological differences between the Saaz and Frohberg strains. These studies confirmed that, while Saaz strains grow faster at low temperatures (10˚C), consistent with a greater genetic contribution from the cold tolerant parent,

Frohberg strains do indeed produce greater amounts of alcohol and use the sugars in brewing wort more rapidly and completely than Saaz strains (Gibson et al. 2013; Walther et al. 2014). Specifically, while Frohberg strains completely consumed all the maltose and maltotriose present in brewing wort, Saaz strains tested were unable to consume maltotriose and left substantial amounts of maltose unconsumed as well (Gibson et al. 2013).

While all lager brewing yeasts whose genomes have been analyzed fall into one of two clades consisting of closely related strains, that is not to say that all strains of a single lineage are genetically or phenotypically identical. Variation in chromosome copy numbers and translocations is particularly striking in the Frohberg lineage (Dunn and Sherlock 2008; van den Broek et al. 2015). In addition, the inability to use maltotriose was previously thought to be one of the defining physiological characteristics of the Saaz lineage. More recent work, however, has identified Saaz strains which can robustly consume this sugar, though the majority of Saaz strains characterized are still unable to do so (Magalhães et al. 2016).

While less essential to the brewing process lager beers are also known for their distinctive flavor profile ("crisp" and "clean"). Group specific differences in flavor compound production have also been observed. Frohberg strains tended to produce more compounds that impart a fruity character, such as isoamyl acetate (banana), ethylacetate (pear) and ethyl octanoate (apple) (Gibson et al. 2013; Walther et al. 2014). Saaz strains in contrast tend to produce more acetaldehyde, which is considered a more floral flavor. (Gibson et al. 2013). As with cryotolerance and the ability to utilize maltotriose, production of different flavor compounds also varies between strains of a single lineage (Gibson et al. 2013; Walther et al. 2014).

Origin of lager-brewing yeasts

In addition to laying the ground work for understanding the genetic basis for the different characteristics of Saaz and Frohberg type yeasts, the results of studies characterizing the genomes of lager-brewing yeasts also suggested that lager yeasts originated from two independent hybridization events (Liti et al. 2005; Dunn and Sherlock 2008) (Fig. 2D & F). This hypothesis was further supported by multilocus sequence comparisons of the *S. cerevisiae* portion of the lager yeast lineages, which found that different haplotypes were carried by the two lineages (Dunn and Sherlock 2008). Lack of genetic diversity at the loci examined in the non-*S. cerevisiae* portion of the genomes, however, made it difficult to draw similar conclusions for the second lager yeast parent.

With the spread of genome sequencing technology, sequence comparisons could be performed across the entire genome of lager yeasts rather than a few select loci. Fine scale detail of the break points of shared translocations cast doubt on the independent origin of the two lineages (Hewitt et al. 2014; Walther et al. 2014). Shared translocations were also observed by Dunn and Sherlock in their 2008 study, but these results were not considered strong evidence for a shared origin of the two lineages (Dunn and Sherlock 2008). This is because translocations in the same regions are consistent with experimental findings that independent populations under the same conditions often evolve the same translocations (Dunham et al. 2002). Several of the shared translocations within lager yeast however, were found to have identical breakpoints, which is unlikely if they occurred independently. This led to the hypothesis that the Saaz and Frohberg lineages did indeed have a shared origin, with the tetraploid Frohberg lineage representing the ancestral state and the Saaz lineage resulting from extensive loss of the *S. cerevisiae* component of the genome (Fig. 2D & E)(Walther et al. 2014; Wendland 2014). Given that extensive variation in chromosome loss is observed both among Frohberg and Saaz strains (Dunn and Sherlock 2008), it is plausible that a lineage of the hypothetical ancestral tetraploid underwent

extensive chromosomal loss and through success in the brewing environment and further distribution by brewers went on to create its own distinct lager-brewing lineage. However, other groups argued that the shared break points represent not a shared history, but rather "fragile sites" within the genome, prone to breakage and subsequent translocation (Hewitt et al. 2014; Monerawela et al. 2015). New evidence now supports a complex model for the origin of lager-brewing yeasts, with both shared and independent hybridization events (Baker et al. 2015; Okuno et al. 2016; Peris & Langdon et al. 2016). However, these new analyses pointing to complex origins were only possible with the discovery of pure strains of the unknown lager yeast parent.

Identification of the parents of lager-brewing yeast hybrids

When the hybrid nature of lager yeasts became evident, it was immediately clear and has never been in dispute that *S. cerevisiae* was one of the parent species (Martini and Kurtzman 1985; Hansen et al. 1994; Hansen and Kielland-Brandt 1994; Tamai et al. 1998). Dunn and Sherlock (2008) demonstrated that the *S. cerevisiae* portion of the genome shares alleles with *S. cerevisiae* strains associated with ale brewing. Since that time, extensive work has been done characterizing the population structure of *S. cerevisiae* (Liti et al. 2009; Schacherer et al. 2009; Strope et al. 2015; Gallone et al. 2016; Gonçalves et al. 2016; Peter et al. 2018). Recent work, using whole-genome sequence data and focusing specifically on strains of *S. cerevisiae* used in fermentations, has solidified the placement of the *S. cerevisiae* portion of lager yeast genomes within the beer (that is ale) lineage of *S. cerevisiae* yeasts (Gonçalves et al. 2016).

In the 1990's and early 2000's, as the species designations and relationships among other *Saccharomyces* became increasingly clarified, how the non-*S. cerevisiae* lager parent fit into the genus remained uncertain. Initially, it appeared that the second parent might be, the then-recognized species *S. bayanus* (Casaregola et al. 2001; Naumova et al. 2005; Nguyen and Gaillardin 2005), but with more

extensive sequence analysis, it was soon realized that the strains identified as *S. bayanus* were a complex set of hybrids of *S. uvarum* and *S. cerevisiae* and a third unknown lineage closely related to, but distinct from, *S. uvarum*. It is clear, based on sequence comparisons that it was this unknown lineage that was the parent of lager yeasts (Nguyen and Gaillardin 2005; Rainieri et al. 2006; Nakao et al. 2009; Nguyen et al. 2011).

Decades of speculation were finally put to rest when, in 2011, *Saccharomyces eubayanus* was isolated from forests in Patagonia. An early draft of the genome sequence of *S. eubayanus* indicated that this species had >99% nucleotide identity to the unknown parent of lager-brewing yeasts. From these results it was clear that it was a strain of *S. eubayanus* that had contributed the second set of genetic material found in lager-brewing yeasts (Libkind et al. 2011).

Origin of the lager *S. eubayanus* genome

Speculation on the origin of hybrid lager yeasts has evolved with the discovery of new *S. eubayanus* isolates and our understanding of *S. eubayanus* population structure. Initially, pure *S. eubayanus* strains were only isolated from South America, where they are found with great frequency and which seems to be the center of *S. eubayanus* diversity (Libkind et al. 2011; Peris et al. 2014; Peris & Langdon et al. 2016). In contrast, in Europe, where a number of other yeast species have been isolated, including *S. uvarum, S. kudriavzevii*, *S. paradoxus,* and of course numerous examples of *S. cerevisiae*, pure strains of *S. eubayanus* remain unidentified (Kuehne et al. 2007; Sampaio and Gonçalves 2008; Almeida et al. 2014; Almeida et al. 2015). It was also noted that, intriguingly, lager brewing originated very near the time that Europeans began traveling to the New World. This lead to speculation that it was the beginning of contact between the Americas and Europe that lead to the fateful rendezvous of *S. eubayanus* with native European yeast strains (Libkind et al. 2011).

Since its initial discovery, however, *S. eubayanus* has been found to exist outside of South America. Though it is much less common and isolated much more infrequently, strains of *S. eubayanus* have been found in North America, New Zealand, and East Asia (Bing et al. 2014; Peris et al. 2014; Gayevskiy and Goddard 2016; Peris & Langdon et al. 2016). Whole-genome sequence analysis of a strain of *S. eubayanus* isolated from Tibet revealed that it was significantly more closely related to the *S. eubayanus* parent of lager yeasts than were any strains found in Patagonia, making a South American origin for the *S. eubayanus* parent of lager yeasts unlikely. It now seemed more likely that *S. eubayanus* had traveled from East Asia, perhaps with traders along the silk road, and from there eventually made its way into Central European breweries.

As ever more *S. eubayanus* strains were isolated, work was done to establish the overall population structure of *S. eubayanus* (Peris et al. 2014; Peris & Langdon et al. 2016). It was found that the *S. eubayanus* subgenomes from lager yeasts and the Tibetan isolate of *S. eubayanus* were part of a small subclade of a population predominantly in South America which included two strains isolated from North Carolina (Peris & Langdon et al. 2016). Based on their geographic distribution, this clade was named the Holarctic subpopulation. Of the three wild Holarctic isolates for which whole-genome sequence data was available, phylogenetic analyses place the Tibetan isolate closest to *S. eubayanus* lager genomes, consistent with an East Asian origin for these strains. Whole-genome sliding window analyses, however, revealed more complexity. It was found that, while most of the genome for lager *S. eubayanus* was most closely related to the Tibetan isolates, other regions were more similar to the North Carolinian isolates. Overall, approximately a third of the lager genomes were more closely related to the North Carolinian isolates than to the Tibetan isolate. Moreover, which regions of the lager genomes were more closely related to the North Carolinian genomes was found to be different for the two lineages of lager yeast, suggesting that different strains of *S. eubayanus* hybridized to produce the

two lager lineages. While this provides further support for multiple hybridization events giving rise to modern lager yeasts it also suggests that it was not the ancestor of the Tibetan lineage that gave rise to lager hybrids, but another set of strains, perhaps from a cryptic European *S. eubayanus* population (Peris & Langdon et al. 2016).

Contribution of *S. eubayanus* to brewing-related traits

Cold tolerance

Saccharomyces yeasts can be broadly divided between thermotolerant (maximum growth temperature ≥37˚C) and cryotolerant (maximum growth temperature ≤35˚C) species (Gonçalves et al. 2011; Salvadó et al. 2011). The thermotolerant group includes *S. cerevisiae*, while *S. eubayanus* has a growth profile matching other cryotolerant *Saccharomyces* (Libkind et al. 2011; Gibson et al. 2013). One of the defining characteristics of lager-brewing yeasts is their ability to ferment at relatively low temperatures (~7-15˚C), a trait which they inherited from their *S. eubayanus* parent. Between the lager-brewing yeast lineages, Saaz strains are the more cryotolerant (Gibson et al. 2013; Walther et al. 2014). This is consistent with Saaz yeasts possessing a relatively greater proportion of genetic material from the cryotolerant parent, *S. eubayanus,* compared to Frohberg lineage strains.

The genetics of cryotolerance are not well understood in *Saccharomyces,* and only a small number of loci have been implicated in cold tolerance (Yamagishi et al. 2010; Paget et al. 2014). In lager yeasts, just a single locus has been specifically described as contributing to cryotolerance (Yamagishi et al. 2010). The molecular role these loci play in supporting low temperature growth has also been poorly characterized. Based on the identity of the few loci that have been identified and some physiological studies in *S. cerevisiae* and *S. kudriavzevii,* there are some implications for the role of glycerol and control of redox balance in tolerance to cold temperatures, but more research is needed at both the

molecular and the genetic levels (Hayashi and Maeda 2006; Panadero et al. 2006; Aguilera et al. 2007; Arroyo-López et al. 2010; Tulha et al. 2010; Paget et al. 2014).

In the last 15 years, work studying mitochondrial DNA (mtDNA) in metazoans, including studies across populations of humans, has suggested a role for thermal environment in shaping variation in mtDNA (Mishmar et al. 2003; Fontanillas et al. 2005; Cheviron and Brumfield 2009; Foote et al. 2011; Willett 2011; Pichaud et al. 2013; Dingley et al. 2014; DuBay and Witt 2014; Quintela et al. 2014; Silva et al. 2014; Consuegra et al. 2015; Baris et al. 2016; Camus et al. 2017). Thermotolerance studies in *Saccharomyces* that have considered mitochondria have largely focused on high temperatures (>36˚C), but these studies have also implicated mtDNA in contributing to thermotolerance or sensitivity (Paliwal et al. 2014; Špírek et al. 2014; Wolters et al. 2018). A recent study by Li and Fay examined changes in gene transcription in hybrids between *S. cerevisiae* and the cryotolerant yeast *S. uvarum* between moderate and high temperatures (Li and Fay 2017). They found that which version of certain mitochondrial genes was expressed switched depending on the temperature the hybrids were grown at. These results implicate mtDNA, not only in adaption to high temperatures, but more moderate or even low temperatures as well. Both lineages of lager yeasts inherited their mtDNA from their cryotolerant *S. eubayanus* parent, however, what role this may play in cryotolerance in lager yeasts is still being explored.

Maltotriose utilization

In brewing wort, the most abundant fermentable sugars, those that can be consumed to produce ethanol, are maltose, maltotriose, and glucose. Of these, maltose, a disaccharide consisting of two glucose moieties, is the most abundant, making up around 60% of the sugars present (Meussdoerffer and Zarnkow 2009). Glucose and maltotriose (three glucose moieties) each make up about 20% of the

available fermentable sugars. Consumption of these sugars proceeds in order of increasing complexity (Briggs et al. 2004). Glucose is consumed first, and an extensive regulatory system is in place in *Saccharomyces* to ensure that the genes needed to consume non-glucose sugars remain repressed until all available glucose has been consumed (Horák 2013). Once glucose is removed from the wort, yeasts proceed to consume maltose. As the most abundant fermentable sugar, utilization of maltose is the most important for the production of fully fermented beer. Consequentially, the genes responsible for maltose utilization have been extensively studied. The genes needed to consume maltose are contained in multiple subtelomerically located clusters referred to as *MAL* loci. These loci consist of a maltose transporter (*MALT*), an α-glucosidase (*MALS*), and a regulator of the first two genes (*MALR*) (Rautio and Londesborough 2003).

Maltotriose is the final major sugar component consumed, if it is consumed at all. Maltotriose is poorly or completely unconsumed by many brewing strains (Gibson et al. 2013; Magalhães et al. 2016). The same genes used to consume maltose are responsible for the utilization of maltotriose as well. While the α-glucosidases that hydrolyze maltose into its glucose subunits can hydrolyze maltotriose as well (Rautio and Londesborough 2003), only a handful of maltose transporters (*MALT* genes) are also able to carry maltotriose from the external environment across the plasma membrane (Brown et al. 2010). In addition, most *MALT* genes have greater activity on maltose than maltotriose, ensuring preferential maltose consumption. As a major component of brewing wort, the inability to ferment maltotriose can lead to beer with an undesirable flavor profile and represents a considerable economic cost.

Three groups of *MALT* genes that can transport maltotriose have been described in *Saccharomyces*, the *MPH* genes, *MTT1* (also known as *MTY1* and unusual for having greater affinity for maltotriose than maltose), and *AGT1* genes (Han et al. 1995; Day et al. 2002; Dietvorst et al. 2005; Salema-Oom et al.

2005; Nakao et al. 2009; Brown et al. 2010). Sometimes the *MALT* gene, *MAL11,* is also erroneously described as being able to transport maltotriose (Brown et al. 2010; Baker et al. 2015; Brickwedde et al. 2017). This is because, despite not being phylogenetically closely related, *MAL11* and *AGT1* are both located at the *MAL1* locus in *S. cerevisiae* and, as such, are considered alleles of each other (Charron and Michels 1988; Han et al. 1995; Vidgren et al. 2005; Vidgren et al. 2009; Brown et al. 2010). All the *MALT* genes that have been identified as transporting maltotriose have been described as occurring in at least some strains of lager yeast (Vidgren et al. 2005; Nakao et al. 2009; Magalhães et al. 2016), though which parent species they originate from is not always obvious.

Initially, *MTT1* was described as a lager-specific gene because it had never been observed in pure strains of *S. cerevisiae* (Dietvorst et al. 2005; Salema-Oom et al. 2005; Wendland 2014). For this reason, *MTT1* was originally thought to originate from the *S. eubayanus* parent of lager yeasts (Dietvorst et al. 2005; Nakao et al. 2009; Cousseau et al. 2013). However, strains of *S. cerevisiae* are highly polymorphic in gene content (Bergström et al. 2014) and hundreds more genome sequences of different strains of *S. cerevisiae* have been published since *MTT1* was first described in 2005 (Liti et al. 2009; Schacherer et al. 2009; Strope et al. 2015; Gallone et al. 2016; Gonçalves et al. 2016; Peter et al. 2018). More recent evidence examining these new genomes now points to the *S. cerevisiae* parent of lager yeasts contributing *MTT1.* (Vidgren et al. 2010; Baker et al. 2015; Nguyen and Boekhout 2017).

Another gene whose origin in lager yeasts has been ambiguous is *AGT1,* the first maltotriose transporter to be identified in *S. cerevisiae* (Han et al. 1995). Lager yeasts carry two alleles of *AGT1* (Nakao et al. 2009). One closely matches the allele found in *S. cerevisiae* (98% nucleotide identity) and was most likely donated by the *S. cerevisiae* lager parent; the other is only 79% identical at the nucleotide level. Based on the sequence divergence of this gene from the *S. cerevisiae* allele and its location in close proximity

to *S. eubayanus* sequences within the lager genome, this allele of *AGT1* (*lgAGT1* for lager-*AGT1*) was considered most likely to have originated from the *S. eubayanus* parent of lager-brewing yeast (Nakao et al. 2009). Examination of the genome assembly of the type strain of *S. eubayanus,* however, failed to find evidence of *AGT1* (Baker et al. 2015). However, the presence/absence of subtelomerically-located genes like *AGT1* can be highly polymorphic, and its absence from the type strain does not necessarily indicate its absence from the *S. eubayanus* species (Bergström et al. 2014; Baker et al. 2015). In 2015, Hebly et al. recovered contigs containing the partial sequence of a gene with high similarity to *lgAGT1* from a genome assembly of a Tibetan isolate of *S. eubayanus* (Hebly et al. 2015). However, thus far, no full-length sequence of *lgAGT1* or an*AGT1*-like gene has been recovered from *S. eubayanus*.

Introduction to the following thesis chapters: The *S. eubayanus* genome and lager brewing

With the discovery of pure strains of *S. eubayanus*, widespread and routine whole-genome sequencing, and the multitude of molecular tools developed in *S. cerevisiae,* we are poised to address many of the outstanding questions regarding the *S. eubayanus* genome in lager brewing. One of the questions that has received considerable attention in recent years is the origin and relationship of the two extant lager-brewing yeast lineages (Liti et al. 2005; Dunn and Sherlock 2008; Hewitt et al. 2014; Walther et al. 2014; Wendland 2014; Monerawela et al. 2015; Okuno et al. 2016; Peris & Langdon et al. 2016). In chapter 2, I use a high-quality assembled genome of *S. eubayanus* to examine the evolution of the subgenomes of lager-brewing yeasts during the course of domestication to brewing conditions with particular emphasis on the *S. eubayanus* subgenomes (Baker et al. 2015). I examine differences in the amount of divergence between the *S. cerevisiae* and *S. eubayanus* subgenomes of the two lager genomes and the implications of these differences for the origin and relationship of the two lineages. In this chapter, I also briefly describe putative *MAL* loci present in the type strain of *S. eubayanus*. Building off the work examining *MAL* loci in the *S. eubayanus* type strain, in chapter 3, I identify more putative *MALT* genes in other

strains of *S. eubayanus* and characterize the ability of the proteins encoded by *MALT* genes found in *S. eubayanus* to transport maltotriose. I also present the results of a directed evolution experiment to evolve several different strains of *S. eubayanus* for maltotriose utilization. This experiment resulted in the surprising formation of a novel chimeric maltotriose transporter from parent proteins that were unable to transport maltotriose. In chapter four, I explore the role of mitochondrial DNA (mtDNA) in determining temperature tolerance in synthetic *S. cerevisiae* x *S. eubayanus* hybrids and industrial lager strains. Using controlled inheritance of mitochondria in synthetic *S. cerevisiae* x *S. eubayanus* hybrids, I measured the relative growth of hybrids across a wide range of temperatures (4-37˚C). I also completely removed the native mtDNA present in lager yeasts, inherited from the *S. eubayanus* parent, and replaced it with mtDNA from strains of *S. cerevisiae* and assayed the relative growth of these strains across various temperatures compared to the lager strain carrying its native mtDNA. The results of these experiments have implications for the role of mitochondria, not only in adaption to high temperature in yeast, but colder temperatures as well. In the final chapter, I summarize the findings of my thesis and discuss future directions.

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Chapter 2

The Genome Sequence of *Saccharomyces eubayanus* and the Domestication of Lager-Brewing Yeasts.

Supplementary information from this chapter can be found in Appendix A.

This chapter is a modification of a manuscript published in MBE.

EmilyClare Baker, Bing Wang, Nicolas Bellora, David Peris, Amanda Beth Hulfachor, Justin A. Koshalek, Marie Adams, Diego Libkind, Chris Todd Hittinger. 2015

B. Wang assembled, annotated, and generated assembly statistics for the nuclear genome of *S. eubayanus*.
N. Bellora assembled the mitochondrial genome of *S. eubayanus*.
D. Peris annotated and analyzed the mitochondrial genome of *S. eubayanus* and lager-brewing yeast and wrote the supplementary material for the mitochondrial genome analyses.
A.B. Hulfachor, J.A. Kshalek and M. Adams prepared *S. eubayanus* genomic DNA and performed whole-genome sequencing.
C.T. Hittinger conceived of this project, assembled the 2μ plasmid, helped write the manuscript, supervised all work, and provided financial support.
I contributed by identifying *MAL* loci, performing genome wide phylogenetic and evolutionary analyses, performing experiments to validate the genome assembly, and wrote the manuscript with input and edits from all authors.

ABSTRACT

The dramatic phenotypic changes that occur in organisms during domestication leave indelible imprints on their genomes. Although many domesticated plants and animals have been systematically compared to their wild genetic stocks, the molecular and genomic processes underlying fungal domestication have received less attention. Here we present a nearly complete genome assembly for the recently described yeast species *Saccharomyces eubayanus* and compare it to the genomes of multiple domesticated alloploid hybrids of *S. eubayanus x S. cerevisiae* (*S. pastorianus* syn. *S. carlsbergensis*), which are used to brew lager-style beers. We find that the *S. eubayanus* subgenomes of lager-brewing yeasts have experienced increased rates of evolution since hybridization, and that certain genes involved in metabolism may have been particularly affected. Interestingly, the *S. eubayanus* subgenome underwent an especially strong shift in selection regimes, consistent with more extensive domestication of the *S. cerevisiae* parent prior to hybridization. In contrast to recent proposals that lager-brewing yeasts were domesticated following a single hybridization event, the radically different neutral site divergences between the subgenomes of the two major lager yeast lineages strongly favor at least two independent origins for the *S. cerevisiae x S. eubayanus* hybrids that brew lager beers. Our findings demonstrate how this industrially important hybrid has been domesticated along similar evolutionary trajectories on multiple occasions.

INTRODUCTION

The molecular evolutionary processes of domestication have been extensively studied in plants and animals (Doebley et al. 2006; Lu et al. 2006; Cruz et al. 2008; Wang et al. 2014), but relatively few examples have been investigated in fungi (Rokas 2009; Borneman et al. 2011). Although domesticated microbes achieve tremendous population sizes, lineages can adaptively lose the ability to reproduce sexually when passaged vegetatively (Lang et al. 2009), while other lineages are derived from sterile interspecies hybrids (Querol and Bond 2009; Borneman et al. 2012; Gibson and Liti 2015). The loss of sexual reproduction and potentially extreme bottlenecks could lead to especially dramatic elevations in the fixation of deleterious alleles through Muller's Ratchet (Muller 1964; Felsenstein 1974), with each lineage of domesticated microbe potentially following its own domestication trajectory.

The yeasts used to ferment lager-style beers are examples of highly successful, domesticated interspecies hybrids. While *S. cerevisiae* has been used for millennia to brew ale-style beers and other alcoholic beverages, lager-brewing originated more recently in 15th century central Europe (Meussdoerffer 2009). Distinguished by their low fermentation temperatures and the settling of yeasts during fermentation, lagers are characterized as having a crisp taste that is distinctive from ale-style beers, which are often associated with relatively pure strains of *S. cerevisiae.* Though ale strains of *S. cerevisiae* are the prototypical brewing yeasts, hybrid lager strains account for 94% of the world market (Riese and Eßlinger 2009).

Historically, lager yeasts have been referred to as *Saccharomyces pastorianus* syn. *S. carlsbergensis*, but, in the early 1980's, they were shown to be interspecies hybrids of *S. cerevisiae* and a second parental species (Nilsson-Tillgren et al. 1981; Martini and Kurtzman 1985; Martini and Martini 1987). Although the phylogenetic placement of this missing parent had been hypothesized since the early 2000's

(Casaregola et al. 2001; Naumova et al. 2005; Nguyen and Gaillardin 2005; Rainieri et al. 2006; Nakao et al. 2009; Nguyen et al. 2011), *Saccharomyces eubayanus* was first described as an independent species in 2011 when non-hybrid strains were found in association with *Nothofagus* trees in Patagonia (Libkind et al. 2011). Since then, rare isolates have also been isolated in North America (Peris et al. 2014) and China (Bing et al. 2014). However, the only evidence of *S. eubayanus* in Europe remains the hybrid lager-brewing strains of *S. cerevisiae x S. eubayanus* and other genetically complex interspecies hybrids (Almeida et al. 2014; Gibson and Liti 2015). *S. eubayanus* has not been definitively associated with human-controlled fermentations, except as interspecies hybrids (Rodríguez et al. 2014; Gibson and Liti 2015).

Lager yeasts consist of two distinct lineages, both of which were isolated in the late 19th century and form the basis of lager brewing today (Gibson et al. 2013; Gibson and Liti 2015). These lineages, Saaz and Frohberg, were named for the areas in which they were isolated, and are also known as Group I and Group II strains, respectively (Dunn and Sherlock 2008). Other authors distinguish Saaz/Group I strains taxonomically as *S. carlsbergensis* (Wendland 2014) and Frohberg/Group II strains as *S. pastorianus*. Each lineage has unique flavor and brewing profiles, as well as genetic compositions, but both lineages are *S. cerevisiae x S. eubayanus* alloploid hybrids (Dunn and Sherlock 2008; Gibson et al. 2013; Walther et al. 2014). Saaz strains have a slightly greater capacity to grow at low temperatures than Frohberg strains, and the two lineages also produce differing amounts of various esters important to the taste of the final product (Gibson et al. 2013; Walther et al. 2014). Saaz strains also have relatively poor fermentation performance compared to Frohberg strains, at least partly due to their poor utilization of maltose and maltotriose, which comprise 45-65% and 16-26% of all available sugars, respectively (Boulton and Quain 2001). This deficiency likely contributes to the predominance of Frohberg strains in modern industrial-scale brewing (Gibson et al. 2013).

Greater tolerance of lower temperatures and poor utilization of maltose and maltotriose make Saaz strains more physiologically similar to their *S. eubayanus* parent than Frohberg strains (Gibson et al. 2013). Perhaps not surprisingly, Saaz strains contain a higher proportion of *S. eubayanus* DNA in their genome, approximately one full diploid *S. eubayanus* genome and a haploid *S. cerevisiae* genome (i.e. allotriploids) (Walther et al. 2014). In comparison, Frohberg strains have a more equitable composition of parental genomes so that Frohberg genomes are comprised of approximately one full diploid *S. eubayanus* genome and one full diploid *S. cerevisiae* genome, making them approximately allotetraploid hybrids (Nakao et al. 2009; Walther et al. 2014).

The origin of these lineages is still contentious. One hypothesis proposes that the Saaz and Frohberg lineages resulted from independent hybridization events (Liti et al. 2005; Dunn and Sherlock 2008; Bond 2009). The most extensive study to date (Dunn and Sherlock 2008) examined the genome composition of multiple lager yeast strains using aCGH (array comparative genomic hybridization) with probes from *S. cerevisiae* S288c and *S. uvarum* CBS 7001 (previously *S. bayanus* var. *uvarum*), the sister species of *S. eubayanus*, as well as limited sequence data. However, concerns have remained about the use of *S. uvarum* as a proxy for *S. eubayanus* and the low resolution of aCGH, and subsequent findings have cast doubt on the multiple origins hypothesis. Specifically, three translocations between the *S. eubayanus* and *S. cerevisiae* chromosomes of lager yeasts share identical breakpoints between the Saaz and Frohberg lineages (Hewitt et al. 2014; Walther et al. 2014). This led Jürgen Wendland and colleagues to propose that both lineages were derived ultimately from a single hybridization event between a diploid *S. eubayanus* parent and a diploid *S. cerevisiae* parent (or two haploids followed by endoreduplication) (Walther et al. 2014; Wendland 2014). Under this hypothesis the Frohberg strains represent the ancestral state of all lager-brewing yeast lineages, while Saaz strains were derived later from a rare viable allodiploid meiotic spore that subsequently mated with a haploid *S. eubayanus* spore to form an

allotriploid lineage. Other groups, however, have argued on the basis of experimental evidence and limited gene sequence data from lager strains that identical breakpoints could have resulted from independent events at recombination hotspots or fragile sites in *Saccharomyces* chromosomes (Hewitt et al. 2014; Monerawela et al. 2015). Consequently, it is still unclear which hypothesis best explains the origins of lager-brewing yeasts.

Understanding the origins and evolution of lager yeasts, as well as the genetics of what makes them such successful industrial strains compared to their non-hybrid parents, is still an active area of research. While a draft assembly of *S. eubayanus* has been available since 2011, this initial assembly has a number of drawbacks (Libkind et al. 2011). Most critically, it was assembled with relatively low coverage of 36 bp single-end reads using the *S. uvarum* genome as a reference. While *S. uvarum* is the sister species to *S. eubayanus*, they are, nevertheless, approximately 7% diverged at the sequence level (Libkind et al. 2011), roughly the same distance as between humans and macaques (Gibbs et al. 2007). This reference-based assembly cannot account for any translocations in the *S. eubayanus* genome relative to the *S. uvarum* genome and lacks crucial information on *S. eubayanus* subtelomeric sequences. Complete and accurate assembly of the subtelomeric regions is of special interest because they often harbor novel and highly polymorphic genes, such as many of those important for brewing (Brown et al. 2010), but these regions have been traditionally difficult to assemble (Brown et al. 2010; Ellegren 2014). Recently, an improved genome assembly for *S. eubayanus* was published by Hebly et al., but this assembly still lacks critical coverage of the *S. eubayanus* subtelomeric sequences and fails to provide scaffolded *S. eubayanus* chromosomes (Hebly et al. 2015).

In the absence of a high-quality reference genome, putative *S. eubayanus* genes within lager-brewing yeasts have been described based on the apparent absence of close homologs within *S. cerevisiae* and,

when their genomic location is known, their physical proximity to non-*S. cerevisiae* portions of lager yeast genomes (Dietvorst et al. 2005; Nakao et al. 2009; Vidgren et al. 2010; Cousseau et al. 2013). With our assembly of the *S. eubayanus* genome, it is now possible to study lager yeasts in the context of the complete genomes of both parental species. Not only does our *S. eubayanus* assembly fill this important information gap, its quality and coverage, both in terms of depth and completeness, exceeds that of any *Saccharomyces* genome outside of *S. cerevisiae*. In addition to setting a new benchmark for genome assembly quality, the *S. eubayanus* genome also demonstrates what is possible with current Illumina sequencing technology and assembly algorithms. Here we analyze the genome evolution of *S. eubayanus* and its domesticated hybrids to begin to infer how these genomes have changed during domestication, yielding unprecedented insights into the complex origins and evolution of these industrially important hybrids.

RESULTS/DISCUSSION

A high-quality annotated *de novo* genome assembly of *Saccharomyces eubayanus*

To study the evolution of the genome sequence and structure of *S. eubayanus* and its hybrid descendants, we assembled *de novo* the genome sequence of FM1318, a monosporic derivative of the species type strain (CRUB 1568^T = PYCC 6148^T = CBS 12357^T). To enable the use of the ALLPATHS-LG genome assembler, we built two specialized Illumina libraries: a “fragment library” with paired-end 300 bp reads (i.e. 2 x 300 bp) and a “jumping library” with mate-pair reads with an average insert size of approximately 6.5 kb. Briefly, ALLPATHS-LG first joins paired-end reads from the fragment library that overlap to create longer reads, from which it builds a de Bruijn graph to construct contigs; the longer insert jumping library is then incorporated into the de Bruijn graph to scaffold the contigs, resolve repeats, and flatten the graph (Ribeiro et al. 2012). Since all *Saccharomyces* genomes contain Ty retrotransposons that are approximately 6 kb, duplicate gene families, and several other large repeats, a

long-read or long-insert scaffolding strategy is critical to providing physical evidence that spans gaps to order and orient contigs. ALLPATHS-LG performed well on the nuclear genome but poorly on the 2-micron plasmid and the mitochondrial genome, both of which are circular and present at higher copy numbers. Instead, we assembled these genomes using SPAdes (Bankevich et al. 2012) which produced complete contigs that we manually circularized. We performed several additional procedures and quality checks to combine and improve the final assembly (see Materials and Methods).

This assembly strategy resulted in a genome assembly of higher quality than any published for a *Saccharomyces* species other than *S. cerevisiae* (Liti et al. 2009; Scannell et al. 2011; Liti et al. 2013; Engel et al. 2014). Specifically, the 11.66 Mb nuclear genome was found on 22 scaffolds, and the scaffold N50 was 896 kb. These scaffolds were built from 144 contigs, with a contig N50 of 198 kb. Manual inspection of gaps suggested that most were due to Ty elements or other repetitive sequences. With the exception of 6 scaffolds ranging from 2 kb to 13 kb in length, all scaffolds were placed onto chromosomes. Only chromosome XII had an internal gap, which corresponded to the *rDNA* repeats and is conserved with *S. cerevisiae*. Indeed, the genome of *S. eubayanus* is nearly syntenic with *S. cerevisiae* with the exception of a handful of small inversions and two previously documented reciprocal translocations that it shares with its sister species, *S. uvarum* (fig. 1) (Fischer et al. 2000; Scannell et al. 2011). One assembled chromosome (chromosome VIII) contained telomeric repeats, and several contained well-resolved multi-copy subtelomeric gene families.

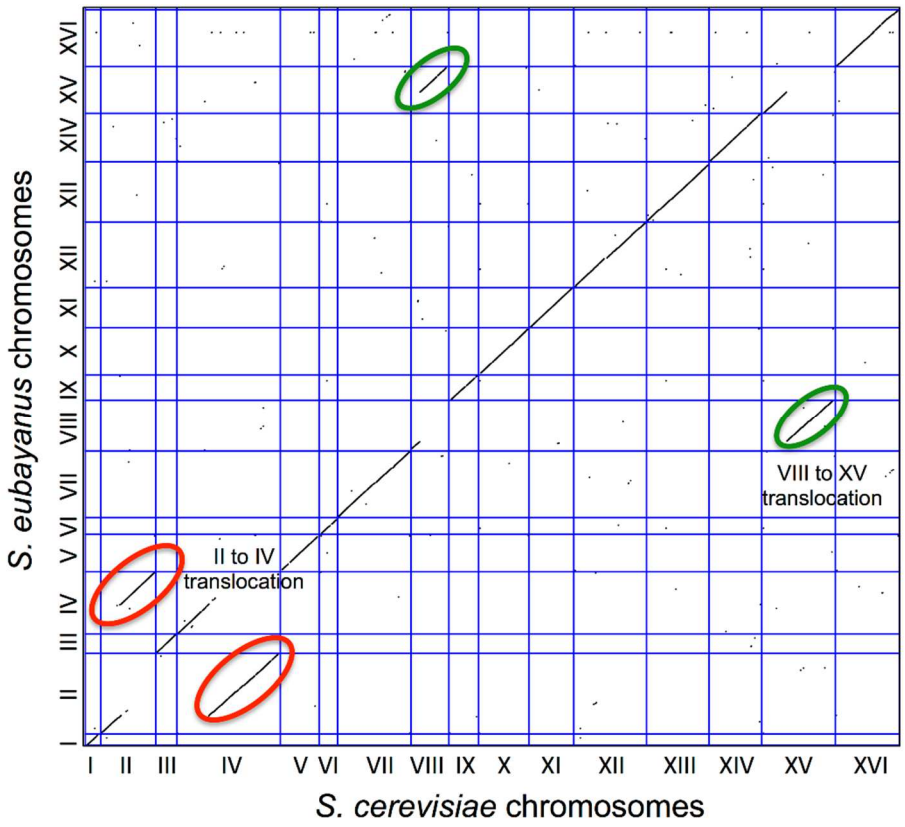

Figure 1. Synteny plot comparing the genome structure of *S. eubayanus* and *S. cerevisiae*. Dot plot comparing the location of genes in the *S. eubayanus* (FM1318) genome assembly to their location in *S. cerevisiae* (S288c). Lines circled in the same color indicate reciprocal translocations.

We adapted the Yeast Genome Annotation Pipeline (YGAP) to annotate our high-quality *de novo* assembly of the *S. eubayanus* genome (see Materials and Methods). This pipeline resulted in 5515 predicted protein-coding genes for *S. eubayanus*, which is similar to the current draft genomes of other *Saccharomyces* species (Liti et al. 2009; Scannell et al. 2011; Liti et al. 2013). Of the predicted protein-coding genes, 4993 were unambiguous 1:1:1 orthologs between *S. cerevisiae*, *S. uvarum*, and *S. eubayanus*. Since the Saaz and Frohberg lineages of lager-brewing yeasts contain largely complete but sometimes non-overlapping copies of both the *S. cerevisiae* and *S. eubayanus* genomes (Walther et al. 2014), we separately considered 1:1:1:1:1 orthologs for each lineage. Specifically, the *S. cerevisiae*:*S. cerevisiae* (Frohberg):*S. uvarum*:*S. eubayanus*:*S. eubayanus* (Frohberg) set had 3649 orthologs, while the *S. cerevisiae*:*S. cerevisiae* (Saaz):*S. uvarum*:*S. eubayanus*:*S. eubayanus* (Saaz) set had only 3102 orthologs due to the loss of more *S. cerevisiae* genes, principally through the loss of chromosomes (Dunn and Sherlock 2008). 2268 orthologs were common between the Saaz 1:1:1:1:1 ortholog set and the Frohberg 1:1:1:1:1 ortholog set. For phylogenetic analyses, we also included *S. paradoxus* sequences (1:1:1:1:1:1 ortholog sets), as an outgroup for *S. cerevisiae* lager and non-lager sequences, leaving 2194 orthologs.

Based on the shared set of 1:1:1:1:1 orthologs (coding sequences) between the Saaz and Frohberg genomes, the *S. cerevisiae* subgenome of the Saaz lineage is 99.57% identical to S288c, while in the Frohberg lineage, the *S. cerevisiae* subgenome is 99.60% identical. The *S. eubayanus* subgenomes of both lager strains are 99.55% identical to FM1318 (see supplementary table S1), similar to a previous estimate of 99.56% (Libkind et al. 2011).

The *S. eubayanus* mitochondrial genome

The *S. eubayanus* mitochondrial genome (mtDNA) is 64 kb, which is 6.6 kb smaller than the mtDNA of Frohberg lager-brewing yeast (Nakao et al. 2009) and 21.8 kb smaller than the mtDNA of *S. cerevisiae*

(S288c). *S. eubayanus* mtDNA has a similar gene order to the Frohberg representative, but it differs in the number of introns in *COB* and the locations of *COX1* introns (fig. 2). Both *S. eubayanus* and Frohberg mtDNA show two rearrangements relative to *S. cerevisiae* (S288c), one involving 15S *rRNA* and *tRNA-Trp* loci and a second involving the *tRNA-Glu* and *COB* loci (supplementary fig. S1). These structural and sequence similarities establish *S. eubayanus* as the main donor of mtDNA for lager yeasts of the Frohberg lineage. In addition, our analyses also revealed a dynamic history of selfish elements and possible localized introgression with other *Saccharomyces* species. Overall the Frohberg mtDNA coding sequences are approximately 98.56% identical to the *S. eubayanus* mtDNA (See supplementary results S1 and supplementary figs. S1 and S2).

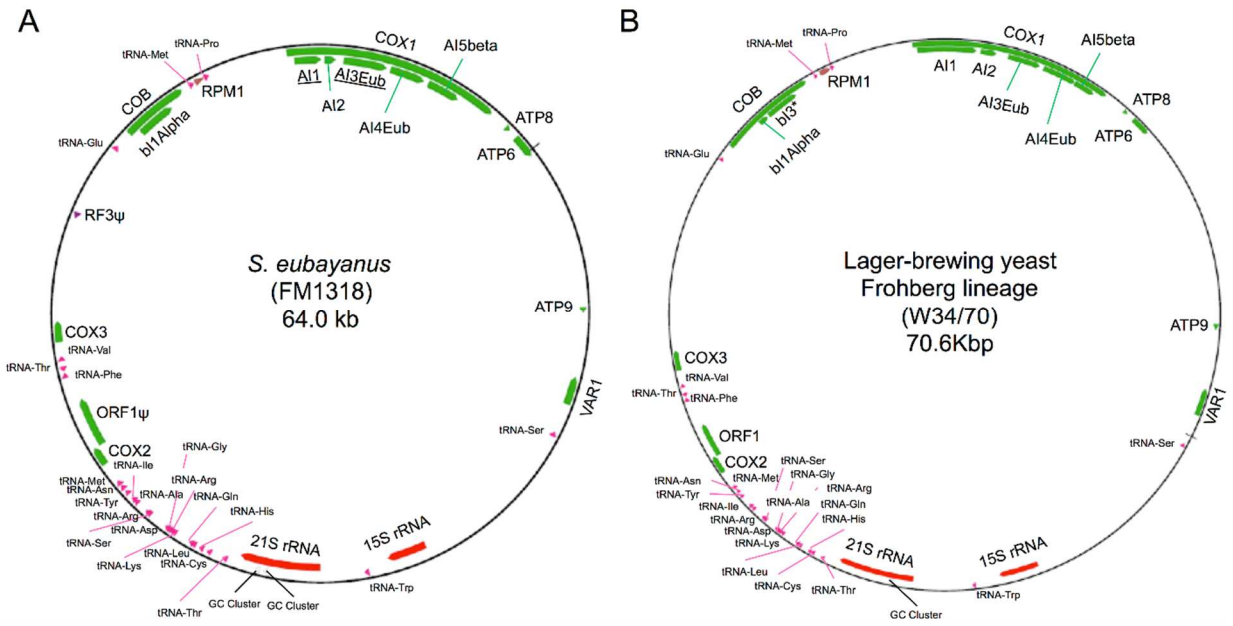

Figure 2. Mitochondrial genome structure of *S. eubayanus* and lager-brewing yeast. Schematic representation of *S. eubayanus* (FM1318) and lager-brewing yeast (Frohberg strain W34/70) annotated mitochondrial genomes. Mitochondrial genes, rRNAs, tRNAs, and non-coding RNAs are represented in green, red, pink, and brown, respectively. Genes with asterisks are elements or gene sequences not shared by both *S. eubayanus* and the lager yeast mitochondrial genomes. Underlined names are intronic regions located in different positions between *S. eubayanus* and Frohberg lager yeast.

Characterization of the maltose (*MAL*) utilization genes of *S. eubayanus*

The *MAL* genes allow the sugars maltose, maltotriose, and related sugars, to be utilized as carbon sources. They are typically subtelomeric and often found in clusters consisting of genes encoding a maltose permease (*MALT*), a maltase (*MALS*), and a transcriptional regulator of the pathway (*MALR*) (Needleman 1991). *S. cerevisiae* also contains additional maltose utilization genes, including genes encoding the isomaltases *IMA1-IMA5* (Teste et al. 2010) and the maltose and maltotriose transporters *MPH2* and *MPH3* (Day et al. 2002). These genes are also subtelomeric but are not necessarily found in close association with other maltose utilization genes. For simplicity, we refer to genes related to maltose utilization collectively as *MAL* genes. The industrial importance of understanding maltose utilization, particularly the *S. eubayanus* versions of *MAL* genes in lager-brewing yeasts, and the historic difficulty of assembling the subtelomeric regions, makes these genes excellent candidates to test the quality and completeness of our *S. eubayanus* assembly.

An extensive search of the *S. eubayanus* genome revealed 14 genes related to maltose utilization spread across 4 chromosomes (fig. 3 and supplementary table S2), as well as two putatively pseudogenized *MAL* genes on an unplaced scaffold (supplementary results S2). For comparison, in the most recent assembly of the *S. eubayanus* genome, which was based entirely on Illumina paired-end reads, only three of these *MAL* genes could be identified, and none could be placed on chromosomes (Hebly et al. 2015). In our analysis, all *MAL* genes were found in subtelomeric clusters (fig. 3) and, in most cases, were the last genes before the end of the assembled chromosome sequence. The *MAL* genes were often found in association with other genes known to be related to fermentation and brewing, such as genes encoding hexose transporters and alcohol dehydrogenases, consistent with the tendency of subtelomeric regions to harbor brewing-related genes (Brown et al. 2010).

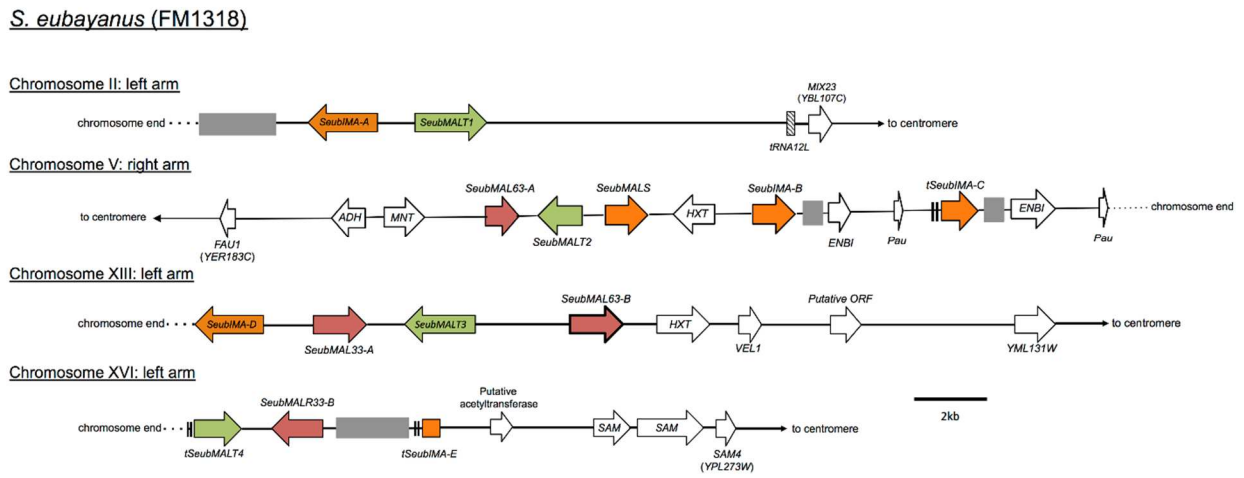

Figure 3. *MAL* gene clusters in reference genome of *S. eubayanus*. Genome regions in *S. eubayanus* (FM1318) with clusters of genes related to maltose (*MAL*) utilization. Regions are represented from chromosome ends to the first gene that is syntenic with *S. cerevisiae* (S288c). Gene sizes and distances are approximately to scale. Arrows show the direction of transcription and direction to the centromeres. Gray boxes represent sequence gaps. *MAL* genes are colored: orange genes encode maltases and isomaltases, green genes encode maltose transporters (permeases), and red genes encode transcription factors that regulate other *MAL* genes. *MAL* genes are named for their closest *S. cerevisiae* homolog in S288c and prefixed with "*Seub*". *S. eubayanus MAL* genes that are most similar to the same *S. cerevisiae MAL* gene are distinguished by letters (*SeubIMA-A, SeubIMA-B*, etc.). Double lines before or after a gene represent incomplete sequence due to poor sequence resolution in those areas and their names are marked with a "t" for truncated. All non-*MAL* genes in *S. eubayanus* are named for their closest gene family in *S. cerevisiae* using standard names with the exception of genes that are the first gene syntenic with *S. cerevisiae* (S288c). The first *S. eubayanus* genes syntenic with S288c are named using the standard names of their *S. cerevisiae* syntenic homologs, where available, along with their systematic names.

The origins of the *MAL* genes of lager-brewing yeasts

To infer the origins of *MAL* genes in lager yeasts, we extracted homologs from the publicly available genome assemblies of the two lager yeast lineages, Saaz and Frohberg (Walther et al. 2014), by using both the *S. cerevisiae* (S288c) (Cherry et al. 2012; Engel et al. 2014) and *S. eubayanus* sequences as BLAST queries. Since ale strains are the most likely *S. cerevisiae* parent of lager-brewing yeasts (Dunn and Sherlock 2008; Querol and Bond 2009; Monerawela et al. 2015), *MAL* genes were also extracted from the publicly available genomes of the ale strains Foster's O and Foster's B (Borneman et al. 2011). Many ale *MAL* genes were incomplete or, in some cases, completely missing from previously published assemblies, so we relied primarily on the *MAL* gene sequences from S288c for our comparisons. Where possible, lager *MAL* genes were assigned as either of *S. cerevisiae* or *S. eubayanus* origin based on sequence similarity (supplementary table S2).

Though many lager *MAL* genes had very high sequence identity with either *S. cerevisiae* (S288c) or *S. eubayanus* (FM1318) genes, in a number of cases the identity was insufficient to identify them as being of *S. eubayanus* or *S. cerevisiae* origin. In most cases, the similarity between the ale strain *MAL* genes and their lager homologs was about the same as for S288c. In several cases, however, the ale strain *MAL* genes were much more similar to the lager yeast genes than the sequences from S288c. Several ale *MAL* genes had over 98% nucleotide identity to their lager yeast counterparts, whereas the closest S288c homolog had less than 85% identity; in one case, the nearest homolog had even been identified as a *S. eubayanus* gene. Since many of the *MAL* genes extracted from the ale genomes were incomplete, were of poor sequence quality, or had frameshifts that were likely 454 sequencing artifacts, these results are probably an underestimate of the true similarity of ale strains to the *S. cerevisiae* genomes of lager-brewing yeasts. In some cases, the percent identity of a lager *MAL* gene to its closest *S. cerevisiae* or *S. eubayanus* homolog was low enough that its true origin remained ambiguous. Despite relatively low

sequence identities, synteny sometimes supported the *S. eubayanus* origin of lager yeast genes where sequence identity was ambiguous (fig. 4A, supplementary table S2). For example, the gene order within *MAL* clusters from *S. eubayanus, S. cerevisiae,* and the Saaz and Frohberg lager yeast lineages revealed one cluster in both lager yeast lineages that is largely syntenic with the *S. eubayanus MAL* cluster on chromosome V and another *MAL* cluster in the Saaz lineage that is syntenic with the cluster on chromosome X of S288c (fig. 4A). The common origin of many genes within these clusters is further supported by phylogenetic analysis (fig. 4B). While we could establish the parent species for a number of lager yeast *MAL* genes, the origin of many *MAL* genes remains ambiguous, including the previously identified lager brewing-yeast specific genes, *MTT1* and *Lager-AGT1* (see supplementary results S3 and supplementary tables S2 and S3) (Dietvorst et al. 2005; Nakao et al. 2009; Vidgren et al. 2010; Cousseau et al. 2013). To improve identification of the source of *MAL* genes and other brewing-related genes in lager yeasts, it will be necessary to identify strains more closely related to the parental strains, both in *S. cerevisiae* and *S. eubayanus*.

A genome-wide signature of domestication in lager-brewing yeasts

To test for the genome-wide consequences of domestication, we compared the genome-wide distributions of the ratio of nonsynonymous substitutions to total substitutions per gene between different lineages of *Saccharomyces* and the subgenomes of lager-brewing yeast hybrids. Specifically, we considered genes with homologs in *S. cerevisiae, S. paradoxus, S. eubayanus, S. uvarum* and where both the *S. cerevisiae* and *S. eubayanus* homologs had been retained in both the lager-brewing yeast lineages (1:1:1:1:1:1 ortholog sets). Using PAML (Yang 2007), the rate of synonymous substitution (d_S) and the rate of nonsynonymous substitution (d_N) were computed for each gene along each lineage, from which

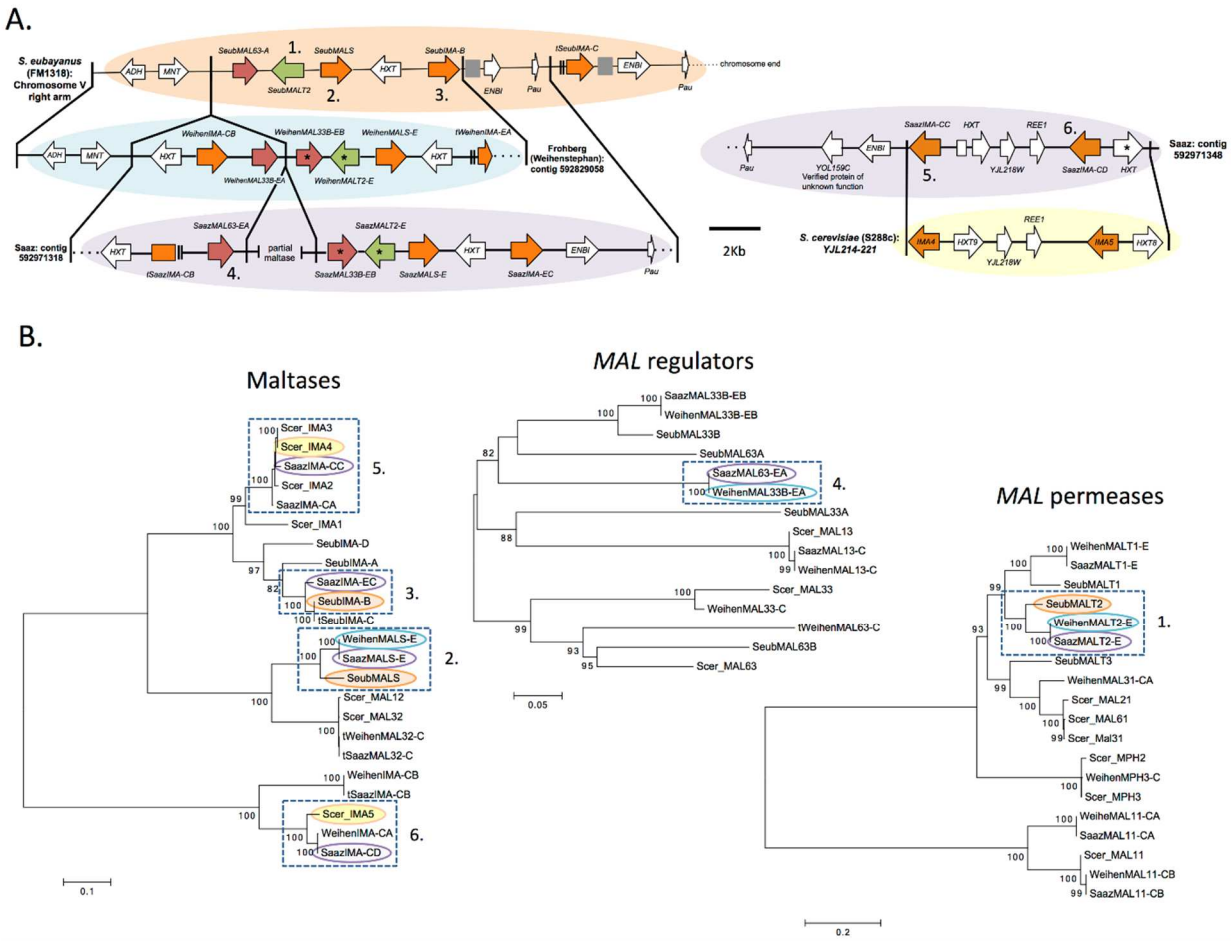

Figure 4. Synteny and phylogenetic analysis of *MAL* genes from *S. eubayanus* and lager-brewing yeasts. Synteny and phylogenetic analysis of *MAL* genes from *S. eubayanus* (FM1318), *S. cerevisiae* (S288c), and the Saaz (CBS 1513) and Frohberg (W34/70) lineages of lager-brewing yeasts. Numbers in (A) and (B) indicate genes whose orthology is supported both by synteny and phylogenetic analysis. (A) Solid lines connecting genomes designate blocks of synteny. Chromosome and contig locations are indicated to the left or right of genome segments. The location of the *S. cerevisiae* segment is indicated by the systematic names of the genes within the syntenic region. Asterisks indicate genes with complete sequences but putatively inactivating mutations. The inactivated Saaz *HXT* in the region syntenic to *S. cerevisiae* is divided in two due to an insertion within the gene. Genes are colored as in fig. 3. Gene sizes and distances are approximately to scale. Arrows show the directions of transcription. Gray boxes

represent gaps in the sequence. Double lines before or after a gene represent incomplete sequence due to poor resolution in those areas, and their names are marked with a “t” truncated. Dotted lines represent the end of a chromosome or contig. (B) Maximum likelihood trees for maltases, regulators of *MAL* genes, and maltose permeases (transporters) based on nucleotide sequences. Branch lengths are based on the number of substitutions per site. Bootstrap support values of 70 or higher are shown at nodes. Genes present in the synteny analysis are highlighted by an oval of the same color as their genome blocks in (A). Dashed boxes indicate groups of genes whose ortholog is also supported by synteny. More details on these genes can be found in supplementary table S2.

we estimated the number of substitutions. Saaz and Frohberg were considered separately as the lager yeast representative (supplementary data S1).

Consistent with domestication having had a genome-wide impact, we found that the proportion of nonsynonymous changes out of all substitutions per gene was significantly greater (p-value < 10^{-21} for both Frohberg and Saaz) in the *S. eubayanus* subgenome of lager lineages than in the wild Patagonian isolate (fig. 5). For all comparisons, the results were not dependent on whether the Saaz or Frohberg lineage was used as the lager yeast representative, and the Saaz and Frohberg lineages themselves did not differ from one another (p-values >> 0.05). These results strongly suggest that purifying selection has played a much more limited role in the evolution of the *S. eubayanus* subgenome of lager yeasts than in the genome of its non-domesticated relative. Although the polyploid state of lager yeast could also have contributed, relaxation of purifying selection under domestication conditions was probably the primary cause of the increase in the fixation of nonsynonymous substitutions.

Demographic factors outside of domestication, such as a hypothesized population bottleneck in the lineage leading to the *S. eubayanus* strains of the Northern Hemisphere (Almeida et al. 2014; Peris et al. 2014), could also have contributed to the observed increase in nonsynonymous substitutions. Prior to their hybridization with *S. cerevisiae*, these strains were likely part of a European subpopulation of *S. eubayanus* with very low genetic diversity. By comparing the number of substitutions since the divergence of these lineages from each other (supplementary data S2) to the number of substitutions since their divergence with FM1318, we estimate that at least 3% of the observed divergence can be attributed to processes that occurred after the *S. eubayanus* parents of the two lager yeast lineages diverged from one another. Importantly, this subset of substitutions appears to show an even stronger bias toward nonsynonymous changes than the rest of the genome.

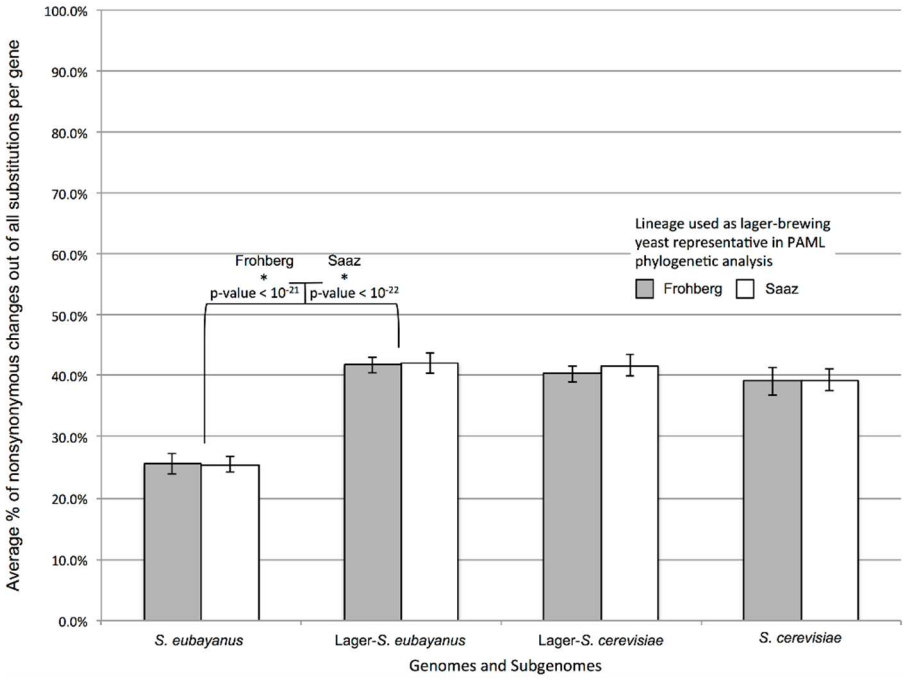

Figure 5. Analysis of nucleotide substitutions in lager-brewing yeasts and parent species genomes. Genome-wide averages of the percent of nonsynonymous substitutions out of all substitutions per gene for the genomes of *S. cerevisiae, S. eubayanus*, and the *S. cerevisiae* and *S. eubayanus* subgenomes of lager-brewing yeasts hybrids when using either the Frohberg (gray) or Saaz (white) lineage as the representative of lager-brewing yeasts. Substitutions were estimated by PAML phylogenetic analysis using orthologs from *S. cerevisiae, S. paradoxus, S. eubayanus, S*. *uvarum* and orthologs that are present in both the *S. cerevisiae* and *S. eubayanus* subgenomes of the Saaz and Frohberg lineages. Only the 2194 genes shared by both 1:1:1:1:1:1 ortholog sets were included in the analyses. Error bars represent 99% binomial confidence intervals. Comparisons between genomes and subgenomes (and between using Saaz versus Frohberg as the lager yeast representative) were made using logistic regression. *'s represent statistically significant comparisons. Note that the enrichment of nonsynonymous changes in lager lineages of *S. eubayanus* is not due to the inverse correlation between ω and d_S: the average d_S for each branch left to right is 0.0045, 0.0046, 0.0061, 0.0062, 0.0046, 0.0050, 0.0046, and 0.0047; the average d_N for each branch is 0.0006, 0.0006, 0.0017, 0.0019, 0.0010, 0.0013, 0.0010, 0.0010.

(nonsynonymous changes represent 55% of substitutions from the common ancestor of Saaz and Frohberg versus 36% from their ancestor with FM1318). Thus, the *S. eubayanus* subgenomes in these interspecies hybrids seem to have experienced a substantial relaxation of purifying selection beyond any demographic factors that may have occurred within wild *S. eubayanus* during the divergence of the relevant Patagonian and Northern Hemisphere subpopulations.

We have likely underestimated the impact of domestication on the evolution of the *S. eubayanus* subgenomes in lager yeasts because our estimate assumes that domestication began only after the divergence of the *S. eubayanus* strains that would give rise to the Saaz and Frohberg lineages. The population of *S. eubayanus* that gave rise to lager hybrids could have already begun to adapt to brewing prior to the divergence of those two lineages. Nonetheless, it is unlikely that *S. eubayanus* strains were as strongly associated with brewing as ale strains of *S. cerevisiae* prior to hybridization. Consistent with the hypothesis that *S. eubayanus* subgenomes were more affected by domestication, the *S. cerevisiae* subgenomes of lager hybrids do not show the same degree of an increase in nonsynonymous changes after the divergence of the two lager lineages (fig. 5).

Some metabolism genes may have been shaped by domestication to brewing

Genes that have experienced elevated rates of protein sequence evolution in both the Saaz and Frohberg lineages but that acquired different nonsynonymous changes are of special interest because they may indicate independent responses to the same environmental and domestication pressures. The *S. eubayanus* alleles of *NOT3* have experienced an independent elevation of the relative rate of nonsynonymous substitution (Saaz ω = 2.10, Frohberg ω=1.57) (ω= rate of nonsynonymous substitution (d_N) / rate of synonymous substitution (d_S)). This global regulator of transcription (Collart et al. 2013) has further acquired a large number of number of changes along both *S. cerevisiae* lager branches, including

five unshared nonsynonymous differences between the Saaz and Frohberg alleles. Similarly, *ADR1* encodes a transcription factor required for the expression of the alcohol dehydrogenase specialized for ethanol oxidation (*ADH2*), rather than fermentation, as well as the regulation of other genes related to the utilization of ethanol (Young et al. 2003). In the Saaz lineage, the *ADR1* allele derived from *S. cerevisiae* appears to have undergone rapid evolution (ω = 2.23), while the *S. eubayanus* allele also appears to have an elevated rate of evolution (ω = 0.94). In the Frohberg lineage, the estimated rate of evolution for both the *S. cerevisiae* allele (ω = 0.87) and the *S. eubayanus* allele (ω = 1.45) are moderately elevated. Finally, *REG2*, an important regulator of glucose-repressed genes (Frederick and Tatchell 1996), especially the *MAL* genes (Jiang et al. 2000), experienced five nonsynonymous substitutions along the *S. eubayanus* Frohberg branch. Many caused radical amino acid changes that may have reduced the function of the protein, allowing it to adjust to the needs of the new glucose-poor but maltose-rich environment.

Multiple independent origins of lager-brewing yeasts

The single origin (Walther et al. 2014; Wendland 2014) and multiple origin (Liti et al. 2005; Dunn and Sherlock 2008; Bond 2009) hypotheses for the origins of lager yeasts make distinct predictions about the divergences that would be expected between the *S. eubayanus* and *S. cerevisiae* subgenomes of the Saaz and Frohberg lineages (fig. 6A). In the case of a single origin, both the *S. eubayanus* and *S. cerevisiae* subgenomes would have begun with identical sequences in the two lineages. Once the *S. eubayanus* and *S. cerevisiae* subgenomes were present in the same hybrid nucleus, they would be expected to have accumulated neutral substitutions at the same rate. As a result, the single origin hypothesis predicts that the neutral divergence between the *S. cerevisiae* subgenome of the Saaz lineage and the *S. cerevisiae* subgenome of the Frohberg lineage should be equivalent to the divergence of the *S. eubayanus* subgenomes of the Saaz and Frohberg lineages. In contrast, the multiple origins

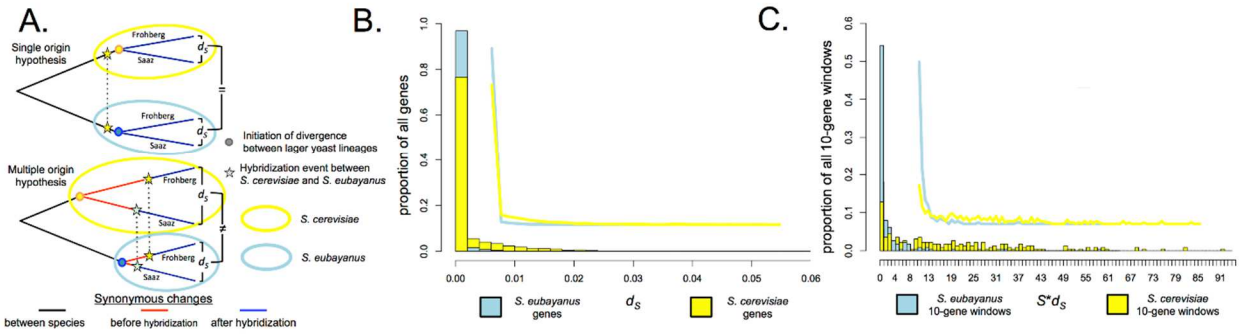

Figure 6. Test of models for the origin of lager-brewing yeast lineages. (A) Hypothetical models for the origin of hybrid lager-brewing yeast lineages and the relative neutral divergence (d_S and S^*d_S) between the subgenomes of each lineage under each model. (B) and (C) are the distributions of the estimated synonymous rates of evolution (*dS*) and the estimated number, respectively of synonymous substitutions (S^*d_S) for all genes and 10 gene windows respectively, between either the *S. cerevisiae* subgenomes (yellow) or the *S. eubayanus* subgenomes (blue) of the two lager-brewing yeast lineages. Line drawings within graphs represent outlines of the histograms to better show their overall distributions.

hypothesis predicts that the parental genomes could have already begun to diverge from each other before hybridizing, leading to potentially different levels of neutral divergence.

Consistent with the multiple origins hypothesis, the rate of synonymous substitution (d_S) and the estimated number of synonymous substitutions ($S*d_S$) was over 10 times higher for the *S. cerevisiae* subgenome than for the *S. eubayanus* subgenome (supplementary data S2 and supplementary table S4). This pattern was not driven by a few genes but was rather distributed across the entire genome (fig. 6B (p-value < 10^{-206}) and fig. 6C (p-value < 10^{-44})). The relatively even distribution of divergence was also inconsistent with more complex models involving the differential loss of heterozygosity between lineages (supplementary fig. S3). This result provides a clear indication that the *S. cerevisiae* subgenomes of the two lineages of lager-brewing yeasts had begun to diverge from each other well before the *S. eubayanus* subgenomes. The directionality of this result is consistent with population genetic studies that suggest that there was very limited genetic diversity among the *S. eubayanus* strains that would have produced lager-brewing yeasts (Peris et al. 2014) and that standing variation among *S. cerevisiae* ale strains was higher (Dunn and Sherlock 2008). In this context, these results suggest that the Saaz and Frohberg lineages were created by at least two distinct hybridization events between nearly identical strains of *S. eubayanus* with relatively more diverse ale strains of *S. cerevisiae.*

CONCLUSIONS

The origins of lager-brewing yeasts

The finding that multiple hybridization events gave rise to alloploid lager-brewing yeasts is in concert with previous observations that suggested complex reticulate evolutionary events for many *Saccharomyces* strains used in human-controlled fermentations, including multiple origins of *S. cerevisiae x S. kudriavzevii* hybrids and the existence of manifold *S. uvarum* strains with introgressions

from multiple species, (Le Jeune et al. 2007; Peris, C. a. Lopes, et al. 2012; Peris, C.A. Lopes, et al. 2012; Peris, C. Belloch, et al. 2012). Monerawela et al. (2015) recently made a similar argument that the amount of diversity seen between the *S. cerevisiae* portions of the Saaz and Frohberg lineages cannot be accounted for by only 500 years of divergence from a single hybridization event at the historical start of lager-brewing. While fungal molecular clocks and the historical record of brewing are open to interpretation, no plausible molecular mechanism is available to explain how the *S. cerevisiae* and *S. eubayanus* subgenomes could have evolved at such different rates at nearly neutral sites once they were present in the same nucleus. Although both the Saaz and Frohberg lineages show similar patterns of relaxed purifying selection, the difference in the amount of neutral divergences between their subgenomes is incompatible with the hypothesis that the lineages arose from a common origin as the result of a single hybridization event.

The existence of shared translocations between the two lineages must still be reconciled with the multiple origins of the lager-brewing yeast lineages. A variety of plausible models have been proposed to explain how shared translocations could have arisen between independent lineages. Mitotic recombination hot spots or fragile sites have been observed in experiments involving pure and hybrid strains, including lager-brewing yeasts (Dunham et al. 2002; Bond et al. 2004; James et al. 2008; Dunn et al. 2013; Hewitt et al. 2014; Monerawela et al. 2015). Indeed, one of the three shared translocations is near the *MAT* locus, a known target of the HO endonuclease. Alternatively, shared translocations might have occurred in one of the parental strains prior to the hybridizations that produced the modern lager-brewing yeast lineages. Hybridization and introgression between different *Saccharomyces* species is common in strains associated with human-controlled fermentation (Le Jeune et al. 2007; Novo et al. 2009; Libkind et al. 2011; Dunn et al. 2012; Peris, C. a. Lopes, et al. 2012; Peris, C.A. Lopes, et al. 2012; Peris, C. Belloch, et al. 2012; Almeida et al. 2014), so a partially domesticated lineage of *S. eubayanus*

could have acquired *S. cerevisiae* genetic material or vice versa. Though the precise mechanism, or mechanisms, for the independent formation of identical translocations remain unknown, the balance of evidence strongly favors multiple origins for lager-brewing yeasts over a single origin.

The genomic response of lager yeasts to domestication

Once hybridization events occurred in the Saaz and Frohberg lineages, both genomes appear to have experienced similar rates of increased evolution. While positive selection likely contributed some number of nonsynonymous substitutions, relaxation of purifying selection was probably the main driver of the increased rates of protein sequence evolution in these lineages. The different responses between the *S. cerevisiae* and *S. eubayanus* subgenomes could reflect the fact that the *S. cerevisiae* parental strains had more previous exposure to brewing environments.

Domestication may have led to a particularly striking relaxation of selection in the case of lager yeasts because hybridization likely prevented the new lineages from mating and recombining with their parental species through conventional meiotic means. During selective sweeps and passaging bottlenecks, the lineages would have been exposed to Muller's Ratchet (Muller 1964; Felsenstein 1974), increasing the average ω for the entire genome. Clonal interference is also known to occur in experimentally evolved populations of yeasts (Kao and Sherlock 2008; Lang et al. 2013) and the meiotic counterpart, the Hill-Robertson Effect (or interference), is often seen in plant and animal domestication along with elevated evolutionary rates (Hill and Robertson 1966; Doebley et al. 2006; Lu et al. 2006; Cruz et al. 2008; Wang et al. 2014). Many questions remain about the genetic process of domesticating lager yeasts, but the analysis of the near complete genome sequence of *S. eubayanus* has substantially clarified the origins of the major lineages of the interspecies *S. eubayanus x S. cerevisiae* hybrids used to brew lagers and provided a roadmap for future research.

MATERIALS AND METHODS

Strains and genomes

The genome for *S. cerevisiae* strain S288c was accessed through *Saccharomyces* Genome Database (SGD) (Cherry et al. 2012; Engel et al. 2014). The ale strain genomes, Foster's O and Foster's B, which were deposited by Borneman et al. (2011), were also accessed through SGD. The lager-brewing yeast genomes CBS 1513 (Saaz lineage) and Weinstephan34/70 (WS34/70, W34/70; Frohberg lineage) deposited by Walther et al. (2014) were accessed from DDBJ/EMBL/GenBank (accessions AZCJ01000000 and AZAA01000000, respectively). The assemblies for *S. uvarum* strain CBS 7001 and *S. paradoxus* strain CBS 432 were accessed through www.saccharomycessensustricto.org (Liti et al. 2009; Scannell et al. 2011). Mitochondrial sequences were for *S. cerevisiae, S. paradoxus*, and the Frohberg lineage of lager yeast were accessed through GenBank (accessions NC_001224, YP_006460229, and NC0012145, respectively) (Foury et al. 1998; Nakao et al. 2009; Procházka et al. 2012). It is unlikely that using a monosporic derivative of the type strain of *S. eubayanus* (FM1318), thereby removing any heterozygosity in the type strain of *S. eubayanus* (CRUB 1568^T = PYCC 6148^T = CBS 12357^T), had a significant impact on our analyses because the type strain itself has very low heterozygosity (0.0021%) (Hebly et al. 2015).

Genome sequencing

The fragment library was prepared according to our previously published Illumina genomic DNA library preparation protocol (Hittinger et al. 2010), with the exception that Illumina paired-end adapters and PCR primers were used. From this paired-end library, 35,394,604 Illumina MiSeq 300-bp reads were obtained with an insert size of 380 +/- 100 bp. The jumping library was prepared from genomic DNA using Illumina's Nextera Mate Pair Sample Preparation Guide (Illumina Part # 15035209, Rev. C, Jan 2013) and Nextera Mate Pair Sample Preparation Kit (Illumina Inc., San Diego, California, USA) with the

following modifications. For each sample, 4.5 µg of high molecular weight DNA was introduced to 12 µL of tagmentation enzyme and incubated at 55C for 27 min. After tagmentation, strand displacement and cleanup was performed as described in the protocol, and the DNA was loaded on a 0.6% agarose gel impregnated with GelGreen Nucleic Acid Stain (Biotinium Inc., Hayward, CA). The DNA was excised from the gel between 6-8 kb. Gel cleanup and size selection was verified using an Agilent Bioanalyzer High Sensitivity Chip (Agilent Technologies, Santa Clara, CA). Purified samples were circularized, and the remaining linear DNA was digested as described in the manufacturer's protocol. After circularization, the DNA was sheared to an average size of 400 bp using a Diagenode Bioruptor (Diagenode Inc, Denville, NJ) for 33 min, with cycles of 30s on, 30s off, on high power. Samples were end-repaired, a 3'-A was added to each fragment, Illumina adapters were ligated, and PCR was performed as described in the protocol. The final products were purified using Agencourt AMPure XP beads. The quality and quantity of the finished library were assessed using an Agilent DNA1000 series chip assay (Agilent Technologies, Santa Clara, CA) and Invitrogen Qubit HS Kit (Invitrogen, Carlsbad, California, USA), respectively, and the library was standardized to 2 µM. The library was sequenced using an Illumina HiSeq 2000, and images were analyzed using CASAVA version 1.8.2. From this jumping library, 14,144,070 100-bp mate-pair reads were obtained with an apparent insert size of 6593 +/- 914 bp.

Nuclear genome assembly

The Illumina mate-pair and paired-end libraries were both trimmed with CUTADAPT v. 1.3 (Martin 2011). Reads below 20 bp were discarded. Reads whose mates were discarded were retained as single-end reads. These processed reads were assembled using ALLPATHS-LG v. r44837 with the following options: frag_size = 500, frag_stddev = 100, insert_size = 3000, insert_stddev = 300, and PLOIDY = 1. Insert sizes were estimated by ALLPATHS-LG from the data during the assembly process. The ALLPATHS-LG assembly used 79.3% of reads from the fragment library, including 3,251,698 valid pairs, while it used

81.5% of reads from the jumping library, including 5,542,587 valid pairs. We also attempted an assembly by adding an additional jumping library that underwent no size selection, but the scaffold N50 declined from 896 kb to 764 kb, and several misassembles were introduced. For these discrepancies, the validity of the original assembly scaffolding was verified by PCR (supplementary fig. S4). In one case, the alternative assembly completely closed a gap, which was verified by PCR; this 2.8 kb segment was the only portion of the second assembly retained in the final assembly. Two adapter sequence contaminants at the edges of contigs were replaced with Ns.

Since there were so few unplaced scaffolds greater than 1 kb in the initial ALLPATHS-LG assembly, we considered each individually. We deleted four scaffolds that were completely contained elsewhere in the final genome, including two ALLPATHS-LG scaffolds that contained partial mitochondrial genomes redundant with the complete mitochondrial assembly generated by SPAdes (see below). BLAST searches against the genomes of *S. cerevisiae* and our *S. eubayanus* assembly suggested that three scaffolds likely belong in gaps at the *GAL2*, *PPH22*, and *PEP1* loci. To evaluate these possible placements, we mapped the mate-pair reads using BOWTIE2 with the default settings and compared how many paired mates supported joining all possible pairs of contigs. Ignoring joins supported by fewer than 100 paired mates, strong support was found to place each of these scaffolds (supplementary tables S5 and S6). Based on this evidence, the *PPH22* scaffold was fully placed into a gap, creating two smaller gaps. The *GAL2* scaffold was placed across and fully closed two small gaps, an assembly challenge caused by recent tandem duplication shared with *S. uvarum* (Hittinger et al. 2004). Partial genes encoding *PEP1/YBL017C* appeared at the edges of two different contigs in the middle of large scaffolds, an apparent segmental duplication of a small portion of chromosome II onto chromosome XIV. The BOWTIE2 mapping provided similar support for placing the unplaced *PEP1* scaffold in both places. Since the BLAST overlap with chromosome II was stronger, we placed the unplaced *PEP1* scaffold there, although the sequence likely

is present in both places. Two unplaced scaffolds contain interesting gene clusters (one with *MAL* pseudogenes and one with *FRE* genes involved in iron metabolism), but the BOWTIE2 mapping supported a handful of alternative placement scenarios that we did not investigate further. The remaining four unplaced scaffolds were short (less than 3500 kb) and only had homology to uncharacterized or dubious ORFs.

Nuclear genome annotation

We annotated the high-quality *de novo* genome assembly of *S. eubayanus* with a pipeline based on the Yeast Genome Annotation Pipeline (YGAP) (Proux-Wéra et al. 2012). After obtaining a draft annotation from YGAP, we imposed several criteria to do the following: 1) remove predicted protein-coding genes that did not encode complete open reading frames 2) to enforce GenBank-compatible annotations, and 3) to make orthology assignments explicit when the evidence was unambiguous. YGAP is designed to annotate yeast species that are closely related to *S. cerevisiae* and were similarly derived from a whole genome duplication event about 100 million years ago, as well as more distant relatives that were not subject to this whole genome duplication. Consequently, YGAP produces a Yeast Gene Order Browser (YGOB) (Proux-Wéra et al. 2012) "pillar" file that does not explicitly assign orthology, but rather lists both ohnologs (paralogs created by the whole genome duplication) that are present in *S. cerevisiae* as possible annotations. We assigned orthology by imposing a strict synteny requirement: orthology was assigned only when the gene with two pillar annotations was adjacent to a gene whose orthology had already been assigned and when synteny was conserved between *S. cerevisiae* and *S. eubayanus*. Only three genes could not be annotated unambiguously by this procedure, all of which are adjacent to translocations or inversions.

We also corrected some annotations to conform to GenBank conventions and standards. Specifically, if a coding sequence (CDS) was predicted to continue into a gap, it was marked as partial to exclude the gap from the CDS region. If a CDS had an internal stop codon, its annotation was deleted. tRNA annotations whose cognate amino acid could not be identified were deleted. If a predicted CDS did not begin with a start codon, we scanned upstream until we found a start codon, encountered a stop codon, or reached a gap; the longest possible open reading frame (ORF) was then annotated; in some cases, the frame was corrected. For a CDS that did not end in a stop codon, we simply scanned downstream until we reached the nearest in frame stop codon. We also deleted annotations for all CDS with fewer than 10 amino acid residues, duplicate annotations, and annotations for which at least half of the sequence was a gap or ambiguous sequence. We modified three predicted introns to match splice donor and acceptor consensus sites and deleted one predicted intron where no splice donor and acceptor consensus sites could be found. We also deleted two predicted novel genes that completely overlapped with *S. cerevisiae* homologs.

To make the dot plot figure showing the synteny between *S. eubayanus* and *S. cerevisiae* (fig. 1), we concatenated every chromosome sequence together to make one continuous sequence and generated an index file to indicate where each chromosome started and ended. We then used the program LASTZ (Harris 2007) with the default settings to perform the genome alignment and generate a tab-delimited file. We filtered out any alignment less than 1 kb and used 'R' to make the dot plot.

Mitochondrial genome assembly and annotation

To recover the mitochondrial genome, the trimmed paired-end reads were assembled with SPAdes 3.1.1 (Bankevich et al. 2012). Since mtDNA has a higher AT content than nuclear DNA and has multiple copies per cell, we analyzed the distribution of GC content and the coverage among the resulting contigs with

lengths >5 kb. One contig of about 64 kb was detected with a GC content of 17.5% and 14.6-fold higher coverage than most other contigs, all of which had >35% GC content (supplementary fig. S5). Since this contig matched the expected size and characteristics of *S. eubayanus* mitochondrial genome, inferred from the Frohberg lager yeast mitochondrial genome (Nakao et al. 2009), the contig was selected as the putative mitochondrial genome. We found evidence of 41 *Saccharomyces cerevisiae* mitochondrial genes using BLASTN (Altschul et al. 1997), including 25 tRNAs and 2 rRNAs. Location of rRNAs were confirmed with RNammer1.2 (Lagesen et al. 2007). Additional searches using BLASTX-Q3 were performed using *Saccharomyces paradoxus* proteins sequences as queries. The genome arrangement of the genes were plotted and compared to *S. cerevisiae* and Frohberg lineage lager-brewing yeast mitochondrial genomes (fig. 2 and supplementary figs. S1 and S2 and supplementary results S1).

Analysis of genome evolution

The sequences used to analyze both nonsynonymous and synonymous changes during genome evolution (fig. 5) were the 1:1:1:1:1:1 ortholog sets produced using YGAP and the procedure described above, while those used to analyze the rate of synonymous change between the lager lineages (fig. 6) were the 1:1:1:1:1 ortholog sets. To prepare sequences for evolutionary analysis the translated amino acid sequences of orthologs were first aligned by MUSCLE v.3.8.31 (Edgar 2004). Alignments with PRANK (Löytynoja 2014) produced nearly identical results for the genes *ADR1, ERT1, FET3*, and *NOT3.* Indels between orthologs were then removed from the original untranslated sequences using PAL2NALv.14 (Suyama et al. 2006). The evolutionary analysis for each set of orthologs was performed using the CODEML package of PAMLv.4.7 (Yang 2007) assuming the F3x4 codon frequency model, and implementing the free ratio branch model. The free ratio branch model assumes an independent ω for each branch and does not assume a molecular clock. The number of synonymous and nonsynonymous substitutions was estimated for each gene by multiplying d_n by the number of nonsynonymous sites in

the gene (*N*) and d_s by the number of synonymous sites in the gene (*S*). We treated each gene as an independent event in order to make the data parametric, and for each gene, we calculated the proportion of nonsynonymous changes out of all substitutions ($N*d_N / (S*d_S + N*d_N)$). The distributions of these values were compared between genomes and subgenomes by logistic regression as implemented in R version 3.1.0. 99% Binomial confidence intervals were calculated in the MSTATv.6.1.1 statistical package (http://mcardle.wisc.edu/mstat/) to establish the statistical difference in the quantity of synonymous changes between the *S. eubayanus* and *S. cerevisiae* subgenomes of the lager-brewing yeasts (supplementary table S4) and to construct error bars in fig. 5.

Gene analyses

For sequences from ale strains and *S. eubayanus,* genome locations were found by BLAST using S288c and, where applicable, known lager yeast gene sequences as queries. The sequences of interest were then extracted manually with custom scripts. BLAST searches were also performed on over 100 publicly available *S. cerevisiae* genomes (Bergström et al. 2014; Strope et al. 2015), but searches of these genomes failed to further clarify the origins of any lager *MAL* genes. The percent identity between gene sequences, either amino acid or nucleotide as applicable, was calculated from MUSCLE alignments using Clustal2.1 accessed from the MUSCLE server (http://www.ebi.ac.uk/Tools/msa/muscle/). Gene trees were made in MEGA 6.06 (http://www.megasoftware.net/) (Tamura et al. 2013). The parameters to construct each tree were determined by the "Find Best-Fit Substitution Model (ML)" package within MEGA for nucleotide sequences using default parameters. Trees were constructed using the indicated best fitting model and parameters; robustness was assessed using 1000 bootstrap replicates.

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Chapter 3

Evolution of a novel chimeric maltotriose transporter in *Saccharomyces eubayanus*.

Supplementary information from this chapter can be found in Appendix B.

This chapter is a modification of a soon-to-be-submitted manuscript.

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C.T. Hittinger conceived of this project, helped write the manuscript, supervised all work, and provided financial support.
I contributed to the conception of this project, designed and performed all experiments and analyses, and wrote this manuscript.

ABSTRACT

At the molecular level, the evolution of new traits can be broadly divided between changes in gene expression and changes in protein structure. Each of these categories of changes have their own factors governing the ways in which they can evolve. Environmental factors, both internal and external, can influence the evolution of changes in gene expression, while protein structure evolution is generally thought to proceed either through sequential point mutations or recombination of whole functional units. In *Saccharomyces* yeasts the uptake of the important brewing related sugar, maltotriose, is known to be the primary limiting factor in its utilization. Within the species *Saccharomyces eubayanus* there are no known strains that are able to consume maltotriose. Here we describe the results of a directed evolution experiment of genetically distinct strains of *S. eubayanus* to maltotriose utilization. We found that our experimental selection conditions likely played a role in inhibiting the evolution of expression of a native maltotriose transporter. In an experimental replicate that did evolve maltotriose consumption, we mapped the causative locus to a novel chimeric transporter protein. Interestingly, this chimeric maltotriose transporter was formed by recombination between two transporters that are unable to carry maltotriose. In addition, the recombination event occurred without regard to functional motifs. While highlighting the constraints on the evolution of gene expression these results also provide important insights into the mutations and mutational pathways that are open to proteins to evolve new functions.

INTRODUCTION

Proteins that can transport maltotriose into the cell are relatively rare in *Saccharomyces* (Han et al. 1995; Dietvorst et al. 2005; Salema-Oom et al. 2005; Brown et al. 2010; Horák 2013). The scarcity of maltotriose transporters suggests that import of maltotriose, a key step in its utilization, is an uncommon function for the sugar transporters present in *Saccharomyces* to evolve. Consumption of this trisaccharide by *Saccharomyces* yeasts is of general interest to the brewing community since a key consideration for any new brewing strain is its ability to rapidly and completely use all brewing-related sugars. Of the sugars present in brewing wort, maltose is the most abundant followed by maltotriose and glucose. Of these, maltotriose is by far the most difficult to ferment (Briggs et al. 2004; Briggs D.E., Brookes P.A., Stevens R. 2004; Eßlinger 2009), though it comprises around 20% of fermentable sugars in wort (Meussdoerffer and Zarnkow 2009). Maltotriose is poorly utilized or completely unutilized by many brewing strains of *Saccharomyces*, and the primary hinderance is the inability of cells to transport the sugar across the plasma membrane (Wang et al. 2002; Rautio and Londesborough 2003). In brewing, this leads to large amounts of unconsumed sugar, lower amounts of ethanol, and a flavor profile that is regarded as undesirable in most beer styles. As a result, there is considerable interest in the identification of proteins that can support maltotriose utilization and of strains carrying such genes.

Recently, special interest has been given to the development of *Saccharomyces eubayanus* for commercial brewing (Hebly et al. 2015; Krogerus et al. 2015; Krogerus, Magalhães, et al. 2017; Hittinger et al. 2018). As a hybrid with *Saccharomyces cerevisiae, S. eubayanus* forms the industrially important lager-brewing yeasts (Libkind et al. 2011), accounting for more than 90% of the total market. The importance of these *S. eubayanus* derived strains to the brewing industry also accounts for the emphasis on the commercial development of this newly discovered addition to the genus *Saccharomyces* (Libkind et al. 2011). So far, no strain of *Saccharomyces eubayanus* isolated from nature has been reported to

consume maltotriose (Gibson et al. 2013; Bing et al. 2014; Peris et al. 2014; Hebly et al. 2015; Peris & Langdon et al. 2016; Gibson et al. 2017), despite evidence for the presence of functional transporters in the *S. eubayanus* subgenome of industrial *S. cerevisiae* x *S. eubayanus*(Nguyen and Boekhout 2017) hybrids (i.e. lager-brewing yeasts) (Dietvorst et al. 2005; Nakao et al. 2009; Vidgren et al. 2010; Cousseau et al. 2013; Baker et al. 2015).

In yeasts that are capable of maltotriose consumption, the sugar is taken up by a small number of genes in the maltose transporter (*MALT*) family (Han et al. 1995; Dietvorst et al. 2005; Salema-Oom et al. 2005; Brown et al. 2010). While a number of transporters in this family have been characterized that can carry maltose and other sugars (Brown et al. 2010), maltotriose transporters are more rare, reflecting the general difficultly of transporting higher molecular weight sugars, such as dextrins and starch (Barnett 1992; Briggs et al. 2004). Work on improving direct uptake of maltotriose in brewing yeasts has focused on expression of this limited set of known maltotriose transporters, through directed evolution for increased expression (Jansen et al. 2004; Brickwedde et al. 2017), introducing maltotriose transporters into new strains through selective breeding (Stewart 1981; Bilinski and Casey 1989; Mukai et al. 2001; Hebly et al. 2015; Krogerus et al. 2015; Mertens et al. 2015; Krogerus et al. 2016; Krogerus, Seppänen-Laakso, et al. 2017; Nikulin et al. 2018), or by heterologous expression (Stewart 1981; Jansen et al. 2004; Yamakawa et al. 2010). These methods all rely on the presence of functional maltotriose transporters, either natively or heterologously expressed and are limited by the number of strains and proteins that are known to be capable of transporting maltotriose. For instance, almost all synthetic lager hybrids that have been reported to date have utilized the type strain of *S. eubayanus* (Hebly et al. 2015; Krogerus et al. 2015; Mertens et al. 2015; Krogerus et al. 2016; Krogerus, Seppänen-Laakso, et al. 2017; Nikulin et al. 2018), which is unable to consume maltotriose (Gibson et al. 2013) and whose genome contains no known maltotriose transporters (Baker et al. 2015). As a result, all synthetic lager hybrids must rely on

the *S. cerevisiae* parent to supply maltotriose utilization, limiting the *S. cerevisiae* parent to strains that already have this capability.

How new maltotriose transporters can form is less well studied than the expression or function of known transporters (Smit and Dissertation 2007; Smit et al. 2008). Proteins with novel functions can arise through a variety of methods (Long et al. 2003). Rarely, new proteins can spring forth from previously non-coding regions of the genome. More commonly, new proteins evolve by duplication and subsequent divergence. Evolution through stepwise point mutations can be a slow and constrained process though. Deleterious epistatic interactions, at the intermediate mutational steps separating the original protein from the derived protein, can make new functions difficult to access by successive point mutations. Mutational events that result in multiple amino acid changes at once can help bridge fitness valleys and speed the evolution of new functionality (Cui et al. 2002; Bittihn and Tsimring 2017). Ectopic gene conversion resulting in chimeric protein sequences are such a mutational event.

Chimeric coding sequences have been found to be an important mechanism by which proteins can evolve new functions (Henikoff et al. 1997; Patthy 1999; Long et al. 2003; Patthy 2003), and they have been implicated in playing a significant role at the cellular level in both infectious and non-infectious diseases in humans (Mitelman et al. 2007; Malfavon-Borja et al. 2013; Rippey et al. 2013; Leffler et al. 2017). Chimeric proteins have been most widely studied in the context of metazoan genomes. One of the first recently evolved chimeric genes to have both its origin and subsequent evolution characterized in depth was the gene *jingwei* in *Drosophila* (Long et al. 2003). The *jingwei* gene combines parts of a testis specific protein with a retrotransposed alcohol dehydrogenase. *Jingwei* has been shown to be expressed in testis and conserved between two species of *Drosophila* across 2 million years (Wang et al. 2000). *Jingwei* exemplifies many of the characteristics usually associated with chimeric proteins

(Henikoff et al. 1997; Patthy 1999; Long et al. 2003; Patthy 2003). Like most other chimeric proteins that have been described in eukaryotes, *jingwei* is a large multidomain protein that was constructed via the movement of whole functional units (domains) facilitated by intronic sequences, a process referred to as domain or exon shuffling. Movement of whole proteins modules has been considered key to the evolution of functional chimeras. Even in the absence of intronic sequences, exchange of whole functional modules is thought to be important for the formation of functional proteins. But exchange of entire, independently functional, units is not the only method by which functional chimeric proteins can be generated.

Alternatively, recombination without regard for functional domains also has the potential to create proteins with novel characteristics. Recombination within domains can lead to functional proteins even between non-homologous protein sequences (Bogarad and Deem 1999; Cui et al. 2002; Rogers and Hartl 2012). However, because functionally important structures are likely to be conserved between related proteins, it has been found that recombination between homologous sequences, which is less likely to disturb essential within-protein interactions, is more likely to result in functional proteins (Cui et al. 2002; Voigt et al. 2002; Rogers and Hartl 2012). Theoretical work has demonstrated the potential of recombination to allow proteins to rapidly bypass fitness minima in the adaptive landscape separating two protein functions (Cui et al. 2002; Bittihn and Tsimring 2017). Making use of this foundation, protein engineering has utilized recombination between homologous sequences (often called DNA shuffling) followed by selection for the function of interest with great success (reviewed in(Giver and Arnold 1998; Minshull and Willem Stemmer 1999; Cole and Gaucher 2011)). Researchers utilizing DNA shuffling have found that the function of chimeric proteins is often unpredictable based on the parent sequences and functions alone (Campbell et al. 1997; Giver and Arnold 1998). Just a small sample of the accomplishments in this area includes: increasing the brightness of green fluorescent protein (Crameri

et al. 1996), the evolution of a recombinase that can efficiently excise integrated HIV provirus (Sarkar et al. 2007), and the construction of hexose transporters with increased specificity to D-xylose (Nijland et al. 2018). More recently, recombination between paralogous sequences have been shown to be selected for in natural populations, suggesting that such sequences have been preserved for specific functional reasons (Thomas 2006; Rogers and Hartl 2012).

In the present study, we characterize the native *MALT* genes found in *S. eubayanus* for their ability to enable the transport of maltotriose and confirm the presence of such genes in some strains of *S. eubayanus.* We also describe a novel chimeric maltotriose transporter that resulted from directed evolution of *S. eubayanus* for maltotriose consumption. Rather than being a gain-of-function mutation through domain swapping, this new maltotriose transporter was formed through ectopic gene conversion between two *MALT* genes whose protein products could not transport maltotriose. In this way, we increase both the number of known maltotriose transporters and the number of strains known to carry them and also provide insight into how proteins gain novel functions.

RESULTS/DISCUSSION

Maltotriose transporters in *S. eubayanus*

In the type strain of *S.* eubayanus, four genes, designated *MALT1-4,* have been identified as having homology to genes encoding known maltose transporters (*MALT* genes) (Baker et al. 2015; Okuno et al. 2016). Because *MALT2* and *MALT4* encode identical amino acid sequence (see Materials and Methods), we refer to these genes jointly as *MALT2/4*. To determine if they could enable maltotriose transport, Malt1, Malt2/4, and Malt3 were individually overexpressed using an inducible promoter in yHRVM108, a strain of *S. eubayanus* isolated from North Carolina that is unable to grow on maltotriose and, unlike

other strains of *S. eubayanus,* has sluggish growth on maltose. None of these genes were able to confer growth on maltotriose when overexpressed (Table 1).

Strain	Background	Transporter	Initial OD	Day 3	Day 6
yHRVM108	North Carolinian strain	-	0.11 (+/- 0.01)	0.36 (+/- 0.02)	0.78 (+/-0.15)
yHEB1870	yHRVM108	*MALT1*	0.13 (+/- 0.03)	0.43 (+/- 0.04)	0.58 (+/-0.04)
yHEB1877	yHRVM108	*MALT2/4*	0.11 (+/- 0.00)	0.39 (+/- 0.01)	0.57 (+/-0.02)
yHEB1872	yHRVM108	*MALT3*	0.13 (+/- 0.01)	0.41 (+/- 0.00)	0.62 (+/-0.5)
yHEB1883	yHRVM108	*ncAGT1*	0.11 (+/- 0.01)	0.54 (+/- 0.07)	1.34 (+/-0.10)

Table 1. Growth on maltotriose of strains expressing MALT genes on a doxycycline-inducible plasmid.

N = 3, standard deviation in parentheses.

Although none of the transporters found in the type strain of *S. eubayanus* were able to support growth on maltotriose, there is compelling evidence from lager-brewing yeasts for the existence of maltotriose transporters within the greater *S. eubayanus* population (Dietvorst et al. 2005; Nakao et al. 2009; Vidgren et al. 2010; Cousseau et al. 2013; Baker et al. 2015). Of particular interest are alleles of *AGT1*. Two versions of *AGT1* are present in the genomes of lager-brewing yeasts. One, which we call *scAGT1* (*S. cerevisiae-AGT1*), was donated by the *S. cerevisiae* parent of lager yeasts, and the other, which we call *lgAGT1* (lager-*AGT1*), was proposed to be of *S. eubayanus* origin (Nakao et al. 2009). Both *lgAGT1* and *scAGT1,* like other *AGT1* alleles, can transport maltotriose (Han et al. 1995; Day, Rogers, et al. 2002; Dietvorst et al. 2005; Vidgren et al. 2005; Vidgren et al. 2009; Vidgren and Londesborough 2012; Cousseau et al. 2013). Thus far, full-length sequences closely related to this *lgAGT1* have not been described in any strain of *S. eubayanus* (Hebly et al. 2015).

Strain CDFM21L.1 and a closely related strain isolated from North Carolina, yHRVM108, belong to the Holarctic subpopulation of *S. eubayanus* and are close relatives of the strains of *S. eubayanus* that hybridized with *S. cerevisiae* to form lager-brewing yeasts (Peris & Langdon et al. 2016). Because of their close phylogenetic relationship, CDFM21L.1, yHRVM108, and the *S. eubayanus* lager parent are more likely to share strain specific genes, like *lgAGT1,* in common (Bergström et al. 2014). From a search of Illumina reads sequences available for CDFM21L.1 and yHRVM108, we were able to assemble two full-length genes with high sequence identity to *lgAGT1,* which we designated *tbAGT1* and *ncAGT1,* for Tibetan-*AGT1* and North Carolinian-*AGT1*, respectively (Fig. 1).

A.

nuc	*lgAGT1*	*ncAGT1*	*scAGT1*	*AGT1*
tbAGT1	99.89	95.74	79.26	80.16
lgAGT1		95.63	79.15	80.05
ncAGT1			79.75	80.66
scAGT1				98.64

aa	lgAgt1	ncAgt1	scAgt1	Agt1
tbAgt1	99.84	95.57	83.74	86.07
lgAgt1		95.41	83.58	85.90
ncAgt1			83.25	85.41
scAgt1				97.24

B.

C.

Figure 1. Alignment of *AGT1*-like genes. A) Tables highlighting the nucleotide (nuc) and amino acid (aa) percent identities between members of the *AGT1* family. Darker colors indicate greater sequence similarity. B) Multiple sequence alignment between nucleotide sequences of *tbAGT1, lgAGT1*, and *ncAGT1*. Black lines indicate nucleotide differences. C) Multiple sequence alignments between protein sequences of *tbAGT1, lgAGT1*, and *ncAGT1*. White gaps indicate amino acid differences.

Two single nucleotide polymorphisms (SNPs) separate *tbAGT1* and *lgAGT1.* One SNP results in a synonymous substitution and the other in a nonsynonymous substitution near the N-terminus of the protein outside of any predicted transmembrane domains (Fig. 1B & C, Fig. S1). Analyses of the predicted effect of this substitution in *lgAGT1* (using STRUM and SIFT mutant protein prediction software (Ng and Henikoff 2001; Quan et al. 2016)) suggest that it is unlikely to significantly impact protein structure or function (Table S1). In contrast *ncAGT1* has 95% nucleotide identity with *lgAGT1,* with nonsynonymous differences distributed throughout the sequence (Fig. 1A-C). Despite the presence of *ncAGT1*, yHRVM108 grows poorly on maltose and is unable to grow on maltotriose, raising the question of whether the ability to transport maltotriose has been conserved between ncAgt1 and lgAgt1. Interestingly, and unlike all *MALT* genes found in the Patagonian type strain of *S. eubayanus*, overexpression of ncAgt1 in yHRVM108 conferred growth on maltotriose (Table 1), suggesting that insufficient *ncAGT1* gene expression, rather than protein function, is likely the main reason for the inability of yHRVM108 to grow on maltotriose.

Phylogenetic relationship among maltose transporters

To put the relationship between *S. eubayanus, S. cerevisiae,* and lager *MALT* genes into a phylogenetic perspective, a gene tree was constructed for these three groups of genes (Fig. 2). Consistent with previous analyses of *MALT* genes in *Saccharomyces* (Brown et al. 2010), the *MALT* genes fell into 3 major clades. *MPH* genes, encoding maltose transporters native to *S. cerevisiae* but also present in some lager yeasts (Day, Higgins, et al. 2002; Vidgren et al. 2005), formed their own clade. The largest clade was made up of *MALT1-4* from *S. eubayanus, MALx1* genes from *S. cerevisiae,* and the lager-specific gene *MTT1* (Dietvorst et al. 2005; Salema-Oom et al. 2005; Baker et al. 2015). This clade was further subdivided into a group containing only *S. eubayanus MALT* genes and their close lager homologs, and a group consisting of *MALx1* genes, *MTT1*, and *MALT3*. The final major clade was significantly divergent

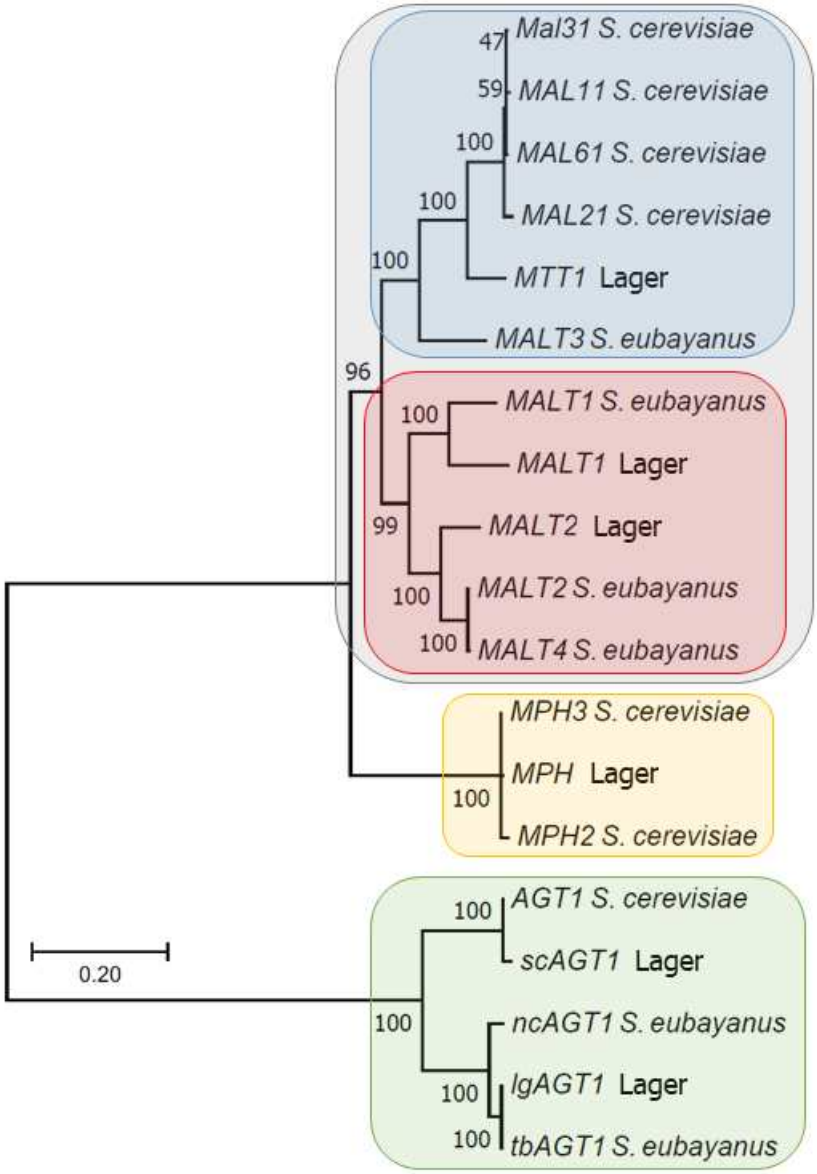


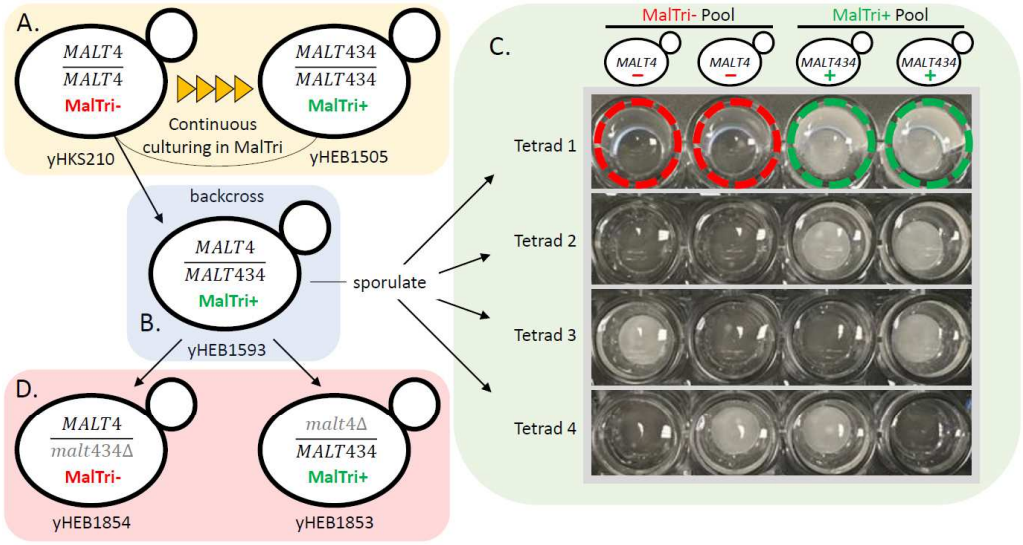
Figure 2. Phylogeny of *Saccharomyces MALT* genes. ML phylogenetic tree of *MALT* genes described in *S. cerevisiae, S. eubayanus*, and lager-brewing hybrids. The scale bar equals the number of nucleotide substitutions per site.

from the other two. This clade consisted of the *AGT1* genes and was further split between *AGT1* genes originating from *S. cerevisiae* and *AGT1* genes originating from *S. eubayanus.*

Evolution of maltotriose consumption

Since yHRVM108 already contains a functional maltotriose transporter, we decided to see if it could readily evolve maltotriose consumption through a directed evolution experiment. We also decided to try to experimentally evolve maltotriose utilization in FM1318 (Libkind et al. 2011) and in yHKS210 (Peris et al. 2014), strains that lack transporters capable of conferring maltotriose utilization, even when overexpressed (Table 1). A search of the available genome sequence reads for FM1318 (Baker et al. 2015; Okuno et al. 2016) and yHKS210 (Peris & Langdon et al. 2016) confirmed that neither of these strains contain genes that are closely related to *AGT1*-like genes or other known maltotriose transporters (Dietvorst et al. 2005; Salema-Oom et al. 2005). Since none of these strains could grow on maltotriose, a small amount of glucose was also added to the media to permit a limited number of cell divisions to allow for mutation and selection to occur. Each strain was set up in triplicate and evolved by serial passaging in liquid media. Strains that could not use the primary carbon source in the directed evolution medium underwent approximately one cell division per day on average.

Over the course of 100 passages, representing approximately 3,150 cell divisions in total between all the strains and replicates, only a single replicate evolved the ability to grow in maltotriose. Surprisingly, it was not a replicate of yHRVM108, but one of yHKS210 that evolved maltotriose consumption. Two single-colony isolates (yHEB1505-6) from this replicate were isolated and confirmed to be able to grow on maltotriose without added glucose (Fig. 3A, Table S2).



E.

Strain	Description	Initial OD	Day 3
yHKS210	Wild admixture strain	0.12 (+/-0.02)	0.38 (+/- 0.01)
yHEB1505	Single-colony isolate of yHKS210 evolved in maltotriose	0.12 (+/-0.02)	1.45 (+/- 0.01)
yHEB1593	yHKS210 x yHEB1505	0.12 (+/-0.01)	1.24 (+/- 0.02)
yHEB1854	yHEB1593 *malt434Δ*	0.13 (+/-0.01)	0.39 (+/- 0.01)
yHEB1853	yHEB1593 *malt4Δ*	0.11 (+/-0.01)	1.33 (+/- 0.04)

Figure 3. Evolution and validation of chimeric maltotriose transporter, Malt434. A) After continuous culturing in maltotriose with a small amount of added glucose, yHKS210, which was originally unable to use maltotriose (MalTri-), gained the ability to consume maltotriose (MalTri+) and replaced both alleles of *MALT4* with a new chimeric gene, *MALT434*. B) Strain yHEB1593, which is a backcross between yHKS210 and yHEB1505, is also MalTri+. C) To test the inheritance of maltotriose utilization, yHEB1593 was sporulated. Figure shows a subset of tetrads screened growing on SC + 2% maltotriose. Examples of MalTri- spores in tetrad 1 are circled in red, and MalTri+ examples are circled in green. Whole genome sequencing of MalTri+ and MalTri- pools showed that maltotriose utilization perfectly correlated with the presence/absence of *MALT434*. D) Reciprocal hemizygosity test of the *MALT4/MALT434* locus in the backcross strain yHEB1593. E) Table of initial and day-three OD_{600} (OD) readings of yHKS210, yHEB1505, yHEB1593, yHEB1853, and yHEB1854 on SC + 2% maltotriose as the sole carbon source. N = 3, standard deviation in parentheses.

Indirect evolution of maltotriose consumption

Initially, we anticipated that it would be simple for yHRVM108 to evolve the ability to utilize maltotriose because it already contained a transporter whose expression allows for strong maltotriose utilization in the parent background (Table 1). However, over the course of 100 passages, representing around 1,050 generations between the three replicates of yHRVM108, no maltotriose-utilizing lineage of yHRVM108 arose. While evolving yHRVM108 under our maltotriose selection regime was not successful, we were surprised to find an alternative and indirect selection regime could evolve maltotriose utilization in this background. When we began directed evolution of *S. eubayanus* to maltotriose, we also started another directed evolution experiment to try to improve yHRVM108's sluggish growth in maltose by selecting for growth on maltose. Here, two of three replicates of yHRVM108 evolved the ability to grow rapidly on maltose within 110 passages in maltose. On average, single-colony isolates from these replicates grew twice as fast as the unevolved parent over two days in maltose (Table S3). Interestingly, these isolates also gained the ability to utilize maltotriose (Table S2), despite never being exposed to maltotriose during the course of directed evolution. The fact that maltotriose consumption independently evolved at least twice under directed evolution for maltose utilization suggests that our maltotriose selection regime itself may have played a role in restraining evolution.

Although we found the difficulty of evolving expression of a functional transporter surprising, such a result is not unprecedented. In a long-term evolution experiment in *E. coli*, a functioning citrate transporter was present in the founding strain. Though expression of this gene would have been highly favored in the citrate-rich experimental environment it took thousands of generations, even after the necessary potentiating mutations had appeared, before a gene amplification/rearrangement event joined the citrate transporter gene to a new promoter, resulting in a novel expression pattern (Blount et al. 2012). Such results display the importance of both the genetic and external environment in

supporting or discouraging evolution along a particular path. In retrospect, what appeared to be a simple request, to turn on the *ncAGT1* gene in the condition being selected for, may in fact have been quite difficult by simple mutations, whereas our indirect selection regime on maltose proved more effective.

Evolution of maltotriose utilization through a chimeric transporter

To determine the genetic architecture of maltotriose utilization in the replicate of yHKS210 that evolved the ability to grow in maltotriose, we set up an F_1 backcross between evolved maltotriose utilizing isolate yHEB1505 and the parent strain (yHKS210), producing strain yHEB1593, a putative heterozygote capable of growth on maltotriose (Fig. 3B & E). In a test of 15 fully viable F_2 tetrads, maltotriose utilization segregated in a perfect 2:2 manner (Fig. 3C). These results suggested that the ability of the evolved strain to utilize maltotriose is conferred by a dominant mutation at a single genetic locus. We performed bulk-segregant analysis (Brauer et al. 2006; Segrè et al. 2006; Ehrenreich et al. 2010) using strains derived from the F_2 spores, dividing them between those that could (MalTri$^+$) and those that could not (MalTri$^-$) utilize maltotriose (Fig. 3C), with a total of 30 strains in each category. Twelve 1-kb regions were identified as containing fixed differences between the MalTri$^+$ and MalTri$^-$ strains. Of these regions, eight mapped to genes encoding ribosomal proteins and most likely represent assembly artefacts due to the presence of many closely related paralogs. Three other regions contained fixed changes between the MalTri$^+$ and MalTri$^-$ groups but had no clear relationship to carbon metabolism. The final 1-kb region mapped to the *MALT4* locus of *S. eubayanus* genome (Baker et al. 2015; Okuno et al. 2016). The coding sequence of *MALT4* from the MalTri$^+$ group contained 52 SNPs relative to the *MALT4* allele found in yHKS210. All SNPs occurred within a single 230-bp region. Of these, 11 were predicted to lead to non-synonymous changes. Closer inspection revealed that the changes within the 230-bp region were the result of an ectopic gene conversion event between *MALT4* and *MALT3,* creating

a chimeric gene (Fig. 4), likely through ectopic gene conversion. We call this chimeric *MALT4* allele *MALT434* after the arrangement of sequences from its parent genes. The sequence of *MALT3* was not impacted by this mutational event.

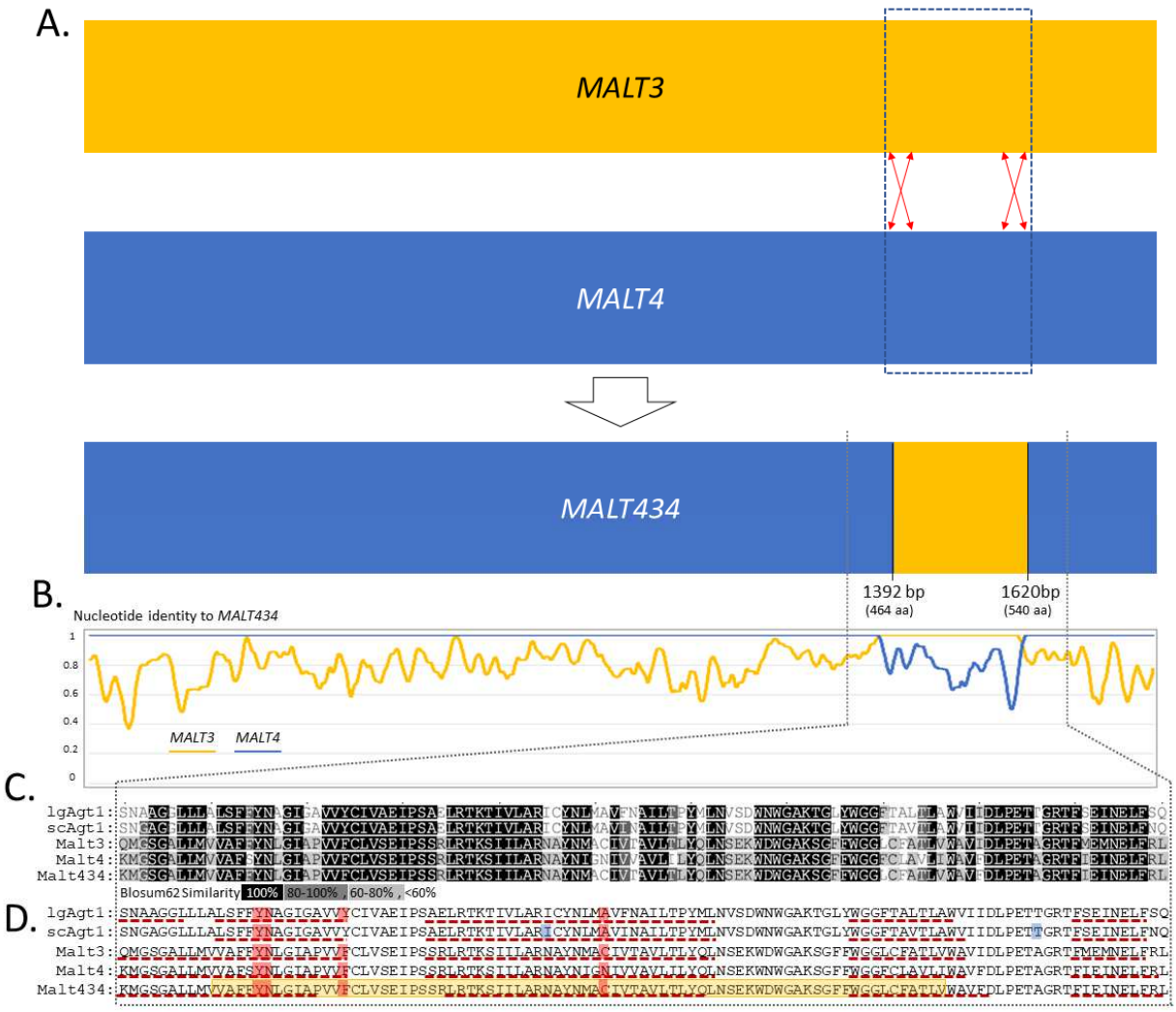
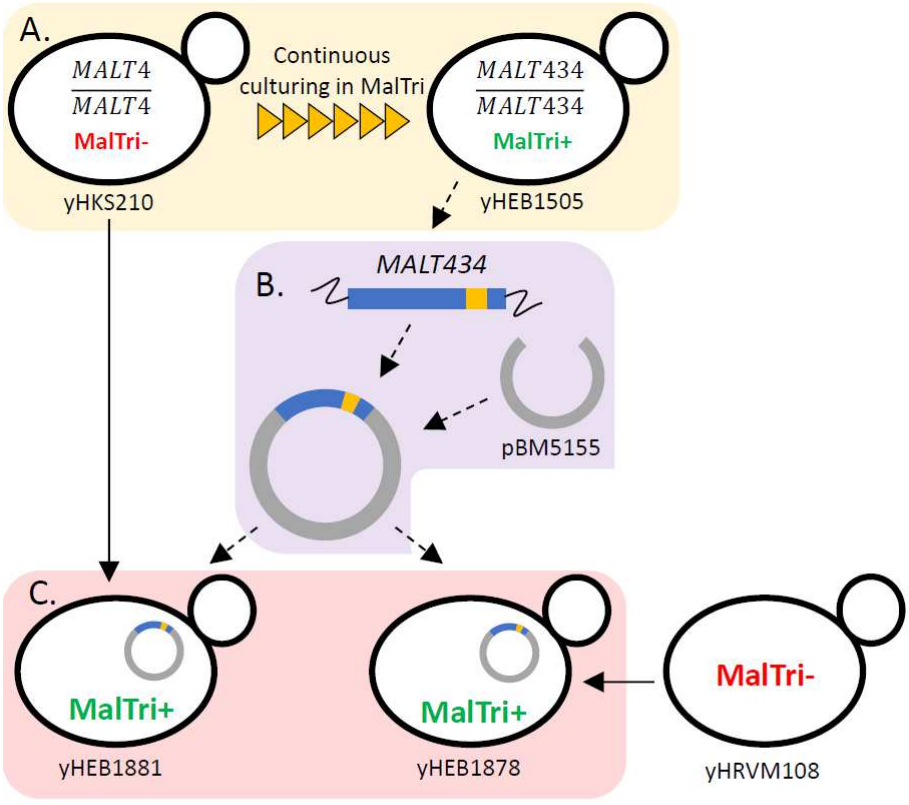


Figure 4. Sequence structure of *MALT434*. A) Schematic of the origin of *MALT434*. B) Line graphs representing the identity between nucleotide sequences of *MALT3* and *MALT4* from yHKS210 to *MALT434* over 10-bp sliding windows. C-D) Segment of the alignment of the chimeric region between Malt3, Malt4, Malt434, scAgt1, and lgAgt1. The region highlighted in yellow in the Malt434 sequence indicates the chimeric region. The regions underlined with a red dashed line are predicted transmembrane domains. The amino acids highlighted in red are predicted maltose binding residues. The residues highlighted in blue were experimentally found to be important for maltotriose transport by Smit *et al.* 2008.

To confirm that *MALT434* was the causative locus of maltotriose utilization, we performed a test of reciprocal hemizygosity in the heterozygous F_1 backcross strain (Fig. 3D). Removal of *MALT434* eliminated the F_1 backcross strain's ability to utilize maltotriose (Fig. 3E), demonstrating that *MALT434* is required for maltotriose utilization. Conversely, replacing the parental, non-chimeric allele of *MALT4* in the heterozygous F_1 backcross strain had no impact on maltotriose utilization. Furthermore, overexpression of Malt434 in both the unevolved parent, yHKS210, and in the yHRVM108 background (Fig. 5) supported growth in maltotriose, demonstrating that overexpression of Malt434 is sufficient to confer maltotriose utilization. These results confirm that the mutant *MALT434* gene encodes a functional maltotriose transporter.



D.

Strain	Plasmid	Initial OD	Day 6
yHKS210	-	0.12 (+/-0.02)	0.55 (+/-0.02)
yHEB1881	*MALT434*	0.11 (+/- 0.01)	1.31 (+/- 0.05)
yHRVM108	-	0.11 (+/-0.01)	0.78 (+/-0.15)
yHEB1878	*MALT434*	0.12 (+/-0.01)	1.46 (+/-0.04)

Figure 5. Heterologous expression of *MALT434* supports growth in maltotriose. A) Evolution of non-maltotriose utilizing strain (MalTri-), yHKS210, to maltotriose utilizing (MalTri+) strain, yHEB1505, by serial passing on maltotriose containing media (same as fig. 3A). B) Insertion of *MALT434* into vector pBM5155 for doxycycline-inducible heterologous expression in MalTri- strains. C) Transformation of *MALT434* expression plasmid in MalTri- *S. eubayanus* strains yHKS210 and yHRVM108. D) Table of initial and day-six OD_{600} (OD) measurements of parent strains and strains carrying the *MALT434* expression plasmid grown in SC media with maltotriose as the sole carbon and doxycycline to induce plasmid expression.

Potential structural impact of Malt434 chimerism

It was surprising that a sequence from *MALT3* permitted *MALT4* to now encode a maltotriose transporter because neither *MALT3* nor *MALT4* supported maltotriose utilization on their own (Table 1). Malt3 and Malt4 share about 80% amino acid identity overall and 85% amino acid identity in the chimeric region specifically (Fig. 4B). Most residues in the chimeric region had high similarity between Malt3 and Malt4, as measured by Blosum62 similarity matrix (Fig. 4C) (Henikoff and Henikoff 1992), but there were a handful of low-similarity amino acids as well. To gain insight into what changes in protein structure may be driving the new functionality of Malt434, we used I-TASSER (Zhang 2008; Roy et al. 2010; Yang et al. 2015) to predict the protein structure of Malt3, Malt4, and Malt434. I-TASSER predicts a protein's structure based on its homology to proteins whose structures have already been solved. Consistent with other studies on the structure of maltose transporters in *Saccharomyces* (Cheng and Michels 1989; Han et al. 1995; Barrett et al. 1999; Yan 2015), I-TASSER predicted that Malt3, Malt4, and Malt434 were similar to members of the Major Facilitator Superfamily (MFS) of transporters, specifically the sugar porter family (Yan 2015). Protein structure is predicted to be conserved between Malt3 and Malt4, including within the chimeric region, which encompasses one full transmembrane domain and parts of two other transmembrane domains (Fig. 4D). Four maltose binding sites were also predicted in the chimeric region. These same domains and predicted binding residues were predicted for Malt434 as well. Interestingly, I-TASSER predicted several of the alpha helices to be shorter in the chimera relative to the parent proteins: two alpha helixes in the chimeric region and two towards the N-terminal end of the protein (Fig. 4D, Fig. S1). The regions covered by these alpha helices were otherwise predicted to be conserved, out to phylogenetically distant Malt proteins lgAgt1 and scAgt1 (Fig. 2, Fig. 4D, Fig. S1). The predicted shortening of some alpha helixes suggests that recombining the *MALT3* region into *MALT4* may have decreased the overall rigidity of the encoded chimeric protein, allowing it to accommodate bulkier substrates, such as maltotriose. Mutations that increase structural flexibility have been

recognized in protein engineering as an important step in accommodating new substrates (Khersonsky Olga and Tawfik 2010; Mannige 2014).

Besides increasing overall flexibility, the specific location of the chimeric region could have also played a role in supporting maltotriose transport. A previous study found two residues that were important for scAgt1's ability to transport maltotriose, while not affecting its ability to transport maltose (Smit et al. 2008). One of these residues lies within the chimeric region we observed in Malt434, and the other is 10 amino acids downstream (Fig. 4D, Fig. S1). Since the overall structure of maltose/maltotriose transporters is conserved (Cheng and Michels 1989; Han et al. 1995; Barrett et al. 1999; Yan 2015), the area in and around the chimeric region in Malt434 may itself be important for substrate specificity.

The chimeric structure of Malt434 may then have facilitated maltotriose transport in two ways. First, it may have increased the overall flexibility of the protein, allowing it to accommodate the larger maltotriose molecule. Second, it could also have specifically altered an important substrate interface for better interaction with maltotriose, possibly by also making this region more flexible. Testing these biophysical and structural models will require future experiments, such as solving the crystal structures for Malt3, Malt4, and Malt434 as complexes with maltose and/or maltotriose.

A non-modular chimeric path to novel substrate utilization

Most of the work on functional innovations by chimeric proteins has focused on the rearrangement of discrete functional units, with or without the benefit of intronic sequences (Doolittle 1995; De Chateau et al. 1996; Patthy 1999; Patthy 2003; Vogel et al. 2004; Bashton and Chothia 2007; Furuta and Kobayashi 2012). However, Malt434 does not fit easily into the framework of new protein creation by the reordering/exchanging of modules, even when considering smaller functional units such as a single

alpha helix. While the chimeric region does completely move one alpha helix from Malt3 into the Malt4 background, the breakpoints of the conversion also result in two other alpha helixes with some residues from the Malt4 parent and some from the Malt3 parent, creating chimeric alpha helixes (Fig. 4D, Fig. S1). In addition, while domains important for sugar specificity probably exist in Malt3 and Malt4 (Barrett et al. 1999; Smit et al. 2008), in regard to maltotriose, the "sugar specificity" domain(s) between Malt3 and Malt4 do not seem to have different functions or specificities in their native backgrounds. In Malt3 and Malt4, there is no specific "maltotriose-transporting" domain to be swapped. Instead, the ability of the residues from Malt3 to facilitate maltotriose transport likely relies on their interaction with one or more residues in Malt4, not on their independent ability to interact with maltotriose.

Rather than the modular framework of novel protein formation, we believe that Malt434 is an example of another framework for how recombination can lead to the evolution of novel functions. Theoretical and experimental work has demonstrated the important role that recombination between related proteins can play in facilitating the evolution of new functions (Cui et al. 2002; Mody et al. 2009; Rogers and Hartl 2012; Bittihn and Tsimring 2017). Indeed, protein engineering has utilized the technique of DNA shuffling since the mid 1990's to recombine closely related coding sequences to efficiently generate proteins with novel or improved functions (Giver and Arnold 1998). More recently, experimental work has begun to demonstrate the importance of recombination between closely related proteins in nature for the evolution of new functions (Thomas 2006; Mody et al. 2009; Rogers and Hartl 2012). In this model, two duplicate proteins accumulate the multiple amino acid changes needed for a new function independently and in a neutral manner. All the mutations that, in combination, are needed for the new function are then brought together at once, en masse, through recombination. This allows proteins to "tunnel" to new functions, bypassing potentially deleterious single-step intermediates (Cui et al. 2002; Bittihn and Tsimring 2017).

While *MALT3* and *MALT4* are not recent duplicates, they do share a distant paralogous relationship with each other (Fig. 2). In addition, as members of the sugar porter subfamily of proteins they share a highly conserved protein structure (Cheng and Michels 1989; Han et al. 1995; Barrett et al. 1999; Yan 2015). The conservative nature of sugar porter family proteins means that recombination events like the one that formed Malt434, which do not fall between clear domains, probably have a relatively high likelihood of creating functional transporters (Drummond et al. 2005), though of unpredictable specificity.

In the case of Malt434, we do not yet know which specific amino acid interactions were important for the gain of maltotriose utilization in the chimera, let alone the function or history of the residues in their native background. It may be that they represent neutral changes in their parent background, but they also could have been selected for as-yet-uncharacterized specificities. Nevertheless, it was the independent accumulation of these changes in a common ancestral background that eventually allowed these sequences to recombine and create a novel function.

CONCLUSION

Our findings suggest that evolution of maltotriose utilization by *Saccharomyces* yeasts is sometimes not a straightforward process. Even when a functioning maltotriose transporter is available in the parent genome, the regulatory changes necessary to support atypical expression may be difficult to evolve under certain experimental conditions. Conversely, when a maltotriose transporter is not already present and must be made de novo, single point mutations are probably insufficient to switch or expand the specificity of available Malt proteins. With relatively high probability that a functional protein will result (Drummond et al. 2005), recombination between paralogous proteins can rapidly do what a single point mutation cannot and in a single rare event introduce the multiple residue changes needed to

support new functions. Our report on the evolution of a chimeric maltotriose transporter from parental proteins that could not transport maltotriose supports the role of recombination outside of functional domains and motifs in the formation of proteins with novel functions.

MATERIALS AND METHODS

Strains

All strains discussed in this paper are listed in Table S4. Briefly, FM1318 is a monosporic derivative of the type strain of *S. eubayanus*, which was isolated from Patagonia (Libkind et al. 2011). yHRVM108 was isolated from Durham, North Carolina, and is closely related to the *S. eubayanus* strains that hybridized with *S. cerevisiae* to give rise to lager-brewing yeasts (Peris & Langdon et al. 2016). yHKS210 was isolated from Sheboygan, Wisconsin, and is an admixture between populations A and B of *S. eubayanus* whose genome is nearly homozygous (Peris et al. 2014). Of these strains, FM1318 and yHKS210 grew well on maltose, but did not grow on maltotriose. yHRVM108 grew sluggishly on maltose and did not grow on maltotriose. yHAB47 is a copy of Weihenstephan 34/70 (Peris & Langdon et al. 2016), a representative of the Frohberg or Group II (Magalhães et al. 2016) lineage of lager-brewing hybrids (*S. cerevisiae* (2n) x *S. eubayanus* (2n)(Nguyen and Boekhout 2017)). CDFM21L.1 is a strain of *S. eubayanus* isolated from Tibet (Bing et al. 2014) and closely related to yHRVM108. Of known *S. eubayanus* strains, CDFM21L.1 is the most genetically similar to the *S. eubayanus* parents of lager-brewing hybrids (Bing et al. 2014; Peris & Langdon et al. 2016).

Identification of *MALT* genes

Previously, we identified four genes with homology to the genes encoding the maltose transporters of *S. cerevisiae* and lager-brewing hybrids in the genome assembly of FM1318 published by Baker *et al.* 2015 (Baker et al. 2015). These genes were previously designated *MALT1-4.* Only a partial contig was available

for *MALT4* in this assembly, but a BLAST (Altschul et al. 1997) search of the Okuno *et al.* 2016 (Okuno et al. 2016) assembly of the type strain of *S. eubayanus* (of which FM1318 is a monosporic derivative) allowed us to annotate the full-length sequence of *MALT4*. *MALT4* has 99.7% identity to *MALT2* at the nucleotide level and 100% identity at the amino acid level. The regions from 900 bp downstream of *MALT2* and *MALT4* and upstream to the ends of chromosomes V and XVI (regions of approximately 12 kb in the Okuno *et al.* 2016 (Okuno et al. 2016) assembly), respectively, share 99.1% nucleotide identity. The 10 kb outside of this region only shares 49.8% nucleotide identity. Thus, *MALT2* and *MALT4* are close paralogs that are likely related by a recent subtelomeric duplication and/or translocation event.

Reads for homologs of *AGT1* were retrieved using the functional *AGT1* sequence from lager yeast (*lgAGT1*) as the query sequence (Nakao et al. 2009) in an SRA-BLAST search of the SRA databases of NCBI for yHRVM108 (SRR2586159) and CDFM21L.1(SRR1507225). All reads identified in the BLAST searches were downloaded and assembled using the de novo assembler in Geneious v. 9.0.3 (http://www.geneious.com) (Kearse et al. 2012). The homologs identified in yHRVM108 and CDFM21L.1 were designated *ncAGT1* (for North Carolinian *AGT1*) and *tbAGT1* (for Tibetan *AGT1*), respectively. The presence and sequence of *ncAGT1* in yHRVM108 was further verified by PCR amplification and Sanger sequencing (Table S5). CDFM21L.1 was not available at the time of this work for further verification of the presence of *tbAGT1*.

Directed evolution

Directed evolution was initiated by growing parent strains overnight in liquid YPD medium (1% yeast extract, 2% peptone, 2% glucose). One mL of maltotriose or maltose medium was inoculated with enough overnight culture to give an OD_{600} reading of ~0.1. Evolution on maltotriose was conducted in synthetic complete (SC) medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.2% complete

drop out mix) with 2% maltotriose and 0.1% glucose. The addition of 0.1% glucose ensured enough growth that mutations could occur and be selected for through the ensuing generations. Directed evolution of yHRVM108 on maltose was carried out in SC with 2% maltose. Because yHRVM108 grew so poorly on maltose alone, an additional 0.1% glucose was supplemented into its medium; after increased growth was observed around generation 110 for replicate A, from which strains yHEB1585-1587 are derived, and around generation 80 for replicate B, from which strains yHEB1588-90 were derived, subsequent generations of yHRVM108 directed evolution in maltose for these replicates was conducted with 2% maltose only. The directed evolution of each strain was carried out in triplicate. Samples were grown on a culture wheel at room temperature (22°C) and diluted 1:10 into fresh media every 3-4 days. Samples of each evolution replicate were taken every other passage and placed into long-term storage by mixing 700uL of culture with 300uL of 50% glycerol in a cryotube and storing it at -80°C. The number of doublings between passages was estimated from cell counts during the second and third passages. Evolution was carried out for a total of 100 passages.

Sporulation and backcrossing

To induce sporulation, strains were grown to saturation, washed twice, and then resuspended in 200µL liquid sporulation (spo) medium (1% potassium acetate, 0.5% zinc acetate). 30µL of this suspension was added to 1.5mL of spo medium and incubated on a culture wheel at room temperature. Cultures were checked for sporulation after 2-5 days. Tetrads were dissected using a Singer SporePlay. For backcrossing, tetrads of the strains to be crossed were dissected on a single YPD plate. A spore from one parent was placed in close proximity to a spore from the other parent; they were observed over several hours for mating and zygote formation. Transformations of the diploid F_1 backcross strain for gene knockouts were carried out as described below in the section describing the construction of gene expression plasmids.

Construction of gene expression plasmids

Genes encoding transporters of interest were cloned via gap repair into the *Not*I site of plasmid pBM5155 (GenBank KT725394.1), which contains the complete machinery necessary for doxycycline induction of genes cloned into this site (Alexander et al. 2016). Transformation was carried out using standard lithium acetate transformation (Gietz and Woods 2002) with modifications to optimize transformation in *S. eubayanus*. Specifically, transformation reactions were heat shocked at 34°C. After 55 minutes, 100% ethanol was added to 10% total volume, and the reactions heat shocked for another 5 minutes before they were allowed to recover overnight and plated to selective media the next day. When necessary, plasmids were recovered and amplified in *Escherichia coli* for transformation into multiple strains. The sequences of genes encoding transporters cloned into pBM5155 were verified by Sanger sequencing. *S. eubayanus MALT1*, *MALT3*, and *MALT4* were amplified from FM1318, *lgAGT* was amplified from yHAB47, and *ncAGT1* was amplified from yHRVM108. Primers used for plasmid construction and sequence verification are listed in Table S5.

Growth assays

Growth was measured in liquid media in 96-well plates using OD_{600} measurements on a FLUOstar Omega microplate reader. To test the abilities of single-colony isolates of yHKS210 evolved in maltotriose to grow on maltotriose, strains were grown overnight in liquid YPD and washed. Cells were inoculated into wells to give an initial OD_{600} reading of ~0.1-0.2. To test the ability of single-colony isolates of yHRVM108 evolved in maltose to use maltose and maltotriose, a single colony was used to inoculate both SC + 2% maltose and SC + 2% maltotriose media. For assays testing the growth of strains carrying *MALT* genes expressed on an inducible plasmid, strains were grown to saturation, washed twice, resuspended in liquid SC without added carbon, and starved for 24 hours. The next day, strains were diluted in SC without added carbon to OD_{600} = 1.9 +/- 0.05 to ensure that all cultures had approximately

the same starting concentration. 15µL of each diluted culture was added to 235µL of the test medium. Three technical replicates, randomly distributed on a 96-well plate to control for position effects, were carried out for each strain. Strains were tested in SC with 2% added carbon source and 50ng/mL doxycycline to induce plasmid gene expression.

Bulk-segregant analysis

As described above, 60 spores from 15 fully viable tetrads of strain yHEB1593 (F_1 of yHKS210 x yHEB1505) were dissected and individually screened for their ability to grow in SC + 2% maltotriose. F_2 segregants that could grow in maltotriose were classified as MalTri$^+$, and those that could not were classified as MalTri$^-$. Each F_2 segregant was then individually grown to saturation in liquid YPD. The saturated cultures were spun down, the supernatant removed, and enough cells resuspended in liquid SC medium to give an OD_{600} measurement of between 1.9 and 1.95. Strains were pooled based on their ability to grow in maltotriose, leading to a MalTri$^+$ pool and a MalTri$^-$ pool. To pool, 1mL of each strain dilution was added to the appropriate pool of cells and vortexed to mix. A phenol-chloroform extraction and ethanol precipitation was used to isolate gDNA from the segregant pools. The gDNA was sonicated and ligated to Illumina TruSeq-style dual adapters and index sequencing primers using the NEBNext® DNA Library Prep Master Mix Set for Illumina® kit following the manufacturer's instructions. The paired-end libraries were sequenced on an Illumina MiSeq instrument, conducting a 2 x 250bp run.

Analysis of bulk-segregant whole-genome sequencing reads

To identify fixed differences between the meiotic segregant pools, whole-genome assemblies were made for the MalTri$^-$ group of segregants using the meta-assembler iWGS with default settings (Zhou et al. 2016). The final de novo genome assembly of the MalTri$^-$ pool was made by DISCOVAR (Weisenfeld et al. 2014) in iWGS. This assembly was used for reference-based genome assembly and variant calling

using reads from the MalTri^{+} pool following the protocol described in Peris and Langdon *et al.* 2016 (Peris & Langdon et al. 2016). Assemblies of the putative chimeric maltotriose transporter were retrieved from the MalTri^{+} pool of reads using the program HybPiper (Johnson et al. 2016). Briefly, HybPiper uses a BLAST search of read sequences to find reads that map to a query sequence; it then uses the programs Exonerate (Slater and Birney 2005) and SPAdes (Bankevich et al. 2012) to assemble the reads into contigs. The sequence and genomic location of the chimeric transporter were further verified by PCR amplification and Sanger sequencing (Table S5), as was the sequence of *MALT4* from yHKS210.

Phylogenetic and protein mutation prediction analyses

Multiple sequence alignments between the proteins encoded by the *MALT* genes were carried out using MUSCLE (Edgar 2004), as implemented in Geneious v.9.0.3 (Kearse et al. 2012)(http://www.geneious.com). Phylogenetic relationships were determined using codon alignments. Codon alignments were made using PAL2NAL (Suyama, Torrents, & Bork, 2006; http://www.bork.embl.de/pal2nal/) to convert the MUSCLE alignments of amino acid sequences to nucleotide alignments. A phylogenetic tree of nineteen *MALT* genes from *S. eubayanus* and *S. cerevisiae* and lager-brewing yeasts was made as described in Baker *et al.* 2015 (Baker et al. 2015) using MEGA v.6. All genes used in the phylogenetic analysis are as follows: *MAL21, MAL31*, and *MAL61* from *S. cerevisiae*; *MALT1* and *MALT3* from *S. eubayanus*; *MALT1, MALT2,* and *MPH* from lager-brewing yeast; *MPH2 and MPH3* from *S. cerevisiae*; *AGT1* (*MAL11* in Baker *et al.* 2015 (Baker et al. 2015)) from *S. cerevisiae*; *scAGT1* (*WeihenMAL11*-CB in Baker *et al*. 2015 (Baker et al. 2015)); and *lgAGT1* (*WeihenMAL11*-CA in Baker *et al*. 2015 (Baker et al. 2015)) were retrieved as previously described in Baker *et al.* 2015 (Baker et al. 2015). Sequences for *MALT2* and *MALT4* were retrieved from the genome assembly of CBS 12357^{T} from Okuno *et al.* 2016 (Okuno et al. 2016). *MAL11* was retrieved from the genome assembly of

S. cerevisiae strain YJM456 (Strope et al. 2015). Sequences for *tbAGT1* and *ncAGT1* were retrieved as described above. *MAL11* and *AGT1* both encode α-glucoside transporters, are located at the *MAL1* locus in *S. cerevisiae*, and as such are considered alleles of each other (Charron and Michels 1988; Han et al. 1995). Their shared genomic location notwithstanding, *MAL11* and *AGT1* are not phylogenetically closely related, with *MAL11* clustering with other *MALx1* type transporters (Fig. 2). In addition, while *AGT1* can support maltotriose transport, *MAL11,* like other known *MALx1* genes, cannot (Han et al. 1995; Brown et al. 2010). Despite their dissimilarity, *AGT1* is recorded in the *Saccharomyces* Genome Database (yeastgenome.org) as *MAL11* since the reference strain carries the *AGT1* allele at the *MAL1* locus (Vidgren et al. 2005; Vidgren et al. 2009). For this reason, *MAL11* is often used to refer to *AGT1* (Brown et al. 2010; Baker et al. 2015; Brickwedde et al. 2017). For clarity, here we use *MAL11* to only refer to the *MALx1*-like allele and *AGT1* to the distinct maltotriose-transporting allele.

Protein structure predictions for *MALT3*, *MALT4*, *lgAGT1*, and *scAGT1* were carried out using the I-TASSER server, and the structure prediction of *MALT434* was carried out using the command line version of I-TASSER (Zhang 2008; Roy et al. 2010; Yang et al. 2015) (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). The potential impact of the single residue difference between *lgAGT1* and *tbAGT1* was analyzed by two different methods. Prediction of the change in free energy (ΔΔ*G)* was carried out using the STRUM server (https://zhanglab.ccmb.med.umich.edu/STRUM/, accessed 3-21-18) (Quan et al. 2016). A ΔΔ*G* score of < +/- 0.5 was considered to be unlikely to affect function (Bromberg and Rost 2009). Homology-based predictions were made using SIFT at http://sift.jcvi.org/ (accessed 3-30-18) (Ng and Henikoff 2001; Ng and Henikoff 2002; Ng and Henikoff 2003; Ng and Henikoff 2006; Kumar et al. 2009). The SIFT Related Sequences analysis was done using the amino acid sequences of *MALT* genes in the phylogenetic analysis above. Several SIFT analyses were also carried out using the SIFT Sequence analysis program. This analysis operates using the same principle as the SIFT Related Sequences analysis,

but rather than being supplied by the user, homologous sequences were provided by a PSI-BLAST search of the indicated protein database. The SIFT Sequence analyses were carried out using default settings and the following databases available on http://sift.jcvi.org/ (accessed 3-30-18) NCBI nonredundant 2011 Mar, 2UniRef90 2011 Apr, 3UniProt-SwissProt 57.15 2011 Apr.

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Chapter 4

The Impact of mitochondria on temperature tolerance in industrial and synthetic lager-brewing yeast hybrids.

Supplementary information from this chapter can be found in Appendix C.

This chapter is a modification of a soon to be submitted manuscript.

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R. Moriarty contributed to the development and troubleshooting of experimental protocols and construction of strains.
D. Peris contributed to the experimental design and to the development of experimental protocols.
X. Li, J. Fay and C.T. Hittinger contributed to the conception of this work.
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I contributed to the experimental design and developing experimental protocols, performed experiments, conducted analyses and wrote the manuscript with input and edits from D. Peris and C. T. Hittinger.

ABSTRACT

A growing body of observational and experimental data supports the role of mitochondria in thermal adaptation. In *Saccharomyces* yeasts, relatively little work has examined the role of mitochondrial DNA (mtDNA) in temperature tolerance and most of this work has focused on tolerance to high temperatures. The yeasts of the *Saccharomyces* genus can be broadly divided between cryotolerant and thermotolerant species. The genetic and molecular basis of the ability to grow at low temperatures in cryotolerant *Saccharomyces* yeasts is still poorly understood and what role mtDNA may play has not been tested. The industrial yeast strains used in brewing lager-style beers were formed by hybridization between the thermotolerant species *Saccharomyces cerevisiae* and the cryotolerant species *Saccharomyces eubayanus* and inadvertently selected by brewers over numerous generations. Lager style beers are fermented at low temperatures and it has been known for some time that lager yeasts inherited the ability to ferment at these low temperatures from their *S. eubayanus* parent. Intriguingly lager hybrids also inherited their mtDNA from the *S. eubayanus* parent. In the following study we assayed the influence of parental mitotype on relative growth in synthetic *S. cerevisiae* x *S. eubayanus* hybrids across a wide range of temperatures. We also examined the impact of exchanging the native mtDNA present in an industrial lager-brewing hybrid, with mtDNA from *S. cerevisiae.* In this way we demonstrate that mtDNA influences the growth of *Saccharomyces* hybrids at both high and low temperatures and show a continuing influence of mitotype in an industrial lager hybrid.

INTRODUCTION

Suitable thermal tolerance is a critical component of how organisms adapt to their environment. Studies have begun to establish the link between variation in mitochondrial DNA (mtDNA) sequence (mitotypes), mitochondrial function and temperature adaptation between populations, particularly in metazoans. The *mitochondrial climatic adaptation* hypothesis (Camus et al. 2017) posits that functional variation between mitotypes plays an important role in shaping adaptation of organisms to their thermal environment. Support for this hypothesis comes from both indirect and, increasingly, direct lines of evidence. Clines of mitotypes along temperature gradients or associations between mitotype and distinct thermal environments have been observed for numerous metazoan species (Cheviron and Brumfield 2009; DuBay and Witt 2014; Quintela et al. 2014; Silva et al. 2014; Baris et al. 2016), including *Drosophila* (Camus et al. 2017), salmon (Consuegra et al. 2015), shrews (Fontanillas et al. 2005), whales (Foote et al. 2011), and humans (Mishmar et al. 2003) among others. Experiments in invertebrates have demonstrated directly that different mitotypes can alter temperature tolerance (Willett 2011; Pichaud et al. 2013), and more recently, direct experimental evidence has emerged for the role of mitotype in thermal adaption in natural environments (Dingley et al. 2014; Camus et al. 2017).

Genetically dissecting mitochondrially encoded traits in metazoans is difficult, due to their obligately sexual lifestyle and uniparental mitochondrial inheritance. In addition, mitochondrial candidate loci are difficult to identify, unless there are only a small number of nucleotide differences between mitotypes, and differences in non-coding regions are even more difficult to detect (Camus et al. 2017). Furthermore, the results of experiments with mitochondria in animals can be compounded by sex and tissue specific differences in mitochondrial function (Fontanillas et al. 2005; Wolff et al. 2016; Camus et al. 2017). For fine scale genetic dissection of traits linked to mtDNA, a more genetically tractable system is desirable.

Recent work has shown that mitotype can also play a role in thermotolerance in the model fungal yeast genus *Saccharomyces* (Paliwal et al. 2014; Špírek et al. 2014; Wolters et al. 2018). The *Saccharomyces* genus consists of eight known species (Liti et al. 2006; Hittinger 2013; Naseeb et al. 2017), which can be broadly divided between cryotolerant and thermotolerant species. Thermotolerant strains (maximum growth temperature ≥36˚C) form a clade that includes the standard model organism *Saccharomyces cerevisiae* (Gonçalves et al. 2011; Salvadó et al. 2011). To date, the genetics of temperature preference, particularly preference for cold temperatures, in *Saccharomyces* yeasts has been difficult to ascertain. Only three candidate loci have been identified as supporting cryotolerance in *Saccharomyces*, two in *S. cerevisiae* and one in a hybrid between the thermotolerant species *S. cerevisiae* and the distantly related cryotolerant species *S. eubayanus* (Yamagishi et al. 2010; Libkind et al. 2011; Gibson et al. 2013; Paget et al. 2014). Recent studies have found that both within and between species variation in mitotype can impact thermotolerance in *Saccharomyces*. Most work in this area has focused on the impact of intraspecies variation in mitotype within *S. cerevisiae* (Paliwal et al. 2014; Wolters et al. 2018) or on interspecies differences between *S. cerevisiae* and its thermotolerant sister species *S. paradoxus* (Leducq et al. 2017), though some work has also investigated more distant genetic relationships within *Saccharomyces* (Špírek et al. 2014). These studies have been largely concerned with mitochondrial function under heat-related stress (~37˚C). However, mitotype could influence temperature tolerance in *Saccharomyces* across a broad range of temperatures, not just at thermal extremes. Indeed, evidence from arctic species suggests that mitochondrial adaption specifically to cold conditions is common (Foote et al. 2011; Garvin et al. 2011; Melo-Ferreira et al. 2014). Furthermore, in a recent study of hybrids of *S. cerevisiae* and the cryotolerant species *Saccharomyces uvarum* when allele specific expression was measured at both 22˚C and 37˚C, an unexpected abundance of mitochondrial genes were identified as having allele-specific differences in expression, not only at 37˚C, but also at 22˚C,

where both *S. cerevisiae*, *S. uvarum,* and their hybrid grow robustly (Li and Fay 2017). These results suggest the importance of mitochondrial DNA, even at moderate temperatures.

Among the other cryotolerant species of the *Saccharomyces* genus are *Saccharomyces eubayanus*, *Saccharomyces arboricola,* and *Saccharomyces kudriavzevii.* Together *S. uvarum* and its sister species *S. eubayanus* form the small "*bayanus"* clade of *Saccharomyces* (Hittinger 2013), which diverged from *S. cerevisiae* roughly 20 million years ago (Kellis et al. 2003). This amount of divergence represents approximately the equivalent genetic divergence between humans and chickens (Dujon 2006). While it is thermotolerant *S. cerevisiae* that is best known for its role in human related fermentations, most commercial brewing occurs using cryotolerant *S. cerevisiae* x *S. eubayanus* hybrids in the production of lager-style beers (Libkind et al. 2011). These lager-brewing hybrids are distinguished by the tendency of the yeast to drop to the bottom of fermentations (bottom fermenting), a distinct flavor profile, and robust fermentation at low temperatures (~7-15˚C) (Tenge 2009). In comparison, ale-brewing yeasts tend to float at the top of fermentations and are used to brew at relatively high temperatures (15-24˚C). Most ale strains have been found to consist of pure *S. cerevisiae* genetic material, though some brewing strains classified as ales and isolated from low-temperature regions of Europe, have been determined to be *S. cerevisiae* x *S. kudriavzevii* hybrids (Peris, Belloch, et al. 2012; Peris et al. 2018). Perhaps not surprisingly, the *S. cerevisiae* component of lager yeasts has been found to be most similar to other strains of *S. cerevisiae* used in beer brewing (Dunn and Sherlock 2008; Gonçalves et al. 2016). Among strains of *S. eubayanus* that have been characterized, strains belonging to the Holarctic lineage have been identified as being closely related to the population of *S. eubayanus* that gave rise to lager yeasts (Bing et al. 2014; Peris & Langdon et al. 2016).

With the discovery of the wild-stock of *S. eubayanus* (Libkind et al. 2011), there is substantial interest in developing novel lager-brewing hybrids (Hebly et al. 2015; Krogerus et al. 2015; Krogerus, Magalhães, et al. 2017; Hittinger et al. 2018) and, therefore, in understanding the genetics of brewing-relevant traits, such as temperature tolerance. Intriguingly, the two lineages of lager-brewing yeast and other industrial hybrids inherited their mtDNA from their cryotolerant parent, *S. eubayanus* (Nakao et al. 2009; Baker et al. 2015; Okuno et al. 2016; Peris et al. 2017), but the influence of mtDNA on cryotolerance in lager-brewing yeast is unknown. Here, to determine whether mtDNA plays a role in temperature tolerance in hybrids of *S. cerevisiae* and *S. eubayanus*, we tested relative growth of newly created synthetic hybrids inheriting different parental mitotypes. We also directly tested the influence of mtDNA in an industrial lager strain by replacing the *S. eubayanus* mitotype with mtDNA from *S. cerevisiae*.

The genetic tools available in *Saccharomyces* yeasts permitted us to manipulate the inheritance of mtDNA in identical nuclear backgrounds. We find that when mtDNA from the thermotolerant parent, *S. cerevisiae*, is inherited, hybrids have superior growth over hybrids with *S. eubayanus* mtDNA when grown at high temperatures. Likewise, hybrids with mtDNA from the cryotolerant parent, *S. eubayanus,* have growth superior to hybrids with *S. cerevisiae* mtDNA at low temperatures. In this way, we show that mitotype directly influences the relative ability of otherwise identical strains to grow at different temperatures.

RESULTS

S. cerevisiae and *S. eubayanus* parent strains are thermotolerant and cryotolerant respectively.

In order to establish relative differences in growth between *S. cerevisiae, S. eubayanus,* and their hybrids carrying different mitotypes, a dilution series of each set of hybrids and parents were spotted onto plates containing either glucose or glycerol as the sole carbon source. As a non-fermentable carbon

source, glycerol forces yeasts to utilize their mitochondria via respiration, rather than relying on alcoholic fermentation, the preferred metabolic process of *Saccharomyces* yeasts under aerobic conditions (Crabtree/Warburg effect) (Dashko et al. 2014). On glucose, ρ^0 strains followed the same patterns of growth as their ρ^+ parent but grew less at all temperatures (Fig. S1), reflecting the well-known "petite" phenotype of respiratorily deficient cells (Merico et al. 2007). ρ^0 strains were completely unable to grow on glycerol.

On both glucose and glycerol, the *S. eubayanus* and *S. cerevisiae* parents had opposite temperature responses (Fig. 1A-B). *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, a temperature at which the *S. eubayanus* strains still grew well (Fig. 1A-B and FigS1-4). These results are consistent with the description of *S. eubayanus* as a cryotolerant species (Libkind et al. 2011), with a similar temperature range to its sister species, *S. uvarum,* and are also consistent with the well-known thermotolerant nature of *S. cerevisiae* strains (Gonçalves et al. 2011; Salvadó et al. 2011). Strain-specific differences were also apparent. *Sc* and *SeNC* both grew relatively weakly compared to the other parental strains (Fig. 1A-B). For *Sc,* poor growth was likely driven by the presence of multiple auxotrophies, but the reason for *SeNC's* poor performance is unknown.

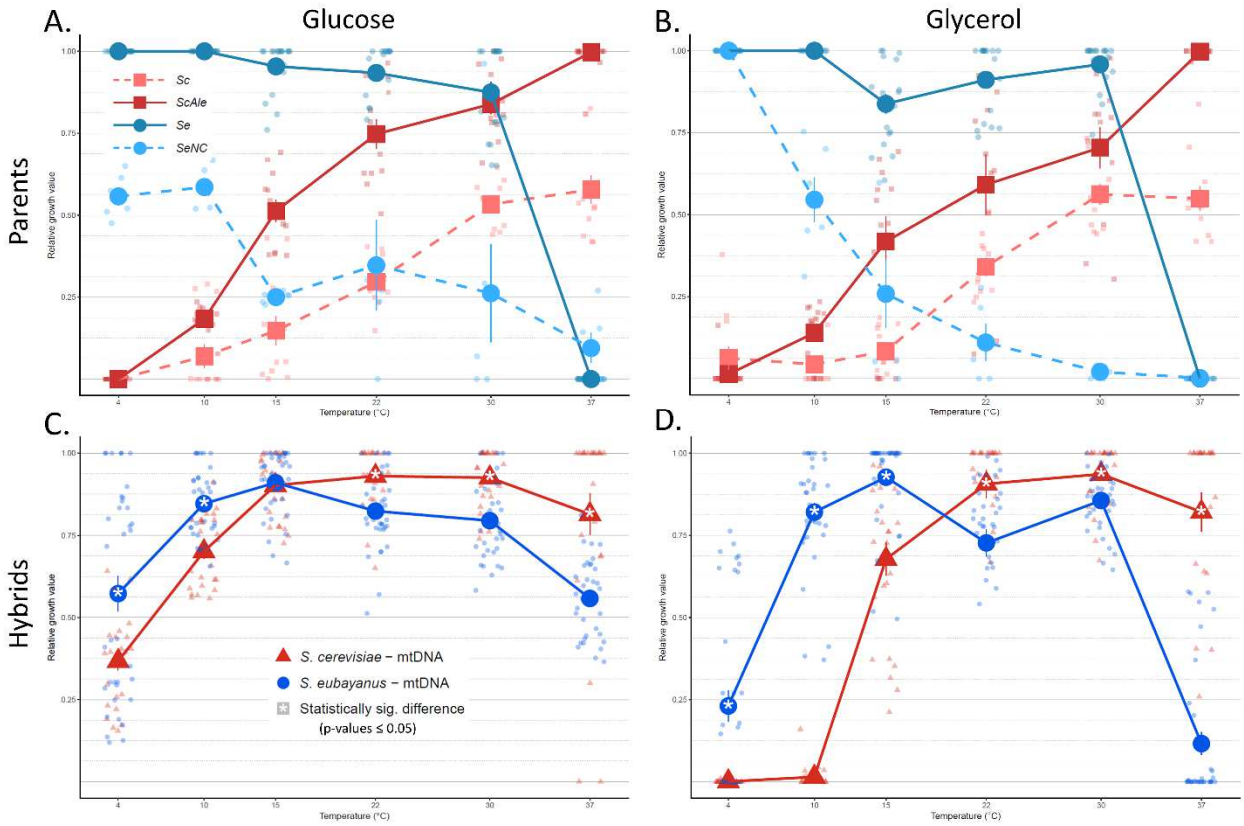


Figure 1. Relative growth of *S. cerevisiae, S. eubayanus* and their hybrids. Graphs of relative growth scores of strains, combined from all tests. A and B) Relative growth of parent strains carrying their native mtDNA on glucose and glycerol. C and D) Relative growth of *S. cerevisiae* x *S. eubayanus* hybrids carrying different parental mtDNA. Error bars represent standard error. Differences in relative growth between hybrids carrying different parental mtDNA with p-values of <0.05 were considered statistically significant and are represented by an asterisk. Parents were not tested for significant differences.

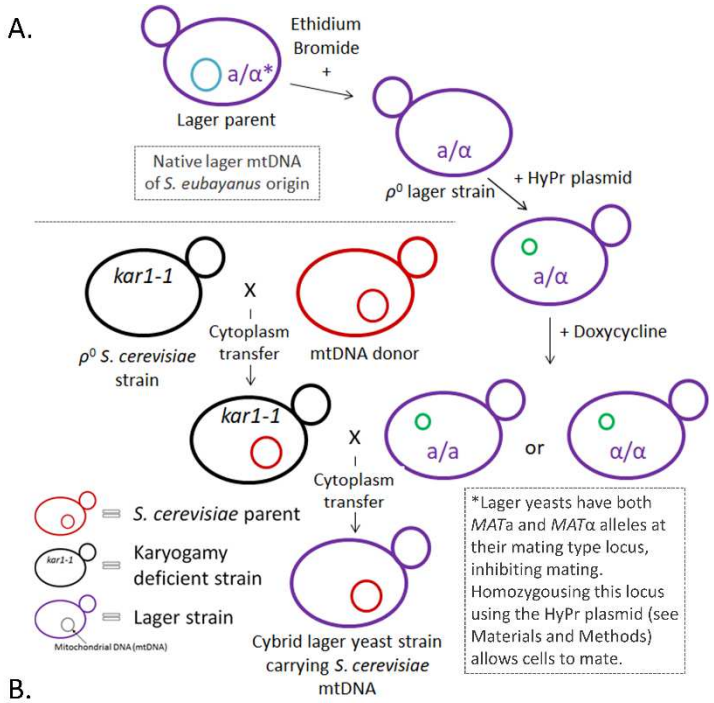
Mitotype influences temperature preference in hybrids according to the parental temperature profile. In general, heterosis was clear for hybrids grown on glucose across all temperatures tested (Fig. 1C and D, Fig. S1), as has been previously observed for synthetic *S. cerevisiae* x *S. eubayanus* hybrids (Hebly et al. 2015). While relative growth was typically high for hybrids of both mitotypes on glucose, subtle differences were apparent (Fig. 1C). Hybrids carrying *S. cerevisiae* mtDNA had significantly greater growth than hybrids carrying *S. eubayanus* mtDNA between 22 and 37˚C, while at 4 and 10°C, it was hybrids with *S. eubayanus* mtDNA that had significantly greater growth. There was no significant difference in growth between hybrids grown at 15˚C on glucose. These same trends were also seen on glycerol but were exaggerated (Fig. 1D), with significant differences between mitotypes at all temperatures. On glycerol, relative growth was greater for *S. eubayanus* mitotype hybrids between 4 and 15°C and was greater for hybrids with *S. cerevisiae* mtDNA between 22 and 37°C.

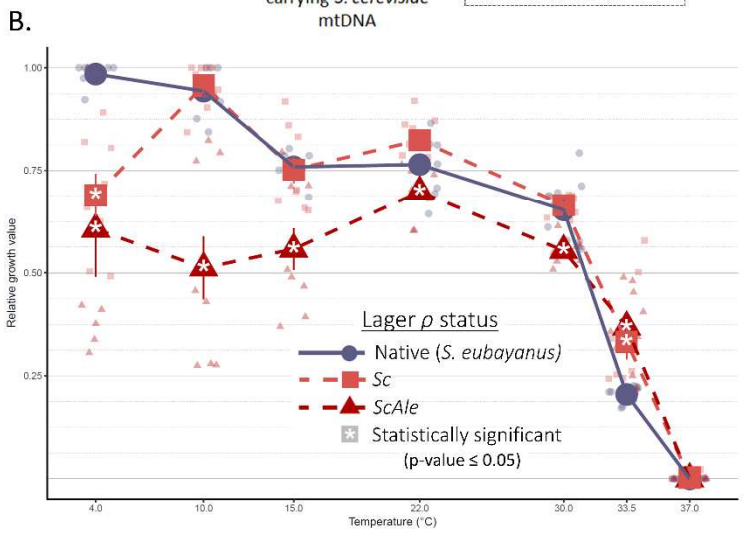
Relative growth patterns for hybrids of different mitotypes were consistent across individual crosses (Fig. S1-4) and when the data were analyzed statistically in aggregate (Fig. 1C and D). Hybrids carrying mtDNA inherited from the *S. eubayanus* parent, whether from the type strain or the North Carolinian strain, had relatively greater growth at low temperatures compared to hybrids carrying mtDNA inherited from either *S. cerevisiae* parent. Conversely, hybrids with *S. cerevisiae* mtDNA, regardless of whether the mtDNA was from the laboratory strain or the ale strain, had relatively more growth at high temperatures compared to hybrids with *S. eubayanus* mtDNA, with the exception of the *ScAle* X *SeNC* ρ^{ScAle} hybrid, which had a substantial growth defect at 37˚C (Fig. S4). The growth of the *ScAle* X *SeNC* ρ^{ScAle} at other temperatures was otherwise consistent with *S. cerevisiae* mtDNA supporting greater growth at high temperatures (Fig. S4B). On glycerol at 37˚C, despite still displaying a clear growth defect, the *ScAle* X *SeNC* ρ^{ScAle} hybrid still had greater growth than the *ScAle* X *SeNC* ρ^{SeNC} hybrid (Fig. S4C). These

results strongly support the contribution of mtDNA to temperature tolerance in *S. cerevisiae* x *S. eubayanus* hybrids, despite strain-specific differences.

S. cerevisiae mitochondrial DNA improves thermotolerance of an industrial lager strain

Unlike the synthetic hybrids assayed above, the *S. cerevisiae* and *S. eubayanus* nuclear genomes of industrial lager-brewing yeast hybrids have been evolving in to lagering conditions for numerous generations (Meussdoerffer 2009; Gibson and Liti 2015). As a result, the effect of mtDNA on temperature tolerance may not be the same in these industrial hybrids as it was for newly generated hybrids. To test if mtDNA still play a role in temperature tolerance in lager-brewing yeast, the native lager mtDNA of *S. eubayanus* origin (Nakao et al. 2009; Peris et al. 2014; Baker et al. 2015; Okuno et al. 2016; Peris et al. 2017) was replaced with *S. cerevisiae* mtDNA from *Sc* and *ScAle* yeasts (Fig. 2A). Consistent with the results for our synthetic hybrids, lager yeasts carrying *S. cerevisiae* mtDNA had greater growth at higher temperatures and increased sensitivity to colder temperatures, with the effect greatly exaggerated on glycerol (Fig. 2B and C). Differences between ρ^{Sc} and ρ^{ScAle} lager cybrids were also clear, especially on glucose. On glucose, there was no difference in growth between lager yeast carrying its native (*S. eubayanus*) mtDNA and those carrying *Sc* mtDNA, except at temperature extremes (Fig. 2B). In contrast, lager ρ^{ScAle} strains grew significantly less than the lager strain with its native mtDNA at most temperatures (Fig. 2B), despite the *ScAle* ρ^{+} strain displaying relatively robust growth across most temperatures in comparison to the industrial lager yeast with its native mtDNA (Fig. S5B).





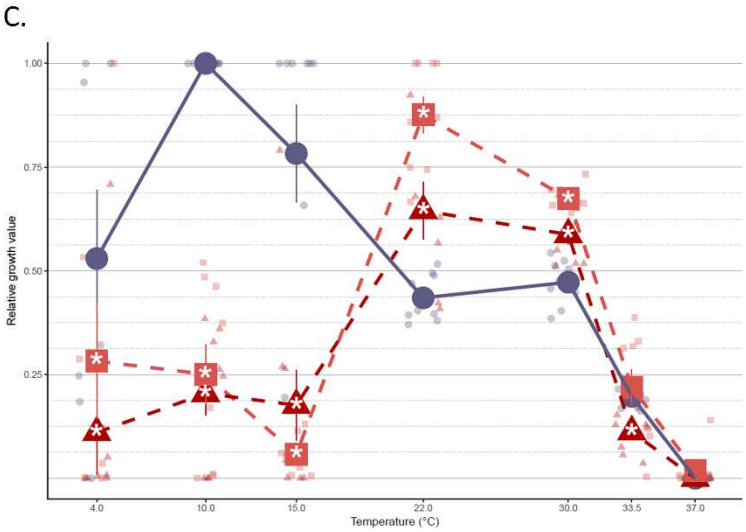


Figure 2. Construction and relative growth of lager cybrids. A) Outline of crosses and strain engineering to produce lager cybrids. Yeast cells represent the nuclear genome, large inner circles represent mtDNA and small green inner circles represent the HyPr plasmid. Lower case "**a**" and "**α**" indicates the mating type of lager yeast. Black indicates genetic material from *S. cerevisiae* karyogamy-deficient strain, red genetic material from *S. cerevisiae* parent, blue of *S. eubayanus* origin, and purple hybrid (i.e. lager) nuclear material. B and C) Growth of lager strain with native (*S. eubayanus*) mtDNA and lager cybrids with *S. cerevisiae* mtDNA. Error bars represent standard error and asterisks indicate statistically significant differences in growth between the cybrid and lager with native mtDNA (p-value <0.05). B) Growth on glucose C) Growth on glycerol.

DISCUSSION

Mitotype influences temperature tolerance in synthetic hybrids

Overall, hybrids had an increased range of temperatures they could tolerate compared to their parent strains, regardless of which mtDNA they carried. This heterosis was most evident at temperature extremes. On glucose at 37˚C, hybrids grew most like their *S. cerevisiae* parent, while under 15°C, they grew like their *S. eubayanus* parent. These results support a strong role for the nuclear genome in temperature tolerance and indicate a certain amount of codominance between thermotolerance and cryotolerance supporting genes. While this overall robustness to temperature was observed regardless of which mtDNA a hybrid carried, there were clear and consistent differences in relative growth between hybrids of different mitotypes. At higher temperatures, the *S. cerevisiae* mitotypes permitted increased growth relative to the *S. eubayanus* mitotypes, while the same was true for *S. eubayanus* mitotypes at lower temperatures, correlating with the relative cryotolerance and thermotolerance of their respective species of origin. Since the nuclear component is identical between hybrids of the same cross, these differences must be due to differences encoded in their mtDNA. While trends in temperature preference were apparent on both respiratory and fermentable carbon sources, the effect was exaggerated on respiratory media where growth through mitochondrial respiration is obligatory. These results were consistent across multiple strain backgrounds, indicating the generality of mtDNA effects on temperature preferences between these species.

Putative strain-specific cytonuclear incompatibilities between *S. cerevisiae* and *S. eubayanus*

Out of six *SeNC* ρ^0 strains tested, we were only able to generate a small number of hybrids with *S. cerevisiae* strains that carried *S. cerevisiae* mtDNA, and then only with a single *SeNC* ρ^0 strain (yHEB1638). Compared to *Se* ρ^+ and ρ^0 strains and the *SeNC* ρ^+ parent, where every mating attempt with *S. cerevisiae* strains produced hybrids, successful mating with yHEB1638 was sporadic, with only one out

of ten mating attempts resulting in respiratorily competent hybrids. Difficulty forming hybrids was not the only unusual characteristic of the *S. cerevisiae* x *SeNC* hybrids with *S. cerevisiae* mtDNA. While the *Sc* X *SeNC* ρ^{Sc} hybrid had high relative growth at 37˚C, like other hybrids carrying *S. cerevisiae* mitochondria, relative growth for the *ScAle* X *SeNC* ρ^{ScAle} hybrid plummeted at 37˚C. Interestingly, even with this severe temperature-related growth defect, the *ScAle* mitotype still supported greater growth at 37˚C on glycerol than the *SeNC* mitotype. Because we were only able to form *S. cerevisiae* mtDNA carrying hybrids with one *SeNC* ρ^0 strain, it is unclear if this temperature-dependent growth defect is specific to the yHEB1638 background or general to all *ScAle* X *SeNC* ρ^{ScAle} crosses. Even if the defect is specific to yHEB1638 and not *SeNC* in general, it is interesting that it was only detrimental in the *ScAle* background, as the *Sc* X *SeNC* ρ^{Sc} hybrid did not have the same sensitivity to 37˚C, despite sharing the same *SeNC* ρ^0 parent. Other studies have also found mitotype-related defects in temperature both within and between species in *Saccharomyces* in the study of cybrids (Paliwal et al. 2014; Špírek et al. 2014), though not interspecies hybrids as we have here. The potential for dominant cytonuclear incompatibilities could explain why, in hybrids of *Saccharomyces*, it has been observed that there is a tendency for there to be greater loss of nuclear genetic material from the parental genome that did not contribute the mtDNA (Marinoni et al. 1999; Peris, Lopes, et al. 2012; Peris et al. 2018). Intriguingly, another study also recently uncovered a strain-specific incompatibility between *S. cerevisiae* and *S. eubayanus* (Mertens et al. 2015). Taken together, these results imply that strain-specific incompatibilities exist between *S. eubayanus* and *S. cerevisiae* that prevent them from mating and/or forming viable offspring, which might be condition specific in some cases.

Influence and origin of mitotype in industrial lager yeasts

The impact of mtDNA on lager strain temperature tolerance was broadly similar to what was observed for synthetic hybrids, with some differences. In synthetic crosses of *S. eubayanus* and *S. cerevisiae,*

hybrids generally experienced robust growth across all temperatures, regardless of which mtDNA they carried, though mtDNA was more important at temperature extremes, especially on glycerol (Fig. 1C-D). This observation supports a strong role of the nuclear genome in supporting general temperature tolerance. In contrast, the industrial lager-brewing hybrid W34/70 was unable to grow at 37˚C and steadily declined in relative growth as temperature increased. Swapping the native *S. eubayanus* mtDNA for *S. cerevisiae* mtDNA, increased tolerance to high temperatures on glycerol and, to a lesser extent, on glucose, but the cybrids relative growth still declined precipitously as the temperature increased, and it was unable to grow at 37˚C. Based on these results, it is likely that, after many generations of selection for cold fermentation, the nuclear genes necessary to support growth at higher temperatures are no longer functional, present, or adequately expressed in the industrial hybrid. As a result, any contribution to thermotolerance from the mtDNA is relatively minor on fermentable carbon and insufficient to rescue growth at temperature extremes on either carbon source.

These results are particularly interesting because W34/70 is part of the Frohberg or Group II (Dunn and Sherlock 2008; Nakao et al. 2009) lineage of industrial lager yeasts. Compared to the Saaz or Group I lineage of industrial lager yeasts, the Frohberg lineage has a relatively larger *S. cerevisiae* contribution to its genome (Dunn and Sherlock 2008). The higher *S. cerevisiae* contribution has been associated with relatively greater thermotolerance among Frohberg lineage strains (Dunn and Sherlock 2008; Gibson et al. 2013; Walther et al. 2014). This correlation suggests that, even in a relatively thermotolerant industrial lager strain, the capacity of the *S. cerevisiae* nuclear genome to provide thermotolerance has been substantially reduced compared to the ancestral hybrid. It is nonetheless noteworthy that, even in a genetic background where the nuclear component of thermotolerance has been greatly diminished, mtDNA still plays a clear role in temperature tolerance.

It is tempting to speculate about what factors might have favored the retention of *S. eubayanus* mtDNA over *S. cerevisiae* mtDNA in present-day industrial lager-brewing hybrids. Given the difference in growth between our synthetic hybrids and the industrial lager hybrid and cybrids, it is likely that substantial changes occurred with regard to temperature tolerance over the course of adaption to lagering conditions. It is also evident that much of this change is attributable to changes within the nuclear genome. Even so, the mtDNA inherited still has a significant impact on temperature tolerance in all strains tested, with the *S. eubayanus* mtDNA favoring growth at lower temperatures. Increased cold tolerance could have given hybrids carrying the *S. eubayanus* mtDNA a selective advantage at the lower temperatures and high population densities at which lagers are brewed.

It is interesting to consider that interspecies incompatibilities, along with the ability to grow at low temperatures, might also have been a factor driving the retention of *S. eubayanus* mtDNA in industrial lagers. Of our synthetic hybrids, the *ScAle* X *SeNC* hybrids are the most genetically similar to the strains that gave rise to industrial lager hybrids. Like other hybrids tested, at low temperatures those that carried *S. eubayanus* type mtDNA had a growth advantage over hybrids that carried *S. cerevisiae* type mtDNA. Unlike other hybrids, the *ScAle* X *SeNC* hybrids with *ScAle* mtDNA had a severe growth defect at 37˚C, the highest temperature assayed. As discussed above, we cannot be certain if this is a strain-specific defect or one general to all *ScAle* X *SeNC* ρ^{ScAle} strains. However, if a high-temperature growth defect is common to hybrids between *S. cerevisiae* ale strains and Holarctic lineage *S. eubayanus* strains that inherit the *ScAle* mitotype, those hybrids that inherited the *S. eubayanus* mitotype could have had another significant advantage above and beyond superior growth at lower temperatures.

One of the initial stages of beer manufacturing is the production of wort by boiling malted grain to extract the sugar component (Krottenthaler et al. 2009). Today, industrial brewers use modern cooling

systems to cool the wort after boiling (Schu 2009), but historically, wort was cooled in open troughs, allowing air to pass freely over the hot liquid (Unger 2004: 167). This process exposed the wort to microbes that could colonize and ferment the wort into beer; a similar process is still used in the manufacture of lambics (Burberg and Zarnkow 2009) and American coolship ales (Bokulich and Bamforth 2013). In this scenario, the hybrids with the *S. eubayanus* mtDNA would not only have had an advantage at the lower temperatures but would have had an immediate advantage in being able to colonize the wort while it was still too hot for hybrids with *ScAle* mtDNA. The ability to colonize the wort early and continue rapid growth as the temperature cooled could have given hybrids with *S. eubayanus* mtDNA an insurmountable advantage, not only over their pure *S. cerevisiae* and *S. eubayanus* parents, but also over other hybrids carrying *S. cerevisiae* mtDNA.

CONCLUSIONS

We have shown that mtDNA can have a significant impact on the thermotolerance of hybrids between *S. cerevisiae* and *S. eubayanus*. The identification of a role for mtDNA in temperature adaptation in *Saccharomyces* yeasts offers a new genetically and experimentally tractable tool outside of metazoan systems with which to investigate the *mitochondrial climatic adaptation* hypothesis (Camus et al. 2017). A particularly exciting possibility from recent work in *S. cerevisiae* is the potential to map differences in thermotolerance, not only to nuclear loci, but to mitochondrially-encoded sequences as well by taking advantage of natural heteroplasmy and mtDNA recombination (Wolters et al. 2018).

While the finding that mtDNA influenced temperature preference in *S. cerevisiae* x *S. eubayanus* hybrids was general across the different strains tested, clear background-dependent difference were also observed. Given the interest in creating new lager hybrids for industrial use (Hebly et al. 2015; Krogerus et al. 2015; Mertens et al. 2015; Krogerus et al. 2016; Krogerus, Seppänen-Laakso, et al. 2017; Nikulin et

al. 2018), it is clear that strain background, not only of the *S. cerevisiae* parent, but also the *S. eubayanus* parent, and the inheritance of mtDNA should all be important considerations in strain construction. In addition to establishing a role for mtDNA in cryotolerance in lager yeast, we found that potential strain-specific incompatibilities suggest that *S. cerevisiae* x *S. eubayanus* hybrids could be a productive system for the study of genetic incompatibilities between species, particularly with regard to within species variation in such incompatibilities.

MATERIALS AND METHODS

Yeast strains and strain construction

Not all strains within a species are equally thermotolerant or cryotolerant, and different strains of *S. cerevisiae* can have 4˚C or more difference between their optimum growth temperatures (Salvadó et al. 2011). Since mitotype has been found to be important, at least at temperature extremes (Paliwal et al. 2014; Špírek et al. 2014; Leducq et al. 2017; Wolters et al. 2018), when determining thermotolerance in different strains of *S. cerevisiae,* we decided to include strains from different populations in our study. In addition to a laboratory strain of *S. cerevisiae* and a monosporic derivative of the type strain of *S. eubayanus,* an ale strain of *S. cerevisiae* and a strain of *S. eubayanus* isolated from North Carolina were also included (Hittinger and Carroll 2007; Libkind et al. 2011; Peris & Langdon et al. 2016). These two additional strains were chosen for their relative similarity to the parents of lager-brewing yeast hybrids.

Specifically, FM1283 (*Sc*) is descended from BY4724, which is itself a derivative of S288C (Brachmann et al. 1998; Hittinger and Carroll 2007). WLP530B (*ScAle*), is a commercial ale strain; its' pure *S. cerevisiae* background was confirmed by whole genome sequencing and assembly of reads to a concatenated pan-*Saccharomyces* reference genome, by use of the program sppIDer (Langdon *et al.* under revision). FM1318 (*Se*) is a monosporic derivative of the type strain of *S. eubayanus,* CBS 12357 (Libkind et al.

2011). The strain yHRVM108 (*SeNC*) was isolated from North Carolina and identified as being a close relative of the *S. eubayanus* parent of lager-brewing yeast hybrids (Peris and Langdon *et al.* 2016). W34/70 (Weihenstephan 34/70 or yHAB47) is an industrial strain belonging to the Frohberg lineage of lager-brewing yeast hybrids (Peris and Langdon *et al.* 2016). All strains used in this study are listed in (Table S1).

To facilitate strain crossing, stable haploid *ScAle*, *Se*, and *SeNC* strains were generated by replacing one allele of the *HO* locus with a selectable marker by standard lithium acetate transformation (Gietz and Woods 2002; Alexander et al. 2014), with modifications made for transforming *S. eubayanus* (see Supplementary Materials and Methods). Successful replacement of the *HO* locus was confirmed by PCR with primers specific to the *HO* locus (Table S2). The resultant strains were sporulated and individual tetrads dissected using a Singer Sporeplay. *ScAle* was sporulated in liquid sporulation medium (1% potassium acetate, 0.005% zinc acetate) and grown at room temperature (~22°C) before dissecting after 4-5 days. To sporulate *Se* and *SeNC,* 200µL of saturated culture were plated onto a YPD (1% yeast extract, 2% peptone, 2% glucose) plate and grown at room temperature for 3-5 days before dissecting tetrads. Strains lacking the *HO* coding sequence were selected for by growth on YPD + antibiotic, and the mating type was identified by mating with tester strains.

Synthetic hybrids

To test the effect of mitotype on temperature tolerance in *S. cerevisiae* x *S. eubayanus* hybrids*,* we made sets of hybrids containing mtDNA from one parent or the other. When two ρ^+ yeast cells mate, the mtDNA of both parents is present in the zygote, but a single mtDNA haplotype is rapidly fixed after only a few cell divisions (Berger and Yaffe 2000). Which mtDNA haplotype is fixed often happens in a non-random manner (Zweifel and Fangman 1991; Marinoni et al. 1999; Hsu and Chou 2017), and

recombinant mtDNAs' are also possible, even common (Berger and Yaffe 2000; Wolters et al. 2018). To control the inheritance of mtDNA in synthetic hybrids, we generated ρ^0 (mtDNA completely absent) strains to mate with ρ^+ strains, so that mtDNA from only the ρ^+ parent would be present in hybrids (Fig. 3). ρ^0 strains were generated by treating ρ^+ parent strains with ethidium bromide (Fox et al. 1991). Respiration-deficient strains were screened for by the absence of growth on glycerol, and the complete removal of mtDNA was confirmed by DAPI staining (Eckert-Boulet et al. 2011). Because of the mutagenic nature of ethidium bromide, to control for the effect of any spurious mutations, we generated ρ^0 strains of each parent strain in triplicate.

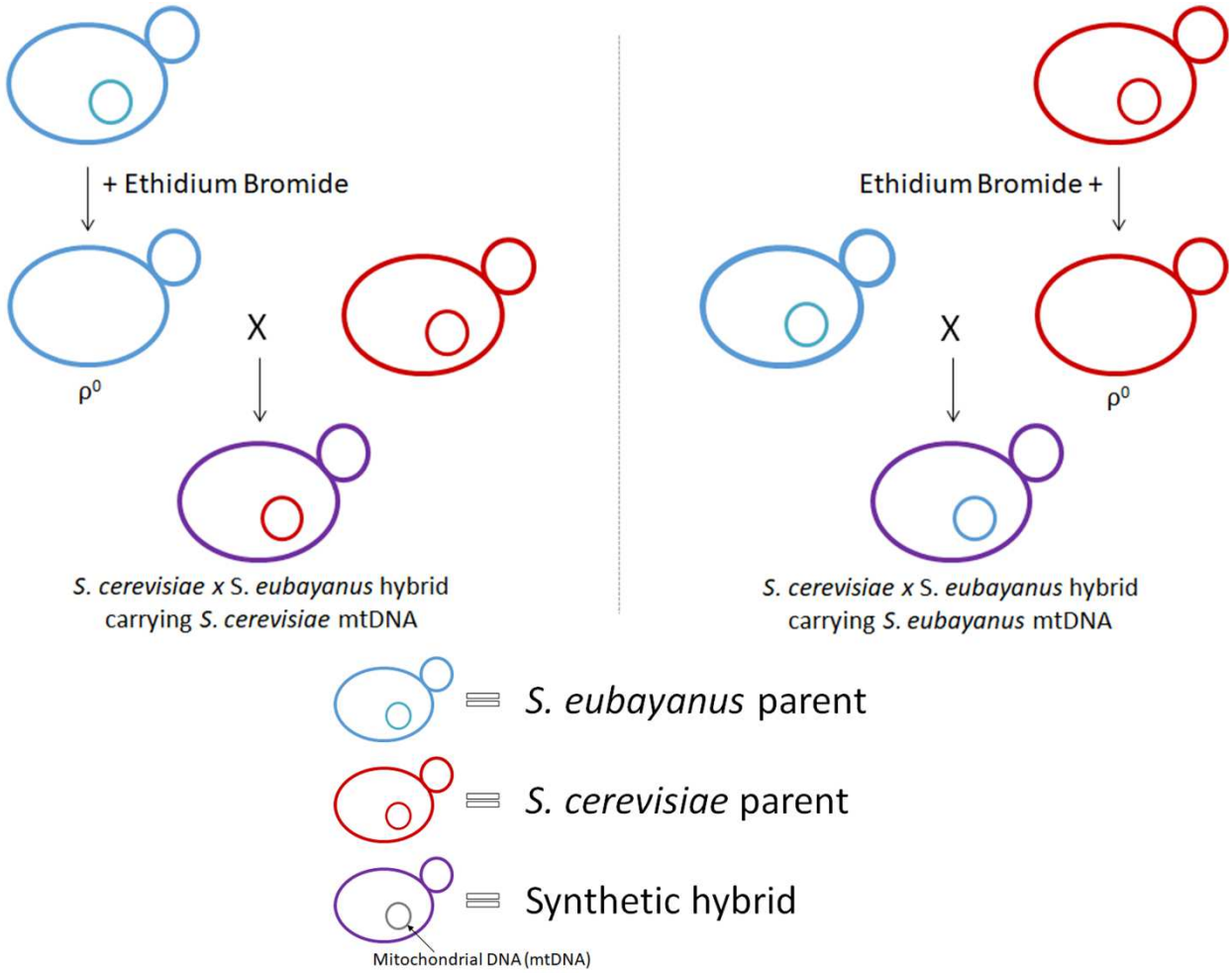


Figure 3. Hybrid mating scheme. Outline of procedure to control the inheritance of mtDNA in crosses of *S. cerevisiae* and *S. eubayanus*. Yeast cells represent the nuclear genome, and inner circles represent mtDNA. Red indicates genetic material of *S. cerevisiae* origin, blue of *S. eubayanus* origin, and purple hybrid nuclear material.

Hybrids were made by mating a ρ^0 strain of one species with a ρ^+ strain of the opposite mating type of the other species. Mating was performed by mixing the parent strains together on a YPD plate and letting them mate overnight. Allowing mating to occur for one or two more days and/or at 30˚C sometimes improved mating efficiency. Hybrids were selected by growth on glycerol and resistance to the appropriate antibiotics. When appropriate drug selection markers were not present in the parental genomes, zygotes were picked manually and tested for growth on glycerol to confirm retention of functional mitochondria. The hybrid nature of all strains was confirmed by ITS sequencing (Table S2) (McCullough et al. 1998; Sylvester et al. 2015). To ensure maintenance of mitochondria, hybrid strains were grown only on media with glycerol as the sole carbon source, except for during experiments.

In general, the different *S. cerevisiae* and *S. eubayanus* backgrounds and mitotypes readily formed hybrids, although mtDNA could be lost if hybrids were not grown on non-fermentable media. The exception was for crosses attempted between *S. eubayanus*-North Carolina (*SeNC*) ρ^0 strains and *S. cerevisiae* ρ^+ strains (both the lab and ale strains). Hybrids between *SeNC* ρ^0 and *S. cerevisae* ρ^+ strains were attempted multiple times (>50 attempts total) with six independently generated *SeNC* ρ^0 strains. Out of these attempts, only 4 successful hybrids were formed, one between yHEB1528 (*ScAle* ρ^+) and yHEB1638 (*SeNC* ρ^0) and three between yHWA117 (*Sc* ρ^+) and yHEB1638 (*SeNC* ρ^0). There was no similar difficulty producing the same hybrids with *S. eubayanus* mitochondria.

It is not clear if the ability to form respiratorily competent hybrids is unique to yHEB1638, as even hybrids with this strain took multiple attempts to achieve. Because the ethidium bromide used to generate ρ^0 strains is broadly mutagenic, it is likely that yHEB1638 has a number of mutations differentiating it from the other *SeNC* ρ^0 strains we generated. It is possible that one of these changes allowed yHEB1638 to maintain functional mtDNA in hybrids with *S. cerevisiae* carrying *S. cerevisiae*

mtDNA, whereas other *SeNC* ρ^0 strains could not. We include the results of growth assays with the hybrids made using yHEB1638 to determine if they follow the same general trends as other hybrid strains, with the caveat that the results from these experiments cannot be verified by hybrids made from independently generated *SeNC* ρ^0 strains.

Mitochondrial transfers

To produce strains with a lager yeast nuclear background and *S. cerevisiae* mtDNA (cybrids), karyogamy deficient (*kar1-1*) ρ^0 strains (Conde and Fink 1976; Costanzo and Fox 1993; Thorsness and Fox 1993) were used to transfer mitochondria from a donor *S. cerevisiae* strain to a lager strain ρ^0 (Fig. 2A), that were constructed as described above. Briefly, the lack of karyogamy in crosses with *kar1-1* mutants allows the mixing of cytoplasm between mated cells, while preventing fusion between the nuclear genomes, ultimately leading to progeny with mixed cytoplasm, but only one nuclear background. In this way, donor mitochondria from *S. cerevisiae* strains were transferred into the *kar1-1* ρ^0 strains by mating yeast as above and selecting for functional mtDNA (by growth on glycerol, a non-fermentable carbon source) and the *kar1-1* background, while selecting against the donor strain background (Fig. 2A). Since the *S. cerevisiae* strains, FM1283 (*Sc*), WLP530B (*ScAle*), and their derived strains used in this work are prototrophic and the *kar1-1* strains (MCC109 and MCC123) are auxotrophic for *ura3*, we were able to select for the *kar1-1* background and simultaneously select against the *Sc* and *ScAle* background by selecting for resistance to 5-fluoro-orotic acid (FOA).

Because lager yeasts contain both *MAT*a and *MAT*α at their mating type locus, mating does not usually occur. To mate polyploid lagers to the *kar1-1* ρ^+ strains for mitochondria transfer, the *MAT* locus had to first be homozygosed. The *MAT* locus of lager ρ^0 strains was homozygosed using a HyPr (Hybrid Production) plasmid (pHCT2) to induce mating type switching (Alexander et al. 2016). Cybrids, strains

with a single nuclear background and mitochondria from a donor strain, were selected for by selecting against the *kar1-1* background. To confirm that only lager genetic material was present in the resulting cybrids, three loci throughout the lager genome were sequenced to confirm that they contained only lager alleles (Table S2). As with hybrids, cybrids were also cultured on glycerol, except for during experiments, to ensure maintenance of mtDNA.

Growth Assays

Each hybrid and cybrid was constructed three times with an independently generated ρ^0 parent. Each of these independent hybrids was tested three times at each temperature. In total, combining biological and technical replicates, each hybrid cross was tested a total of nine times at each temperature, with some exceptions. Since there was only one *SeNC* ρ^0 strain with which we were able to successfully form hybrids containing *S. cerevisiae* mtDNA, only one biological replicate for each *S. cerevisiae* strain was formed with *SeNC*, which each had three technical replicates at each temperature. Consequently, these hybrids (*Sc* X *SeNC* ρ^{ScAle} and *ScAle* X *SeNC* ρ^{ScAle}) only had three replicates total at each temperature. In addition, because of contamination or poor photo quality a small number of replicates (n = 5) had to be discarded. These were: two for *Sc* X *SeNC* experiments on glycerol, one at 22˚C and one at 37˚C and three for lager cybrid experiments at 4˚C, one replicate growing on glucose and two growing on glycerol.

Yeast strains were grown in liquid synthetic complete (SC) medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.2% complete drop out mix). Strains containing their native mtDNA and ρ^0 strains were grown with 2% glucose, while hybrids and cybrids were grown with 2% glycerol and 2% ethanol to force the maintenance of mtDNA. After reaching saturation, cells were washed in either water or defloculation buffer (20mM citrate, 5mM EDTA) and resuspended in either SC (without carbon) or defloculation buffer to an OD_{600} of 1 +/- 0.05. Due to the extremely flocculent nature of *ScAle*, cultures

had to be washed and resuspended in defloculation buffer. For consistency, all strains used in experiments with *ScAle* were treated identically with buffer. Yeast strains were plated in a dilution series of OD_{600} = 1.0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}. Dilutions were plated onto SC plates containing either 2% glucose or 2% glycerol as the sole carbon source. Plates were grown at 4, 10, 15, 22, 30, and 37°C. Tests with lager cybrids were also grown at 33.5°C. Plates were grown until at least one strain on a plate showed growth at all five dilutions or after they had been allowed to grow for more than two months, whichever came first.

Analysis of growth assays.

To determine how well different strains grew relative to each other, the combined intensity (a proxy for growth) of the first and second dilutions (OD_{600} = 1 and 10^{-1}) were measured using custom CellProfiler pipelines (Lamprecht, Sabatini, & Carpenter, 2007;www.cellprofiler.org), and the values were combined. To be able to compare growth between plates, which may have differences in absolute intensity, growth on each plate was normalized by dividing by the strain with the highest measured combined intensity on each plate. This procedure created a relative growth score for each strain that was used to compare growth across different replicates. Statistically significant differences in growth were tested for using the Wilcoxon rank-sum test, as implemented in R version 3.4.3 (R Development Core Team 2017), and corrected for multiple tests using the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995), as implemented in R version 3.4.3. P-values ≤ 0.05 were considered significant.

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Chapter 5

Conclusions and Future Directions

The genetic basis of adaptation is a fundamental question of biological research. Studying how organisms have and/or can evolve to their environments at the genetic level is the first step in understanding the molecular basis of adaptation. Evolutionary genetics can be studied at a variety of scales, from the whole genome to a single gene and across millions of years in natural environments to a directed evolution experiment during the course of a graduate career. By studying evolution across these different scales, the whole picture of how and why evolution occurs is enhanced.

Just as studying evolution at a single scale can constrain our understanding, by limiting ourselves to a limited set of experimental organisms we could be limiting our ability to fully comprehend the complex ways that molecular, genetic, and evolutionary processes can achieve their ends (Gasch et al. 2016). Research using *Saccharomyces* yeasts exemplifies how the tremendous body of knowledge developed in a single, well-studied model system can inform studies in related organisms, which in turn enhances and extends our understanding of discoveries in the model system. As an example, the model yeast *Saccharomyces cerevisiae* is a thermotolerant member of the *Saccharomyces* genus (Gonçalves et al. 2011), and likely as a result, we have a poor understanding of the genetic and molecular basis of cryotolerance. What understanding we do have, has come through comparative studies with related *Saccharomyces* species (see introduction of chapter 4), which in turn were only possible because of techniques developed in *S. cerevisiae* and our broad understanding of the genetics of this model system.

In this thesis, I have discussed the genetics of evolution and adaptation in the non-model yeasts, *Saccharomyces eubayanus* and its hybrids with *Saccharomyces cerevisiae,* at various timescales and in different environments. I used a genome-wide comparative analysis of evolution to address how the *S. cerevisiae* and the *S. eubayanus* subgenomes of lager-brewing yeasts have evolved since hybridization and domestication to the brewing environment. This analysis provided insight into the evolutionary

history and origin of modern lager-brewing yeasts and also a fuller understanding of the impact of domestication on microbial genomes. Moving from the scale of the whole genome to the mitochondrial genome, I showed that differences between the mitochondrial DNA of *S. cerevisiae* and *S. eubayanus* result in significant changes in the ability of their hybrids to grow across a wide range of temperatures. This work established a role for mitochondria, not only in tolerance to high temperatures in *Saccharomyces* yeasts, but also to low temperatures as well. Finally, at the level of a single gene, a directed evolution experiment resulted in the identification of a novel maltotriose transporter through the unexpected evolution of a chimeric protein. The position of the breakpoints in this chimeric protein support the importance of recombination outside of functional domains in producing chimeric proteins with novel functions. Outstanding questions still remain from the original research that inspired these projects and the results of this work have in turn raised new questions. In the following sections, I discus several of the outstanding questions that remain in relation to the work of this thesis.

How has the *S. cerevisiae* subgenome of lager yeasts adapted to growth at cold temperatures?

Appropriate thermal tolerance is essential for organisms to thrive in their given habitat. In chapter 4, from growth assays of synthetic *S. cerevisiae* x *S. eubayanus* hybrids and lager-brewing yeast strains it was observed that while mtDNA played a role in the temperature preference of hybrids the nuclear genome played a large role as well. In addition, the nuclear component of temperature tolerance was co-dominant between the *S. cerevisiae* and *S. eubayanus* subgenomes in synthetic hybrids. Interestingly, while synthetic hybrids retained the ability, like their *S. cerevisiae* parent, to grow robustly at high temperatures, the industrial lager yeast tested had lost much of this ability and was much more sensitive to high temperatures. These results suggest that the nuclear encoded thermotolerance genes inherited from the *S. cerevisiae* parent are no longer active in lager yeasts, probably due to relaxed selection for their maintenance.

In chapter 2, analysis of the rate of evolution across the lager genome established that some genes in both the *S. cerevisiae* and *S. eubayanus* subgenomes have experienced increased rates of evolution. If *S. cerevisiae* thermotolerance genes have indeed been evolving under relaxed selection in industrial lager yeasts it would be expected that they would be among the genes that display increased rates of evolution in the analysis from chapter 2. Future work could build on the observations in chapter 4 and the methods used for whole genome analysis in chapter 2 to determine if specific genes in the *S. cerevisiae* subgenome of lager yeasts can be linked to the decreased ability of lager yeasts to tolerate high temperatures by examining the rates of evolution of genes within the *S. cerevisiae* subgenome of lager yeasts.

To attempt to identify genes related to heat tolerance in the *S. cerevisiae* subgenome of lager yeasts in this manner, it will be important to expand the whole-genome evolutionary analysis to include at least one ale strain. Because the *S. cerevisiae* subgenome of lager yeasts is most similar to the genomes of ale yeasts, comparison with an ale genome will be important for identifying increased rates of evolution specifically along the lager lineage, rather than increased evolution that has occurred within ale yeasts in general. This is an important consideration as the genomes of *Saccharomyces* yeasts associated with human fermentations seem to be evolving under more relaxed selective pressure, leading to a general increase in the rate of evolution, than those yeasts found in non-anthropogenic settings (Baker et al. 2015). Since the ale strain tested in growth assays in chapter 4 retained the ability to grow robustly at high temperatures, it is likely that the genes related to heat tolerance in ale strains have been under more constrained evolution compared to the same *S. cerevisiae* genes in lager yeasts. Similarly, including a strain of *S. eubayanus* more closely related to the *S. eubayanus* subgenome of lager-brewing yeasts could also establish another point of comparison that could help identify if certain types of genes experienced different rates of evolution in the *S. cerevisiae* subgenome versus the *S. eubayanus*

subgenomes of lager yeasts. Overall, this approach could help identify genes key for thermotolerance in *Saccharomyces* yeasts.

Can alternative selection regimes better select for maltotriose utilization?

In chapter 3, it was found that, under directed evolution for maltotriose consumption, supplemented with a small amount of glucose, evolution of maltotriose utilization was extremely rare for a strain *S. eubayanus* (yHRVM108) carrying a functional, but presumably, unexpressed maltotriose transporter (*ncAGT1*). However, when yHRVM108 was placed under directed evolution for improved maltose consumption, not only did multiple replicates evolve the ability to rapidly utilize maltose, but concurrently they also evolved the ability to utilize maltotriose, despite no maltotriose being present in the selection media. As no other known maltotriose transporters are present within the genome of yHRVM108, this result implies that the replicates independently evolved improved maltose consumption through increased expression of *ncAGT1*.

These results suggest that the conditions of the directed evolution experiment may have constrained rather than promoted evolution of maltotriose consumption. In other experiments to evolve *Saccharomyces* yeasts to carbon limitation or maltotriose consumption, the parent strains already had some capacity to use the supplied carbon source (Dunham et al. 2002; Brickwedde et al. 2017). For our experiments, this was true for directed evolution for improved maltose utilization, but it was not the case for directed evolution for maltotriose utilization. This difference in initial ability to take up the target sugar could have important implications for the observed differences in evolvability of maltotriose utilization.

Upregulation of *MAL* genes first requires maltose (or presumably maltotriose) to enter the cell (Wang et al. 2002). In the absence of glucose, at least one of the Malt transporters is ostensibly expressed at some level to allow this to occur (Wang et al. 2002). As yHRVM108 is unable to grow in maltotriose, it seems unlikely that appreciable ncAgt1 is expressed. Nonetheless, under the maltose evolution conditions, cells started with some limited capacity to take up maltose, presumably from another Malt gene that was expressed, and thereby likely induced expression of other *MAL* genes. This may have loosened the repression of *ncAGT1* as a result of the cascade of regulatory changes that occur upon the cell sensing the absence of glucose and the presence of maltose (Horák 2013). As a result, relatively simple mutations may have been sufficient to allow for *ncAGT1* expression during evolution in maltose. Such changes would have been selected for since expression of *ncAGT1* could help increase maltose uptake and only inadvertently would provide cells with the ability to consume maltotriose (Brown et al. 2010).

In contrast, during evolution in maltotriose, the *MAL* genes as a whole would have remained fully repressed since no maltose was present in the culture medium and maltotriose was unable to enter the cell. In consequence, rarer (and never observed) mutations might have been necessary to achieve expression of *ncAGT1.* Such a result is not unprecedented. In the long-term evolution of *E. coli* experiment, a functioning citrate transporter was present in the founding strain. Even though expression of this gene would have been highly favored in the citrate rich experimental environment, it took thousands of generations, even after the necessary potentiating mutations had appeared, before such expression arose (Blount et al. 2012).

In our directed evolution experiments, maltotriose utilization was an unintentional side effect of evolution for improved maltose consumption and the question remains, how to improve selective conditions specifically for the utilization of maltotriose? Based on the hypothesis stated above, that the

presence of maltose lead to a general derepression of *MAL* genes which facilitated the evolution of *ncAGT1* expression, adding a small amount of maltose to maltotriose selection media should increase the probability of the evolution of maltotriose utilization. The addition of maltose to the directed evolution of other strains of *S. eubayanus,* which do not carry functional but unexpressed maltotriose transporters, could also potentially improve the outcomes of directed evolution. It is possible that other mutations that confer maltotriose utilization will also be more accessible when the genes whose mutation is most likely to confer maltotriose utilization are under less repression.

These experiments could help address two basic questions. First, how does the genetic environment, here the general derepression of *MAL* genes, influence the evolvability of new traits, such as the expression of a gene in a novel environment or evolution of a protein with a new function? Second, if adding maltose to the selective environment does make the evolution of novel maltotriose transport proteins more likely, how do these proteins gain this new functionality? Are they always chimeras between related proteins? Do they always have mutations or recombination events in the same regions of the protein? Can transporters outside of the *MALT* family gain the ability to transport maltotriose? These are interesting questions with implications for understanding the relationship between amino acid sequence, tertiary structure, and what ultimately determines the function of a protein.

What is the genetic and molecular basis of mitochondrial DNA's influence on temperature preference?

In chapter 4, I provided evidence that differences in mitochondrial DNA (mtDNA) between *S. cerevisiae* and *S. eubayanus* influence the tolerance of their hybrids to hot and cold temperatures. These results raise two related questions. First, what are the genetic differences between *S. cerevisiae* and *S. eubayanus* mtDNA that cause differing responses to temperature in the same nuclear background? And second, what are the molecular/physiological underpinnings of changes in temperature sensitivity that

result from these genetic differences. There are several methods that could be used to begin to address these questions.

Beginning with physiological differences between hybrids of different mitotypes, direct measurements of the physiological characteristics of hybrids could be informative about what differences the mitotypes cause and how these differences are actually protective to cells at different temperatures. Previous studies have implicated glycerol as a cryoprotectant, and the ability to balance redox at a particular temperature as important for determining thermal preferences (Hayashi and Maeda 2006; Panadero et al. 2006; Aguilera et al. 2007; Arroyo-López et al. 2010; Tulha et al. 2010; Paget et al. 2014). Physiological studies could determine if measures of these traits differ between hybrids carrying different mitotypes. Such characteristics could be measured using established techniques for the study of yeast physiology such as gas or liquid chromatography or enzymatic assays (Arroyo-López et al. 2010; Gibson et al. 2013; Su et al. 2015). Understanding what changes occur could also help focus the search for causative loci.

To map causative genetic loci, RNA sequencing experiments could potentially suggest causative alleles by detecting mitochondrial genes whose expression differs between *S. cerevisiae* and *S. eubayanus* mtDNA in hybrids. A similar method was recently used to identify genes with differential expression at moderate and high temperatures in hybrids between *S. cerevisiae* and *S. uvarum* carrying the *S. cerevisiae* mtDNA (Li and Fay 2017). However, different thermal responses between hybrids carrying *S. cerevisiae* versus *S. eubayanus* mtDNA might not be caused by differences in expression, but from functional changes at the protein level. Even if differences in the relative abundance of different mitochondrial genes were not observed, changes in the expression level of other genes impacted by the mitochondrial genes could inform which genes in the mtDNA are influencing the hybrids response to

temperature. In addition to helping map causative loci, these changes in expression could also implicate molecular and physiological changes important for cold or heat adaptation.

One exciting possibility is to utilize natural mtDNA recombination that occurs in *Saccharomyces* zygotes to map the causative alleles for temperature preference. After mating between two yeast cells, the mtDNA from both parents is initially present, but within several cell divisions one mitotype is fixed in cells (Berger and Yaffe 2000). Prior to fixation of a single mitotype, recombination between the parental mitotypes can occur and has been found to be common in intraspecies matings of *S. cerevisiae* (Wolters et al. 2018). Introgressions from other species are also common in mtDNA in *Saccharomyces* (Peris et al. 2017). Such introgressions have been recorded in strains from a number of different species, suggesting that recombination between mtDNA in hybrids is not infrequent. While the utility of recombination for fine scale mapping of mitochondrial loci is just beginning to be explored (Wolters et al. 2018) and the frequency of interspecies mtDNA recombination is unknown, this tendency toward recombination could potentially be leveraged to carry out traditional genetic mapping studies for mitochondrial loci important to temperature preference in *Saccharomyces*.

Can we leverage intraspecies variation to study interspecies incompatibilities?

An incidental finding of the experiments presented in chapter 4 was the discovery of putative strain and condition-specific incompatibilities in *S. cerevisiae* x *S. eubayanus* hybrids. Specifically, I observed that hybrids attempted with nuclear DNA (nucDNA) from the North Carolinian strain of *S. eubayanus* (*SeNC*) and the mtDNA from either of the *S. cerevisiae* strains tested (*Sc* and *ScAle*) formed respiration-competent hybrids much more infrequently than other hybrid crosses. Additionally, in the one successful *ScAle* x *SeNC* ρ^{ScAle} cross, it was further observed that, while this hybrid had growth patterns similar to other hybrids carrying *S. cerevisiae* mtDNA between 4 and 30°C, there was a significant defect

in its ability to grow at 37°C. This defect was specific to the *ScAle* x *SeNC* ρ^{ScAle} hybrid and was not observed for any other crosses including *Sc* x *SeNC* ρ^{Sc}, which had the same *SeNC* ρ^0 parent as the *ScAle* x *SeNC* ρ^{ScAle} hybrid. Because only a single *SeNC* ρ^0 strain successfully formed respiratorily competent hybrids with *ScAle* mtDNA, it is not clear if the temperature related growth defect is general to all *ScAle* x *SeNC* ρ^{ScAle} hybrids or is specific to the *SeNC* ρ^0 parent strain used. As a result, to understand this temperature-related growth defect, a better understanding of the general difficulty forming respiratorily competent hybrids with *SeNC* nucDNA and *S. cerevisiae* mtDNA is first needed. For this reason, I will only be discussing the putative cytonuclear incompatibility between *SeNC* nucDNA and mtDNA from *S. cerevisiae* for the remainder of this section.

What is interesting about the putative cytonuclear incompatibility between *SeNC* nucDNA and *S. cerevisiae* mtDNA is that, unlike other interspecies cytonuclear incompatibilities that have been identified in *Saccharomyces,* the presence of this incompatibility is variable within a species (Chou and Leu 2010). While respiratorily competent hybrids were rarely observed when the North Carolinian *S. eubayanus* strain was the parent, such hybrids formed readily with the reference strain of *S. eubayanus*. Another difference between this and other known cytonuclear incompatibilities in *Saccharomyces* is that the incompatibility is dominant and is apparent in F_1 hybrids (see Fig. 2 in chapter 1) when the full nuclear complement from both parent species is present. Other cytonuclear incompatibilities that have been identified between *Saccharomyces* species are recessive and the incompatibility is only expressed when some or all of the nuclear genetic material from the parent that did not supply the mtDNA is missing (Lee et al. 2008; Chou and Leu 2010). Multiple experiments will be needed to disentangle the genetic and molecular basis of this incompatibility, but certain experiments will be important for laying the ground work.

Before addressing the ultimate underlying genetic and molecular mechanism of this cytonuclear incompatibility it will be important for its effects in hybrids to be fully characterized. In the course of attempting to produce hybrids between the incompatible genotypes, several relevant observations were noted. For all of the *SeNC* ρ^0 strains that were tested, zygotes were regularly observed, viable hybrids were common on glucose media, and respiratory competent hybrids were extremely rare. Of the six independently generated *SeNC* ρ^0 strains that were used to attempt these matings, only one strain, yHEB1638, successfully formed hybrids carrying the *Sc* or the *ScAle* mtDNA. However, even this strain failed to produce respiratorily competent hybrids at a high rate.

The above observations have not been rigorously tested, and it is not known whether the ability to form respiratorily competent hybrids with *S. cerevisiae* mtDNA is unique to yHEB1638 or if the production of respiratorily competent hybrids is simply very low for all *SeNC* ρ^0 strains. Carrying out a rigorous test of these anecdotal observations will establish if yHEB1638 is unique in its ability to form respiratorily competent hybrids with *S. cerevisiae* mtDNA and also the extent to which these crosses differ from crosses that easily produced respiratorily competent cells. For this reason, I propose measuring mating efficiency, zygote viability on non-respiratory media, and the frequency of respiratorily competent hybrids among the viable zygotes. These measurements should be made for matings between the parent strains, compatible crosses and incompatible crosses as well. For the incompatible crosses, yHEB1638 should be tested alongside one or more of the *SeNC* ρ^0 strains for which respiratorily competent hybrids were not observed. Crosses can be carried out either by mass mating or cell-to-cell matings. In either case, cells will need to be observed periodically by microscopy during the course of mating to observe zygote formation in order to determine mating efficiency.

Another assay that would be immediately informative about the mechanism of this cytonuclear incompatibility is imaging mtDNA in incompatible crosses. In those hybrids that are unable to respire two things could be happening to the mtDNA that result in loss of respiration. The first is that mtDNA might fail to be transferred from mother cells to daughter cells during mitosis, resulting in the absence of any mtDNA in daughter cells. The second is that mtDNA could be present in daughter cells but be nonfunctional. These two scenarios are easily distinguishable by visualizing mtDNA by DAPI staining and fluorescence microscopy. Information on the presence/absence of mtDNA from incompatible hybrids will directly offer evidence of the molecular mechanism of the incompatibility.

The results of these assays will be important for determining follow-up experiments to eventually determine what genetic and molecular interactions are causing this incompatibility and why it is experienced by crosses with the North Carolinian strain of *S. eubayanus* but not the *S. eubayanus* reference strain. Answering these questions could inform our understanding of the molecular variation found within species and the origin of between species incompatibilities. The observation of strain and condition-specific interspecies incompatibilities in *S. eubayanus* offers a unique opportunity to map the genetic basis of incompatibilities between distantly related species and understand within species variation in these incompatibilities.

General Conclusions

The study of non-model *Saccharomyces* yeasts and their hybrids has the potential to expand our understanding of the complexity and the evolutionary potential of organisms. The studies presented here explored the impact of evolution over millions of years (the time separating mtDNA in *S. cerevisiae* and *S. eubayanus*), a few hundred years (the origin of lager yeast hybrids), and a single year (the time frame of the directed evolution experiments) and at genetic scales ranging from the whole genome to a

single gene. The work described here has demonstrated the genetics of adaptation across these scales with regard to a variety of environmental conditions both broad (the brewing environment) and specific (temperature and carbon source utilization). In conclusion, this work has demonstrated the power and potential of using *S. eubayanus* and its hybrids with *S. cerevisiae* to address a variety of evolutionary and genetic questions and provides the ground work for future studies using *S. eubayanus* and its hybrids with *S. cerevisiae* and other *Saccharomyces* species.

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Appendix A

Supplementary Information from Chapter 2

Table S1. Nucleotide diversity between lager4brewing yeast genomes and their parental strains

	Saaz			Frohberg		
	N*dN	S*dS	all	N*dN	S*dS	all
***S. cerevisiae* (S288c)**	2268.4	4279.7	6548.1	2196.2	4129.6	6325.8
Lager,*S. cerevisiae*	2944.8	4415.6	7360.4	2362.2	3954.4	6316.6
Total changes between lineages	5213.2	8695.3	13908.5	4558.4	8084.0	12642.4
% of bp changed between lineages	0.22%	0.99%	0.43%	0.20%	0.93%	0.40%
% identity between lineages	99.78%	99.01%	**99.57%**	99.80%	99.07%	**99.60%**
changes per 1000 bp	2.2	9.9	4.3	2.0	9.3	4.0
Average # of differences per gene	2.30	3.83	6.13	2.01	3.56	5.57

	Saaz			Frohberg		
	N*dN	S*dS	all	N*dN	S*dS	all
***S. eubayanus* (FM1318)**	1232.3	3880.3	5112.6	1224.2	3827.0	5051.2
Lager,*S. eubayanus*	3835.8	5463.8	9299.6	3783.2	5430.6	9213.8
Total changes between lineages	5068.1	9344.1	14412.2	5007.4	9257.6	14265.0
% of bp changed between lineages	0.22%	1.06%	0.45%	0.22%	1.06%	0.45%
% identity between lineages	99.78%	98.94%	**99.55%**	99.78%	98.94%	**99.55%**
changes per 1000 bp	2.2	10.6	4.5	2.2	10.6	4.5
Average # of differences per gene	2.23	4.12	6.35	2.21	4.08	6.29

	Total # nonsynonymous sites	**Total # synonymous sites**	**Total # sites**
Saaz	2324071	881426	3205497
Frohberg	2301842	872845	3174687

Total # of genes: 2268
N*dN : estimated total number of nonsynonymous changes S*dS: estimated total number of synonymous changes Saaz: Saaz lineage of lager4brewing yeasts
Frohberg: Frohberg lineage of lager4brewing yeasts
Values discussed in the main text

Table S2. Maltose utilization genes in *S. eubayanus* and lager-brewing yeasts

S. eubayanus (FM1318)

Transporters	Location	Length (aa)	Closest S. cerevisiae homolog	Identity (%)[a]
SeubMALT1	ChrII –left arm (subtelomeric)	614	*MAL21*	80.36
SeubMALT2[H]	ChrV – right arm (subtelomeric)	614	*MAL31*	82.19
SeubMALT3[H]	ChrXIII – left arm (subtelomeric)	615	*MAL31*	85.15
tSeubMALT4[H]	ChrXVI – left arm (subtelomeric)	425 (t)	*MAL31*	84.16
Maltases	**Location**	**Length (aa)**	**Closest S. cerevisiae homolog**	**Identity (%)[a]**
SeubIMA-A	ChrII –left arm (subtelomeric)	590	*IMA1*	90.49
SeubIMA-B	ChrV – right arm (subtelomeric)	590	*IMA1* and *IMA4*	92.19
tSeubIMA-C	ChrV – right arm (subtelomeric)	510 (t)	*IMA2*	92.14
SeubMALS	ChrV – right arm (subtelomeric)	586	*MAL32*	84.76
SeubIMA-D	ChrXIII – left arm (subtelomeric)	590	*IMA1*	91
tSeubIMA-E	ChrXVI – left arm (subtelomeric)	181 (t)	*IMA2*	90.61
Regulators	**Location**	**Length (aa)**	**Closest S. cerevisiae homolog**	**Identity (%)[a]**
SeubMAL63A	ChrV – right arm (subtelomeric)	472	*MAL63*	73.83
SeubMAL63B	ChrXIII – left arm (subtelomeric)	471	*MAL63*	84.68
SeubMAL33A	ChrXIII – left arm (subtelomeric)	462	*MAL33*	70.72
SeubMAL33B	ChrXVI – left arm (subtelomeric)	469	*MAL33*	72.44

lager-brewing yeast-Saaz lineage (CBS 1513)

Transporters	Length (aa)	Likely Origin	Closest homolog	Identity (%)[a]
Lager – MTT1[M, H] *(tSaazMAL61-C)*	370	*inconclusive*	*MAL61*	92.14
Lager – AGT1 [M, H] *(SaazMAL11-CA)*	611	*inconclusive*	*MAL11*	85.9
SaazMALT1-E	595	*inconclusive*	*SeubMALT1*	87.88
SaazMALT2-E[i, H]	612	*S. eubayanus*[S]	*SeubMALT2*	89.18
SaazMAL11-CB[i, H]	617	*S. cerevisiae*	*MAL11*	96.45
Maltases	**Length (aa)**	**Likely Origin**	**Closest homolog**	**Identity (%)[a]**
tSaazMAL32-C	542	*S. cerevisiae*	*MAL32*	99.63
SaazIMA-CA	590	*S. cerevisiae*	*IMA2*	99.83
SaazIMA-EC	596	*S. eubayanus*[S]	*tSeubIMA-C*	98.62
SaazMALS-E	586	*S. eubayanus*[S]	*SeubMALS*	95.21
tSaazIMA-CB	575	*inconclusive*	*IMA5*	87.63
SaazIMA-CC	590	*S. cerevisiae*[S]	*IMA2*	99.49
SaazIMA-CD	582	*S. cerevisiae*[S]	*IMA5*	97.42
tSaazIMA1-C	166	*S. cerevisiae*	*IMA1*	100
tSaazIMA-EA	166	*S. cerevisiae*	*SeubIMA-A* (Ale)	83.03 (>99)[A]
Regulators	**Length (aa)**	**Likely Origin**	**Closest homolog**	**Identity (%)[a]**
tSaazMAL63-C	181	*S. cerevisiae*	*MAL63*	96.13
SaazMAL13-C[i]	474	*S. cerevisiae*	*MAL13* (Ale)	99.58 (100)[A]
SaazMAL63-EA	478	*inconclusive*	*SeubMAL63A*	71.49
SaazMAL33B-EB[i]	499	*S. eubayanus*[S]	*SeubMAL33B*	86.75

t – truncated sequence; [i] likely to be inactivated; [M] previously identified as a maltotriose transporter; [H] *S. cerevisiae* homolog can transport maltotriose; [S] origin supported by synteny; [a] based on amino acid and corrected for frameshift if necessary; [A] based on comparison to Foster's O and Foster's B base pair sequence

Table S2 – continued

lager-brewing yeast-Frohberg lineage (W34/70)

Transporters	Length (aa)	Likely Origin	Closest homolog	Identity (%)[a]
Lager – MTT1[M, H] *(WeihenMAL31-CA)*	616	*inconclusive*	*MAL31*	90.72
Lager – AGT1[M, H] *(WeihenMAL11-CA)*	611	*inconclusive*	*MAL11*	85.9
WeihenMALT1-E	595	*inconclusive*	*SeubMALT1*	87.88
WeihenMPH3-C[H]	603	*S. cerevisiae*	*MPH3*	99.34
tWeihenMAL31-CB[H]	363	*S. cerevisiae*	*MAL31*	96.69
WeihenMALT2-E[i,H]	612	*S. eubayanus*[S]	*SeubMALT2*	89.18
WeihenMAL11-CB[i,H]	617	*S. cerevisiae*	*MAL11*	96.45
Maltases	**Length (aa)**	**Likely Origin**	**Closest homolog**	**Identity (%)[a]**
tWeihenMAL32-C	573	*S. cerevisiae*	*MAL32*	99.48
tWeihenIMA-EB	338	*S. eubayanus*	*SeubIMA-B*	97.92
WeihenIMA-CA	582	*S. cerevisiae*	*IMA5*	97.25
WeihenMALS-E	586	*S. eubayanus*[S]	*SeubMALS*	95.21
WeihenIMA-CB	582	*inconclusive*	*IMA5*	87.78
tWeihenIMA-CC	198	*S. cerevisiae*	*IMA1*	99.49
tWeihenIMA-CD	198	*S. cerevisiae*	*IMA1*	99.49
tWeihenIMA-CE	198	*S. cerevisiae*	*IMA1* (Ale)	86.8 (>97)[A]
tWeihenIMA-EA	264	*S. eubayanus*[S]	*SeubIMA-A*	96.98
Regulators	**Length (aa)**	**Likely Origin**	**Closest homolog**	**Identity (%)[a]**
tWeihenMAL63-C	460	*S. cerevisiae*	*MAL63* (Ale)	81.96 (>98)[A]
WeihenMAL33-C	471	*S. cerevisiae*	*MAL33*	91.24 (>99)[A]
WeihenMAL33B-EA	478	*inconclusive*	*SeubMAL33B*	71.79
WeihenMAL13-C[i]	474	*S. cerevisiae*	*MAL13*	100
WeihenMAL33B-EB[i]	499	*S. eubayanus*[S]	*SeubMAL33B*	86.75

t – truncated sequence; [i] likely to be inactivated; [M] previously identified as a maltotriose transporter; [H] *S. cerevisiae* homolog can transport maltotriose; [S] origin supported by synteny; [a] based on amino acid and corrected for frameshift if necessary; [A] based on comparison to Foster's O and Foster's B base pair sequence

Table S3. WU-BLAST results and amino acid alignment of *MTT1*-like sequences from non-S288c strains of *S. cerevisiae*

WU-BLAST results and amino acid alignment of *MTT1*-like sequences from non-S288c strains of *S. cerevisiae*

		From WU-BLAST						
S. cerevisiae strain	amino acid ID to *MTT1*[a]	E-value	ID (%)	Location (contig/scaffold accession #)	start	end	Strain Description[b]	Notes
T7	96.42	0	95	AFDE01000045.1	83091	84938	Missouri oak tree exudate isolate.	Extracted sequence is reverse complement of *MTT1*.
DBVPG6044	96.09	0	95	JRIG01000448.1	8583	10430	Haploid derivative of West African bili wine isolate.	Extracted sequence is reverse complement of *MTT1*. In order to put the sequence into frame what is likely an extra A was removed from a poly A tract and a likely missing T in a poly T tract put in. This modified sequence still has an early stop codon.
YPS163	91.38 (overall)	0	95	JRIC01000169.1	81	1964	Pennsylvania woodland isolate.	Part of the end of this sequence is obscured by poor sequence (a tract of N's). When the part before this is translated and aligned to *MTT1* the percent ID is 96.32%, while for the remaining sequence after the obscured portion the percent ID for the translated sequence is 92.86%.
Sigma1278b	95.12	0	94	ACVY01000024.1	161442	163289	Laboratory strain.	Extracted sequence is reverse complement of *MTT1*.
SK1	96.31	0	95	JRIH01000332.1	1265	2975	Laboratory strain.	Sequence is incomplete because the contig ends. Like DBVPG6044, in order to put the sequence into frame what is likely an extra A in a poly A tract was removed and a likely missing T from a poly tract put in. This modified sequence also still has an early stop codon.
FL100	93.2	0	94	JRIT01000039.1	100613	102381	Laboratory strain.	Extracted sequence is reverse complement of *MTT1*. Part of the end of this sequence is obscured by poor sequence (a tract of N's). When the translated sequences before the poor sequence is aligned to *MTT1* the percent ID is 94.69%, while for the remaining sequence after the obscured portion the percent ID for the translated sequence is 92.86%.

Nucleotide sequence of *MTT1* from lager yeast of the Frohberg lineage (strain W34/70) used as WU-BLAST query sequence against "OTHER S. cerevisiae STRAINS: GENOMIC SEQUENCE" database in *Saccharomyces* Genome Database (www.yeastgenome.org) on 5-12-2015.

[a]pairwise alignment with *MTT1* WS34/70 in MUSCLE

[b]description from *Saccharomyces* Genome Database (http://www.yeastgenome.org/download-data/sequence)

Table S4. Number of synonymous substitutions in the *S. eubayanus* vs. *S. cerevisiae* subgenomes of lager-brewing yeasts

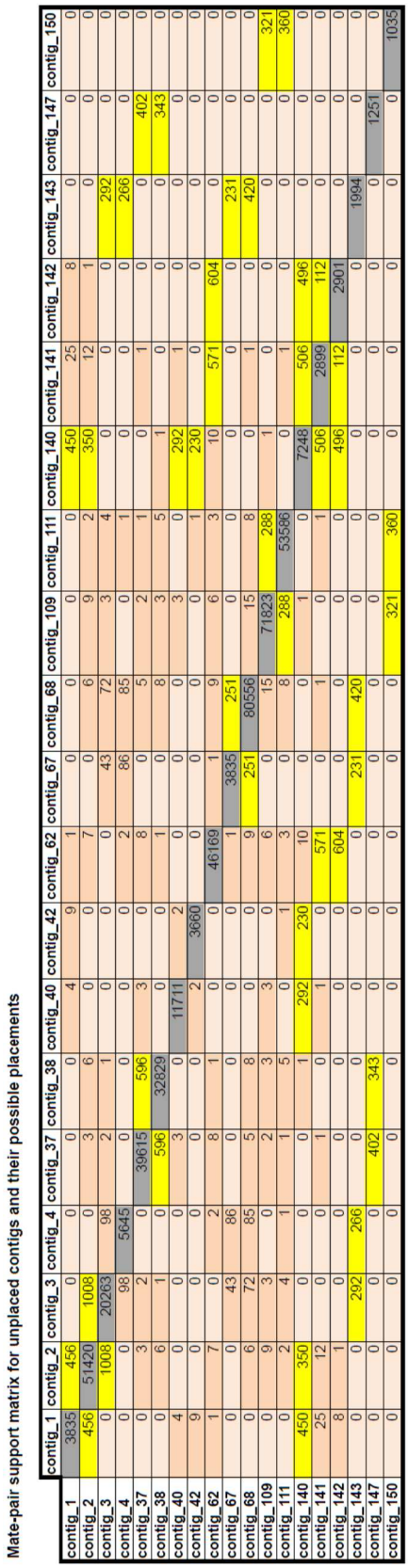
Number of synonymous substitutions in the *S. eubayanus* vs. *S. cerevisiae* subgenomes of lager-brewing yeasts

		sites with synonymous substitutions in *S. eubayanus* subgenome of lager-brewing yeasts			sites with synonymous substitutions in *S. cerevisiae* subgenome of lager-brewing yeasts		
Lineage	**Total # of synonymous sites**	**total # of substitutions**	**proportion of sites**	**99% binomial confidence interval**	**total # of substitutions**	**proportion of sites**	**99% binomial confidence interval**
Saaz	2310707	194	8.40E-05	(6.924e-5, 0.0001008)	2240	9.69E-04	(0.0009175, 0.001023)
Frohberg	2310707	191	8.27E-05	(6.807e-5, 9.935e-5)	1919	8.31E-04	(0.0007825, 0.0008805)
combined	2310707	385	1.67E-04	(0.0001456, 0.0001898)	4159	1.80E-03	(0.001729, 0.001873)

Table S5. BOWTIE2 mapping for placement of unplaced scaffolds

unplaced scaffold	unplaced contig	scaffold-possible placement	contig-possible placements	mate support count>100
MALψ cluster scaffold_18	contig_140	chrII-L	contig_1	450
		chrII-L	contig_2	350
		chrXIII-R	contig_40	292
		chrXIII-R	contig_42	230
		self	contig_140	7248
		FRE cluster(scaffold_19)	contig_141	506
		fully contained in conitg_141	contig_142	496
FRE cluster scaffold_19	contig_141	chrXV-R	contig_62	571
		MAL cluster(scaffold_18)	contig_140	506
		self	contig_141	2899
		fully contained in conitg_141	contig_142	112
PEP1 scaffold_21	contig_143	chrII	contig_3	292
		chrII	contig_4	266
		chrXIV	contig_67	231
		chrXIV	contig_68	420
		self	contig_143	1994
PPH22 scaffold_25	contig_147	chrIV	contig_37	402
		chrIV	contig_38	343
		self	contig_147	1251
GAL2 scaffold_28	contig_150	chrXII	contig_109	321
		chrXII	contig_111	360
		self	contig_150	1035

Table S6. Mate-pair support matrix for unplaced contigs and their possible placements



Mate-pair support matrix for unplaced contigs and their possible placements

	contig_1	contig_2	contig_3	contig_4	contig_37	contig_38	contig_40	contig_42	contig_62	contig_67	contig_68	contig_109	contig_111	contig_140	contig_141	contig_142	contig_143	contig_147	contig_150
contig_1	3835	456	0	0	0	0	4	9	1	0	0	0	0	450	25	8	0	0	0
contig_2	456	51420	1008	0	3	6	0	0	7	0	6	9	2	350	12	1	0	0	0
contig_3	0	1008	20263	98	2	1	0	0	0	43	72	3	4	0	0	0	292	0	0
contig_4	0	0	98	5645	0	0	0	0	2	86	85	0	1	0	0	0	266	0	0
contig_37	0	3	2	0	39615	596	3	0	8	0	5	2	1	0	1	0	0	402	0
contig_38	0	6	1	0	596	32829	0	0	1	0	8	3	5	1	0	0	0	343	0
contig_40	4	0	0	0	3	0	11711	2	0	0	0	3	0	292	1	0	0	0	0
contig_42	9	0	0	0	0	0	2	3660	0	0	0	0	1	230	0	0	0	0	0
contig_62	1	7	0	2	8	1	0	0	46169	1	9	6	3	10	571	604	0	0	0
contig_67	0	0	43	86	0	0	0	0	1	3835	251	0	0	0	0	0	231	0	0
contig_68	0	6	72	85	5	8	0	0	9	251	80556	15	8	0	1	0	420	0	0
contig_109	0	9	3	0	2	3	3	0	6	0	15	71823	288	1	0	0	0	0	321
contig_111	0	2	4	1	1	5	0	1	3	0	8	288	53586	0	1	0	0	0	360
contig_140	450	350	0	0	0	1	292	230	10	0	0	1	0	7248	506	496	0	0	0
contig_141	25	12	0	0	1	0	1	0	571	0	1	0	1	506	2899	112	0	0	0
contig_142	8	1	0	0	0	0	0	0	604	0	0	0	0	496	112	2901	0	0	0
contig_143	0	0	292	266	0	0	0	0	0	231	420	0	0	0	0	0	1994	0	0
contig_147	0	0	0	0	402	343	0	0	0	0	0	0	0	0	0	0	0	1251	0
contig_150	0	0	0	0	0	0	0	0	0	0	0	321	360	0	0	0	0	0	1035

self pairing
0 mate-pair support
0 < mate-pair support < 100
mate-pair support > 100

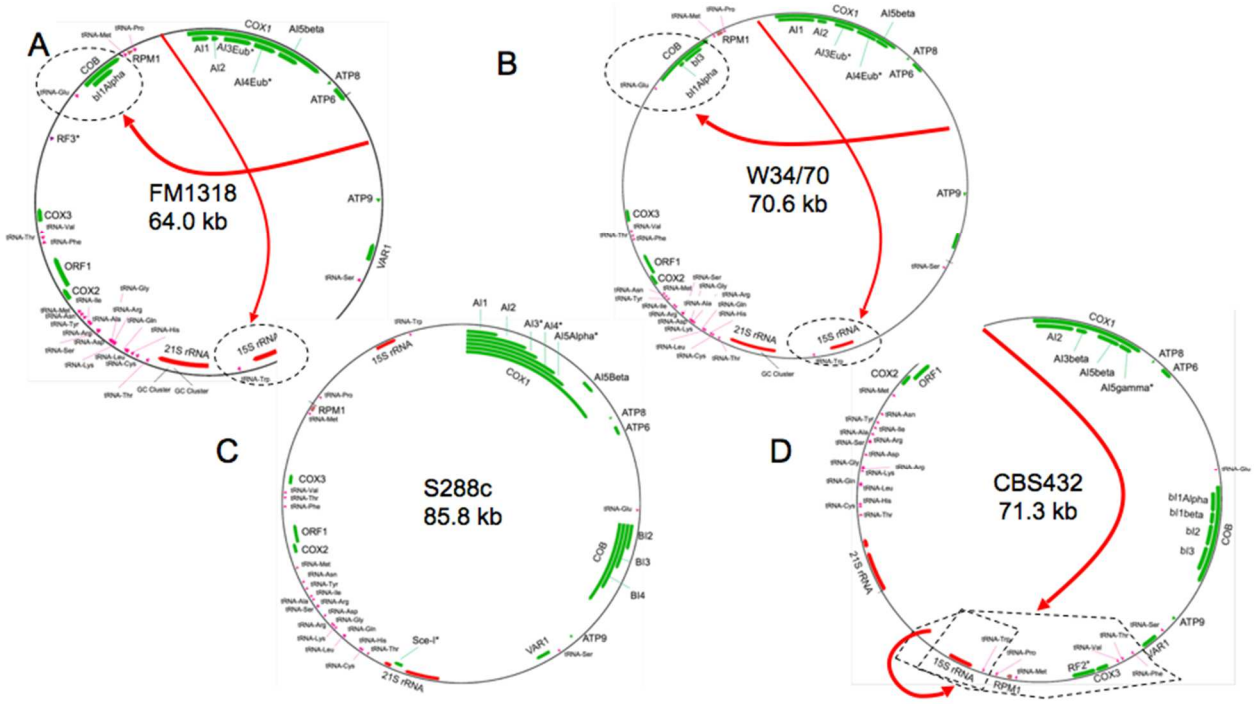


Figure S1. Lager related mitochondrial genomes. Schematic representation of *S. eubayanus* (FM1318), lager-brewing yeast (Frohberg strain W34/70), *S. cerevisiae* (S288c), and *S. paradoxus* (CBS 432) mitochondrial genome annotations. Mitochondrial genes, rRNAs, tRNAs, and non-coding RNAs are represented in green, red, pink, and brown, respectively. Genes with asterisks are additional elements or gene sequences in each mitochondria compared with *S. cerevisiae* S288c mtDNA. Red arrows represent rearrangements compared to S288c.*S. paradoxus RF2* was annotated in this study.

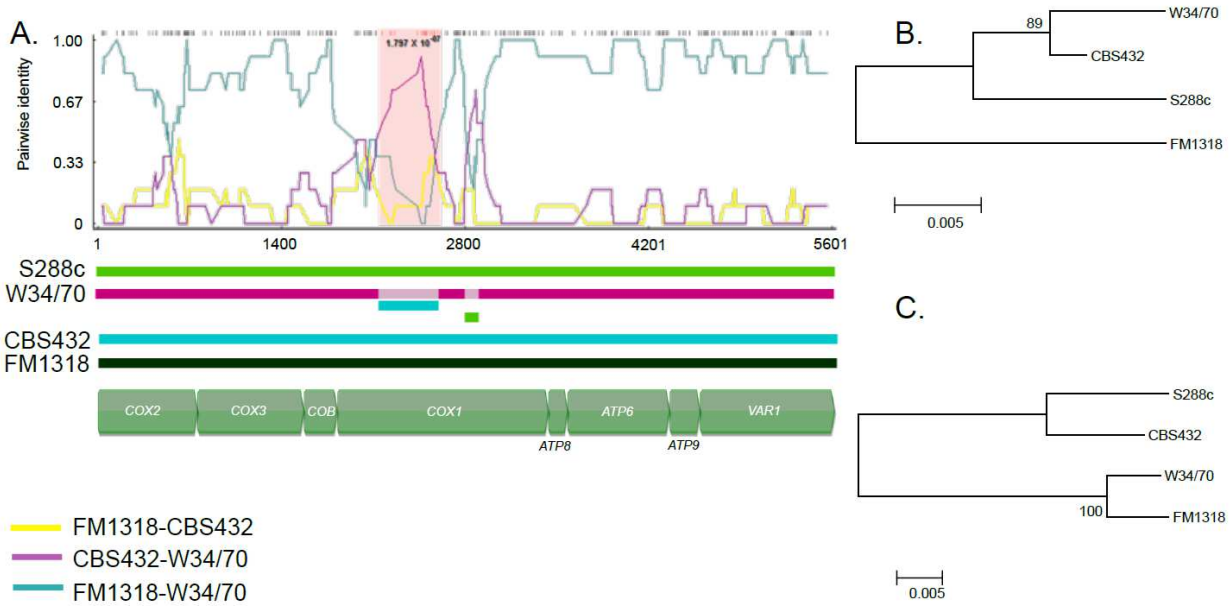


Figure S2. Introgression within the lager yeast mitochondrial genome. Lager-brewing yeast (Frohberg strain W34/70) mtDNA shows an introgression from *S. paradoxus* in the *COX1* gene. A) The output results from RDPv4 shows an introgression in the *COX1* gene in W34/70 involving exon 4. Other potential introgressions, such as in *COB* and the 5' end of *COX1,* were also detected but not well supported. A Neighbor- Joining tree for the introgressed (B) and non-introgressed regions (C) is shown.

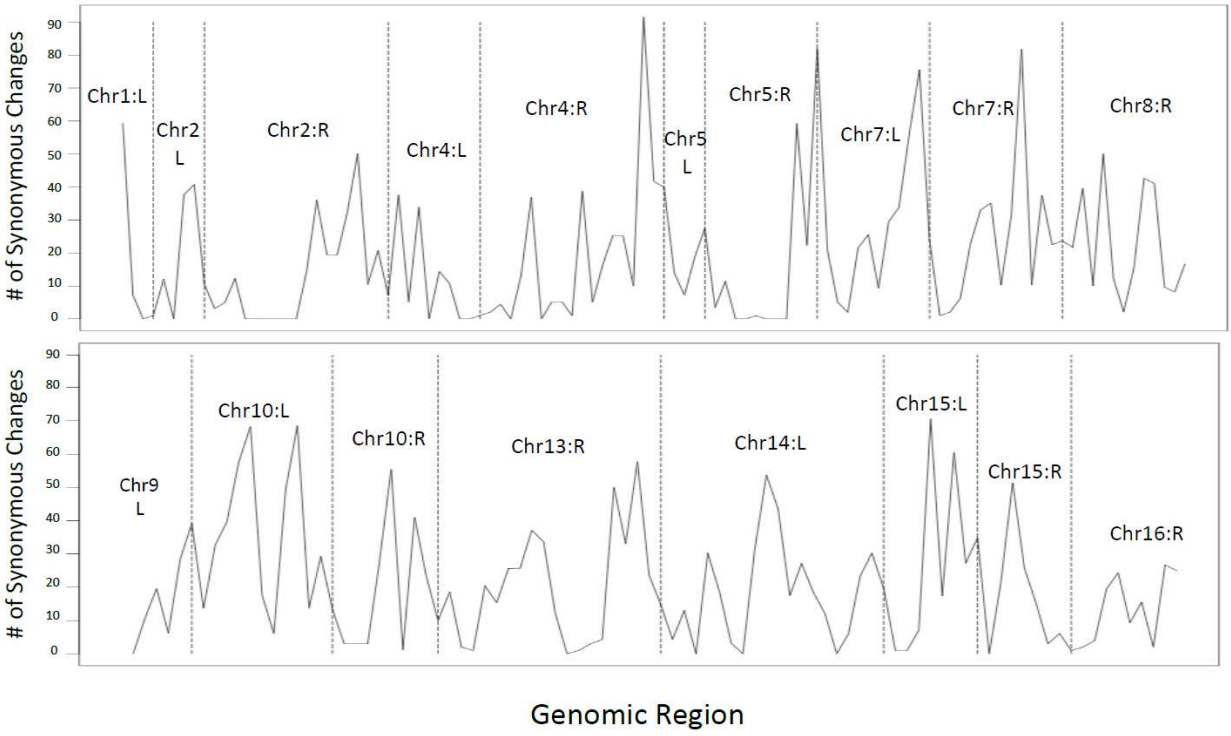


Figure S3. Synonymous changes across the Saaz and Frohberg *S. cerevisiae* subgenomes. Sliding window (10 gene window) of the number of synonymous changes between the *S. cerevisiae* subgenomes of the Saaz and Frohberg lineages of lager-brewing yeasts. Only chromosome arms with at least 40 conserved genes are shown. Due to differences in genome content between the two lineages, not all genomic regions are represented. With multiple origins of lager yeasts from haploid or low heterozygosity *S. cerevisiae* parents drawn from a meiotically reproducing population, comparisons of the genomes would be expected to reveal a range of diversity values, including some haplotype blocks of low diversity. In contrast, widespread loss of heterozygosity could explain the previous observations of low heterozygosity (Dunn and Sherlock 2008) if a single heterozygous *S. cerevisiae* individual had given rise to the both lineages. Under this model, approximately half of all sites that were heterozygous in the parent would have had the same alleles fixed in both lineages by chance. As a result, some segments of the genome would have high diversity between the Saaz and Frohberg lineages, and others would have almost no diversity. Examination of d_S along shared portions of the Saaz and

Frohberg *S. cerevisiae* genomes failed to demonstrate the expected proportion of segments with low diversity expected under this scenario, indicating that a single diploid heterozygous individual was not the *S. cerevisiae* donor for the lager-brewing yeast lineages.

Dunn B, Sherlock G. 2008. Reconstruction of the genome origins and evolution of the hybrid lager yeast Saccharomyces pastorianus. Genome Res. 18:1610–1623.

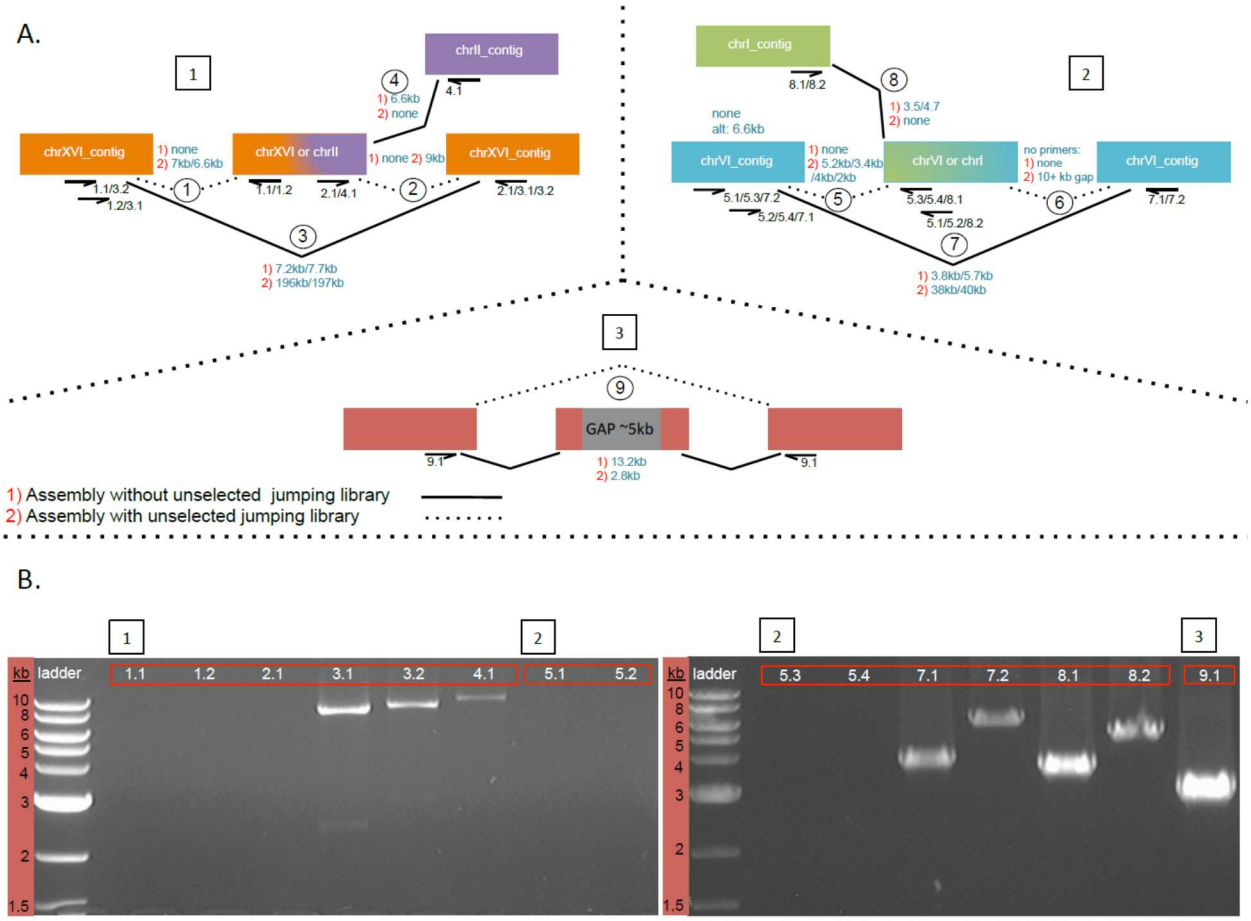


Figure S4. Verification of alternative *S. eubayanus* genome assemblies by PCR. A) shows the regions, numbered 1-3 (boxed numbers), of the *S. eubayanus* genome with alternative configurations between the assembly made without the unselected jumping library (solid line) and the one made with the unselected jumping library (dashed line). Each alternative connection is numbered (circled numbers), and primer pairs are numbered after the connection they test. Combinations of primers and their directions are indicated by half arrows, and the size of resulting bands that would occur under different assemblies are placed where alternative configurations are possible. B) shows the resulting bands from each pair of primers. Reactions that test the same set of alternative connections are outlined in red with the number of the region they represent from part A) placed above. For alternative configuration 3 (covered by PCR reaction 9.1), the assembly with the unselected jumping library fully closed the gap.

S. eubayanus contig

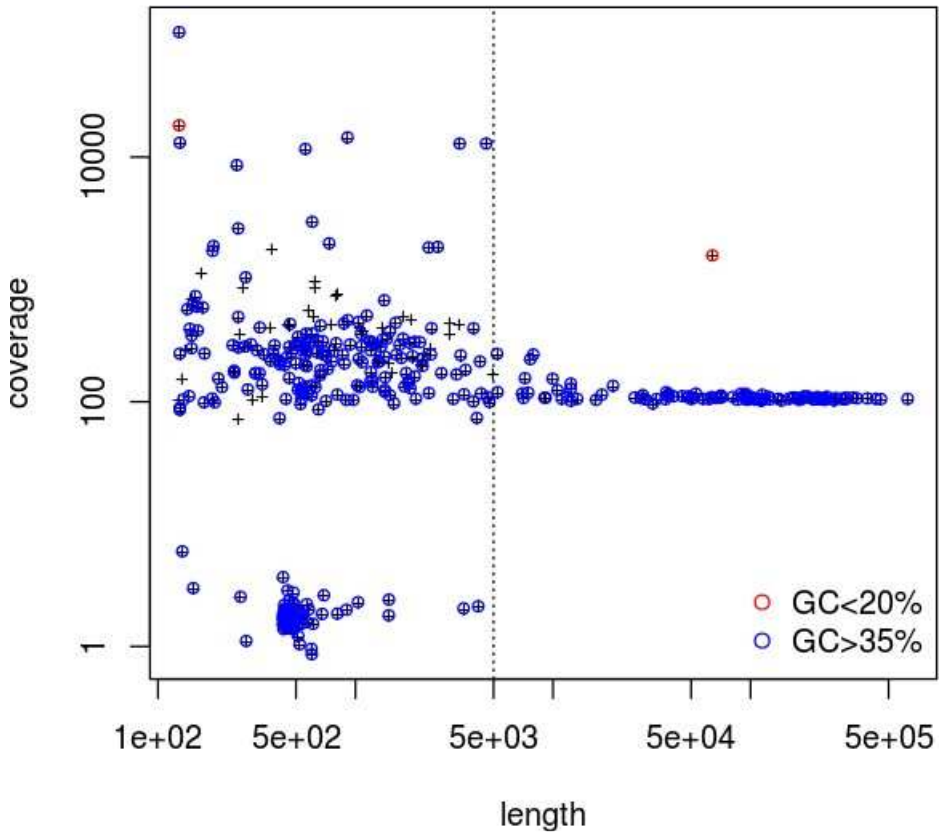


Figure S5. GC content, length, and coverage of SPAdes contigs. Distribution of contigs (>200 bp) relative to their read coverage and length. Contigs with GC content > 35% and <20% marked in blue and red, respectively. The mtDNA contig of 64 kb was detected with GC content 17.5% and 14.6-fold greater coverage relative to the remaining contigs >5 kb (vertical dashed line).

Appendix B

Supplementary Information from Chapter 3

	SIFT[b]			
STRUM[a]	Related Sequences[c]	Sequence[d]		
		NCBI[1]	UniRef90[2]	SwissProt[3]
-0.4	1.00	1.00	0.57	1.00
# of sequences analyzed	19	59	18	8

Table S1. **Results of mutation prediction analyses for E18V, the sole non-synonymous substitution in the lgAGT1 protein-coding sequence, relative to tbAGT1.**

[a]predicted ΔΔG (<0.5 likely no change in function, Bromberg and Rost 2009)

[b]provides amino acid probability score (<0.5 predicted to be deleterious)

[c]sequences used for for SIFT analysis are the same as used for the phylogenetic analysis (Fig. 2)

[d]sequences used for analysis provided by a PSI-BLAST of the indicated protein database

Protein Databases: [1]NCBI nonredundant 2011 Mar, [2]UniRef90 2011 Apr, [3]UniProt-SwissProt 57.15 2011 Apr

Strain	Background	Evolved in	Initial OD	Day 3	Day 6
yHKS210	Admixture strain	-	0.244	0.498	0.664
yHEB1505	yHKS210	maltotriose	0.145	1.666	1.757
yHEB1506	yHKS210	maltotriose	0.15	1.735	1.81
yHRVM108	North Carolinian strain	-	0.133	0.499	0.679
yHEB1585	yHRVM108	maltose	0.213	0.938	1.414
yHEB1586	yHRVM108	maltose	0.133	0.801	1.36
yHEB1587	yHRVM108	maltose	0.117	0.686	1.299
yHEB1588	yHRVM108	maltose	0.139	0.649	1.033
yHEB1589	yHRVM108	maltose	0.129	0.731	1.059
yHEB1590	yHRVM108	maltose	0.147	0.736	1.096

Table S2. Growth on maltotriose of single-colony isolates from directed evolution experiments. Strains were evolved with either maltotriose or maltose as the primary carbon source (2%) with 0.1% added glucose. N = 1.

Strain	Background	Evolved in	Initial OD	Day 2
yHRVM108	North Carolinian strain	-	0.144	0.52
yHEB1585	yHRVM108	maltose	0.249	1.767
yHEB1586	yHRVM108	maltose	0.184	1.628
yHEB1587	yHRVM108	maltose	0.209	1.753
yHEB1588	yHRVM108	maltose	0.234	1.692
yHEB1589	yHRVM108	maltose	0.366	1.272
yHEB1590	yHRVM108	maltose	0.244	1.405

Table S3. Growth on maltose of single-colony isolates. Isolated from directed evolution of yHRVM108 in 2% maltose + 0.1% glucose. N = 1.

Table S4. Strains and plasmids used in this work

Strain	Species	Background	Relavent genotype	MAL	MalTri	Description	Source
FM1318	*S. eubayanus*	-	-	+	-	monosporic derivative of the type strain of *S. eubayanus*	Libkind & Hittinger *et al*. (*PNAS*,2011)
yHRVM108	*S. eubayanus*	-	-	(+/-)	-	Member of the holoartic subpopulation population of isolated from Durham, North Carolina	Peris & Langdon *et al.* (*PLOS Genetics*,2016)
yHKS210	*S. eubayanus*	-	*MALT4/MALT4*	+	-	Nearly homozygous wild admixture of *S. eubayanus* populations A and B isolated from Sheboygan, Wisconsin	Peris *et al.* (*Molecular Ecology*, 2014)
yHEB1403	*S. eubayanus*	yHKS210	-	+	+	Sample taken from the 86th passage of yHKS210.	This study
yHEB1505	*S. eubayanus*	yHKS210	*MALT434/MALT434*	+	+	single colony isolate taken from the evolution of yHKS210 in maltotriose after 86 passages.	This study
yHEB1506	*S. eubayanus*	yHKS210	-	+	+	single colony isolate taken from the evolution of yHKS210 in maltotriose after 86 passages.	This study
yHEB1593	*S. eubayanus*	yHKS210 x yHEB1505	*MALT434/MALT4*	+	+	Backcross between yHEB1505 and yHKS210	This study
yHEB1853	*S. eubayanus*	yHKS210 x yHEB1505	*MALT434/malt4Δ::NatMX*	+	+	yHEB1593 with original *MALT4* allele replaced with *NatMX* marker	This study
yHEB1854	*S. eubayanus*	yHKS210 x yHEB1505	*malt434Δ::NatMX/MALT4*	+	-	yHEB1593 with chimeric *MALT434* allele replaced with *NatMX* marker	This study
yHEB1881	*S. eubayanus*	yHKS210	*MALT4/MALT4* [pBM5155-*MALT434*]	+	+	yHKS210 with pBM5155 based plasmid for *MALT434* expression	This study
yHEB1878	*S. eubayanus*	yHRVM108	[pBM5155-*MALT434*]	+	+	yHRVM108 with pBM5155 based plasmid for *MALT434* expression	This study
yHEB1870	*S. eubayanus*	yHRVM108	[pBM5155-*MALT1*]	(+/-)	-	yHRVM108 with pBM5155 based plasmid for *MALT1* expression	This study
yHEB1872	*S. eubayanus*	yHRVM108	[pBM5155-*MALT3*]	(+/-)	-	yHRVM108 with pBM5155 based plasmid for *MALT3* expression	This study
yHEB1877	*S. eubayanus*	yHRVM108	[pBM5155-*MALT2/4*]	(+/-)	-	yHRVM108 with pBM5155 based plasmid for *MALT2/4* expression	This study
yHEB1885	*S. eubayanus*	yHRVM108	[pBM5155-*lgAGT1*]	+	+	yHRVM108 with pBM5155 based plasmid for *AGT1* from lager brewing yeast expression	This study
yHEB1882	*S. eubayanus*	yHKS210	[pBM5155-*lgAGT1*]	+	+	yHKS210 with pBM5155 based plasmid for expression of the lager allele of *AGT1*	This study
yHEB1883	*S. eubayanus*	yHRVM108	[pBM5155-*ncAGT1*]	+	+	yHRVM108 with pBM5155 based plasmid for expression of the North Carolinian alllele of *AGT1*	This study
yHAB47	*S. cerevisiae* (2n) *x S. eubayanus* (2n)	-	-	+*	+*	Weihenstephan 34/70; Frohberg lineage of lager brewing yeast hybrids *(Magalhães *et al.* 2016)	Peris & Langdon *et al.* (*PLOS Genetics*,2016)
yHEB1585	*S. eubayanus*	yHRVM108	-	+	+	single colony isolate taken from replicate A of the evolution of yHRVM108 in maltose after 86 passages.	This study
yHEB1586	*S. eubayanus*	yHRVM108	-	+	+	single colony isolate taken from replicate A of the evolution of yHRVM108 in maltose after 86 passages.	This study
yHEB1587	*S. eubayanus*	yHRVM108	-	+	+	single colony isolate taken from replicate A of the evolution of yHRVM108 in maltose after 86 passages.	This study
yHEB1588	*S. eubayanus*	yHRVM108	-	+	+	single colony isolate taken from replicate B of the evolution of yHRVM108 in maltose after 86 passages.	This study
yHEB1589	*S. eubayanus*	yHRVM108	-	+	+	single colony isolate taken from replicate B of the evolution of yHRVM108 in maltose after 86 passages.	This study
yHEB1590	*S. eubayanus*	yHRVM108	-	+	+	single colony isolate taken from replicate B of the evolution of yHRVM108 in maltose after 86 passages.	This study
pBM5155	plasmid	-	*NatMX*	na	na	plasmid for doxycycline-inducible gene expression (GenBank KT725394.1)	Alexander *et al*. 2016 (*Fungal Genet. Biol*)
pHEB7	plasmid	-	-	na	na	pBM5155 with *ncAGT1* gap repaired into the *Not*I site	This study
pHEB11	plasmid	-	-	na	na	pBM5155 with *lgAGT1* gap repaired into the *Not*I site	This study
pHEB16	plasmid	-	-	na	na	pBM5155 with *MALT1* gap repaired into the *Not*I site	This study
pHEB17	plasmid	-	-	na	na	pBM5155 with *MALT3* gap repaired into the *Not*I site	This study
pHEB18	plasmid	-	-	na	na	pBM5155 with *MALT4* gap repaired into the *Not*I site	This study
pHEB19	plasmid	-	-	na	na	pBM5155 with *MALT434* gap repaired into the *Not*I site	This study

Alexander WG, Peris D, Pfannenstiel BT, Opulente DA, Kuang M, Hittinger CT. 2016. Efficient engineering of marker-free synthetic allotetraploids of Saccharomyces. Fungal Genet. Biol. 89:10–17.

Krogerus K, Arvas M, De Chiara M, Magalhães F, Mattinen L, Oja M, Vidgren V, Yue J-X, Liti G, Gibson B. 2016. Ploidy influences the functional attributes of de novo lager yeast hybrids. Appl. Microbiol. Biotechnol. 100:7203–7222.

Libkind D, Hittinger CT, Valério E, Gonçalves C, Dover J, Johnston M, Gonçalves P, Sampaio JP. 2011. Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. Proc. Natl. Acad. Sci. U. S. A. 108:14539–14544.

Peris D, Langdon QK, Moriarty R V., Sylvester K, Bontrager M, Charron G, Leducq J-B, Landry CR, Libkind D, Hittinger CT. 2016. Complex Ancestries of Lager-Brewing Hybrids Were Shaped by Standing Variation in the Wild Yeast Saccharomyces eubayanus. PLOS Genet. 12:e1006155.

Peris D, Sylvester K, Libkind D, Gonçalves P, Sampaio JP, Alexander WG, Hittinger CT. 2014. Population structure and reticulate evolution of Saccharomyces eubayanus and its lager-brewing hybrids. Mol. Ecol. 23:2031–2045.

Table S5: Oligonucleotides used in this work.

Name	Sequence	Description
oHECPB75	TATAGACACGCAAACACAAATACACACACTAAATTAATGAAGGGATTATCCTCATTAATA	amplifies *MTT1* for cloning into pBM5155 over *Not*I site by gap repair
oHECPB76	TCAAGAAATTCGCTTATTTAGAAGTGGCGCGAATTCACTATCATTTGTTCACAACAGATG	amplifies *MTT1* for cloning into pBM5155 over *Not*I site by gap repair
oHECPB77	CACGCAAACACAAATACACACACTAAATTAATGAAAAATATACTTTCGCTGGTAGGAAGA	amplifies *lgAGT1* for cloning into pBM5155 over *Not*I site by gap repair
oHECPB78	CAAGAAATTCGCTTATTTAGAAGTGGCGCGAATTCACTATCATAACGCCTGTTGACTCGC	amplifies *lgAGT1* for cloning into pBM5155 over *Not*I site by gap repair
oHECPB104	ACACGCAAACACAAATACACACACTAAATTAATGAAGAATATCATTTCGCTGGTAAGAAG	amplifies *ncAGT1* for cloning into pBM5155 over *Not*I site by gap repair
oHECPB105	GAAATTCGCTTATTTAGAAGTGGCGCGAATTCACTATTATAATGCCTGCTGACTCATGCT	amplifies *ncAGT1* for cloning into pBM5155 over *Not*I site by gap repair
oHECPB124	CCTATGCTTCTGAAGTTTGC	primer to confirm sequence of *lgAGT1* and *ncAGT1* in pBM5155
oHECPB125	CCTGCCAAACCAAGACAG	primer to confirm sequence of *lgAGT1* and *ncAGT1* in pBM5155
oHECPB128	GCTTGTTTATGTTGGGTCGGTC	primer to confirm sequence of *MTT1* in pBM5155
oHECPB129	GACCGACCCAACATAAACAAGC	primer to confirm sequence of *MTT1* in pBM5155
oHECPB138	ACACGCAAACACAAATACACACACTAAATTAATGAAGGGTCTATCCTCAATGATAAATAG	amplifies *MALT4* (and or *MALT2*) from *S. eubayanus*for cloning into pBM5155 over *Not*I site by gap repair
oHECPB139	CGCTTATTTAGAAGTGGCGCGAATTCACTATCAATCCATTAGAGATGGGGTGCTTTGCTC	amplifies *MALT4* (and or *MALT2*) from *S. eubayanus*for cloning into pBM5155 over *Not*I site by gap repair
oHECPB140	CACGCAAACACAAATACACACACTAAATTAATGAAGGGTCTATCTTCAATATTGAATAGA	amplifies *MALT1* from *S. eubayanus*for cloning into pBM5155 over *Not*I site by gap repair
oHECPB141	CGCTTATTTAGAAGTGGCGCGAATTCACTATCAAATCGTAAGAGATGGGGTAGTTAATTC	amplifies *MALT1* from *S. eubayanus*for cloning into pBM5155 over *Not*I site by gap repair
oHECPB142	CACGCAAACACAAATACACACACTAAATTAATGAAGGGCTTATCCTCACTGATAAACAGA	amplifies *MALT3* from *S. eubayanus*for cloning into pBM5155 over *Not*I site by gap repair
oHECPB143	CGCTTATTTAGAAGTGGCGCGAATTCACTATCATAAATTCGTAATAGATGGTGTGCTTCG	amplifies *MALT3* from *S. eubayanus*for cloning into pBM5155 over *Not*I site by gap repair
oHECPB156	ATATTATGGTAGATATGACCTTTACG	primer to confirm sequence of *MALT2/4* in pBM5155
oHECPB157	GGTTGATTTCATCAGAGATTGCTTTGG	primer to confirm sequence of *MALT2/4* in pBM5155
oHECPB158	CGTAAAGGTCATATCTACCATAATAT	primer to confirm sequence of *MALT2/4* in pBM5155
oHECPB159	CCAATGTCTGACTGTGTCTTATGCCTC	primer to confirm sequence of *MALT2/4* in pBM5155
oHECPB160	GAGGCATAAGACACAGTCAGACATTGG	primer to confirm sequence of *MALT2/4* in pBM5155
oHECPB161	CTGTCTTGGTTTGGCAGG	primer to confirm sequence of *lgAGT1* and *ncAGT1* in pBM5155
oHECPB162	CTGTGAAAGTTTAGGGATGATTGCGG	primer to confirm sequence of *MALT1* in pBM5155
oHECPB163	CCGCAATCATCCCTAAACTTTCACAG	primer to confirm sequence of *MALT1* in pBM5155
oHECPB164	CCTGTATATGTTGGATCGGTCAAAC	primer to confirm sequence of *MALT1* in pBM5155
oHECPB165	GTTTGACCGATCCAACATATACAGG	primer to confirm sequence of *MALT1* in pBM5155
oHECPB166	CCTTTGGCTTTAAGATACTACC	primer to confirm sequence of *MALT3* in pBM5155
oHECPB167	GGTAGTATCTTAAAGCCAAAGG	primer to confirm sequence of *MALT3* in pBM5155
oHECPB168	GGATGCTCTGATACTCACGG	primer to confirm sequence of *MALT3* in pBM5155
oHECPB169	CCGTGAGTATCAGAGCATCC	primer to confirm sequence of *MALT3* in pBM5155
oHECPB170	GGARAGTGATACCTTATCATCTGCTGCGCTAAGAGTCAAGATCTGTTTAGCTTGCCTT	amplifies MX-driven drug markers with overhanges to the *MALT4* and *MALT2* loci for allele replacement by homologous recombination
oHECPB171	ACTCAAAAAAAATTCCAAAAGCTATTAGGTAACTGAGCTCGTTTTCGACACTGGAT	amplifies MX-driven drug markers with overhanges to the *MALT4* and *MALT2* loci for allele replacement by homologous recombination
oHECPB172	CGATATTTCCGCCGCAGCCCGAG	specifically amplifies the *MALT2* locus when used with primers oHECPB159 or oHECPB156
oHECPB173	CTTAGTAGCAGCGACATATTCAAG	specifically amplifies the *MALT4* locus when used with primers oHECPB159 or oHECPB156
oHCT770	AACTCTTGTTTTCTTCTTTTCTCTAAA	amplifies genes inserted over *Not*I site in pBM5155
oHCT771	GGGACCTAGACTTCAGGTTGTC	amplifies genes inserted over *Not*I site in pBM5155

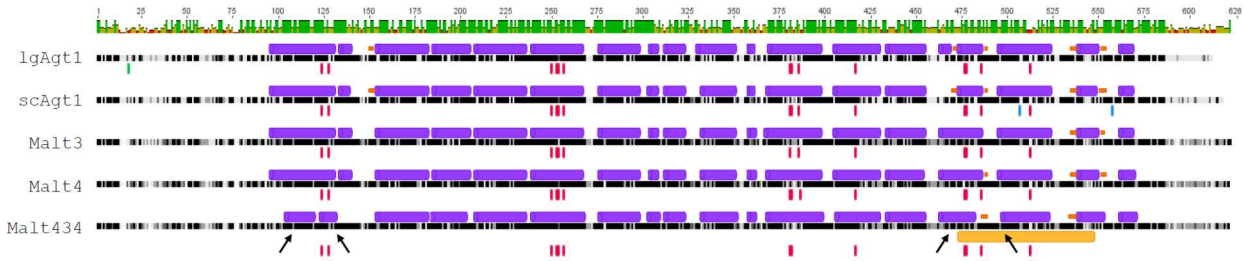


Figure S1. Protein structural alignment between Malt3, Malt4, Malt434, scAgt1, and lgAgt1. The purple blocks represent predicted alpha helices, and the orange lines represent predicted beta strands. Red ticks mark predicted maltose binding sites. Blue ticks mark residues found to be important for maltotriose transport by Smit *et al.* 2008. A green tick marks the location of the single non-synonymous substitution between *lgAGT1* and *tbAGT1*. Arrows point to alpha helices in Malt434 whose predicted sizes are reduced compared to other transporters in the alignment.

Appendix C

Supplementary Information from Chapter 4

Table S1. Strains and plasmid used in this work

Strain	Synonym	Species	Background	*ρ* status	*MAT*	Marker(s)	Description	Source
FM1283	yHWA117	*S. cerevisiae*	BY4724 (S288C)	native mtDNA	**a**	*ura3-Δ lys2-Δ* P_{TDH3}-*yEBFP*-T_{CYC1}	Laboratory strain	Hittinger & Carroll (*Nature*,2007)
yHRVM481	-	*S. cerevisiae*	FM1283	null	**a**	*ura3-Δ lys2-Δ* P_{TDH3}-*yEBFP*-T_{CYC1}	ρ^0 laboratory strain	This study
yHRVM483	-	*S. cerevisiae*	FM1283	null	**a**	*ura3-Δ lys2-Δ* P_{TDH3}-*yEBFP*-T_{CYC1}	ρ^0 laboratory strain	This study
yHRVM485	-	*S. cerevisiae*	FM1283	null	**a**	*ura3-Δ lys2-Δ* P_{TDH3}-*yEBFP*-T_{CYC1}	ρ^0 laboratory strain	This study
WLP530B	yHAB48	*S. cerevisiae*	-	native mtDNA	**a/α**	-	Brewing isolate (Belgian Ale)	White Labs
yHWA245	-	*S. cerevisiae*	RM11-1a	native mtDNA	**a**	*ade2-Δ::HERP1 leu2-Δ ura3-Δ hoΔ::KanMX*	Laboratory strain used to amplify the *KanMX* cassette with overhangs to the *HO* locus for allele replacement in WLP530B	Alexander *et al.* (*Genetics*, 2014)
yHEB1528	-	*S. cerevisiae*	WLP530B	native mtDNA	**a**	*hoΔ::KanMX*	Stable haploid of brewing isolate	This study
yHEB1632	-	*S. cerevisiae*-ale	WLP530B	null	**a**	*hoΔ::KanMX*	ρ^0 stable haploid of brewing isolate	This study
yHEB1621	-	*S. cerevisiae*-ale	WLP530B	null	**a**	*hoΔ::KanMX*	ρ^0 stable haploid of brewing isolate	This study
yHEB1623	-	*S. cerevisiae*-ale	WLP530B	null	**a**	*hoΔ::KanMX*	ρ^0 stable haploid of brewing isolate	This study
FM1318	yHEB10	*S. eubayanus*	-	native mtDNA	**a/α**	-	Monosporic derivative of the type strain	Libkind & Hittinger *et al.* (*PNAS*,2011)
yHEB162	-	*S. eubayanus*	FM1318	native mtDNA	**α**	*hoΔ::NatMX*	Stable haploid of FM1318	This study
yHEB1611	-	*S.eubayanus*	yHEB162	null	**α**	*hoΔ::NatMX*	ρ^0 stable haploid of FM1318	This study
yHEB1613	-	*S.eubayanus*	yHEB162	null	**α**	*hoΔ::NatMX*	ρ^0 stable haploid of FM1318	This study
yHEB1614	-	*S.eubayanus*	yHEB162	null	**α**	*hoΔ::NatMX*	ρ^0 stable haploid of FM1318	This study
yHRVM108	yHEB449	*S.eubayanus*	-	native mtDNA	**a/α**	-	Holarctic population isolate	Peris & Langdon *et al.* (*PLOS Genetics*,2016)
yHEB1606	-	*S. eubayanus*	yHRVM108	native mtDNA	**α**	*hoΔ::NatMX*	Stable haploid of yHRVM108	This study
yHEB1633	-	*S. eubayanus*	yHEB1606	null		*hoΔ::NatMX*	ρ^0 stable haploid of yHRVM108; potentially *S. cerevisiae ρ* incompatible	This study
yHEB1634	-	*S. eubayanus*	yHEB1606	null		*hoΔ::NatMX*	ρ^0 stable haploid of yHRVM108; potentially *S. cerevisiae ρ* incompatible	This study
yHEB1635	-	*S. eubayanus*	yHEB1606	null		*hoΔ::NatMX*	ρ^0 stable haploid of yHRVM108; potentially *S. cerevisiae ρ* incompatible	This study
yHEB1636	-	*S. eubayanus*	yHEB1606	null		*hoΔ::NatMX*	ρ^0 stable haploid of yHRVM108; potentially *S. cerevisiae ρ* incompatible	This study
yHEB1637	-	*S. eubayanus*	yHEB1606	null		*hoΔ::NatMX*	ρ^0 stable haploid of yHRVM108; potentially *S. cerevisiae ρ* incompatible	This study
yHEB1638	-	*S. eubayanus*	yHEB1606	null	**α**	*hoΔ::NatMX*	ρ^0 stable haploid of yHRVM108; *S. cerevisiae ρ* compatible	This study
MCC123	yHEB879	*S. cerevisiae*	-	null	**a**	*ade2-1, ura3-52, kar1-1*	ρ^0 karyogamy deficient	Thorsness & Fox (*Genetics*, 1993)
MCC109	yHEB880	*S. cerevisiae*	-	null	**α**	*ade2-1, ura3-52, kar1-1*	ρ^0 karyogamy deficient	Costanzo & Fox (*Mol. Cell Biol.*, 1993)
yHEB1752	-	*S. cerevisiae* x *S. eubayanus*	FM1283 x *S. cerevisiae* yHEB1611	mtDNA	**a/α**	*ura3-Δ lys2-Δ* P_{TDH3}-*yEBFP*-T_{CYC1} *hoΔ::NatMX*	synthetic hybrid	This study
yHEB1749	-	*S. cerevisiae* x *S. eubayanus*	FM1283 x *S. cerevisiae* yHEB1613	mtDNA	**a/α**	*ura3-Δ lys2-Δ* P_{TDH3}-*yEBFP*-T_{CYC1} *hoΔ::NatMX*	synthetic hybrid	This study
yHEB1746	-	*S. cerevisiae* x *S. eubayanus*	FM1283 x *S. cerevisiae* yHEB1614	mtDNA	**a/α**	*ura3-Δ lys2-Δ* P_{TDH3}-*yEBFP*-T_{CYC1} *hoΔ::NatMX*	synthetic hybrid	This study
yHEB1127	-	*S. cerevisiae* x *S. eubayanus*	yHRVM481 x *S. eubayanus* yHEB162	mtDNA	**a/α**	*ura3-Δ lys2-Δ* P_{TDH3}-*yEBFP*-T_{CYC1} *hoΔ::NatMX*	synthetic hybrid	This study
yHEB1737	-	*S. cerevisiae* x *S. eubayanus*	yHRVM483 x *S. eubayanus* yHEB162	mtDNA	**a/α**	*ura3-Δ lys2-Δ* P_{TDH3}-*yEBFP*-T_{CYC1} *hoΔ::NatMX*	synthetic hybrid	This study
yHEB1740	-	*S. cerevisiae* x *S. eubayanus*	yHRVM485 x *S. eubayanus* yHEB162	mtDNA	**a/α**	*hoΔ::NatMX*	synthetic hybrid	This study
yHEB1768	-	*S. cerevisiae* x *S. eubayanus*	yHEB1528 x *S.cerevisiae*-ale yHEB1611	mtDNA	**a/α**	*hoΔ::KanMX /hoΔ::NatMX*	synthetic hybrid	This study

Table S1 – continued

Strain	Synonym	Species	Background	*ρ* status	*MAT*	Marker(s)	Description	Source
yHEB1766	-	*S. cerevisiae x S. eubayanus*	yHEB1528 x yHEB1613	*S.cerevisiae*-ale mtDNA	**a/α**	*hoΔ::KanMX /hoΔ::NatMX*	synthetic hybrid	This study
yHEB1764	-	*S. cerevisiae x S. eubayanus*	yHEB1528 x yHEB1614	*S.cerevisiae*-ale mtDNA	**a/α**	*hoΔ::KanMX /hoΔ::NatMX*	synthetic hybrid	This study
yHEB1761	-	*S. cerevisiae x S. eubayanus*	yHEB1632 x yHEB162	*S. eubayanus* mtDNA	**a/α**	*hoΔ::KanMX /hoΔ::NatMX*	synthetic hybrid	This study
yHEB1758	-	*S. cerevisiae x S. eubayanus*	yHEB1621 x yHEB162	*S. eubayanus* mtDNA	**a/α**	*hoΔ::KanMX /hoΔ::NatMX*	synthetic hybrid	This study
yHEB1755	-	*S. cerevisiae x S. eubayanus*	yHEB1623 x yHEB162	*S. eubayanus* mtDNA	**a/α**	*hoΔ::KanMX /hoΔ::NatMX*	synthetic hybrid	This study
W34/70	yHAB47	*S. cerevisiae* (2n) *x S. eubayanus*(2n)	Weihenstephan 34/70	native mtDNA	**a/α**	-	industrial lager-brewing strain; Frohberg (Group II) lineage	Peris & Langdon *et al.* (*PLOS Genetics*,2016)
yHEB1626	-	*S. cerevisiae* (2n) *x S. eubayanus*(2n)	yHAB47	null	**a/α**	-	ρ^0 industrial lager-brewing strain	This study
yHEB1627	-	*S. cerevisiae* (2n) *x S. eubayanus*(2n)	yHAB47	null	**a/α**	-	ρ^0 industrial lager-brewing strain	This study
yHEB1628	-	*S. cerevisiae* (2n) *x S. eubayanus*(2n)	yHAB47	null	**a/α**	-	ρ^0 industrial lager-brewing strain	This study
pHCT2	pHEB12	plasmid	-	-	-	*NatMX*	HyPr plasmid; for doxycycline-inducible mating type switching in *Saccharomyces*; GenBank KT725395	Alexander *et al.* 2016 (*Fungal Genet. Biol*)
yHEB1793	-	*S. cerevisiae* (2n) *x S. eubayanus*(2n)	yHEB1626	null	**a/α**	[pHCT2 (HyPr)]	mating competent ρ^0 lager strain	This study
yHEB1797	-	*S. cerevisiae* (2n) *x S. eubayanus*(2n)	yHEB1627	null	**a/α**	[pHCT2 (HyPr)]	mating competent ρ^0 lager strain	This study
yHEB1798	-	*S. cerevisiae* (2n) *x S. eubayanus*(2n)	yHEB1628	null	**a/α**	[pHCT2 (HyPr)]	mating competent ρ^0 lager strain	This study
yHEB1800	-	*S. cerevisiae* (2n) *x S. eubayanus*(2n)	yHEB1628	null	**a/α**	[pHCT2 (HyPr)]	mating competent ρ^0 lager strain	This study
yHEB1835	-	*S. cerevisiae* (2n) *x S. eubayanus*(2n)	yHEB1793	*S.cerevisiae*-ale mtDNA	**a/α**	[pHCT2 (HyPr)]	lager cybrid	This study
yHEB1827	-	*S. cerevisiae* (2n) *x S. eubayanus*(2n)	yHEB1797	*S.cerevisiae*-ale mtDNA	**a/α**	[pHCT2 (HyPr)]	lager cybrid	This study
yHEB1828	-	*S. cerevisiae* (2n) *x S. eubayanus*(2n)	yHEB1798	*S.cerevisiae*-ale mtDNA	**a/α**	[pHCT2 (HyPr)]	lager cybrid	This study
yHEB1839	-	*S. cerevisiae* (2n) *x S. eubayanus*(2n)	yHEB1793	*S.cerevisiae*-ale mtDNA	**a/α**	[pHCT2 (HyPr)]	lager cybrid	This study
yHEB1841	-	*S. cerevisiae* (2n) *x S. eubayanus*(2n)	yHEB1797	*S.cerevisiae*-ale mtDNA	**a/α**	[pHCT2 (HyPr)]	lager cybrid	This study
yHEB1843	-	*S. cerevisiae* (2n) *x S. eubayanus*(2n)	yHEB1800	*S.cerevisiae*-ale mtDNA	**a/α**	[pHCT2 (HyPr)]	lager cybrid	This study

Table S2. Oligonucleotides used in this work.

Name	Sequence	Description
oHECPB15	AATACATACAACTTACTTTTTCAAAATTAATTTACATACTAGATCTGTTTAGCTTGCCTT	amplifies MX-driven drug markers with overhangs to the *S. eubayanus HO* locus for allele replacement by homologous recombination.
oHECPB16	TCTATATAGACAACAACCACTTCCACTAGCCTTTAAGCGAGCTCGTTTTCGACACTGGAT	amplifies MX-driven drug markers with overhangs to the *S. eubayanus HO* locus for allele replacement by homologous recombination.
oHWA565	CTATGGTTTACGAAATGATCCACG	primer specific to 450bp upstream of *S. cerevisiae HO* locus; used to amplify region around *HO* locus with selection marker for allele replacement by homologous recombination, to confirm allele replacement, and to confirm the absence of *ρ* carrier DNA in lager cybrids.
oHWA566	CACTGACCCAGTCTTGTCTTC	primer specific to 540bp downstream of *S. cerevisiae HO* locus; used to amplify region around *HO* locus with selection marker for allele replacement by homologous recombination, to confirm allele replacement, and to confirm the absence of *ρ* carrier DNA in lager cybrids.
oHWA568	TTTGCAAATCGAAGACCCAT	primer internal to oHWA565 and OHWA566; used for sequencing to confirm the absence of *ρ* carrier DNA in lager cybrids.
oHMB3	GTTTCTGGCCGAGCTACAAG	primer specific to 260bp upstream of *S. eubayanus HO* locus; used to confirm allele replacement.
oHMB4	CAAGGCCATGTCTTCTCGTT	primer specific to 410bp upstream of *S. eubayanus HO* locus; used to confirm allele replacement.
oHECPB148	TTTGAATATCAATGAAAATGCC	primer specific to the *KAR1* locus of *S. cerevisiae*; used to amplify the *KAR1* locus to confirm absence of *ρ* carrier DNA in lager cybrids.
oHECPB149	TTAAAACCTATAATACACATATATATTGC	primer specific to the *KAR1* locus of *S. cerevisiae*; used to amplify and sequence the *KAR1* locus to confirm absence of *ρ* carrier DNA in lager cybrids.
oHDP25	TGCGCCAAGTGTCTGAAGAACAACTGGGA	primer general to the *GAL4* locus of *Saccharomyces*; used to amplify and sequence the *GAL4* locus to confirm absence of *ρ* carrier DNA in lager cybrids (Peris et al. 2012).
oHDP26	GCGATTTCAATCTGGTTATTATACAACATCAT	primer general to the *GAL4* locus of *Saccharomyces*; used to amplify the *GAL4* locus to confirm absence of *ρ* carrier DNA in lager cybrids (Peris et al. 2012).
ITS1	TCCGTAGGTGAACCTGCGG	standard primer to amplify out the 5.8S rDNA (*ITS*) sequence from fungi; used to amplify *ITS* region to confirm successful creation of synthetic hybrids (McCullough et al. 1998).
ITS4	TCCTCCGCTTATTGATATGC	standard primer to amplify out the 5.8S rDNA (*ITS*) sequence from fungi; used to amplify and sequence *ITS* region to confirm successful creation of synthetic hybrids (McCullough et al. 1998).

Peris D, Lopes C a., Arias A, Barrio E. 2012. Reconstruction of the Evolutionary History of Saccharomyces cerevisiae x S. kudriavzevii Hybrids Based on Multilocus Sequence Analysis. PLoS One 7:e45527.

McCullough M. J., Clemons K. V, McCusker J. H., Stevens D. A., 1998 Intergenic transcribed spacer PCR ribotyping for differentiation of Saccharomyces species and interspecific hybrids. J. Clin. Microbiol. 36: 1035–8.

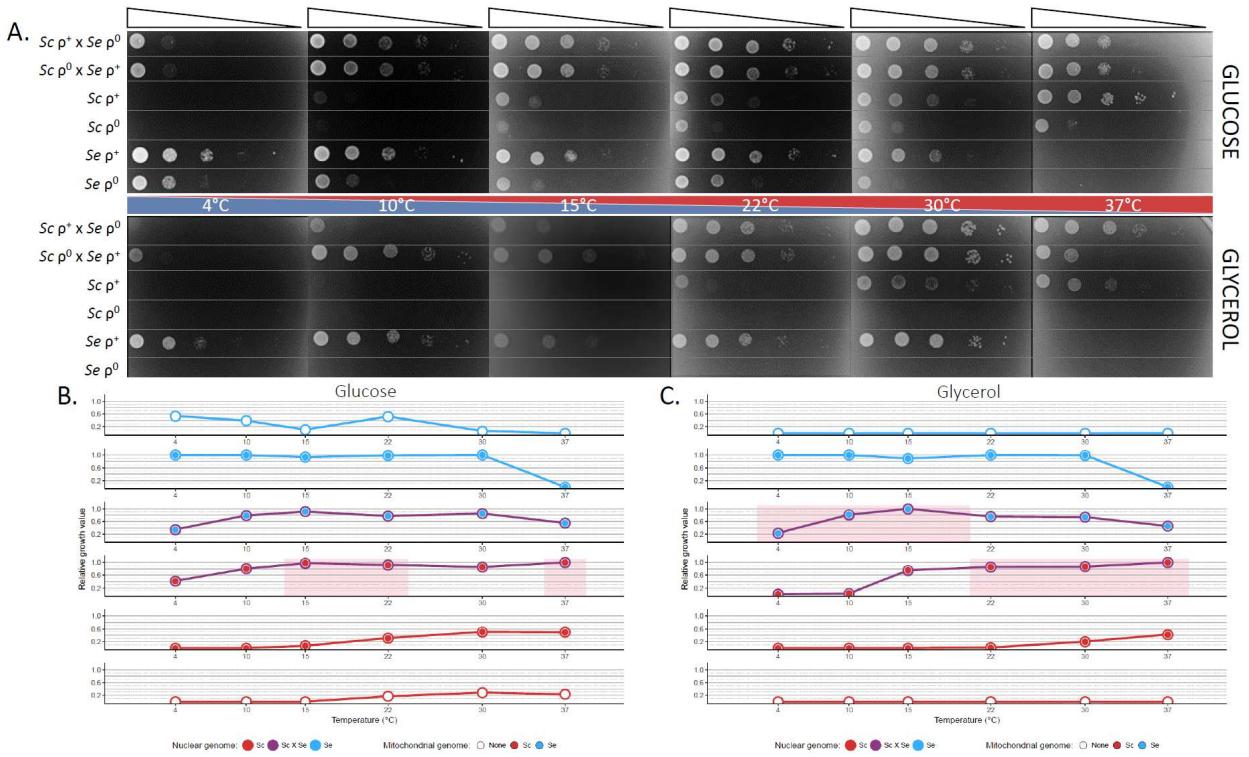


Figure S1. *Sc* x *Se* growth assay. Growth assay for *S. cerevisiae* (laboratory strain) x *S. eubayanus* (type strain) hybrids and parental strains. A) Representative spot assay plates grown at various temperatures on plates containing glucose or glycerol as the sole carbon source. The following strains were tested: *S. cerevisiae* (*Sc*) ρ^+ parent, *Sc* ρ^0 parent, *S. eubayanus* (*Se*) ρ^+ parent, *Se* ρ^0 parent, *Sc* ρ^+ x *Se* ρ^0 hybrid, and *Sc* ρ^0 x *Se* ρ^+ hybrid. B and C) Relative growth of tested strains across all temperatures combining all replicates. Outer circles and lines represent nuclear genotype, while inner circles represent mtDNA. Highlighted regions represent temperatures where a hybrid of one mitotype had significantly greater relative growth than the hybrid with the alternative mitotype. B) Relative growth of tested strains on glucose. C) Relative growth of tested strains on glycerol.

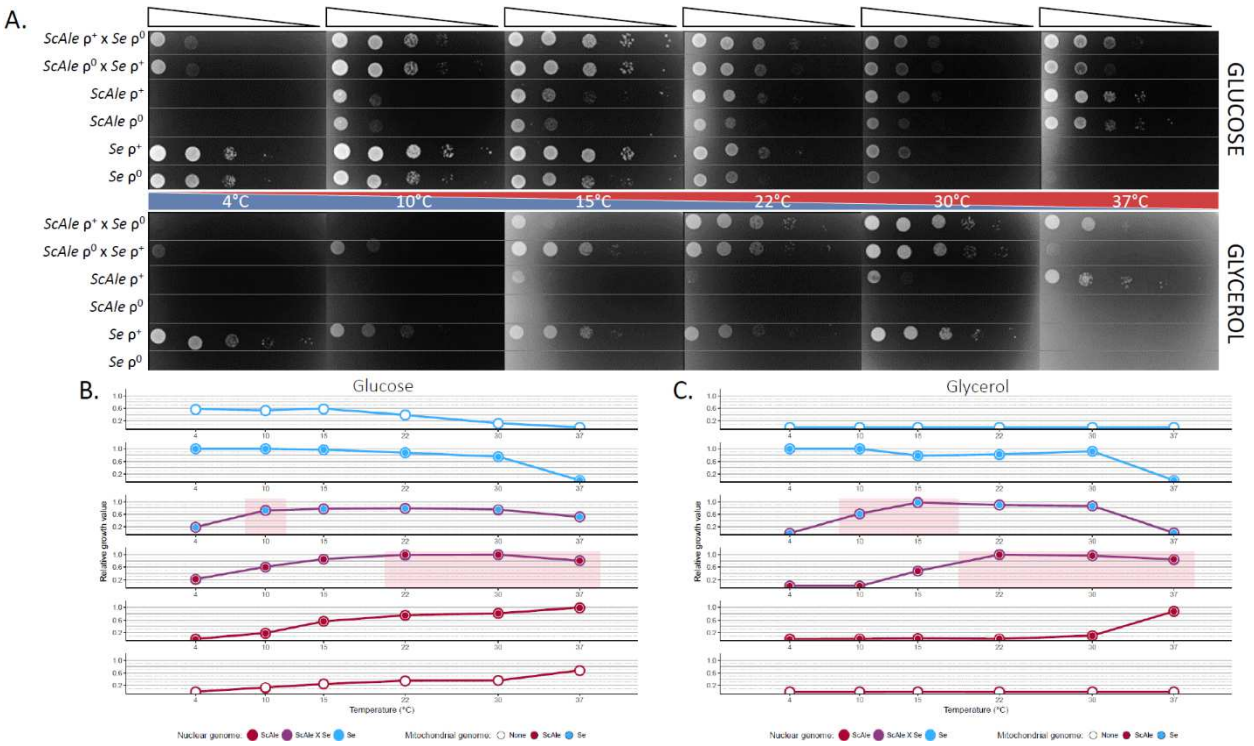


Figure S2. *ScAle* X *Se* growth assay. Growth assay for *S. cerevisiae*-ale x *S. eubayanus* (type strain) hybrids and parental strains. A) Representative spot assay plates grown at various temperatures on plates containing glucose or glycerol as the sole carbon source. The following strains were tested: *S. cerevisiae*-ale (*ScAle*) ρ^+ parent, *ScAle* ρ^0 parent, *S. eubayanus* (*Se*) ρ^+ parent, *Se* ρ^0 parent, *ScAle* ρ^+ x *Se* ρ^0 hybrid, and *ScAle* ρ^0 x *Se* ρ^+ hybrid. B and C) Relative growth of tested strains across all temperatures combining all replicates. Outer circles and lines represent nuclear genotype, while inner circles represent mtDNA. Highlighted regions represent temperatures where a hybrid of one mitotype had significantly greater relative growth than the hybrid with the alternative mitotype. B) Relative growth of tested strains on glucose. C) Relative growth of tested strains on glycerol.

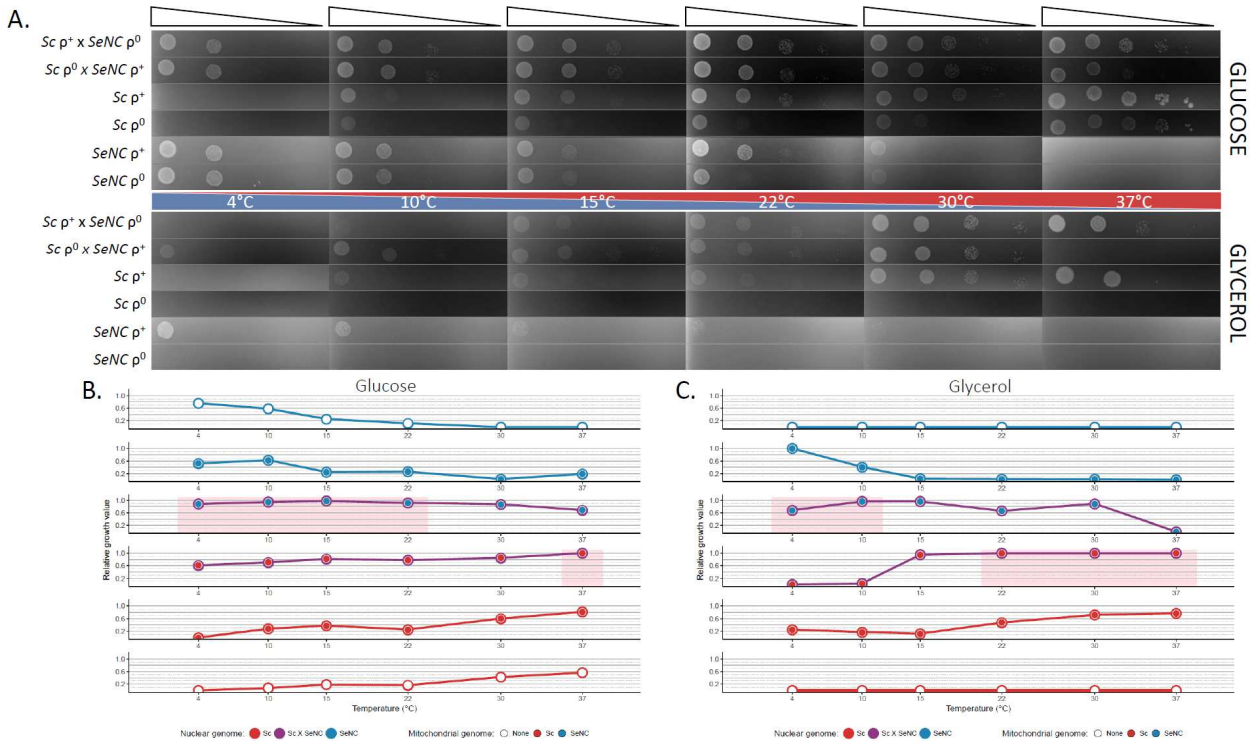


Figure S3. *Sc* X *SeNC* growth assay. Growth assay for *S. cerevisiae* (laboratory strain) x *S. eubayanus* – North Carolina hybrids and parental strains. A) Representative spot assay plates grown at various temperatures on plates containing glucose or glycerol as the sole carbon source. The following strains were tested: *S. cerevisiae* (*Sc*) ρ^+ parent, *Sc* ρ^0 parent, *S. eubayanus* – North Carolina (*SeNC*) ρ^+ parent, *SeNC* ρ^0 parent, *Sc* ρ^+ x *SeNC* ρ^0 hybrid, and *Sc* ρ^0 x *SeNC* ρ^+ hybrid. B and C) Relative growth of tested strains across all temperatures combining all replicates. Outer circles and lines represent nuclear genotype, while inner circles represent mtDNA. Highlighted regions represent temperatures where a hybrid of one mitotype had significantly greater relative growth than the hybrid with the alternative mitotype. B) Relative growth of tested strains on glucose. C) Relative growth of tested strains on glycerol.

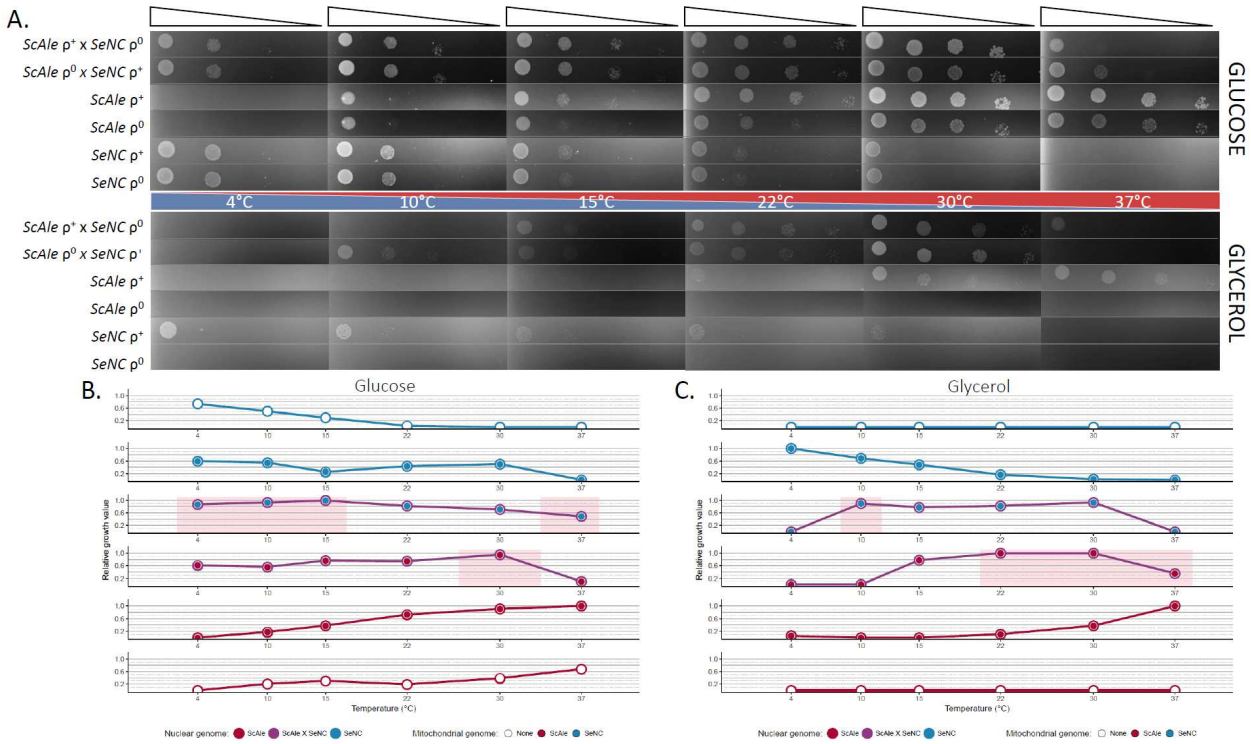
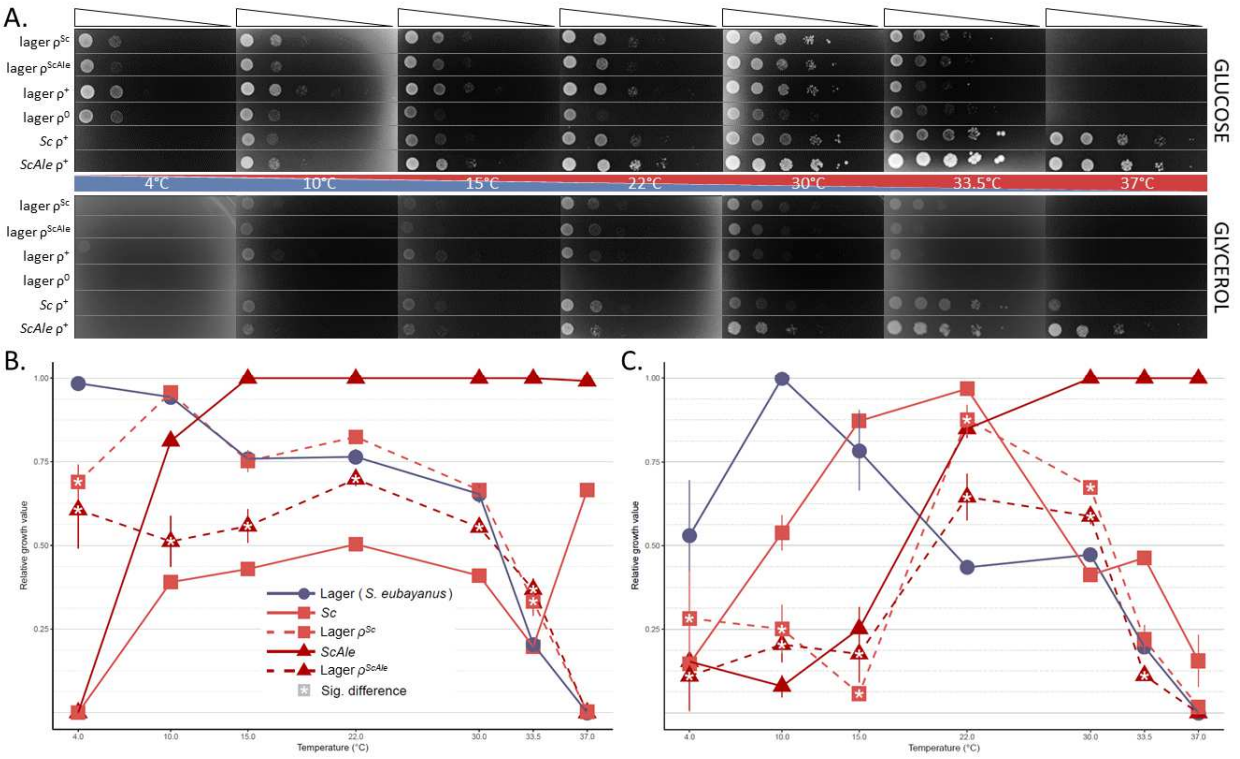


Figure S4. *ScAle* X *SeNC* growth assay. Growth assay for *S. cerevisiae*-ale x *S. eubayanus* – North Carolina hybrids and parental strains. A) Representative spot assay plates grown at various temperatures on plates containing glucose or glycerol as the sole carbon source. The following strains were tested: *S. cerevisiae*-ale (*ScAle*) ρ^+ parent, *ScAle* ρ^0 parent, *S. eubayanus* – North Carolina (*SeNC*) ρ^+ parent, *SeNC* ρ^0 parent, *ScAle* ρ^+ x *SeNC* ρ^0 hybrid, and *ScAle* ρ^0 x *SeNC* ρ^+ hybrid. B and C) Relative growth of tested strains across all temperatures combining all replicates. Outer circles and lines represent nuclear genotype, while inner circles represent mtDNA. Highlighted regions represent temperatures where a hybrid of one mitotype had significantly greater relative growth than the hybrid with the alternative mitotype. B) Relative growth of tested strains on glucose. C) Relative growth of tested strains on glycerol.



FigureS5. Lager cybrid growth assay. Growth assay for lager cybrids and parental strains. A) Representative spot assay plates grown at various temperatures on plates containing glucose or glycerol as the sole carbon source. The following strains were tested: lager ρ^+ parent, lager ρ^0 parent, *S. cerevisiae* (laboratory strain) (*Sc*) ρ^+ parent, *S. cerevisiae*-ale (*ScAle*) ρ^+ parent, lager ρ^{Sc} cybrid, and lager ρ^{ScAle} cybrid. B and C) Relative growth of tested strains across all temperatures combining all replicates. Error bars represent standard error and asterisks indicate statistically significant differences in growth between the cybrid and lager with native mtDNA ($p < 0.05$). B) Relative growth of tested strains, excluding lager ρ^0, on glucose. C) Relative growth of tested strains, excluding lager ρ^0, on glycerol.

Saccharomyces lithium acetate transformation protocol (Gietz and Woods 2002) adapted for *S. eubayanus*

Materials:

- 1 M lithium acetate
- 100 mM lithium acetate
- 50% (w/v) PEG-4000
- Boiled single-stranded salmon sperm DNA (ssssDNA) (10mg/mL)
- 100% Ethanol

Procedure:

1. Grow yeast overnight in 3 mL of YPD or other culturing medium.
2. The next day, inoculate 50 mL of fresh YPD with enough overnight culture to bring the OD to 0.25 and shake at 250 rpm at room temperature.
    - Some strains of *S. eubayanus* can tolerate growth at 30°C and will grow faster at this temperature, but other strains are sensitive and will grow much more slowly.
3. After four hours, take an OD_{600} reading. If between 0.75 and 1.0 OD, continue to step 4; otherwise, allow to continue shaking.
    - For slow growing strains, it may take several more hours to reach the appropriate OD.
4. Wash yeast cells in H_2O.
5. Resuspend yeast cells in 950 µL 100 mM lithium acetate.
6. Aliquot 100 µL of cell suspension into Eppendorf tubes.
7. Spin at max speed in microcentrifuge for 1 min, then remove supernatant.
8. Add in this order:
    - 240 µL 50% PEG-4000,
    - 36 µL 1 M lithium acetate,
    - 43 µL of DNA solution to be transformed,
    - 5 µL boiled ssssDNA.
9. Gently resuspend pellet in transformation mixture.
10. Heat shock for 55 minutes at 34°C.
    - 37°C works almost as well.
11. Add 36 µL 100% Ethanol.
12. Heat shock for another 5 minutes.
13. After heat shock, briefly spin down and remove supernatant.
14. Immediately resuspend in 600 µL of YPD.

<u>Recovery Method 1</u>

1) Incubate at room temperature for 3 hours on wheel or shaker.
2) Plate 200 µL of transformation suspension to each of three selective media plates.
3) Incubate at room temperature.
    - The strain can also be grown at 30°C if the strain is not heat sensitive.

<u>Recovery Method 2</u>

You **MUST** use this method for counter-selection (e.g. selecting for the loss of *URA3* by 5-FOA resistance) to allow for protein turnover.

1) Plate immediately to YPD and allow to grow overnight.
2) The next day, replica-plate to selective media.
   Note: The spatial separation provided by this method guarantees that transformants are independent.

If successful, colonies will generally appear after two or three days.

- Depending on the sensitivity of a strain to a given selection regime, it could take a week or more for colonies to appear, with new colonies appearing up to two weeks after transformation.

Gietz D. R., Woods R. A., 2002 Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol. 350: 87–96.