On the origins and industrial applications of *Saccharomyces cerevisiae* x *Saccharomyces kudriavzevii* hybrids

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ABSTRACT

Companies based on alcoholic fermentation products, such as wine, beer, and biofuels, use yeasts to make their products. Each industrial process utilizes different media conditions, which differ in sugar content, the presence of inhibitors, and fermentation temperatures. *Saccharomyces cerevisiae* has traditionally been the main yeast responsible for most fermentation processes. However, the market is changing due to the consumer demands or external factors, such as climate change. Some processes, such as biofuel production or winemaking, require new yeasts to solve specific challenges, especially those associated with sustainability, novel flavors, and altered alcohol contents. One of the proposed solutions is the application of yeast hybrids. The lager beer market has been dominated by *S. cerevisiae* x *Saccharomyces eubayanus* hybrids. However, several less thoroughly studied hybrids have been isolated from other diverse industrial processes. Here we focus on *S. cerevisiae* x *Saccharomyces kudriavzevii* hybrids, which have been isolated from diverse industrial conditions that include wine, ale beer, cider, and dietary supplements. Emerging data suggest an extended and complex story of adaptation of these hybrids to traditional industrial conditions. *S. cerevisiae* x *S. kudriavzevii* hybrids are also being explored for new industrial applications, such as biofuels. This review describes the past, present, and future of *S. cerevisiae* x *S. kudriavzevii* hybrids.

Keywords: industrial applications, yeasts, *Saccharomyces cerevisiae*, *Saccharomyces kudriavzevii*, hybrids, phylogenetics.

The wine and ale beer industries have been dominated by the yeast species *Saccharomyces cerevisiae*. These industries constitute 64.8% of the alcoholic beverage market (Bailey 2015). Consumer demands are in constant flux, which requires innovative ways to release new wine and beer products. To fulfill these new demands, researchers are mining yeast diversity to apply them to alcoholic fermentation.

S. cerevisiae has seven sister congeneric based on the biological species concept (BSC), which defines species as a group of interbreeding organisms that produce fertile offspring and is reproductively isolated from other such groups (Mayr 1970). Interspecific hybridization experiments have been extensively performed to delimitate the eight *Saccharomyces* species according to the BSC (Naumov 1987), although chromosomal rearrangements can suggest additional species that are inconsistent with phylogenetic and other data (Fischer et al. 2000; Naumov et al. 2000). Due to the fact that reproductive isolation in the genus *Saccharomyces* is mainly postzygotic, viable interspecific hybrids can easily be formed. Although they are totally or partially sterile, these hybrids can be maintained by asexual reproduction. For that reason, the application of the BSC in yeast in general, and in the genus *Saccharomyces* in particular is fuzzy. As an example, the two closest relatives, *S. eubayanus* and *S. uvarum* can interbreed and generate hybrids with levels of spore viability between 7.3% and 18% (Libkind et al. 2011; Bing et al. 2014), although hybrid depression is also indicative of postzygotic reproductive isolation.

The ability of *Saccharomyces* species to generate interspecies hybrids and the capacity of these hybrids to spread by clonal divisions (Le Jeune et al. 2007) have generated a collection of interspecific hybrids with interesting industrial properties. The most studied hybrids are the lager strains of *Saccharomyces pastorianus* (Liti et al. 2005; Dunn and Sherlock 2008; Bond 2009; Nakao et al. 2009; Gibson et al. 2013; Bing et al. 2014; Peris et al. 2014, 2016a; Walther et al. 2014; Wendland 2014; Baker et al. 2015; Gibson and Liti 2015; Monerawela et al. 2015; Okuno et al. 2016). Following the nomenclature suggested by Nguyen and Boekhout (2017), these lager strains are more properly referred to as allopolyploid hybrids of *S. eubayanus* x *S. cerevisiae* (Libkind et al. 2011). In addition to these lager hybrids, there is a collection of other allopolyploid hybrids with more diverse industrial applications, from brewing and winemaking to cider and biofuel production, among others.

This review is intended to give a picture about what we know of the story, isolation, genome composition, and industrial applications of the successful *S. cerevisiae* x *S. kudriavzevii* hybrids. Our intention is that this review can serve as a motivation for the clarification of the remaining open questions related to these interesting hybrids.

History of *S. cerevisiae* x *S. kudriavzevii* hybrid isolation

An interspecies hybrid is defined as an organism with relevant genomic contributions from two or more different species (Bisson 2017). AMH and Vin7, strains isolated from wine (Supplementary Table 1), were the first hybrids to be molecularly identified containing genome contributions from both *S. cerevisiae* and *S. kudriavzevii* species (Bradbury et al. 2005). The application of a diverse set of molecular techniques, such as Amplified Fragment Length Polymorphisms (AFLPs), Restriction Fragment Length Polymorphisms (RFLPs), Pulsed-Field Gel Electrophoresis (PFGE), microsatellites, array Comparative Genome Hybridization (aCGH, macroarrays or microarrays), mitochondrial DNA restriction profiles, and Polymerase Chain Reactions (PCRs) of delta or multilocus dataset together with Sanger-sequencing, has unraveled a collection of fifty-three hybrids (Supplementary Table 1) with nuclear genome contributions of *S. cerevisiae* and *S. kudriavzevii* (Barros Lopes et al. 2002; Naumova et al. 2005; Bradbury et al. 2005; González et al. 2006, 2008; Heinrich 2006; Lopandic et al. 2007; Belloch et al. 2009; Dunn et al. 2012; Erny et al. 2012; Peris et al. 2012a, 2012b, 2012c, 2017b). The genome composition of eleven previously described hybrids, mostly from wine, as well as the CID1 hybrid from cider, have been reanalyzed by short-read whole genome sequencing (WGS) molecular methods (Hittinger et al. 2010; Borneman et al. 2012; Almeida et al. 2014; Borneman et al. 2016). The most recent WGS survey has also uncovered the existence of new *S. cerevisiae* x *S. kudriavzevii* hybrids (Borneman et al. 2016) (Supplementary Table 1). The application of WGS, together with the development of user-friendly bioinformatics tools that integrate genome content analyses (Borneman and Pretorius 2015; Gallone et al. 2016), will allow reanalyses of the genomes of commercial yeast strains.

The isolation sources of the fifty-nine industrial hybrids isolated so far are much more diverse than lager hybrids of *S. eubayanus* x *S. cerevisiae*. Forty-nine hybrids have been isolated from wine fermentations, seven from ale-brewing environments, one from cider, one from a dietary supplement, and one from a clinical source (Supplementary Table 1). These diverse environments suggest that *S. cerevisiae* x *S. kudriavzevii* hybrids might be more tolerant to a broader range of stress conditions than other industrial hybrids.

Better together – the nuclear genome composition of *S. cerevisiae* x *S. kudriavzevii* hybrids

Industrial fermentations are characterized by a broad number of stresses that depend on the phase of the fermentation (Ivorra et al. 1999; Carrasco et al. 2001). For example, alcoholic fermentations have three phases, one is the propagation step where oxidative and osmotic stress are the most important. During the tumultuous fermentation phase, the fluctuation of osmotic, oxidative, oxygen and nutritional levels, as well as ethanol, impacts the survival of yeast strains

(Attfield 1997; Pretorius 2000). The last step involves the storage of the final product, imposing a cold shock (Gibson et al. 2007). The mechanisms developed by wine strains of *S. cerevisiae*, which were domesticated from wild Mediterranean oak tree strains (Almeida et al. 2015), are the capacity to better modulate the expression profile of stress-related genes compared to laboratory strains (Querol et al. 2003; Kvitek et al. 2008). Additionally, some *S. cerevisiae* strains, such as the wine strain EC1118, have acquired genomic regions from non-*Saccharomyces* species via Horizontal Gene Transfer (HGT). These regions contain genes related to oxidative stress and nutrient utilization (Novo et al. 2009; Marsit et al. 2015).

Fermentation at low temperatures is a common practice in modern alcoholic beverage production to preserve the aromatic compounds generated during fermentation (Torija et al. 2003; Beltran et al. 2006, 2008). In low temperature fermentations, *S. cerevisiae* is not well adapted (Arroyo-Lopez et al. 2009), and the process can get stuck or sluggish (Fleet and Heard 1993). *S. cerevisiae* x *S. kudriavzevii* have been frequently isolated from low temperature fermentations, and these hybrids can be dominant in alcoholic fermentations from European regions with cooler Oceanic and Continental climates. The reason is that the second partner of *S. cerevisiae* x *S. kudriavzevii* hybrids, *S. kudriavzevii* grows optimally at lower temperatures (Belloch et al. 2008; Sampaio and Gonçalves 2008; Salvadó et al. 2011). The combination of both parental genomes by hybridization might be the key for exploiting the particularities of both species to generate strains tolerant to fermentation conditions at lower temperatures (Pérez-Torrado et al. 2017).

Genome composition analyses of the fifty-nine *S. cerevisiae* x *S. kudriavzevii* hybrids by different molecular techniques indicate that the *S. kudriavzevii* genome is complementary to *S. cerevisiae* and can mostly be lost (Belloch et al. 2009; Dunn et al. 2012; Peris et al. 2012a, 2012b, 2012c; Almeida et al. 2014; Borneman et al. 2016). The percentage of the *S. kudriavzevii* genome contributed to the hybrid varies from 100% to just 23% (Figure 1A). The *S. kudriavzevii* regions maintained in the hybrids might encode the genetic traits contributing to low temperature tolerance and other important physiological traits. An aCGH, RFLPs and gene ontology enrichment analysis of common *S. kudriavzevii* regions from beer and wine *S. cerevisiae* x *S. kudriavzevii* hybrids detected a significant enrichment of genes involve in fatty acid transport, N-glycosylation of proteins, and ergosterol biosynthesis, terms related with low temperature adaptation (Peris et al. 2012a, 2012c). A few *S. cerevisiae* x *S. kudriavzevii* strains, such as CID1, CBS2834 and W27 (Almeida et al. 2014) have genomic contributions from other *Saccharomyces* species, suggesting either recent secondary hybridizations when genomic contributions are still prevalent, or ancestral hybridizations where small regions (introgressions)

are retained mainly in subtelomeric regions (Figure 1A). For example, the small fraction contributed by *S. paradoxus* to W27 might have already been present in the parental genome of *S. cerevisiae*, as suggested by frequent introgressions between *S. cerevisiae* and *S. paradoxus* (Liti et al. 2006; Muller and McCusker 2009; Dunn et al. 2012). Similarly, the small fraction contributed by *S. eubayanus* to several strains are likely coming from an ancestral introgression in the *S. uvarum* parental donor (Almeida et al. 2014). In organisms with frequent sexual reproduction, introgressions result from hybridization, followed by successive backcrossing with one of the parental species (Rieseberg and Welch 2002). However, in *Saccharomyces* yeasts, clonal division is more frequent than sexual reproduction (Ruderfer et al. 2006; Tsai et al. 2008), and hence, frequent backcrosses are unlikely. Other mechanisms have been proposed, such as the transfer of a complete or partial chromosome from one nucleus to the other in a hybrid zygote before karyogamy takes place (Morales and Dujon 2012), or a hybridization event followed by the loss of most of the genome contributed by one of the parental species (Marinoni et al. 1999), a process recently termed GARMi (Genome AutoReduction in Mitosis) (Karanyicz et al. 2017).

The genetic contributions of each *Saccharomyces* species to stress adaptation during alcoholic fermentation are still unclear in most cases, but the presence of rearrangements can generate genetic innovations. For example, there are two *S. cerevisiae* *PMT1* alleles in Swiss hybrids (W27, W46, SPG16-91, and SPG16-91), but there are none from *S. kudriavzevii*; however, one of the protein-coding sequences is under the control of the *S. kudriavzevii* promoter (Belloch et al. 2009). The combination of different genomes also affects proteins involved in the formation of protein complexes. In *Saccharomyces* species, those proteins show sequence divergences comparable to those found between human and birds (Dujon 2006). In synthetic *S. cerevisiae* x *S. uvarum* hybrids, those hybrids with the chimeric complex *SuTrp2p* (Trp2p protein from *S. uvarum*) x *ScTrp3p* (Trp3p protein from *S. cerevisiae*) grew better than other hybrids, slightly better than the *S. cerevisiae* parent, and significantly better than *S. uvarum* parent (Piatkowska et al. 2013). *S. kudriavzevii* proteins can complement the absence of the *S. cerevisiae* orthologs (Leducq et al. 2012), but there are no studies screening the effect of chimerical protein complexes on the fitness of *S. cerevisiae* x *S. kudriavzevii* hybrids. Cis (promoters, UTRs) and trans (transcription factors) elements that affect gene regulation (Borneman et al. 2007) doubtlessly also affect expression of genes related to stress tolerance, but these effects remain unexplored in hybrids.

Most of the *S. cerevisiae* x *S. kudriavzevii* hybrids are allotriploids, with two genome copies of *S. cerevisiae* and one copy of *S. kudriavzevii* (Borneman et al. 2012; Erny et al. 2012; Peris

et al. 2012b, 2012c; Borneman et al. 2016). In a recent adaptive evolution experiment of an allodiploid *S. cerevisiae* x *S. kudriavzevii* hybrid, after 50 generations of experimental evolution, the hybrid evolved to become approximately triploid; fourteen *S. cerevisiae* chromosomes had two or more copies, while nine *S. kudriavzevii* chromosomes were present in one or fewer copies (Peris et al. 2017a). This trend suggests that the adaptation to the stressful condition might be associated with maintaining the correct homeostasis of protein complexes composed mainly of *S. cerevisiae* proteins. The presence of two copies might also speed-up the accumulation of mutations generating heterozygous sites in the *S. cerevisiae* counterpart which might be beneficial during the adaptation to the stressful conditions. Few hybrid strains are allodiploids or allotetraploids (Supplementary Table S1). Moreover, hybrids display several aneuploidies that might contribute to improved fitness under stressful conditions, as described in aneuploid *S. cerevisiae* strains (Pavelka et al. 2010; Dodgson et al. 2016).

The mitochondrial genome influences the nuclear genome and organismal phenotype

The nuclear genome of some *S. cerevisiae* x *S. kudriavzevii* hybrids are unstable. The Vin7 genomic architecture published by different authors showed a handful of differences, the most important being the loss of *S. kudriavzevii* chromosome III in our Vin7 (Peris et al. 2012c) compared to the sequenced strain (Borneman et al. 2012), suggesting the instability of the Vin7 genome during propagation in the lab (Ibañez et al. in preparation).

Instability might also be promoted by mitochondrial DNA inheritance. The nuclear genome content differs substantially between strains with a *S. cerevisiae* mitochondrial genome and those who inherited the *S. kudriavzevii* mitochondrial genome. Hybrid strains with the *S. cerevisiae* version have fewer *S. kudriavzevii* genomic contributions than those bearing the *S. kudriavzevii* mitochondrial genome (Peris et al. 2012c, Figure 1B) (t-test, *p*-value 0.02975). The genes encoded by the mitochondrial genome interact closely with many genes encoded by the nuclear genome (Merz and Westermann 2009). Indeed, the most important Bateson-Dobzhansky-Muller genetic incompatibilities, responsible for postzygotic isolation between species of *Saccharomyces*, are found between nuclear and mitochondrial genes (Lee et al. 2008; Chou et al. 2010; Hou et al. 2015). The mitochondrial genome is important for several cellular functions, such as the maintenance of redox balance (NADH/NAD⁺), levels of respiration, production of fatty acids, and adaptation to stress; indeed, some metabolic pathways have reaction steps that occur inside the organelle, including those related to aroma production (Bakker et al. 2001; Starkov 2008; Procházka et al. 2012; Avalos et al. 2013; Pires et al. 2014). A gene ontology term enrichment analysis found that those *S. cerevisiae* x *S. kudriavzevii* hybrids with *S. kudriavzevii* mtDNA had significant enrichments of genes performing

mitochondrial functions (Peris et al. 2012c), suggesting that the presence of *S. kudriavzevii* mitochondrial genes promotes the maintenance of *S. kudriavzevii* nuclear genes with mitochondrial functions, which might help properly perform the cellular functions.

As described above, the mitochondrial genome has a direct effect in the phenotype. In hybrids generated by crosses between *S. cerevisiae* and the cryotolerant species *S. uvarum*, hybrids that differ in the inherited mitotype had differences in the levels of respiration, growth rate kinetics, and reactive oxygen response (Solieri et al. 2008; Albertin et al. 2013; Picazo et al. 2014). Most hybrids of *S. cerevisiae* x *S. kudriavzevii* (27 out of 32 hybrids, Supplementary Table S1), all known *S. cerevisiae* x *S. uvarum*, and all known *S. cerevisiae* x *S. eubayanus* hybrids have inherited the mitochondrial genome from the cryotolerant species (*S. eubayanus*, *S. kudriavzevii* or *S. uvarum*) (Peris et al. 2012a, 2014, 2016b, 2017b; Pérez-Través et al. 2014b; Okuno et al. 2016). Further phenotypic investigation is required to clarify why hybrids have inherited the mitochondrial genome of cryotolerant species. Additionally, after the hybridization event, both parental mitochondrial genomes can interact during the first generations, which might result in the presence of recombinant mitochondrial genomes mediated by selfish elements (Figure 1B), such as the homing endonucleases, introns, or AT repetitive elements and GC clusters that are prevalent in mitochondrial genomes (Fritsch et al. 2014; Wu et al. 2015; Peris et al. 2017a; Peris et al. 2017b). How these introgressions might influence in the final phenotype is also still unclear.

Multiple hybridization events explain the origins of *S. cerevisiae* x *S. kudriavzevii* hybrids

The diversity in the nuclear and mitochondrial genome composition of *S. cerevisiae* x *S. kudriavzevii* hybrids (Figure 1), as well as the different locations and environmental conditions in which they were isolated (Supplementary Table 1) support the hypothesis that these hybrids originated by different independent hybridization events (Erny et al. 2012; Peris et al. 2012a, 2012b; Borneman et al. 2016). Loss of heterozygosity (LOH), defined as the conversion of heterozygous regions to homozygous, is an important mechanism that can drive adaptation in the hybrid genome (Heil et al. 2017). LOH represents a huge challenge in the reconstruction of the origin of hybrids when parental genomes show low nucleotide diversity or when potential close relatives to compare are not available (Peris et al. 2016a). However, in the case of *S. cerevisiae* x *S. kudriavzevii* hybrids, LOH might had little or no influence in the conclusion reached by different authors, and there are no reports claiming a single unique origin of *S. cerevisiae* x *S. kudriavzevii* hybrids.

The integration of data from multiple studies, using multilocus, microsatellites, and whole genome sequencing (Erny et al. 2012; Peris et al. 2012a, 2012b; Borneman et al. 2016), as well

as the mitochondrial inheritance (Figure 1B) (Peris et al. 2017b), suggests at least three well-supported independent origins for industrial *S. cerevisiae* x *S. kudriavzevii* hybrids (Figure 2). Additional possible independent origins might be supported for specific strains, but further investigation will clarify this issue (Figure 2, Supplementary Table S1).

The most abundant group of related hybrids with a common origin is group A, with 28 isolates from three different continents (Figure 2). Group A integrates yeast hybrids designated as 1/1, *S. cerevisiae* clade 1/*S. kudriavzevii* clade 1 (Eg8 hybrid as the representative strain for Erny et al. (2012)), *S. cerevisiae* C1 clade/*S. kudriavzevii* K1 clade (Peris et al. 2012b) or *S. cerevisiae* Vin7 clade (Vin7 hybrid as the representative strain for Borneman et al. (2016)). Hybrids from this group A were isolated from wine fermentations, except the cider triple hybrid, CID1. However, the introgressions detected in the mitochondrial genome of CID1 and the difference in its *S. cerevisiae* parental genome might suggest an independent origin for this strain (Erny et al. 2012; Peris et al. 2012b, 2017b), Figure 1B). Group A hybrids are mostly allotriploids, with two copies of *S. cerevisiae* chromosomes, one copy of *S. kudriavzevii* chromosomes, and low levels of heterozygosity (Borneman et al. 2012; Peris et al. 2012c; Borneman et al. 2016). The source of isolation and the molecular data support a wine *S. cerevisiae* strain as the closest relative of the *S. cerevisiae* subgenome of the hybrids (Peris et al. 2012b).

Group C is the second most abundant group, with European isolates from Swiss wine and Belgian Trappist beer (Supplementary Table S1). Group C was named as the *S. cerevisiae* 2 clade/*S. kudriavzevii* 2 clade by Erny et al. (2012), *S. cerevisiae* C2 clade/*S. kudriavzevii* K2 clade by Peris et al. (2012c), and the *S. cerevisiae* Wine clade by Borneman et al. (2016). Mitochondrial genome inheritance is congruent with a single origin for this group, with the exception of EP2, which inherited a *S. cerevisiae* mitochondrial genome. A different mitochondrial inheritance is not enough to support an independent origin due to the biparental inheritance of mitochondrial genome in yeast (Berger and Yaffe 2000). This group is also mostly allotriploid with two copies of *S. cerevisiae* chromosomes, one copy of *S. kudriavzevii*, and low levels of heterozygosity (Belloch et al. 2008; Peris et al. 2012c; Borneman et al. 2016). The sequence data indicated that the *S. cerevisiae* parent involved in the origin of this group also was a wine yeast, which is congruent with the low levels of heterozygosity observed in the *S. cerevisiae* subgenome of these hybrids (Peris et al. 2012b; Borneman et al. 2016).

The third group with the fewest isolates is group B (Supplementary Table S1), which has been isolated from two continents (Figure 2). Group B includes strains with similar nuclear genomes but two different mitotypes (Peris et al. 2012b, 2017b). Multilocus sequencing data

showed higher levels of heterozygosities in their *S. cerevisiae* subgenomes (Peris et al. 2012b). These higher heterozygosities, together with the fact that these hybrids are associated with brewing, suggests that the parental *S. cerevisiae* close relative might belong to one of the described group of *S. cerevisiae* beer strains, which are characterized by high levels of heterozygosity (Gallone et al. 2016; Gonçalves et al. 2016; Borneman et al. 2016).

On the origin of the *S. cerevisiae* x *S. kudriavzevii* hybrids

When, how and where these hybrids originated are questions that remain unanswered. Some authors postulate that hybridization might have occurred by spore-spore conjugations in insects, based on the fact that some hybrids can originate in *Drosophila* (Pulvirenti 2002) and wasp guts, as described for *S. cerevisiae* x *S. paradoxus* (Stefanini et al. 2016). But this hypothesis lacks direct support for *S. cerevisiae* x *S. kudriavzevii* hybrids because these hybrids have not been isolated from wild environments. Nonetheless, recent research has inferred ancestral hybridization events among *Saccharomyces* species, a conclusion supported by both nuclear (Liti et al. 2006; Muller and McCusker 2009; Dunn et al. 2012; Almeida et al. 2014) and mitochondrial introgressions (Wu and Hao 2014; Wu et al. 2015; Leducq et al. 2016, 2017; Peris et al. 2017b), which suggests that hybridizations are occurring in natural environments and might be an important mechanism to generate new lineages (Leducq et al. 2016, 2017; Peris et al. 2017b).

Other authors have suggested that rare mating (Figure 3, see below) between a mating-competent diploid *S. cerevisiae* strain and a haploid *S. kudriavzevii* might be the main mechanism to generate allotriploid *S. cerevisiae* (2n) x *S. kudriavzevii* (n) hybrids (Erny et al. 2012; Peris et al. 2012c) (Model B in Figure 4). This hypothesis clearly explains the origin of group B hybrids (Model B in Figure 4) due to the heterozygosity observed in the *S. cerevisiae* subgenome. Recent studies mating diploid strains of *S. cerevisiae* and *S. kudriavzevii* were able to generate allotriploid and allotetraploid *S. cerevisiae* x *S. kudriavzevii* hybrids (Pérez-Través et al. 2014a), which might suggest rare-mating could also occur in nature.

It is less clear whether group A and C originated by the rare mating mechanism (Model B in Figure 4) or by spore-spore crosses (Model A in Figure 4). Recent adaptive evolution experiments under stressful conditions or laboratory conditions, using hybrids generated by crossing spores from *S. cerevisiae* and *S. kudriavzevii* or spores from *S. uvarum* and *S. kudriavzevii*, resulted in an increase in the number of copies of the parental chromosomes, with no evidence of back-crossing (Karanyicz et al. 2017; Peris et al. 2017a).

Once hybrids are formed by either mating mechanism, insects are attracted by the flavors produced by yeasts (Christiaens et al. 2014). These insect vectors might have spread the newly

generated hybrids to the open containers utilized by Romans during wine fermentations or the beer containers used by Germanic and Celtic tribes for brewing (Corran 1975). Those alcoholic fermentation processes were probably mostly performed with domesticated *S. cerevisiae* yeasts from Mediterranean oaks (Almeida et al. 2015). However, the expansion of these artisanal alcoholic fermentations to regions of Oceanic and Continental climate in Europe (This et al. 2006), characterized by low temperatures, might have offered an opportunity for *S. cerevisiae* x *S. kudriavzevii* hybrids to outcompete the *S. cerevisiae* parental strains (Belloch et al. 2008; Salvadó et al. 2011; Alonso-del-Real et al. 2017). The industrialization of alcoholic beverages, mainly performed by Christian monks (Peris et al. 2012b) in Europe, retained these strains, which were secondarily spread to other continents, such as North America, Africa, and Oceania by the colonial period or yeast commercialization.

Industrial applications of *S. cerevisiae* x *S. kudriavzevii* hybrids

Climatic change and consumer's current demands require new strains for winemaking

In recent years, the wine industry is facing several challenges that depend on two main factors: climatic change and consumer demands (Borneman et al. 2013). Temperature increase in wine-growing regions accelerates the maturation of grapes (White et al. 2006). The sugar content of grape musts is now much higher, resulting in wines with higher ethanol content (Jones et al. 2005). The increase ethanol in the final wine product is not accepted by many consumers due to a decrease in the sensorial quality and a harmful impact on human health. These new situation is a central topic for wine yeast researchers (Contreras et al. 2014; Tilloy et al. 2014, 2015; Morales et al. 2015; Ciani et al. 2016; Alonso-del-Real et al. 2017; Pérez-Torrado et al. 2017). One of the proposed solutions is to harvest grapes earlier in the season, when sugar is at optimal levels to minimize ethanol production. However, earlier ripening generates astringent wines due to the presence of immature tannins and phenols (Varela et al. 2015). An alternative strategy with much greater potential application, is the utilization of single or mixed cultures of wine yeasts starters with the capacity to reroute carbon flux from the production of alcohol, while maintaining the organoleptic quality of the final wine product (Tilloy et al. 2014, 2015; Alonso-del-Real et al. 2017). At the same time, to improve the perception of quality, the wine industry is exploring methods to enhance aroma content and complexity. One well-known mechanism is the application of lower temperatures during wine fermentation, which increases the aroma profile by preventing evaporation and stabilizing aroma and flavor compounds (Torija et al. 2003; Molina et al. 2007).

The potential multiple origins and the high frequency of isolation of *S. cerevisiae* x *S. kudriavzevii* hybrids from wine environments highlights the success of these strains for wine

applications (Erny et al. 2012; Peris et al. 2012a, 2012b) (Supplementary Table S1). This success might be due to the winemaking properties these hybrids offer (Pérez-Torrado et al. 2017). Enological characterization of *S. cerevisiae* x *S. kudriavzevii* demonstrated good adaptation of these hybrids to wine fermentations, especially at low and intermediate temperatures (González et al. 2007; Gamero et al. 2013). Additionally, wines produced by these hybrids have good enological properties, such as high glycerol content, decreased ethanol, improved taste, and a lower production of undesirable acetic acid compared to other wine strains (González et al. 2007; Gamero et al. 2013; Goold et al. 2017). Additionally, *S. cerevisiae* x *S. kudriavzevii* hybrids offer a diverse panel of aromatic compounds (González et al. 2007; Gamero et al. 2013).

As described above, the contributions of *S. cerevisiae*, a species better adapted to alcoholic fermentation, and *S. kudriavzevii*, a cryotolerant species, to their hybrids allow them to ferment at much lower temperatures than their parental species, making them good candidates for the development of new and improved winemaking methods (González et al. 2007; Pérez-Torrado et al. 2017). The molecular mechanisms behind hybrid tolerance to these new conditions correlates with the expression of genes related to cold stress, ergosterol and glycerol metabolism (Combina et al. 2012; Gamero et al. 2015; Pérez-Torrado et al. 2016). The comparison of expression profiles of the parental species, *S. cerevisiae* and *S. kudriavzevii*, demonstrated significant differences in genes involved in glycerol biosynthesis (*PGII* and *TIP1*), genes related to cold shock (*PAU* and *DAN/TIR* families), and genes related to sterol and aroma synthesis (*ARE1*, *ARO10*, and *ATF2*). A substitution of *ScARO10* gene (a phenylpyruvate decarboxylase related with ethanol and ester production) in the *S. cerevisiae* background with the *S. kudriavzevii* ortholog enhanced the production of isobutanol (wine, solvent, and bitter flavors), isoamyl alcohol (whiskey, malt, and burnt flavors), and their esters, all of which impact the aromatic profile (Stribny et al. 2016b). Differences in the production of those compounds might be related to the amino acid differences observed between *ARO10* orthologs (Stribny et al. 2016b). Similarly, the replacement of *ScATF1* and *ScATF2*, genes encoding two alcohol acetyltransferases, with *S. kudriavzevii* alleles increased the levels of 2-phenylethyl acetate (banana flavor) (Stribny et al. 2016a). The increase of glycerol levels might be linked to the different enzymatic activity of Gpd1p, a NAD-dependent glycerol-3-phosphate dehydrogenase protein, between *S. cerevisiae* and *S. kudriavzevii* (Oliveira et al. 2014). Replacement of *ScGPD1* with the *SkGPD1* increased glycerol levels at 12°C and 28°C (Oliveira et al. 2014).

The results observed in industrial *S. cerevisiae* x *S. kudriavzevii* hybrids have promoted the generation of synthetic hybrids (Supplementary Table S1). The development of new synthetic

S. cerevisiae x *S. kudriavzevii* hybrids has also revealed as an interesting approach to solve the new demands of the wine industry (Pérez-Torrado et al. 2017). These newly created hybrids were able to increase the levels of compounds related to flavors, such as fruit, perfume, and flowers (Bellon et al. 2011). In addition, sensory analysis agreed about the complexity of flavors and aroma traits of wines generated with these new synthetic *S. cerevisiae* x *S. kudriavzevii* hybrids (Bellon et al. 2011).

Trappist ale beer fermentations are another niche for S. cerevisiae x S. kudriavzevii hybrids

The two main beer products are lager or ales. The production of ale and lagers are dominated worldwide by multinational companies (Bon 2014). Lagers constitute 94% of the beer market, and they are mainly performed by yeast hybrids of *S. cerevisiae* x *S. eubayanus* (synonyms *S. pastorianus*, *S. carlsbergensis*, and *S. monascensis*) (Wendland 2014). In the case of ale beers, *S. cerevisiae* dominates most of the fermentation production (Gallone et al. 2016; Gonçalves et al. 2016), but there are some beer products associated with *S. cerevisiae* x *S. kudriavzevii* hybrids, most of them related to beers produced at Trappist Abbeys (see above, Supplementary Table S1) in Belgium. Other *S. cerevisiae* x *S. kudriavzevii* hybrids isolated from beer production in Germany, United Kingdom, and New Zealand are phylogenetically related to these Trappist hybrids (Figure 2) (González et al. 2008; Peris et al. 2012a, 2012b).

Trappist beers, as well as other Abbey beers, are first fermented in tanks at a temperature between 15 and 23°C, and subsequently, a second fermentation at 15°C is performed in bottles. These temperatures are closer to the optimum growth temperature of *S. kudriavzevii* than they are to that of *S. cerevisiae* (Salvadó et al. 2011). However, *S. kudriavzevii* is not especially tolerant to the ethanol (Belloch et al. 2008) suggesting temperature and ethanol might be the main reason for selecting *S. cerevisiae* x *S. kudriavzevii* hybrids, instead of the beer *S. cerevisiae* strains. Lager beer producers often want to avoid the production of a phenolic off-flavor (POF) associated with the presence of 4-vinyl guaiacol (4-VG). This off-flavor has been associated with functional *PADI* and *FDCI* genes (Mukai et al. 2010). Most brewing strains of *S. cerevisiae* are characterized by the presence of loss-of-function mutations in *PADI* and/or *FDCI* genes (Mukai et al. 2014; Gallone et al. 2016; Gonçalves et al. 2016) (Supplementary Figure S1). However, the flavors associated with 4-VG (clove-like, medicinal, phenolic, or smoky) are appreciated in some beer styles, such as Belgian wheat beers and German Weizen and Rauch beers (Narziss et al. 1990). Levels of 4-VG in these kind of beers are 10 times higher than lager beers (Venhoenacker et al. 2016). *S. cerevisiae* x *S. kudriavzevii* hybrids are associated with Belgium (Trappist beers) and some Germans beers (Supplementary Table S1).

Unfortunately, the only brewing hybrid of *S. cerevisiae* x *S. kudriavzevii* that is sequenced is WLP500 (a Trappist Ale yeast, [Supplementary Table S1](#)). Manual inspection of the *PADI* and *FDC1* genes of the WLP500 hybrid and the sequenced wine hybrids suggests that those two genes are potentially active. Neither WLP500 *PADI* nor *FDC1* genes contain premature stop codons. Representative *S. cerevisiae* beer strains that do not produce appreciable 4-VG are characterized by the presence of loss of function mutations ([Supplementary Figure S1](#), [Gallone et al 2016](#)). The potential activity of these two genes in WLP500 and probably in other Trappist and ale *S. cerevisiae* x *S. kudriavzevii* might be the responsible of the higher production of 4-VG in Belgium and German wheat beers.

Another important trait is the capacity to consume maltose and maltotriose, the main sugars in wort ([Hornsey I 2003](#)). Ale strains of *S. cerevisiae* from the Beer 1 Group have the genes responsible for maltose and maltotriose utilization, the high-affinity allele (*AGT1*) of *MAL11* (encoding a maltose transporter), while the Beer 2 Group contains an unknown mechanism to facilitate maltotriose utilization ([Gallone et al. 2016](#)). *S. kudriavzevii* is able to consume maltose ([Sampaio and Gonçalves 2008](#)), but it cannot metabolize maltotriose ([Pfliegler et al. 2014](#)). The sequences of the genes related to off-flavor production and sugar utilization remain uncharacterized in these *S. cerevisiae* x *S. kudriavzevii* brewing hybrids.

Fructophilic and cold tolerant yeasts for cider production

In recent years, cider has attracted the interest of more consumers because it is a fermented beverage with a lower ethanol content and less bitter taste than beer or wine. The production of cider has been extended from countries with older traditions, such as Ireland, France, and Spain, to Eastern European countries, such as Poland ([Grebinskij 2017](#)). Cider is the end-product of spontaneous apple must fermentation conducted at low temperatures, 12-20°C ([Morrissey et al. 2004](#)). Cider fermentations is mainly performed by *Saccharomyces* species, but indigenous *Brettanomyces*, *Candida*, *Dekkera*, *Hanseniaspora*, *Metschnikowia* and *Pichia* species also participate during the process ([Morrissey et al. 2004](#); [Valles et al. 2007](#)). *Saccharomyces* cider yeasts belong to *S. cerevisiae*, *S. uvarum*, or a diverse collection of double and triple hybrids, traditionally named *S. bayanus* ([Naumov et al. 2001](#); [Suárez Valles et al. 2007](#); [Pando Bedriñana et al. 2010](#); [Libkind et al. 2011](#); [Nguyen et al. 2011](#); [Pérez-Través et al. 2014b](#)). CID1, a triple hybrid *S. uvarum* x *S. kudriavzevii* x *S. cerevisiae* with small introgressions of *S. eubayanus* ([Figure 1A](#)) has also been isolated from cider fermentations ([Supplementary table S1](#)).

The low temperature conditions of cider fermentations, similar to those of some wine and beer fermentations, favor the participation of hybrids between *S. cerevisiae* and the cryotolerant

species *S. uvarum*, *S. eubayanus*, and *S. kudriavzevii* (Magalhães et al. 2016; González Flores et al. 2017). These industrial hybrids and newly created synthetic hybrids can ferment apple juice at low temperatures (Masneuf et al. 1998; Groth et al. 1999; Naumova E. et al. 2005; Magalhães et al. 2017). One particularity of apple juice is the high fructose concentration compared to wort or grape must (Ye et al. 2014). One of the desired traits these hybrids must retain is the ability to assimilate fructose. Wild *S. kudriavzevii* strains and *S. cerevisiae* x *S. kudriavzevii* hybrids are fructophilic and can ferment this monosaccharide at low temperatures (Gangl et al. 2009; Tronchoni et al. 2009), which is an attractive trait for cider applications. The *FSY1* gene encodes a fructose/H⁺ symporter that actively transports fructose into the cell in some *Saccharomyces* strains. The Fsy1 transporter facilitates the incorporation of fructose when it is found at low concentration, an ability hexose transporters are not able to perform (Rodrigues De Sousa et al. 1995; Gonçalves et al. 2000). The presence of *FSY1* has been not detected in *S. kudriavzevii* strains. It appears that *S. uvarum* and *S. eubayanus* are the only species of *Saccharomyces* that have it (Gonçalves et al. 2000), with the exception of some *S. cerevisiae* wine strains that acquired *FSY1* by HGT from a non-*Saccharomyces* donor (Novo et al. 2009; Galeote et al. 2010). Although, it has been not explored, the main contributor of the *FSY1* gene in CID1 hybrid might be *S. uvarum*.

Yeast hybrids for dietary supplements

Some *S. cerevisiae* strains, traditionally but incorrectly named *Saccharomyces boulardii*, are often applied as a dietary supplement (probiotic) to patients treated with antibiotics to prevent diarrhea after medical treatments (Szajewska and Kolodziej 2015) or to activate the gut immune system (Vieira et al. 2013). A hybrid of *S. cerevisiae* x *S. kudriavzevii* has also been isolated from a brewer's yeast dietary supplement (Supplementary Table S1) (de Llanos et al. 2004; Peris et al. 2012a, 2012b). The source of this probiotic hybrid strain might be yeasts used in Trappist Abbeys, as suggested by its close relationship with brewing hybrids group B (Figure 2). The differences between applying pure *S. cerevisiae* strains and hybrids as probiotics has not been tested, but different strains could potentially lead to differences in antimicrobial activity and the capacity to stimulate immune system (Rima et al. 2012).

New hybrid applications: biofuels

The ability to generate new strains with the best traits of different parents has created new potential industrial applications of yeast hybrids. The biofuel industry has a particular interest. The production of cellulosic ethanol as a renewable substitute for fossil fuels requires the application of yeast strains that are tolerant to high levels of ethanol and “hydrolysate toxins”, inhibitors of yeast growth and fuel production, derived from the deconstruction of

lignocellulosic material while releasing the sugars (Piotrowski et al. 2014). *S. cerevisiae* is the main strain applied to the production of ethanol due to its robustness and stress-tolerance compared to bacteria and other fermenting microbes (Olsson and Hahn-Hägerdal 1996). However, it is difficult to find a strain with all the necessary traits to deal with the stress generated by hydrolysate toxins, ethanol, temperature, and the ability to consume pentose sugars, which are prevalent in lignocellulosic hydrolysates (Jeffries 2006). Hybrids may provide a potential short term solution to integrate the stress-tolerant traits of different species into one strain. In two different studies, *S. cerevisiae* x *S. kudriavzevii* hybrids generated by spore-spore crosses (Supplementary Table S1) have been studied for their tolerance to stressful conditions found in hydrolysates of biomass derived from wheat straw and corn stover (Wimalasena et al. 2014; Peris et al. 2017a). These hybrids showed intermediate phenotypic traits compared to their parents, but in some situations, they outperformed both parents, indicative of hybrid vigor. In addition, adaptive evolution of these F₁ hybrids improved key traits that were affected by hybridization. For example, the *S. cerevisiae* x *S. kudriavzevii* hybrid strain yHDPN5 was evolved for 50 generations under Ammonia Fiber Expansion (AFEX)-corn stover hydrolysate at 30°C in microaerobic conditions (Peris et al. 2017a). Its evolved version, yHDPN379, improved its xylose consumption and tolerance to some individual hydrolysate toxins, perhaps due to an increase in the genome content and the presence of several aneuploidies (Peris et al. 2017a). The identification of the most tolerant *S. cerevisiae* and *S. kudriavzevii* strains (Peris et al. 2017a) could allow researchers to generate new hybrids with even better biofuel outcomes.

A collection of breeding methods for the generation of yeast hybrids

All of these diverse industrial applications of *S. cerevisiae* x *S. kudriavzevii* hybrids demand the generation of new and better strains, with specific traits to be implemented in each particular condition or industrial processes. The development of better methods for yeast isolation (Sampaio and Gonçalves 2008) is increasing the number of available strains, which can have diverse phenotypes, even when they have few genetic differences (Peris et al. 2016b, 2017a). New wild strains, might even lead to the discovery of new traits. One method to generate hybrids is protoplast fusion (Curran and Bugeja 1996), but most of the other available methods exploit the molecular mechanisms of sexual reproduction (Figure 3). In *Saccharomyces*, diploids contain two idiomorphs for the mating-type locus (*MAT*), termed *MATa* and *MATα* (Haber 2012). Once haploid *Saccharomyces* spores are generated with a single mating type (say *MATa*), they rapidly mate with haploids of the opposite mating-type (*MATα*), yielding diploid zygotes. The breeding mechanisms based on the yeast mating system are spore-spore mating

(Mortimer 1993) and mass-mating (Nakazawa et al. 1999). However, *Saccharomyces* yeast are homothallic, and by the expression of an endonuclease (*HO*), a mating-type switch can only occur in haploids. Rarely, mating-type switch can also occur in diploids, such as by a gene conversion event, resulting in two *MAT* alleles of the same idiomorph (*MAT α /MAT α* or *MAT a /MAT a*). These rare diploid cells are then compatible to mate with other converted diploids or haploid spores of the opposite mating type. These events are the basis of the so-called rare-mating method (Spencer and Spencer 1996) (Figure 3). Synthetic *S. cerevisiae* x *S. kudriavzevii* hybrids have been generated by spore-spore crosses or rare mating (Supplementary Table S1) using auxotrophic or drug markers in the parents because only hybrids are able to grow in selective media (Pérez-Través et al. 2012; Steensels et al. 2014). Recently, we have successfully used transformation of the parental diploid cells with antibiotic-resistance plasmids to obtain artificial *S. cerevisiae* x *S. kudriavzevii* hybrids by rare mating (Ortiz-Tovar et al in preparation). Once hybrids are selected by their resistance to both antibiotics, they are grown on non-selective media to promote plasmid loss. This way, hybrids are not considered GMOs (Genetically Modified Organisms) by some researchers and jurisdictions because they do not contain any transgenic DNA. Similarly, Alexander et al. (2016) developed a method based on a set of plasmids, HyPr (Hybrid Production) method, to facilitate the gene conversion of both *MAT* alleles in diploid parents, converting the rare-mating into a frequent-mating event (Figure 3). This method has successfully generated allotetraploids, which then can be exposed to adaptive evolution to generate genomic diversity. In this way, we can now fully exploit the original genomic diversity of the diploid parents, and allotetraploids evolve faster than diploid and haploids (Selmecki et al. 2015), facilitating the adaptation and improvement of the new strains to the stressful conditions.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The interesting collection of *S. cerevisiae* x *S. kudriavzevii* hybrids tell a diverse evolutionary story and offer industrial applications that provide a compelling rationale to continue research on these understudied hybrids. The low number of *S. kudriavzevii* isolates, compared to *S. cerevisiae*, needs to be balanced to improve the population genomic resources so that we can infer close relatives of the *S. kudriavzevii* parental genomes. The high number of *S. cerevisiae* genomes also offers a good opportunity to determine the number of *S. cerevisiae* x *S. kudriavzevii* groups and their potential origins. Additionally, hybrids offer a excellent opportunity to study the genetic traits associated with particular phenotypes. For that reason, we expect that hybrids will continue to be a valuable tool for the study of the genetic differences between *Saccharomyces* species, as well as the evolution of new species.

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COMPETING INTERESTS

The Wisconsin Alumni Research Foundation has filed a provisional patent application entitled “Synthetic yeast cells and methods of making and using same” (describing the HyPr method with William G. Alexander, DP, and CTH as inventors). The other authors declare that there is no conflict of interest.

TABLES AND FIGURES

Figure 1. *Saccharomyces* species contributions to the nuclear and mitochondrial genomes. A) *Saccharomyces* species contributions to the nuclear genome of industrial hybrids. The percentage numbers represent the proportion of the reference genome detected in the hybrid, i.e. a 100% for *S. cerevisiae* suggests that 100% of the *S. cerevisiae* genome was detected in the hybrid. To calculate the percentage of each contribution, we followed a previous pipeline (Peris et al. 2016a) with a window size of 8.1Kb. Each window with a mean mapping coverage value higher than 5 was selected as positive, and values above 1% of the genome are represented. Plots were reconstructed with DiagrammeR package for R (R Development Core Team 2010). Sarb: *S. arboricola*, Scer: *S. cerevisiae*, Seub: *S. eubayanus*, Skud: *S. kudriavzevii*, Smik: *S. mikatae*, Spar: *S. paradoxus*, Suva: *S. uvarum*. B) A median-joining phylogenetic network was reconstructed using a concatenated alignment of 10.7 Kb, consisting of the coding sequence of *COX2-COX3-15S rRNA-COX1-ATP8-ATP6-COB-ATP9-VAR1-21S rRNA* genes. Illumina reads (Supplementary Table S1) for each mitochondrial gene were pulled out using HybPiper (Johnson et al. 2016). Gene alignments were constructed, using ClustalW as implemented in MEGA v7 (Kumar et al. 2016) and manually curated. To identify the incongruent data shown in the phylonetwork, maximum likelihood individual gene trees (Supplementary Figure S2) were reconstructed in MEGA v7 (Stamatakis 2014) under the GTR+GAMMA with 4 discrete gamma categories, and 100 pseudoreplicates to assess branch support. Introgressions are represented and colored according to the legend. Reference *Saccharomyces* strains are colored according to the legend. The scale is given in nucleotide substitutions per site.

Figure 2. Geographic and substrate distribution of *S. cerevisiae* x *S. kudriavzevii* hybrids. The sizes of the symbols represent the number of hybrids from each location. Symbol colors classify the hybrids into potential origins based on previous studies (Supplementary Table 1). Unknown

origin might suggest a different origin group, but further investigation is required. Symbol shapes are according to the isolation source of the hybrids. Maps were built with ggmap package for R ([R Development Core Team 2010](#)).

Figure 3. Overview of breeding methods. The four yeast breeding methods described in the text are represented. Vegetative yeasts and spores are described in the figure legend. Different colors in yeasts and spores highlight the utilization of two different species. Although, auxotrophic and drug markers are not represented they are required for the selection of successful matings before the submission of the hybrid to adaptive evolution. The difference in the thickness of the arrow in the mating step represents the probability of successful matings.

Figure 4. Models of hybridization for *S. cerevisiae* x *S. kudriavzevii* hybrids. Both models, A and B, might originate the hybrids from group A and C. The origin of group B might be the consequence of model B or model A with a second hybridization event with a different haploid *S. cerevisiae* strain.

Supplementary table S1. List of *Saccharomyces cerevisiae* x *Saccharomyces kudriavzevii* hybrids.

Supplementary figure S1. A) *FDC1* and B) *PAD1* maximum-likelihood phylogenetic gene trees. *S. cerevisiae*, *S. kudriavzevii* and *S. uvarum* allele sequences are represented by C, K and U, respectively. Incomplete *PAD1* genes were removed from the tree reconstruction ([Supplementary Table S1](#)). Reference *Saccharomyces* strains are colored according to the legend. Asterisks indicate the inactive gene sequences due to the described premature stop codons ([Gallone et al. 2016](#)). C) and D) show the amino acid alignments of *FDC1* and *PAD1*, respectively. Black and red arrows indicate premature stop codons and nucleotide insertions, respectively. Question marks are the result of incomplete gene sequences likely due to the low coverage. We could not resolve the *PAD1* sequence region of bps 329-341 of IFO1802 which might be the result of an incomplete sequence or a real deletion. An improved genome sequence for IFO1802 will clarify its sequence. BE012 and BE100 *PAD1* sequences were previously reported to have a premature stop codon in Q86 ([Gallone et al 2016](#)); however, we did not detect it.

Supplementary figure S2. Concatenated and individual maximum-likelihood phylogenetic gene trees. Alignments and phylogenetic reconstructions were performed as in the concatenated dataset ([Figure 1B](#)). Introgressions are represented and colored according to the legend. Reference *Saccharomyces* strains are colored according to the legend. The scale is given in nucleotide substitutions per site.

Figure 1.

Figure 2.

Figure 3.

Figure 4.