A comparison of the performance of natural hybrids *Saccharomyces cerevisiae* x *Saccharomyces kudriavzevii* at low temperatures reveals the crucial role of their *S. kudriavzevii* genomic contribution

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Abstract

Fermentation performance at low temperature is a common approach to obtain wines with better aroma, and is critical in industrial applications. The isolation of natural hybrids *S. cerevisiae* x *S. kudriavzevii*, associated with fermentations done in cold countries, has provided an understanding of the mechanisms of adaptation to grow at low temperature. In this work, 23 hybrids between *S. cerevisiae* and *S. kudriavzevii* isolated from different regions were assayed to know their behavior at low temperature (8, 12 and 24 °C). Kinetic parameters and dropping-plate results revealed a very different ability to grow at low temperature. Interestingly, a moderate correlation between the higher content of the *S. kudriavzevii* genome in hybrids and the shorter lag phase at low temperature was observed. The influence of the genetic origin of hybrids on their performance at low temperature was found (8 °C). Finally, strains were selected according to their performance at low temperature, and the expression of cold marker genes (*NSR1*, *GUT2* and *GPD1*) was tested by focusing on the contribution of allele *S. kudriavzevii versus* allele *S. cerevisiae*. The results showed that the relative expression of *S. kudriavzevii* allele was higher that *S. cerevisiae* allele in the strains that grew well at low temperature, and the expression was considerably lower in the strains selected for bad performance at low temperature. These results suggest that the genomic contribution of *S. kudriavzevii* to hybrids is key for improving the fitness of these strains at low temperature.

Keywords: *S. cerevisiae, S. kudriavzevii,* hybrids, cold stress; genomic contribution

Introduction

Several species from the genus *Saccharomyces* are involved in different industrial processes: baking, brewing, winemaking or bioethanol production. *S. cerevisiae* is considered to play a dominant role as a starter strain in wine fermentations, and to be dominant in the final stages of the process (Pretorius, 2000). However, natural hybrids between *S. cerevisiae* and *S. kudriavzevii* are found in several cold-climate European countries (Erny et al., 2012; González et al., 2006; Lopandic et al., 2007; Peris et al., 2012a), and even as a dominant yeast in fermentations in these regions (Lopandic et al., 2007). These natural hybrids have been confirmed as a very useful biotechnological tool thanks to their adaptation to low temperature (Belloch et al., 2008). This is very useful for wineries in northern countries where fermentation takes place at low temperature, and also for winemakers to enhance the aromatic profiles of wine in low-temperature fermentations (Torija et al., 2003). Furthermore, aroma production studied in several *Saccharomyces* hybrids suggests an enhanced aroma in wines at low temperature (Gamero et al., 2013).

We are still quite uncertain about the role of *S. kudriavzevii's* genomic contribution in these natural hybrids. This species has been isolated from decaying leaves in Japan (Naumov et al., 2000), from oak bark samples in Portugal (Sampaio and Gonçalves, 2008) and from oak trees in Spain (Lopes et al., 2010), but not in fermentative industrial environments. A survey conducted by Arroyo-López et al. (2009) showed that temperature was the most important variable to affect the growth rate of *S. cerevisiae*, *S. kudriavzevii* and their interspecific hybrids, and resulted in better *S. cerevisiae* adaptation for improved growth at higher temperature, while *S. kudriavzevii* has been better adapted to grow at lower

63 temperatures. Low temperature-related physiological effects, like plasmatic membrane
64 fluidity, heat-shock protein regulation, RNA secondary structure stability, translation rate
65 and protein folding rate, have been associated with the cryophilic nature of *S. kudriavzevii*
66 (Oliveira et al., 2014; Salvadó et al., 2012; Tronchoni et al., 2014). The assessment by
67 González et al. (2007) emphasizes the larger amount of glycerol produced by
68 *S. kudriavzevii* during low-temperature fermentations compared with *S. cerevisiae*, while
69 the data obtained by Izawa et al. (2004) suggests that glycerol can be a cryoprotectant
70 inside yeast cells. Further research by Oliveira et al. (2014) studied the relationship
71 between glycerol synthesis at low temperature in different *S. kudriavzevii* and *S. cerevisiae*
72 strains, and reported better production of this metabolite from many *S. kudriavzevii* strains
73 regardless of their isolation origin. These authors consequently suggested that this
74 relationship was a species-specific trait. Beside, changes in gene expression have been
75 observed in the adaptation of yeasts to low temperature and found specific cold stress
76 marker genes in *S. cerevisiae* (Aguilera et al., 2007; López-malo et al., 2014; Sahara et al.,
77 2002). Tronchoni et al. (2014) discovered that, compared with *S. cerevisiae*, *S. kudriavzevii*
78 increased the expression of cold marker gene *NSR1*, which encodes a nuclear protein
79 required for pre-rRNA processing and ribosome biogenesis. Paget et al. (2014) disrupted
80 gene *GUT2* in *S. kudriavzevii* and observed major changes in the reduction of
81 cryo-tolerance and enhanced fitness at warm temperatures, which suggests that *GUT2* is
82 involved in the maintenance of the cold phenotype in *S. kudriavzevii*. Oliveira et al., (2014)
83 observed a higher expression of the *GPD1* gene in *S. kudriavzevii* compared to *S. cerevisiae*
84 under low-temperature micro-vinification conditions.

The purpose of this research was to determine the impact of *S. kudriavzevii's* genome to the phenotypical characteristics of the natural hybrids between *S. cerevisiae* and *S. kudriavzevii*. We also explored the influence of genes *NSR1, GUT2* and *GPD1* from the *S. kudriavzevii* genome on the cryo-tolerance of natural hybrid yeasts *S. cerevisiae* x *S. kudriavzevii*.

Materials and Methods

Strains and media

Strains name, species, country of origin, source of isolation and percentage of *S. kudriavzevii* genome present are indicated in Table 1. For the growth curves assay, liquid rich medium (GPY; peptone 0.5%, yeast extract 0.5%, and glucose 2%) was used. The same composition as GPY medium was added with 2% European agar to perform the drop-plate assays.

Drop-plate assays under different temperatures

All the strains were grown overnight in liquid rich medium (GPY) and the OD at 600 nm was adjusted to 1.0. The agar plates with GPY were inoculated with 5 µL serial dilutions from 10^{-1} to 10^{-6}, and were incubated at 8, 12 and 24 °C. Growth was recorded at 36 h, and on days 5 and 9, from the highest to the lowest temperature.

Growth parameters determination

Microplates wells were filled with 0.25 ml of GPY and inoculated with a cell suspension to achieve an OD_{600} of approximately 0.1 to then be monitored with a SPECTROstar Omega instrument (BMGLabtech, Offenburg, Germany). Each microplate included uninoculated wells to observe a noise signal as the control. All the experiments were performed in triplicate. At 24 ºC, the culture was monitored every half hour over 3 days after pre-shaking for 20 s. At 8 ºC and 12 ºC, plates were placed in a refrigerated stove by stirring constantly at 100 rpm and measuring OD_{600} 3 times a day for 7 days. The growth curves were adjusted to the modified Gompertz equation to obtain the specific growth rate and lag phase for each strain (Arroyo-López et al. 2009).

Gene expression measurements

Total RNA was isolated from the cells grown in liquid GPY medium at 12 °C, collected at the middle of exponential phase by centrifugation (4000 rpm/min, 5 min). Cell pellets were resuspended in 500 µl of lysis buffer LETS (100 mM LiCl, 10 mM EDTA, 0.2 % SDS, 10 mM Tris-HCl [pH 7.4]). Cells were mechanically disrupted with 500 µl of glass beads and 500 µl of acid phenol (citrate-saturated, pH 4.3). Contaminants were removed by several extraction steps with acid phenol, phenol-chloroform (5:1, pH 4.3) and chloroform-isoamyl alcohol (24:1). The extracted RNA was precipitated twice during the night at -20 °C. The first precipitation was done by adding 5 M LiCl and the second by adding 3 M sodium acetate and 100% ethanol. After centrifuging at the maximum speed for 15 min, RNA was washed once with 70% ethanol, dried at room temperature and finally resuspended in RNAse-free water. RNA purity and concentration were determined spectrophotometrically in a NanoDrop ND-1000 (Thermo-Scientific) and the integrity of all the samples was

checked by electrophoresis in agarose gel (1%). The gene expression analysis of the target genes in natural hybrid yeasts was performed by the two-step real-time quantitative PCR method. The first cDNA strand was synthesized from 400 ng of each RNA isolated and suspended in 20 μl by the Reverse Transcriptase Core kit from EUROGENTEC, oligo $d(T)_{15}VN$ and random nonamers by following the manufacturer's instructions. The synthesized cDNA was 5-fold diluted with RNAse-free water. Subsequent qPCRs were performed in a LightCycler® 480 Real-Time PCR System (96-wells; Roche Applied Science, Germany) using gene-specific primers (250 nM) in a 10 μl reaction with the LightCycler 480 SYBR Green I Master kit (Roche Applied Science). The PCR primers used to study the relative expression of target genes *NSR1*, *GUT2* and *GPD1* in natural hybrid strains (VIN7, CECT11011, AMH, W46, CECT1990, SPG14-91 and SPG441) and species (T73 and CR85) were designed from the available genomic sequence of *Saccharomyces cerevisiae* strain S288c (*Saccharomyces* genome database, http://www.yeastgenome.org/) and *Saccharomyces kudriavzevii* CR85 strain (C. Toft, personal communication). Two pairs of primers were designed for each gene (one to amplify allele *S. cerevisiae* in the hybrid and the other to amplify allele *S. kudriavzevii*), except for *NSR1* as the forward primer was useful for both species. The design was devised using a region in which the sequences of the two species differed in order to discriminate the expression of the two alleles in the hybrid strains. The sequences of PCR primer pairs used in this study are: NSR1c/k forward (CAAGAAGGAA GTTAAGGCTTCCAA), NSR1c reverse (GAAGATGAAGATTCAGATTCAGACTCA), NSR1k reverse (TCGGAGGAAGAAGAGGTGCTT), GPD1c forward (CAATTGAAAGG TCATGTTGATTCA), GPD1c reverse (TCAGTGATGTAAGAGGATAGCAATTG), GP

D1k forward (GAAAGGCCACGTTAACCCTC), GPD1k reverse (GGATAGAGCACCAC ATTGGATG),GUT2c forward (GGGGACGCTGTACTGGATG), GUT2c reverse (ATCA ACACGTCGAATTGATGC), GUT2k forward (GGATCCGTGTACTGGGCG), GUT2k re verse (CAGCACATCGAATTGGTGC) , ACT1 forward (TGGATTCCGGTGATGGTGTT and ACT1 reverse (CGGCCAAATCGATTCTCAA). The amplicons in all the samples were sized between 100 and 200 bp to ensure maximal PCR efficiency, and thus the strictest quantification. The PCR conditions were previously optimized. The specificity of the primers was tested using the genomic DNA from the two pure strains as a template. Normalization of the expression results was performed using the expression levels of gene *ACT1*, used as the reference gene, which displayed excellent uniformity in the expression levels under the studied growth conditions. All the samples were analyzed in triplicate. A melting curve analysis was included at the end of each amplification program to confirm the presence of a single PCR product of all the samples with no primer-dimers. The results were analyzed by the LightCycler 480 software 1.5.0, and the efficiency of all the primer pairs was previously determined and included in the analysis.

Results

To observe the performance of the diverse populations of natural hybrids between *S. cerevisiae and S. kudriavzevii*, we studied the consequences of a suboptimal growth condition, such as cold stress. The effect of low temperature on plate growth and the specific growth rate (μ) and lag phases (h^{-1}) was studied in 23 natural hybrids of different

origins and isolation sources, and in two representative strains of their parental species. The results are illustrated in Figures 1-3.

Drop-plate assays

The plates that contained the serial dilutions of the 23 natural hybrids and the two pure yeast strains were incubated at 8, 12 and 24 °C. Figure 1 shows the natural hybrid yeast strains seeded from the left to the right column at decreasing concentrations. Strains were ordered according to cold susceptibility at 8°C, at which the effect of low temperature was stronger than at 12 and 24 °C. This comparison was made visually by taking into account the maximum dilution dropped on the plate at which they were able to grow, but also the number and size of the colonies from the last dilution. *S. cerevisiae* strain T73 and *S. kudriavzevii* strain CR85 were used as growth references.

It was difficult to observe changes in drop-plate growth at 12 °C, and especially so at 24 °C. In contrast at 8 °C, the changes that occurred in both parental strains and the natural hybrids were visible. We noticed that PB7 was the hybrid that displayed the best growth on drop-plates at this temperature, but was also one of the best growers at 12 °C and 24 °C. *S. kudriavzevii* strain CR85 also exhibited good growth on drop-plates at 8 °C and 12 °C.

However at 24 °C, its growth was visibly affected compared with other strains such as T73, which grew very well at this temperature, whereas pure strain CR85 grew slightly better than T73 at 12 °C. The lowest temperature (8 ºC) also affected the natural hybrids like IF6 and MR25, both of which were isolated from a non alcoholic origin (dietary complement and respiratory tract, respectively). In Figure 1 shows two different groups according to

192 growth at 8 ºC. Group A includes the strains that grew better at 8 ºC and all the natural
193 wine hybrids, except for HA1835 and HA1841. Group B heads the list of strains whose
194 growth at 8°C was more affected, and it comprises the majority of beer strains and strains
195 IF6 and MR25.

196 **Microbial variability in growth rate**

197 Since differences in growth in a drop test are hard for classifying natural hybrids according
198 to their ability to growth at low temperature, we decided to measure growth parameters to
199 achieve this goal. At first glance, we can observe a wide range of results at 8 °C in Figure
200 2a. The results obtained at 12 ºC and at 24 ºC are shown in Figure 2b and c, respectively.
201 At 8 ºC we observed differences among hybrids, but a 12 °C and at 24 ºC the variability in
202 the results narrows. In Figure 2a we can see that *S. kudriavzevii* CR85 is the strain with the
203 highest μ (0.048 h^{-1}) at 8ºC. The strain with the lowest μ are the natural hybrids VIN7 and
204 IF6, which presented around 16% of the *S. kudriavzevii* CR85 value. In the same graph, we
205 observe that the hybrids SPG14-91 and CECT1990 shown the highest μ at this temperature.
206 Figure 2b shows that SPG441 is the strain with the highest μ at 12 °C and with a value of
207 0.048 h^{-1}. The lowest μ was achieved by strain HA1842. Only 8% of all the strains
208 accomplished values over 80% of the maximum value, hybrids W46 and CECT11004. At
209 the highest temperature applied in this survey (24 °C), the growth rate results achieved for
210 the studied strains were 10-fold higher than those results for the lowest temperatures. Thus
211 Figure 2c shows that the μ for the studied natural hybrids ranges from 0.24 to 0.47 h^{-1}. At
212 this temperature, we can see that SPG172 is the strain with the highest growth rate and IF6
213 present the lowest μ. Half the studied strains presented values between 70% and 90% of the
214 maximum value achieved at this temperature.

To determine the representative strains that displayed good (green bars) or bad (red bars) performance for further tests and comparisons, according to the data in Figure 1 and Figure 2 we selected four good strains (W46, CECT1990, SPG441 and SPG14-91) and three bad ones (AMH, VIN7 and CECT11011). The principal criterion is that significant differences were found in the growth rates for these strains at 8 ºC (Supplementary Figure 1).

Lag phase

Figure 3a shows the lag phase (λ) results, where they show a narrower variability between the natural hybrids than for the specific μ. However, we can observe that the biggest differences occurred at the lower growth temperature (8 °C), at which a very short λ is seen for both species compared to natural hybrids. At this temperature, strain AMH had the longest λ, approximately 67 h. Besides pure species, strains VIN7 and HA18-42 present a very short λ. Most strains have values between 40% and 50% of the maximum value achieved at this temperature (strains CECT11011, CECT11003, HA18-35, HA18-41, W46, W27, and IF6 HA1837). A comparison of Figure 3a and 3b shows that the difference between the λ of the strains analyzed at 12 °C tends to decrease as temperature rises. Conversely for the result obtained at 8 °C, VIN7 has the longest λ among the hybrids studied at 12 ºC followed by the strains HA1842, HA1835 and CECT1990. As Figure 3c shows, a big difference is observed at 24 °C for the estimated λ values at 8 and 12 °C because the minimum λ at this temperature was about 1.5 h and the maximum λ was approximately 3.5 h. At the lower temperatures of 8 and 12 °C, the minimum λ values were about 9 h for both temperatures, and the maximum estimated λ were maximum at 67 h for 8 °C and at 32 h for 12 °C. At 24 °C, VIN7 was also one of the strain with the longest λ, while the strain showing the shortest λ was CR85, which corresponds to 38% of the

maximum value obtained by VIN7 at this temperature. The strains with shorter λ are the hybrids SPG319, SPG14-91, CECT1103; even lowest that the T73 *S. cerevisiae* strain. The strain with lowest short λ at 24ºC, present longest λ at 8ºC (SPG319 and SPG14-91).

To determine if the content of *S. kudriavzevii* genome in the hybrids is influencing their physiological behavior, we studied correlations between the percentage of *S. kudriavzevii* content and three parameters at each temperature: maximal growth rate, lag phase and cold resistance (based on the quantification of the drop test growth). From all these comparisons, we observed only one significant correlation ($r= -0.5770$, $p= 0.0049$) between the percentage of *S. kudriavzevii* content and the lag phase at 8 °C, which confirms the important contribution of *S. kudriavzevii* genome for the cryophilic character of the *S. cerevisiae* x *S. kudriavzevii* hybrids.

Physiological performance and origin of hybrids

According to a previous study (Peris et al., 2012b), *S. cerevisiae* x *S. kudriavzevii* hybrids can be distributed into six groups according to their genetic origin. Thus we wondered whether the physiological abilities for cold tolerance could be related to their genetic origin or not. To test this, we ran a principal component analysis with the growth rate and lag phase data at 8, 12 and 24 ºC. The known percentage of *S. kudriavzevii* present in the strain was also included (Table 1). The results presented in Figure 4 show that the strains of different origins can be grouped together (groups A, B, C, D, E and F). We can also observe that the different groups behave differently from one another. The only exception is Group C, which overlaps Groups A and B, and contains strains with good (green) and bad (red) cold stress tolerances. This is because this group shows far more significant genomic

changes (chromosomal recombinations and losses), which occur from their origin, and there are many differences found among the different strains (Peris et al., 2012b).

The PCA analysis showed that the strains genetically included in the Group A, which were isolated from cold climate regions (Switzerland and Belgium) and characterized by us as the best adapted to cold stress, are also grouped together in the present analysis to the right of the graph (see Figure 4). Interestingly, this group are located on the right, influenced by their μ at 8 ºC (see Supplementary Figure 2). However, the strains in Groups B, D, E and F are located on the left of the graph, influenced by their lag phase at 8 ºC (see Supplementary Figure 2). It is also interesting to observe that CR85, a strain from the cryotolerant species *S. kudriavzevii*, is located in Group A, but the *S. cerevisiae* representative strain T73 is located in Group C.

Relative expression of genes *NSR1, GPD1* and *GUT2* at low temperature

To evaluate the impact of the different *S. cerevisiae* and *S. kudriavzevii* alleles on the performance of hybrids at low temperature, we determined the relative mRNA levels of previously defined *S. kudriavzevii*-related cold stress markers in relation to alleles *S. kudriavzevii* (see introduction). Figure 5 shows the relative expression of genes *NSR1*, *GPD1* and *GUT2* in the natural hybrids selected according to their low (red) or high (green) growth rates at 8 ºC. The results showed that the strains with a high μ at 8 ºC had a higher Sk/Sc ratio than the strains with a low μ at 8 ºC for all the cold stress gene markers. For *NSR1,* we observed that the Sk/Sc ratio was higher for the majority of strains considered good (green), where the Sk/Sc ratio was approximately twice that of the strains classified as bad (red). However, it is noteworthy that the ratio value of Sk/Sc for CECT1990 was

approximately half the ratio obtained for SPG14-91, W46 and SPG441. VIN7 was not included because *S. kudriavzevii* allele was not present in this hybrid strain. The ratios of the expression of *GPD1* between alleles *S. kudriavzevii* and *S. cerevisiae* (Sk/Sc) present in natural hybrids are also shown in Figure 5. For this gene, a Sk/Sc ratio of 6.12 is observed for hybrid CECT1990, and the other two hybrids, VIN7 and CECT11011, respectively give values of 2.85 and 2.90. It was not possible to calculate the ratio for the other strains due to the absence of either of allele. The ratio of the relative *GUT2* expression between alleles *S. kudriavzevii* and *S. cerevisiae* for *GUT2* expression indicated that the strains considered "bad" due to their μ at 8 °C (AMH, VIN7, and CECT11011) had values of 0.65, 0.72 and 1.30, respectively. Hybrid CECT1990 (considered "good") presented a Sk/Sc ratio of 4.34, which is more than 3-fold the ratio of CECT11011. Once again, the other strains were not included because one of the alleles was absent.

Discussion

In the last few years, many research efforts have been made to study yeast hybrids. One important aspect of these special yeasts is that they have show interesting properties which are useful for industrial uses like winemaking. Moreover, the construction of artificial hybrids has opened up a new era to generate new yeast starters as it allows the possibility to either generate new non GMO strains with beneficial characteristics provided by the parental or to enhance *bona fide* strains in specific weak aspects. Despite all the performed studies (for a review see Pérez-Torrado et al., 2017), one open question remains: how do the two genomes interact to generate the final phenotypical outcome? In this work, we

centered on studying natural strains *S. cerevisiae* and *S. kudriavzevii,* and focused on their ability to grow at low temperature. After analyzing the activation of cold stress marker genes, we observed that the strains with a higher μ at the lowest temperature displayed significantly more activation of allele *S. kudriavzevii* compared to allele *S. cerevisiae*. This suggests that some hybrid strains, isolated from cold climate regions and with a common origin (Peris et al., 2012b), have evolved to adapt to low-temperature environments by readjusting the gene expression regulation of promoters *S. kudriavzevii*, especially cold stress markers (Combina et al., 2012; Tronchoni et al., 2014). One intriguing observation was that despite the relation noted with allele *S. kudriavzevii's* contribution to μ in the more cryophilic strain, alleles *S. cerevisiae* were more frequently present than alleles *S. kudriavzevii* in most strains. We hypothesize that this effect could be a consequence of the genetic constrains generated during the genetic origin of the hybrids that limit the levels of certain transcription factors which affect essential genes.

Another important aspect of natural hybrids' adaptation to low temperature is the relative content of cryophilic species *S. kudriavzevii* in their genomic compositions. We observed an interesting negative correlation between λ and the percentage of *S. kudriavzevii* genome present in the hybrid strain, which indicates that the *S. kudriavzevii* genome contributes to better cold stress adaptation. A previous study (Oliveira et al., 2014; Paget et al., 2014; Tronchoni et al., 2014) has suggested that another adaptation mechanism to low temperature environments occurs in natural hybrids during their genetic life history. The hybrids that evolve under cold stress pressure tend to keep alleles *S. kudriavzevii*, whereas those without cold stress tend to lose them. One interesting question is why does genomic composition affect λ more, while μ depends more on the contribution of different alleles to

gene expression? This will require more research, but one initial hypothesis that we propose is that growth-related genes, such as central carbon metabolism, like *GPD1*, need fine-tuned regulation to perform an optimized and coordinated function. This could be done by adapting the levels of different alleles, whereas the consequence presence/absence of genomic reorganization is less precise.

This knowledge can be most interesting for industrial applications, especially to optimize and comprehend one of the techniques that has attracted more attention in recent years in applied industrial biotechnology: generating new starters by artificial hybridization. Recent studies have suggested that artificial hybrids tend to maintain at least one copy of each parental genome (Pérez-Través et al., 2014). In light of our data, this suggests that artificial hybrids could be more efficient to adjust μ under the desired condition. However, the stabilization process used for the artificial generation of hybrids takes place after a few generations, whereas natural hybrids have evolved over thousands of years.

Conclusions

In this work we studied the ability of natural hybrids *S. cerevisiae* x *S. kudriavzevii* to grow at low temperature. The results showed that the hybrids with a high μ at low temperature contain a higher expression of the cryophilic *S. kudriavzevii* alleles of cold stress markers. Furthermore, the strains with a low λ at low temperature correlate with the presence of a high percentage of the *S. kudriavzevii* genome. This knowledge could be of potential interest to interpret and optimize the results obtained by the non GMO artificial hybrid generation techniques followed to generate new industrial starters.

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440

441 **Table 1.** List of strains used in this study. Geographic origins, source of isolation and
442 genetic constitution of *Saccharomyces cerevisiae* x *Saccharomyces kudriavzevii* hybrids.

Strain	Species	Country of origin	Source of isolation	% of *S. kudriavzevii* (a)	Origin group (b)
HA 1835	Sc x Sk	Austria	Wine	-	B
HA 1837	Sc x Sk	Austria	Wine	-	B
HA 1841	Sc x Sk	Austria	Wine	95	B
HA 1842	Sc x Sk	Austria	Wine	100	B
VIN7	Sc x Sk	South Africa	Wine	99	B
W27	Sc x Sk	Switzerland	Wine	100	A
W46	Sc x Sk	Switzerland	Wine	85	A
SPG 14-91	Sc x Sk	Switzerland	Wine	80	A
SPG 16-91	Sc x Sk	Switzerland	Wine	80	A
SPG 126	Sc x Sk	Switzerland	Wine	85	A
SPG 172	Sc x Sk	Switzerland	Wine	85	A
SPG 319	Sc x Sk	Switzerland	Wine	85	A
SPG 441	Sc x Sk.	Switzerland	Wine	82	A
AMH	Sc x Sk	Germany	Wine	29	F
PB7	Sc x Sk	Spain	Wine	100	E
CECT 1388	Sc x Sk	England	Beer	98	C
CECT 1990	Sc x Sk	Germany	Beer	92	C
CECT 11002	Sc x Sk	Belgium	Beer	60	C
CECT 11003	Sc x Sk	Belgium	Beer	85	A
CECT 11004	Sc x Sk	Belgium	Beer	85	A

CECT 11011	Sc x Sk	New Zealand	Beer	98	C
MR25	Sc x Sk	Spain	Respiratory tract	82	C
IF6	Sc x Sk	Spain	Dietary complement	70	D
T73	Sc	Spain	Wine	-	-
CR85	Sk	Spain	Oak bark	100	-

Sc: *Saccharomyces cerevisiae*; Sk: *Saccharomyces kudriavzevii*

[a] Genomic composition analysed by RFLP analysis (Peris et al. 2012a) and CGH arrays (Peris et al. 2012b) (Erny et al. 2012). [b]Origin based on (Peris et al. 2012a)

Figure legends

Figure 1. Drop-plate assays at 25, 12 and 8 °C. Hybrid strains *S. cerevisiae* x *S. kudriavzevii*, including a *S. cerevisiae* (T73) and *S. kudriavzevii* (CR85) representative, are ordered from top to bottom. The best drop-plate growth at the top (A) and the worst growth at the bottom (B).

Figure 2. The growth rate (h^{-1}) of natural hybrids *S. cerevisiae* x *S. kudriavzevii*, including a *S. cerevisiae* (T73) and *S. kudriavzevii* (CR85) representative, at 8 °C (a), 12 °C (b) and 24 °C (c). The average and standard deviation from biological triplicates are shown. The strains selected as good (green) or bad (red) cold stress performance were color-labeled accordingly.

Figure 3. The lag phase (h) of natural *S. cerevisiae* x *S. kudriavzevii*, including a *S. cerevisiae* (T73) and *S. kudriavzevii* (CR85) representative, at 8°C (a), 12°C (b) and 24°C (c). The average and standard deviation from biological triplicates are shown. The strains selected as good (green) or bad (red) cold stress performance were color-labeled accordingly.

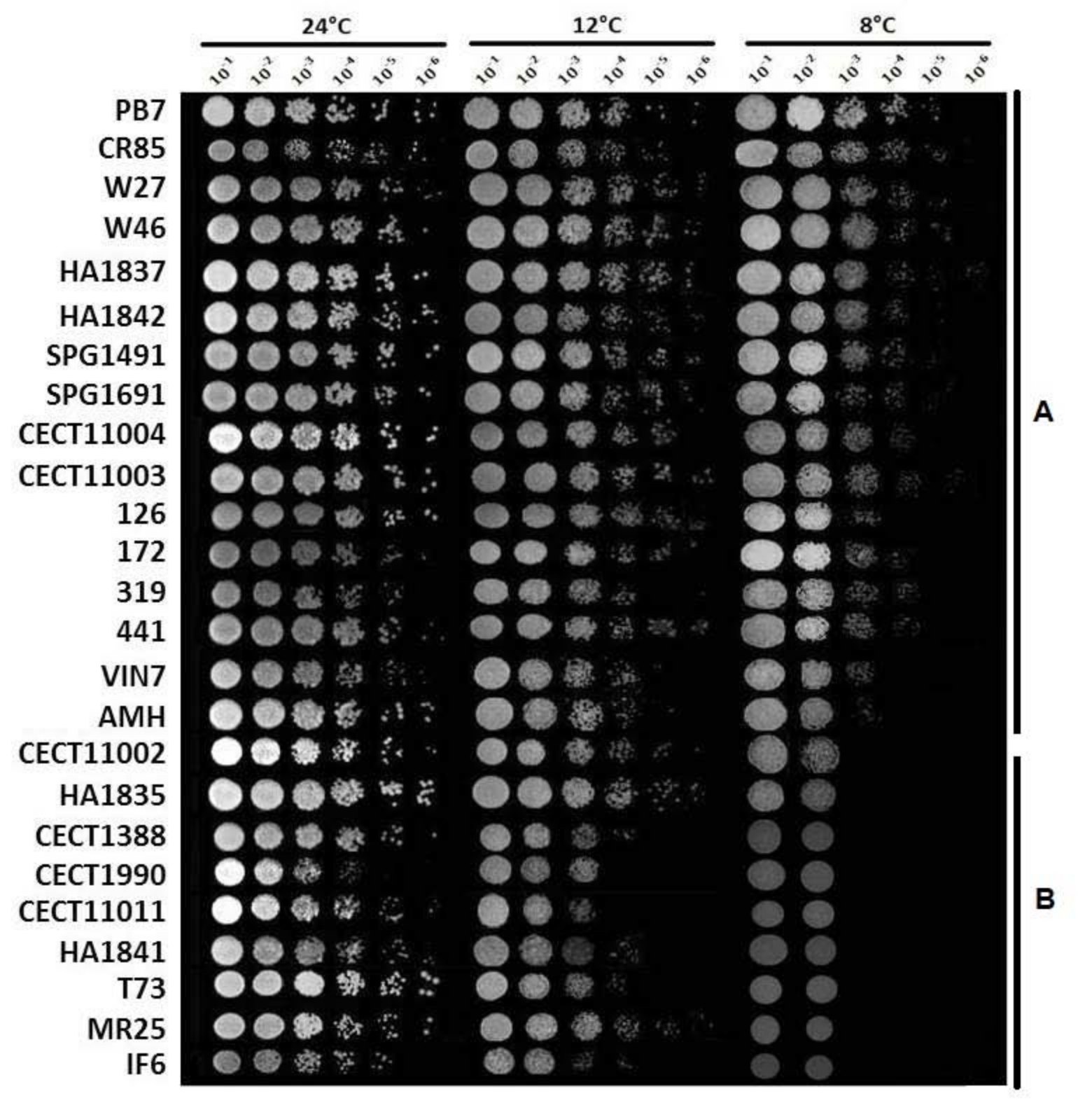
Figure 4. Principal component analysis of hybrid strains according to their physiological data. The growth rates and lag phase data of hybrid strains *S. cerevisiae* x *S. kudriavzevii* (streaked circles), including a *S. cerevisiae* (T73) and *S. kudriavzevii* (CR85) representative (solid circles), were included in the analysis. In addition, the percentage of the *S. kudriavzevii* genome (Table 1) was included. The strains selected as good or bad cold stress performance were color-labeled accordingly. The strains with the same origin were

surrounded by a dashed line to form Groups A to F, as described in Peris et al. (2012). Strain SPG319 was excluded from Group A and labeled with a solid circle.

Figure 5. Relative expression of allele *S. kudriavzevii* of genes *NSR1*, *GUT2* and *GPD1* related to allele *S. cerevisiae* in natural hybrid yeast at 12°C. The average and standard deviation from biological triplicates are shown. The data from the strains selected as good (green) or bad (red) cold stress performance were color-labeled accordingly.

Supplementary Figure 1. Statistical analysis of the strains selected as good (green) or bad (red) cold stress performance regarding growth rate and lag phase. An ANOVA analysis was performed for both grow rate and lag phase at each temperature. Statistically significant differences were labeled with different letters.

Supplementary Figure 2. Impact of the different variables on the PCA analysis. The growth rate (m) and lag phase (l) at different temperatures (8, 12 or 24 ºC) were included along with the percentage of the *S. kudriavzevii* genome content (genome).


24°C
12°C
8°C
10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6}
PB7
CR85
W27
W46
HA1837
HA1842
SPG1491
SPG1691
CECT11004
CECT11003
126
172
319
441
VIN7
AMH
CECT11002
HA1835
CECT1388
CECT1990
CECT11011
HA1841
T73
MR25
IF6
A
B

a)

Grow th rate (h-1)
0.00
0.02
0.04
0.06
VIN7
IF6
HA1842
HA1837
CECT11011
HA1835
AMH
W27
HA1841
MR25
PB7
SPG16-91
CECT1388
CECT1103
W46
SPG172
SPG319
CECT1104
T73
SPG126
CECT1102
CECT1990
SPG14-91
SPG441
CR85

b)

Grow th rate (h-1)
0.00
0.02
0.04
0.06
HA1842
IF6
SPG319
CECT11011
AMH
CECT1388
HA1841
VIN7
CECT1102
MR25
T73
PB7
HA1835
CR85
HA1837
CECT1990
SPG172
SPG16-91
SPG14-91
W27
SPG126
CECT1103
W46
CECT1104
SPG441

c)

Grow th rate (h-1)
0.0
0.2
0.4
0.6
CR85
IF6
VIN7
HA1842
CECT11011
PB7
HA1841
HA1835
HA1837
MR25
SPG14-91
SPG319
SPG126
W27
SPG16-91
AMH
CECT1104
T73
CECT1102
W46
CECT1103
CECT1990
CECT1388
SPG441
SPG172

a)

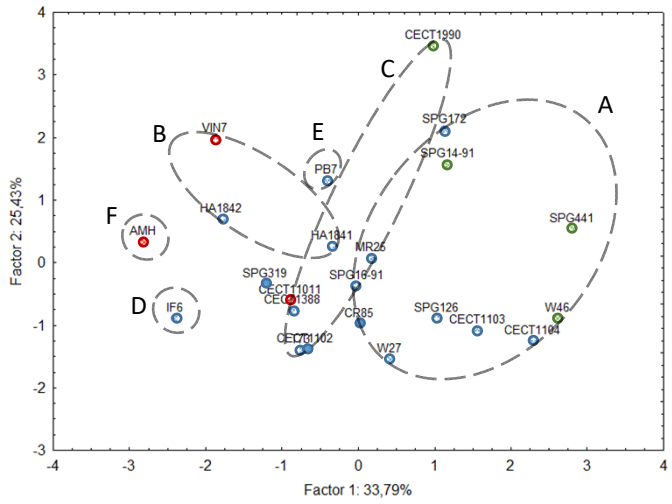
Lag phase (h-1)
100
80
60
40
20
0
CR85
T73
VIN7
HA1842
CECT11011
CECT1103
HA1835
HA1841
W46
W27
HA1837
IF6
CECT1104
SPG441
PB7
SPG126
MR25
SPG16-91
CECT1102
CECT1990
SPG172
SPG14-91
CECT1388
SPG319
AMH

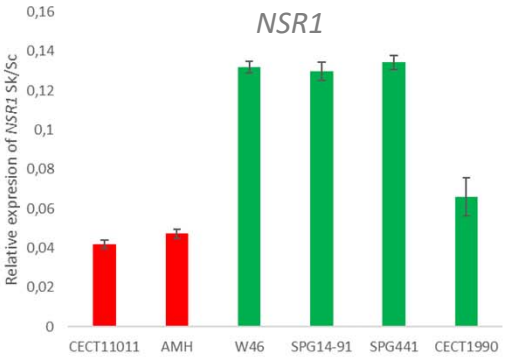
b)

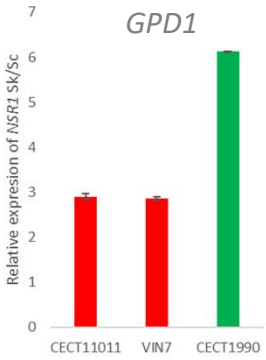
Lag phase (h-1)
40
30
20
10
0
MR25
CECT1102
CECT1388
CR85
T73
IF6
W46
SPG126
W27
CECT1104
CECT1103
CECT11011
HA1841
SPG319
AMH
SPG16-91
HA1837
SPG14-91
PB7
SPG441
SPG172
HA1842
HA1835
CECT1990
VIN7

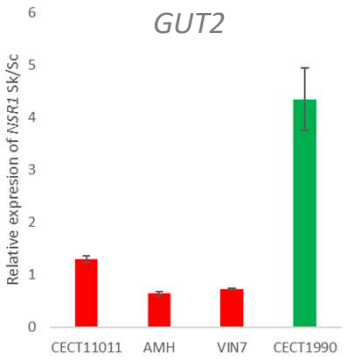
c)

Lag phase (h-1)
4
3
2
1
0
SPG319
SPG14-91
CECT1103
T73
SPG126
SPG16-91
CECT1104
HA1841
W27
HA1842
CECT1102
HA1837
CR85
CECT11011
IF6
W46
MR25
SPG441
HA1835
CECT1388
AMH
SPG172
VIN7
CECT1990
PB7


A
B
C
D
E
F
CECT1990
VIN7
SPG172
SPG14-91
PB7
HA1842
AMH
HA1841
MR25
SPG441
SPG319
SPG18-91
CECT1011
CECT1388
IF6
CR85
SPG126
CECT1103
W46
CECT1104
CECT6102
W27
Factor 2: 25,43%
Factor 1: 33,79%
4
3
2
1
0
-1
-2
-3
-4


NSR1
Relative expresion of NSR1 Sk/Sc
0,16
0,14
0,12
0,1
0,08
0,06
0,04
0,02
0
CECT11011
AMH
W46
SPG14-91
SPG441
CECT1990


GPD1
Relative expresion of NSR1 Sk/Sc
7
6
5
4
3
2
1
0
CECT11011
VIN7
CECT1990


GUT2
Relative expresion of NSR1 Sk/Sc
6
5
4
3
2
1
0
CECT11011
AMH
VIN7
CECT1990