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Complete List of Authors:	Peris, David; Universitat de València, Inst. Cavanilles de Biodiversitat i Biologia Evolutiva Belloch, Carmela; CSIC, Universitat de Valencia, Departamento de Biotecnologia de Alimentos, Instituto de Agroquimica y Technologia de Alimentos Lopandić, Ksenija; University of Natural Resources and Applied Life Sciences, Viena, Institute of Applied Microbiology Álvarez-Pérez, José; Universidad de León, Instituto de Investigación de la Viña y el Vino, , Campus de Ponferrada Querol, Amparo; CSIC, Universitat de Valencia, Departamento de Biotecnologia de Alimentos, Instituto de Agroquimica y Technologia de Alimentos Barrio, Eladio; Universitat de València, Inst. Cavanilles de Biodiversitat i Biologia Evolutiva
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The molecular characterization of new types of *S. cerevisiae* x *S. kudriavzevii* hybrid yeasts unveils a high genetic diversity.

David Peris[1], Carmela Belloch[2], Ksenja Lopandić[4], José Manuel Álvarez-Pérez[3], Amparo Querol[2] and Eladio Barrio[1]

[1]Institute Cavanilles of Biodiversity and Evolutionary Biology, University of Valencia, Valencia, Spain.

[2]Department of Biotecnology, Institute of Agrochemistry and Food Technology (CSIC), Valencia, Spain.

[3]Vine and Wine Research Institute, University of León, 24400-Ponferrada, Spain

[4]Austrian Center of Biological Resources and Applied Mycology, Institute of Applied Microbiology, University of Natural Resources and Applied Life Sciences, Muthgasse 18, 1190 Vienna, Austria.

Corresponding author:

Dr. Eladio Barrio
Institut 'Cavanilles' de Biodiversitat i Biologia Evolutiva
Universitat de València
P.O. Box 22085
E- 46071 València
SPAIN
Tel.: +34 963 543 667
Fax: +34 963 543 670
E-mail: eladio.barrio@uv.es

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Abstract

New double and triple hybrid *Saccharomyces* yeasts were characterized by using PCR-restriction fragment length polymorphism of 35 nuclear genes, located at different chromosome arms, and the sequencing of one nuclear and one mitochondrial genes. Most of these new hybrids were originally isolated from fermentations, however, two of them correspond to clinical and dietary supplement isolates. This is the first time that the presence of double hybrids *S. cerevisiae* x *S. kudriavzevii* in non-fermentative substrates is reported and investigated.

The phylogenetic analysis of the *MET6* nuclear gene confirmed the double or triple parental origin of the new hybrids. The restriction analysis of gene regions in these hybrids revealed a high diversity of genome types. From these molecular characterizations, a reduction of the *S. kudriavzevii* fraction of the hybrid genomes is observed in most hybrids.

Mitochondrial inheritance in hybrids was deduced from the analysis of the mitochondrial *COX2* gene sequences, which showed that most hybrids received the mitochondrial genome from the *S. kudriavzevii* parent. However, two strains inherited a *S. cerevisiae COX2*, being the first report of *S. cerevisiae* x *S. kudriavzevii* hybrids with *S. cerevisiae* mitochondrial genomes. These two strains are those showing a higher *S. kudriavzevii* nuclear genome reduction, especially in the wine hybrid AMH. This may be due to the release of selective pressures acting on the other hybrids to maintain *kudriavzevii* mitochondria-interacting genes.

Introduction

The genus *Saccharomyces* consists of eight species, three of them associated with industrial fermentation processes (*S. bayanus*, *S. cerevisiae*, and *S. pastorianus*), and five isolated from natural habitats (*S. arboriculus*, *S. cariocanus*, *S. kudriavzevii*, *S. mikatae* and *S. paradoxus*) (Kurtzman 2003; Wang and Bai 2008). *S. cerevisiae*, the predominant species responsible for the alcohol fermentation, has been found associated to diverse fermentation processes including baking, brewing, distilling, wine making, cider production, etc. and also in different traditional fermented beverages and foods around the world . The species *S. bayanus* includes two recognized varieties, *bayanus* and *uvarum* (Vaughan-Martini and Martini 2011). *S. bayanus* var. *uvarum* is present in wine and cider fermentations from cold regions of Europe (as examples see Naumov *et al*. 2001; Demuyter *et al*. 2004). The *S. pastorianus* taxon includes hybrid strains between *S. bayanus* and *S. cerevisiae*, which are responsible for the production of lager beer (Kodama *et al*. 2005). The rest of the species are only associated with natural habitats, with the exception of some *S. paradoxus* strains isolated from Croatian vineyards (Redzepović *et al.* 2002), that show a good winemaking performance (Orlić *et al.* 2010).

During their evolution, yeasts have suffered diverse selective processes to become adapted to the fermentation conditions (Querol *et al.* 2003). Diverse molecular mechanisms were involved in the generation of the evolutionary novelties that allowed the adaptation of yeasts to the fermentation processes (for a review see Barrio *et al.* 2006). In the case of the genus *Saccharomyces*, one of the most interesting mechanisms involved in their adaptation to industrial

processes, is the generation of interspecific hybrids (Querol and Bond 2009). Hybrids between *S. cerevisiae* and *S. bayanus* were already identified several decades ago (for a review see Kodama *et al.* 2005). In the last years, a new type of hybrids, between *S. cerevisiae* x *S. kudriavzevii*, have been found both in winemaking and brewing (Bradbury *et al.* 2006; González *et al.* 2006, 2008; Lopandić *et al.* 2007).

In the present study we characterize the genome composition of new *S. cerevisiae* x *S. kudriavzevii* hybrids. These new hybrids include two strains isolated from wine regions located in the southernmost limits of the Oceanic and Continental Europe, and two hybrids isolated for the first time from non-fermentative sources, such as a human respiratory tract isolate (deLlanos *et al.* 2004) and a strain employed as dietary supplement. Other hybrids, molecularly characterized for the first time in this study, are some commercial wine strains described as such by Bradbury *et al.* (2006) and some of the Austrian wine hybrids (Lopandić *et al.* 2007), as well as two triple hybrids *S. bayanus* x *S. cerevisiae* x *S. kudriavzevii* CID1 (Groth *et al.* 1999) and CBS2834 (González *et al.* 2006). The genetic characterization was performed by restriction analysis of 35 nuclear genes located in different chromosomes, and by sequencing the nuclear gene *MET6* and the mitochondrial *COX2* genes. Accordingly, these new hybrids were compared to those characterized in our previous study (González *et al.* 2008).

Materials and methods

Yeast strains and culture media

The natural yeast hybrids *S. cerevisiae* x *S. kudriavzevii* used in this study were originally isolated from different sources and locations as described in Table 1. Yeast strains were grown at 28ºC in GPY medium (2% glucose, 0.5% peptone, 0.5% yeast extract).

PCR amplification and restriction analysis of 35 nuclear gene regions

Characterization of the hybrids was performed by PCR amplification and restriction of 35 gene regions located in different chromosome arms (Fig. 2). DNA was extracted following the procedure described by Querol *et al.* (1992). Amplification and digestion of the nuclear genes was performed by using the methodology described in González *et al.* (2008) except for the subtelomeric *MNT2* gene, that failed to amplify the *S. kudriavzevii* gene and, hence, it was replaced by *GCN1*. Primers used for amplification of *GCN1* gene were *GCN1*-5 (GGTTTRGTKAAAGGTTAYGG) and *GCN1*-3' (CACCAGCYAAAATRGTTGG) and PCR conditions were as in González *et al.* (2008), but using an annealing temperature of 55.5 ºC.

Amplification, sequencing and phylogenetic analysis of COX2 *and* MET6 *genes*

The genes *COX2* and *MET6* were amplified by PCR using the primers and conditions described in Belloch *et al.* (2000) and González *et al.* (2006), respectively. PCR products were cleaned with the Perfectprep Gel Cleanup kit (Eppendorf, Hamburg, Germany) and both strands of the DNA were directly sequenced using the BigDyeTM Terminator V3.0 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK), following the manufacturer's instructions, in an

Applied Biosystems automatic DNA sequencer Model ABI 3730l (Life Technologies Coorporation, Carlsbad, California).

COX2 and *MET6* sequences obtained for the present study are listed in Table 1 with their accession numbers. Other sequences from hybrids were retrieved from sequence databases (accession numbers for *MET6* sequences AJ973280-AJ973295 and AJ973305-AJ973322; and for *COX2* sequences AJ938037-AJ93844, AJ938047, AJ938048 and AJ966727-AJ966733).

Finally, *MET6* sequences from reference or type strains of *S. bayanus* var. *uvarum* (MCYC 623), *S. cerevisiae* (S288C), *S. kudriavzevii* (IFO 1802^{T}), *S. mikatae* (IFO 1815^{T}) and *S. paradoxus* (CECT 1939^{NT}) were retrieved from the fungal alignment viewer of the *Saccharomyces* Genome Database (http://db.yeastgenome.org/cgi-bin/FUNGI/showAlign).

Each set of homologous sequences was aligned in MEGA 4 (Tamura *et al.* 2007). The sequence evolution model that fits our sequence data best was optimized using the corrected Akaike Information Criterion (AICc) with a BioNJ tree as the initial tree, implemented in jModelTest program (Posada 2008). The best fitting model of evolution for *MET6* sequences was TIM1 model (Posada 2003) with a gamma distribution (G) of substitution rates with a shape parameter α= 0.35; and for *COX2* gene sequences the TVM model (Posada 2003) with a gamma distribution (G) of substitution rates with a shape parameter α= 0.123 and 46.2% of invariable sites (I). The parameters of each model, estimated in the previous analysis, were used to obtain the best trees under optimality criterion of maximum-likelihood (ML). Tree reliability was assessed using non-parametric bootstrap re-sampling of 1000 pseudo-

replicates. Phylogenetic analyses were performed using PhyML 3.0 program (Guindon *et al.* 2010).

In the case of COX2 sequences, due to evidences of recombination obtained from sequence comparisons, a Neighbor-net network analysis was also performed with SPLITSTREE4 program (Huson and Bryant 2006).

Results

Analysis of the hybrid nature of the strains by the phylogenetic analysis of MET6 *gene sequences.*

To confirm the hybrid nature of the strains under study and their genealogical relationships, we performed phylogenetic analyses of partial sequences of a nuclear (*MET6*) and a mitochondrial (*COX2*) genes, because such sequences are also available for other hybrids (González *et al.* 2006, 2008).

Three different *MET6* sequence types were found in hybrids that correspond to those of the reference strains of the parental species *S. bayanus*, *S. cerevisiae* and *S. kudriavzevii* (Fig. 1). Thus, the average number of nucleotide subtitutions among *S. cerevisiae* alleles is 0.97 ± 0.88 (from 0 to 3 differences), among *S. kudriavzevii* alleles is 0.23 ± 0.59 (from 0 to 3) and among *S. bayanus* var. *uvarum* is 0 ± 0. In contrast, average numbers of nucleotide differences between species are 83.89 ± 0.78 (*S. cerevisiae* vs. *S. kudriavzevii* alleles, 83 to 87 nucleotide differences), 66.78 ± 0.84 (*S. bayanus* var. *uvarum* vs. *S. cerevisiae* alleles, 65-68 differences) and 60.11 ± 0.42 (*S. bayanus* var. *uvarum* vs. *S. kudriavzevii* alleles, 60-62).

All double and triple hybrids included in the analysis contain two or three *MET6* alleles coming from their parental species (B, C and K), except double hybrids HA1841 and AMH that lost the *S. kudriavzevii MET6* allele. These results confirm the hybrid nature of the new strains.

Nuclear genome characterization of Saccharomyces *hybrids*

The restriction patterns of the 35 genes for the differentiation of the *S. cerevisiae* and *S. kudriavzevii* alleles were described in González *et al.* (2008) with the exception of *MNT2* which was replaced in the present study by *GCN1*. The restriction analysis of the *GCN1* gene region yielded the following fragments: *Hae*III, *S. cerevisiae* 462 + 302 + 144 + 114 bp, and *S. kudriavzevii* 450 + 366 + 206 bp; *Msp*I, *S. cerevisiae* 514 + 508 bp, and *S. kudriavzevii* 1022 bp; and *Cfo*I, *S. cerevisiae* 766 + 256 bp, and *S. kudriavzevii* 634 + 388 bp. Hybrids characterized in a previous study (González *et al.* , 2008) were also assayed for this gene, resulting in the presence of both parental copies in all of them.

The PCR-RFLP patterns of the 10 newly characterized *S. cerevisiae* x *S. kudriavzevi* hybrids, and the 2 triple hybrids are depicted in Figures 2 and 3, respectively. The specific alleles present in each hybrid strain are given in the Supplementary Table S1 and the new restriction patterns in Supplementary Table S2.

Since the *S. cerevisiae* and *S. kudriavzevii* genomes are colineal (Kellis *et al.* 2003), the locations of the gene regions under analysis were chosen to obtain information about the presence of possible chromosomal rearrangements in the hybrid genomes, as described in other hybrids (González

et al. 2008; Belloch *et al.* 2009). This way, the absence in the hybrids of *S. kudriavzevii* alleles for genes located in the same chromosome likely resulted from the loss of the whole chromosome. However, the loss of one gene located in a chromosome but not the other genes of the same chromosome can be postulated as a result of recombination between homeologous chromosomes, as demonstrated for some hybrids (Belloch *et al.*, 2009). This resulted in the replacement of the missing segment by the homologous segment from the other chromosome of different parental origin (see Figures 2 and 3).

This way, chromosomal rearrangements can be postulated as occurred in chromosomes IV (AMH), V (IF6), VII (AMH, VIN7, IF6 and MR25), IX (IF6, MR25), X (IF6, MR25), XI (PB7 and IF6), XIII (IF6, MR25), XIV (MR25), XV (AMH) and XVI (IF6). In four wine hybrids (SOY3, from Croatia, and HA 1835, HA 1837 and HA 1842 from Austria) no rearrangement can be deduced because they contain both parental alleles for all genes.

In general, the *S. cerevisiae* genome fraction is maintained in all these double hybrids whereas a progressive loss of the *S. kudriavzevii* genes is observed. This reduction is more evident in the case of hybrid AMH, which has lost most of the *S. kudriavzevii* chromosomes.

In the case of the triple hybrids (Fig. 3), the typical restriction pattern of *S. bayanus var. uvarum* was found in addition to those of *S. cerevisiae* and *S. kudriavzevii* alleles, indicating that they contain chromosomes from the three parental species. The *S. cerevisiae* and *S. kudriavzevii* chromosomes are co-lineal (synthenic), however, the chromosomes of *S. bayanus* var. *uvarum* contain 4 differential reciprocal translocations (Kellis *et al.* 2003), as depicted in

Figure 3. In the case of triple hybrids, a higher preservation of the *S. bayanus* var. *uvarum* fraction is observed.

The comparison of the RFLP patterns obtained in this study for the new hybrids and those described by González *et al.* (2008, see their figure 3) reveals a considerable diversity in the genome structure of *S. cerevisiae* x *S. kudriavzevii* hybrids, although certain similarities among strains are observed as well. Accordingly, double hybrid strains can be classified in three groups according to the parental genome rearrangements. The first group includes hybrids that maintain the complete genome from both parents (most HA strains and SOY3) or have independently lost from 1-2 chromosomes or chromosome regions from *S. kudriavzevii* (wine strains PB7, VIN7 and most brewing hybrids), the second group comprises strains with a moderate loss (3-4) of *S. kudriavzevii* chromosomes or chromosome regions, including 3 shared events (Swiss wine hybrids and the brewing strain CECT 11003), and the third group includes strains with moderate (MR25, 6 losses) to large *S. kudriavzevii* gene losses (CECT 11002, IF6 and AMH, with 9, 11 and 13, respectively).

Mitochondrial inheritance in hybrids

The analysis of mitochondrial *COX2* gene sequences has been shown as useful to decipher which parental species contributed with their mitochondria to the hybrid strains (González *et al.* 2006).

The comparative analysis of *COX2* sequences with those previously described (González *et al.* 2006), showed the presence of new haplotypes in hybrids PB7, AMH and IF6 (Fig. 2 and 3). The wine hybrids AMH and IF6 contain COX2 sequences more related to *S. cerevisiae* (1 and 14 differences,

respectively being the first description of *S. cerevisiae* x *S. kudriavzevii* hybrids that received their mitochondrial genomes from a *S. cerevisiae* parent.

The other new hybrids contain *COX2* sequences that correspond to previously described haplotypes. Thus, with the exception of PB7, all new wine hybrids contain haplotype K4, already described in the triple hybrid CBS 2834. This haplotype is closely related to haplotypes K2 and K3 from Swiss wine hybrids (1 and 2 nucleotide differences, respectively) and haplotypes exhibited by the Japanese type (haplotype K1, 5 differences) and European strains from *S. kudriavzevii* (haplotypes K8 and 9, with 1 and 3 differences, respectively). The clinical isolate MR25 exhibits the same haplotype K6 described in brewing hybrids, which is related to haplotype K10 present in the wine hybrid PB7 (6 nucleotide differences).

However, a detailed analysis of the *COX2* sequence alignment suggested the possibility of reticulate evolution due to recombination (Table 2). This way, haplotypes K5 (triple hybrid CID1), K6 (brewing hybrids CECT 1388, 1990, 11002, 11011 and the clinical strain MR25) and K10 (wine hybrid PB7) appear as putative recombinant sequences with similarities to *S. kudriavzevii*, *S. cerevisiae* and *S. paradoxus* sequences in their 5'-end, central and 3'-end regions, respectively (see Table 2).

In the case of reticulate evolution due to recombination, a better representation of the phylogenetic relationships is obtained by a Neighbor-net network analysis (Figure 4). Most wine hybrids (except PB7 and AMH) and two Trappist beer hybrids (CECT 11003 and 11004) inherited their mitochondrial genomes (haplotypes K2, K3 and K4) from *S. kudriavzevii*, AMH and IF6 received their mitochondrial genomes from *S. cerevisiae*, although IF6 *COX2*

appears in a striking intermediate position between *S. cerevisiae* and *S. paradoxus-S.mikatae* clades, likely due to its highly divergent 3' end. Finally, most brewing hybrids and the clinical isolate (haplotype K6), the cider CID1 (K5) and the wine PB7 (K10) hybrids appear in an intermediate position due to their chimerical *COX2* sequences.

Different groups of hybrids according to their nuclear and mitochondrial genome constitutions

The combined analysis of the nuclear and mitochondrial genome compositions of *S. kudriavzevii* double and triple hybrids indicates a higher genetic diversity. Strains that differed in a few chromosomal rearrangements contain different mitochondrial haplotypes (e.g. PB7 and the Austrian and Croatian hybrids) and others showing important chromosomal differences share the same mitochondrial sequences (e.g. MR25 and brewing hybrids).

In other cases, there is a certain association between the nuclear and mitochondrial diversities. This way, the two hybrids with a *S. cerevisiae* mitochondrial DNA are those that lost a higher fraction of *S. kudriavzevii* nuclear genome. As well, with the mentioned exception of PB7, wine hybrids appear in two closely related clusters, the Austrian-Croatian cluster (also including VIN7) with low number of chromosomal rearrangements and the sharing the same *S. kudriavzevii*-like mitochondrial haplotype K4, and the Swiss cluster (also including Trappist hybrids CECT11003 and 11004), which share several fixed rearrangements (Belloch *et al.* 2009) and the *S. kudriavzevii*-like mitochondrial haplotype K2 (including the derived K3).

In the case of the two triple hybrids known so far, they also show important differences both in their mitochondrial and nuclear genomes. Thus, these strains do not share any common chromosomal rearrangements indicating independent losses in the three fractions of their hybrid genomes. Moreover, the wine triple hybrid inherited a *S. kudriavzevii* mitochondrial genome similar to that present in wine double hybrids, whilst the cider hybrid contains a mitochondrial *COX2* closely related to that present in most brewing, the clinical and a wine hybrid with similarities intermediate between *S. cerevisiae* and *S. kudriavzevii*.

Discussion

New strains expanding the distribution range of Saccharomyces kudriavzevii *hybrids*

It is more than a decade since an unusual *S. bayanus* x *S. cerevisiae* hybrid, CID1, isolated from home-made Breton cider, was identified as bearing a mitochondrial genome coming from *S. kudriavzevii* (Masneuf *et al.* 1998; Groth *et al.* 1999). Later, a *S. kudriavzevii* contribution to a fraction of the chimerical nuclear genome of this strain was demonstrated (Naumova *et al.* 2005; González *et al.* 2006).

Some years later, a new type of natural hybrid strains between *S. cerevisiae* x *S. kudriavzevii* was described in wine fermentations (Bradbury *et al.*, 2006; González *et al.*, 2006; Lopandić *et al.*, 2007). and brewing environments (González *et al.*, 2008).

In the present study, new *S. cerevisae* x *S. kudriavzevii* hybrid yeasts are described and molecularly characterized. These hybrids contribute to expand

the geographical distribution range of this type of hybrids as well as the sources whence they can be isolated.

This way, the new wine hybrids (PB7 and SO3) were isolated from wine fermentation in the southernmost locations where this kind of hybrids has been isolated so far (Pajares de los Oteros, in Northwestern Spain, and Daruvar, in Central Croatia, respectively). These new descriptions extend the distribution limits of *S. cerevisiae* x *S. kudriavzevii* hybrids to the Southern limit of the European wine regions of Oceanic and Continental climate, where these hybrids have been found so far associated to fermentation processes. In these wine regions, hybrids can be predominant (Schütz and Gafner 1994; González *et al.* 2006; Lopandić *et al.* 2007) likely due to a better adaptation to lower temperatures compared to *S. cerevisiae* (González *et al*. 2007).

The molecular characterization of PB7 showed that, although its nuclear genome composition is similar to other wine hybrids, exhibits a recombinant mitochondrial genome different but closely related to brewing hybrids. Its marginal distribution and its peculiar genome characteristics are indicative of a putative independent origin from other wine hybrids. However, the genome composition of the Croatian SOY3 hybrid was identical to Austrian hybrids, predominant in another wine region of the same Pannonian basin (Lopandić *et al.* 2007), with similar climatologic characteristics as well as historical links in the development of viticulture and enology.

In these Southern locations where the new wine hybrids were isolated, hybrids did not appear as predominant. In both cases, these wine hybrids were found at low frequencies and coexisting with the dominant *S. cerevisiae* strains during the first stages of the wine fermentations. Perhaps the milder

temperatures at which spontaneous fermentations occur in these Southern regions still allow *S. cerevisiae* to outcompete these hybrids.

The present study also describes for the first time *S. cerevisiae* x *S. kudriavzevii* hybrids isolated from non-fermentative environments. Strain MR25 is a human respiratory isolate from 'Hospital del Vall d'Hebron', Barcelona, Spain; and IF6, is commercialized as a dietary supplement. These hybrids are quite different at the genome level, particularly in their mitochondrial genomes. The clinical isolate MR25 shares a *COX2* sequence identical to that present in 4 brewing hybrids, indicating that beer could likely be the source of infection, and the dietary supplement IF6 exhibits a S. *cerevisiae* mitochondrial DNA.

The high genetic diversity among Saccharomyces kudriavzevii *hybrids suggests independent hybridization origins*

The analysis of the nuclear and mitochondrial genome compositions of *S. kudriavzevii* double and triple hybrids unveiled a high diversity, which likely is indicative of independent primary, as well as secondary, hybridization events.

The fact that hybrids inherited 3 types of mitochondrial genomes (*S. cerevisiae*-like, *S. kudriavzevii*-like and recombinant) from their parental ancestors is indicative of at least 3 different origins. Moreover, the important differences in their nuclear genome compositions could also be taken as evidences of independent primary hybridization events.

The presence of recombinant mitochondrial genomes in hybrids can be explained by recombination events occurring after the fusion of mitochondria observed in conjugating *Saccharomyces* spores or cells. This kind of recombination events were already described in *S. cerevisiae* at the within-

species level (Berger and Yaffe 2000), but this is the first time that is described in hybrids at the between-species level. However, we suspect that these recombination events are limited to this *COX2* region because sequences from the next downstream gene, *COX3*, correspond to *S. kudriavzevii* (data not shown).

In addition, the existence of natural triple *S. bayanus* var. *uvarum* x *S. cerevisiae* x *S. kudriavzevii* hybrids can be explained by secondary hybridization between either *S. cerevisiae* x *S. kudriavzevii* hybrids with *S. bayanus* var. *uvarum* strains or *S. bayanus* var. *uvarum* x *S. cerevisiae* hybrids with *S. kudriavzevii* strains. Although both types of double hybrids have been found associated to fermentation environments, the first type of secondary hybridization event could be more probable because *S. kudriavzevii* seems to be present only in natural environments (Sampaio and Gonçalves 2008; Lopes *et al.* 2010) and is outcompeted by *S. cerevisae* in experimental wine fermentations (Arroyo-López *et al.* 2011), whilst *S. bayanus* var. *uvarum* coexists with, or even replaces, *S. cerevisiae* in wine fermentations from cold regions of Europe (Torriani *et al.* 1999; Naumov *et al.* 2000; 2002; Rementería *et al.* 2003; Demuyter *et al.* 2004). However, a secondary hybridization event in natural environments, involving a *S. kudriavzevii* and a *S. bayanus* x *S. cerevisiae*, cannot be totally discarded.

After hybridization, the hybrid genome suffers random genomic rearrangements mediated by crossing-over between homeologous chromosomes (Belloch *et al.* 2009). If these rearrangements were randomly fixed, hybrids with a higher number of rearrangements should derive from older hybridization events, and hybrids with no rearrangements should be very

recent. However, double hybrids showed a trend to maintain the *S. cerevisiae* genome and to reduce the *S. kudriavzevii* that can only be explained by selection acting under the strong restrictive conditions prevailing during fermentation (nutrient depletion, osmotic stress, fermenting temperature, increasing levels of ethanol, etc.). The better adaptation of *S. cerevisiae* to these prevailing conditions constrains the loss of the *S. cerevisiae* fraction of the hybrid genome, and only the *S. kudriavzevii* genome fraction of selective importance for the hybrid (e.g. involved in adaptation to low fermentation temperatures) would be maintained. The fact that hybrids with a *S. kudriavzevii* mitochondrial genome maintain a larger fraction of the *S.kudriavzevii* genome than hybrids with a *S. cerevisiae* mitochondrial DNA, such as AMH and IF6, is also indicative that the inheritance of a *S. kudriavzevii* mitochondrial genome constrains to maintain those *S. kudriavzevii* genes involved in the proper function and maintenance of the mitochondria. Incompatibility between nuclear and mitochondrial genes has been reported for artificial *S. cerevisiae* x *S. bayanus* hybrids (Lee *et al.* 2008). Accordingly, strains possessing *S. cerevisiae*-inherited mitochondria overcome this restriction and may lose these *S. kudriavzevii* mitochondrial-related genes from their nuclear genome.

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Figure legends

Figure 1. Phylogenetic tree obtained with partial sequences of the nuclear *MET6* gene from hybrid strains and reference strains of *Saccharomyces*. The new hybrids are indicated in bold gray characters. Hybrid strains contain one, two or three different *MET6* alleles named C (*S. cerevisiae*), B (*S. bayanus var. uvarum*) or K (*S. kudriavzevii*) according to the closest parental relative. Numbers at the nodes correspond to bootstrap values based on 1000 pseudo-replicates. The scale is given in nucleotide substitutions per site.

Figure 2. RFLPs analysis of 35 nuclear genes from double hybrids. Each square corresponds to a copy of each gene region according to its chromosome location, indicated on the left map. Alleles of *S. cerevisiae* are indicated as white squares and *S. kudriavzevii* alleles are represented as black squares.

Figure 3. RFLPs of 35 nuclear genes from triple hybrids. Each square corresponds to a copy of each gene region according to its chromosome location, indicated on the left map. Alleles of *S. cerevisiae* are indicated as white squares, *S. kudriavzevii* alleles are represented as black squares and *S. bayanus* alleles are depicted in grey squares. Squares filled with two colors indicate that the presence of any of these alleles is possible. Gene orders are the same for *S. cerevisiae* and *S. kudriavzevii* because their genomes are colineal, however, gene orders differ for *S. bayanus* var. *uvarum* because this species exhibits a series of reciprocal translocations as depicted.

Figure 4. Phylogenetic Neighbor-net network obtained with partial sequences of the mitochondrial *COX2* gene from hybrid strains and reference *Saccharomyces* strains. The new hybrids are indicated in bold gray characters. The different *COX2* sequence haplotypes are named by the initial of the species name of the closest parental (C, for *S. cerevisiae*; and K, for *S. kudriavzevii*) followed by a number, according to González *et al.* (2008). The new *COX2* haplotypes described in the present study are indicated in italics. Strains sharing the same haplotype are given at the left.

Table 1. List of strains used in this study. Double hybrids correspond to *S. cerevisiae* x *S. kudriavzevii* hybrids and triple hybrids to *S. bayanus* x *S. cerevisiae* x *S. kudriavzevii* hybrids. Accession numbers of new gene sequences are indicated.

Strain type	Strain reference	Isolation source	*COX2*	*MET6*-C	*MET6*-K
Double hybrids	AMH	Commercial strain, Pinot noir wine, Assmanshausen, Germany	HQ414035	HQ414054	
	HA1835	Weißer Burgunder (Pinot blanc) grapes, Perchtoldsdorf, Austria	HQ414039	HQ414049	HQ414059
	HA1837	Weißer Burgunder grapes, Perchtoldsdorf, Austria	HQ414040	HQ414050	HQ414060
	HA1841	Weißer Burgunder grapes, Perchtoldsdorf, Austria	HQ414041	HQ414051	
	HA1842	Weißer Burgunder grapes, Perchtoldsdorf, Austria	HQ414042	HQ414052	HQ414061
	IF6	Brewer's yeast dietary supplement, Barcelona, Spain	HQ414034	HQ414057	HQ414072
	MR25	Human respiratory tract isolate, Barcelona, Spain	HQ414033	HQ414058	HQ414065
	PB7	Pietro Picudo wine, Los Oteros Winery, León, Spain	HQ414036	HQ414056	HQ414064
	SOY3	Graševina (Welschriesling) must fermentation, Daruvar, Croatia	HQ414032	HQ414055	HQ414063
	VIN7	Commercial strain of unknown origin, Anchor, South Africa	HQ414031	HQ414053	HQ414062
Triple hybrids	CBS 2834	Wine, Wädenswil, Switzerland			
	CID1	Home-made cider, Brittany, France			
S. kudriavzevii	ZP542	Oak bark, Adagoi, Portugal	HQ414038		
	ZP591	Oak bark, Castelo de Vide, Portugal	HQ414037		

Table 2. Comparison of *COX2* haplotype sequences from hybrid and type and reference strains of *Saccharomyces* species. A dot indicates nucleotides identical to that from the type strain of *S. cerevisiae* CECT 1942^{T}. *COX2* regions in hybrids that exhibit a higher similarity to *S. cerevisiae*, *S. kudriavzevii* and *S. paradoxus COX2* sequences are indicated in squared white, black and grey backgrounds, respectively.

Species	Strains	*COX2* haplotype	*COX2* variable nucleotide positions (in vertical)
			1111111123333333444444455555555555555555555555555
			4467902333557501279992335688001111112222223333444556
			0643424046475343801474037347280147890345692589014036
S. cerevisiae	CECT1942^{T}	C1	ATTAATTTATTTTATATTCTATTATTTTACTCTAGCATTCTGGTGACATATGGC
Hybrids	AMH	C2	.....G.....
	IF6	C3	.....A.....CA...AAA.ACT.ATC.AT
	CID1	K5	TACT..CAGACA.TC.A.T..CC.....T....CA.C..A..C...TACCAAT
	PB7	K10	TACT..CAGACA.TC.A.T..CC.....T....CA.C..A..C.CTGACCAAT
	MR25 & brewing	K6	TACT..CAGACA.T...T..CC.....T...GCA.C..A..C.CTGACCAAT
	Swiss & 11003-4	K2	TACT..CAGACA.T...A.AT.CTAAAAG.AT.T..TCAG.A..T.....A.
	W46	K3	TACT..CAGACA.T...A.AT.CTAAAAG.AT.T..TCAG.A..T....C..A.
	HAs, SOY3, VIN7	K4	TACT..CAGACA.T...A.AT.CTAAAAG.AT.T..TCAG.A..A.....A.
S. kudriavzevii	IFO1802^{T}	K1	TACT..CAGACA.TC..A.AT.CT.AAAG.ATCT..TCAG.....A.
	ZP542	K8	TACT..CAGACA.T...A.AT.CTAAAAG.AT.T..CCAG.A..A.....A.
	ZP591	K9	TACT.CCAGA.A.T...A.AT.CTAAAA..AT.T..TCAG.A..A.....A.
S. paradoxus	CECT1939^{NT}	K2	.............C.A.T..CC.....T...G...CAG.A.C.CTGACCAAT
S. mikatae	IFO1815^{T}	K2	....G.........TA.T...C...CA.T...G...CAG...CACTGACC.AT

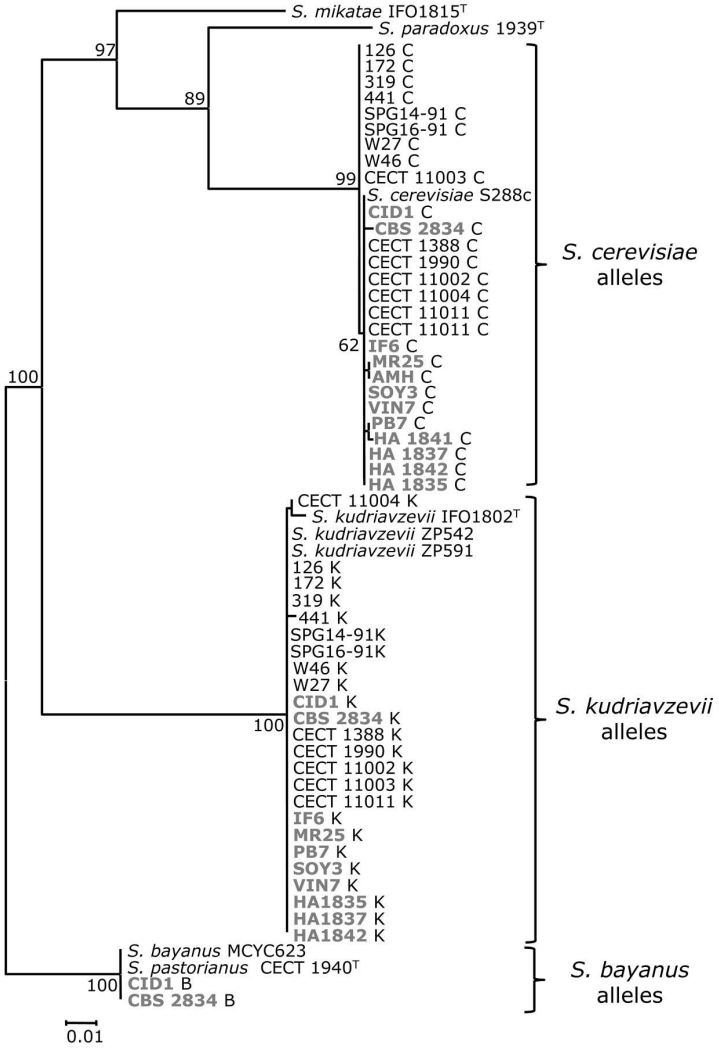

Figure 1. Phylogenetic tree obtained with partial sequences of the nuclear *MET6* gene from hybrid strains and reference strains of *Saccharomyces*. The new hybrids are indicated in bold gray characters. Hybrid strains contain one, two or three different *MET6* alleles named C (*S. cerevisiae*), B (*S. bayanus* var. *uvarum*) or K (*S. kudriavzevii*) according to the closest parental relative. Numbers at the nodes correspond to bootstrap values based on 1000 pseudo-replicates. The scale is given in nucleotide substitutions per site.
119x175mm (600 x 600 DPI)

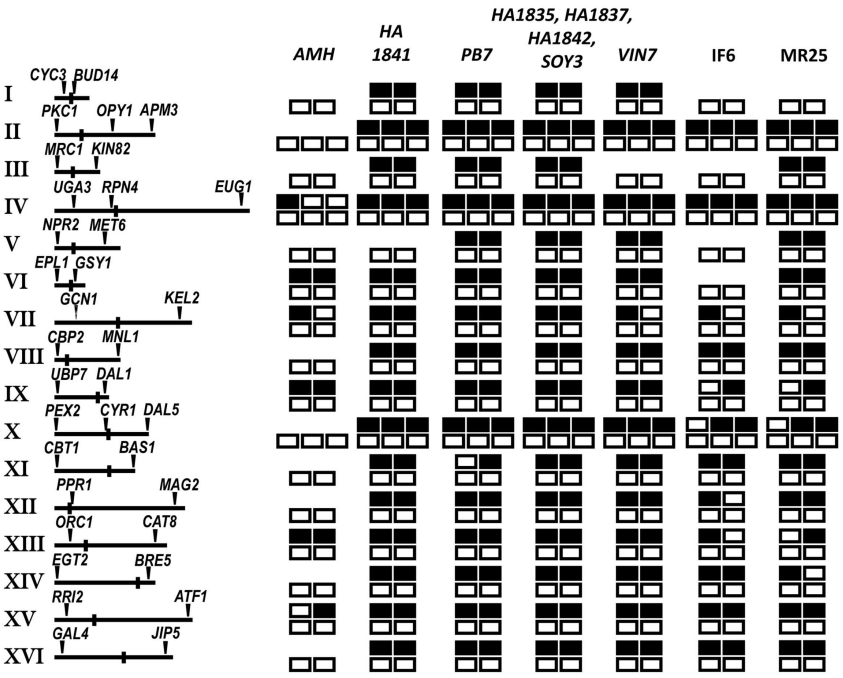

Figure 2. RFLPs analysis of 35 nuclear genes from double hybrids. Each square corresponds to a copy of each gene region according to its chromosome location, indicated on the left map. Alleles of *S. cerevisiae* are indicated as white squares and *S. kudriavzevii* alleles are represented as black squares.
115x86mm (600 x 600 DPI)

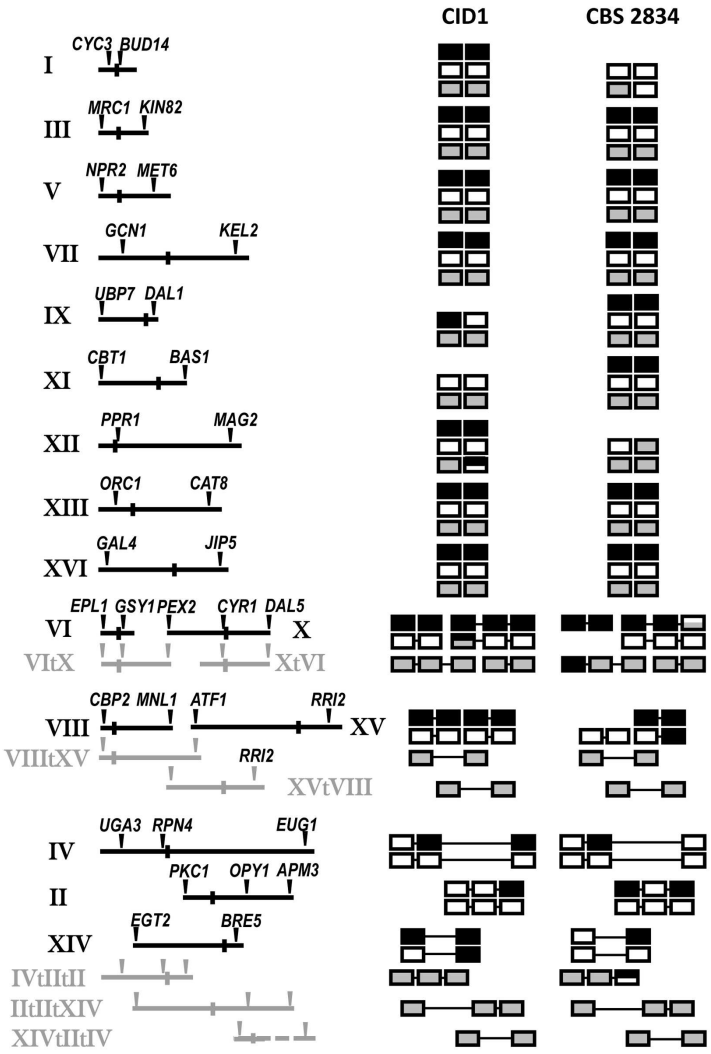

Figure 3. RFLPs of 35 nuclear genes from triple hybrids. Each square corresponds to a copy of each gene region according to its chromosome location, indicated on the left map. Alleles of *S. cerevisiae* are indicated as white squares, *S. kudriavzevii* alleles are represented as black squares and *S. bayanus* var. *uvarum* alleles are depicted in grey squares. Squares filled with two colors indicate that the presence of any of these alleles is possible. Gene orders are the same for *S. cerevisiae* and *S. kudriavzevii* because their genomes are colineal, however, gene orders differ for *S. bayanus* var. *uvarum* because this species exhibits a series of reciprocal translocations as depicted.
160x232mm (600 x 600 DPI)

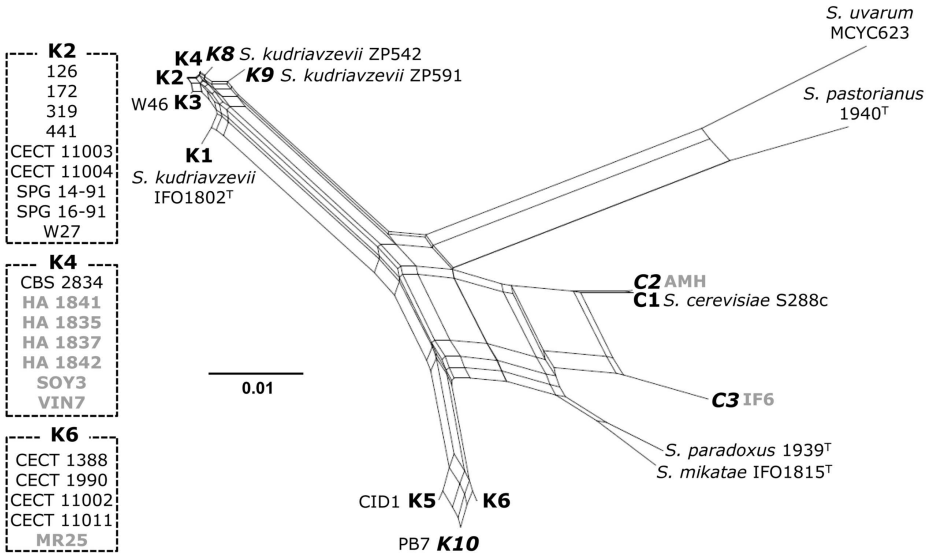

Figure 4. Phylogenetic Neighbor-net network obtained with partial sequences of the mitochondrial *COX2* gene from hybrid strains and reference Saccharomyces strains. The new hybrids are indicated in bold gray characters. The different *COX2* sequence haplotypes are named by the initial of the species name of the closest parental (C, for *S. cerevisiae*; and K, for *S. kudriavzevii*) followed by a number, according to González *et al.* (2008). The new *COX2* haplotypes described in the present study are indicated in italics. Strains sharing the same haplotype are given at the left.
115x86mm (600 x 600 DPI)

Table S1. Conformation of the *S. cerevisiae x S. kudriavzevii* hybrids for each gene region according to the composite restriction patterns exhibited. For a description of the composite restriction patterns, see Gonzalez *et al.* (2008); Lopes *et al. (*2010) and Table S2. C: *S. cerevisiae* alleles, K: *S. kudriavzevii* alleles, B: *S. bayanus* var*. uvarum* alleles

Chromosome	Gene	Doble hybrids										Triple hybrids	
		PB7	1835	1842	1837	1841	SOY3	AMH	Vin7	IF6	MR25	CBS 2834	CID1
I	***CYC3***	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1	C1	C1B1	C1B1K1
	BUD14	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1	C1	C1	C1B1K1
II	***PKC1***	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1K1	C1K1	C1K1	C1B1
	OPY1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1K1	C1K1	C1B1	C1B1
	APM3	C1K2	C1K2	C1K2	C1K2	C1K2	C1K2	C1	C1K2	C1K2	C1K1	C1B1K2	C1B1K1
III	***MRC1***	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1	C1	C1K1	C1B1K1	C1B1K1
	KIN82	C1K1	C1K1	C1K1	C1K1	C1K1	C1K2	C1	C1	C1	C1K1	C1B1K1	C1B1K1
IV	***UGA3***	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1B1	C1B1
	RPN4	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1K1	C1K1	C1B1K1	C1B1K1
	EUG1	C1K2	C1K2	C1K2	C1K2	C1K2	C1K2	C1	C1K2	C1K2	C1K2	C1B1	C1B1K1
V	***NPR2***	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1	C1K1	C1	C1K1	C1B1K1	C1B1K1
	MET6	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1	C1K1	C1	C1K1	C1B1K1	C1B1K1
VI	***EPL1***	C1K1	C1K1	C1K1	C1K1	C1K1	C1K2	C1K1	C1K1	C1	C1K1	K1	C1B1K1
	GSY1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	B1K1	C1B1K1
VII	***GCN1***	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1B1K1	C1B1K1
	KEL2	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1	C1	C1	C1B1K1	C1B1K1
VIII	***CBP2***	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1K1	C1K1	C1B1	C1B1K1
	MNL1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1K1	C1K1	C1B1	C1B1K1
IX	***UBP7***	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1	C1B1K1	B1K1

	DAL1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1B1K1	C1B1
X	***PEX2***	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1	C2	C1B1K1	B2K1
	CYR1	C1K2	C1K2	C1K2	C1K2	C1K2	C1K2	C1	C1K2	C1K1	C1K1	C1B1K2	C1B1K2
	DAL5	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1K1	C1K1	C1B1	C1B1K1
XI	***CBT1***	C1	C1K2	C1K2	C1K2	C1K2	C1K2	C1	C1K2	C1K2	C1K2	C1B1K2	C1B1
	BAS1	C1K2	C1K2	C1K2	C1K2	C1K2	C1K2	C1	C1K2	C2K3	CK2	C1B1K2	C1B1
XII	***PPR1***	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1K1	C1K1	C1B1	C1B1K1
	MAG2	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1	C1K1	B1	C1K1
XIII	***ORC1***	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1B1K1	C1B1K1
	CAT8	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1B1K1	C1B1K1
XIV	***EGT2***	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1K1	C1K1	C1B1	C1B1K1
	BRE5	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1K1	C1	C1B1K1	B2K1
XV	***RRI2***	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1K1	C1K1	C1B1K1	C1B1K1
	ATF1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	B1K1	C1B1K1
XVI	***GAL4***	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1K1	C1K1	C1B1K1	C1B1K1
	JIP5	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1K1	C1K1	C1B1K1	C1B1K1

Table S2. New restriction patterns found in *S. cerevisiae* x *S. kudriavzevii* hybrids deriving from those described in González *et al*. (2008) and Lopes *et al*. (2010).

Chromosome	Gene	Restriction enzyme	New patterns	
X	***PEX2***	*Hae* III	330 210 120 40	B2
XI	***BAS1***	*Hae* III	690 380	B2
XI	***BAS1***	*Hae* III	485 485 110	C2
XI	***BAS1***	*Hae* III	700 180 140 110	K4

Reference List

González SS, Barrio E, Querol A. 2008. Molecular characterization of new natural hybrids between *Saccharomyces cerevisiae* and *Saccharomyces kudriavzevii* from brewing. *Appl Environ Microbiol* **74:** 2314-2320.

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