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x *S. kudriavzevii* hybrid yeasts unveils a high genetic
diversity**

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The molecular characterization of new types of *S. cerevisiae* x *S. kudriavzevii* hybrid yeasts unveils a high genetic diversity.

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Running Title: A high genetic diversity among *S. cerevisiae* x *S. kudriavzevii* hybrids

Keywords: *Saccharomyces* hybrids, *S. cerevisiae*, *S. kudriavzevii*, wine, dietary, clinical yeasts.

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.

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55 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

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104 The natural yeast hybrids *S. cerevisiae* x *S. kudriavzevii* used in this
105 study were originally isolated from different sources and locations as described
106 in Table 1. Yeast strains were grown at 28°C in GPY medium (2% glucose,
107 0.5% peptone, 0.5% yeast extract).

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109 *PCR amplification and restriction analysis of 35 nuclear gene regions*

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

120
121 *Amplification, sequencing and phylogenetic analysis of COX2 and MET6 genes*

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

128 Applied Biosystems automatic DNA sequencer Model ABI 3730I (Life
129 Technologies Corporation, Carlsbad, California).

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

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

140 Each set of homologous sequences was aligned in MEGA 4 (Tamura *et*
141 *al.* 2007). The sequence evolution model that fits our sequence data best was
142 optimized using the corrected Akaike Information Criterion (AICc) with a BioNJ
143 tree as the initial tree, implemented in jModelTest program (Posada 2008). The
144 best fitting model of evolution for *MET6* sequences was TIM1 model (Posada
145 2003) with a gamma distribution (G) of substitution rates with a shape
146 parameter $\alpha = 0.35$; and for *COX2* gene sequences the TVM model (Posada
147 2003) with a gamma distribution (G) of substitution rates with a shape
148 parameter $\alpha = 0.123$ and 46.2% of invariable sites (I). The parameters of each
149 model, estimated in the previous analysis, were used to obtain the best trees
150 under optimality criterion of maximum-likelihood (ML). Tree reliability was
151 assessed using non-parametric bootstrap re-sampling of 1000 pseudo-

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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 substitutions 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: *HaeIII*, *S. cerevisiae* 462 + 302 + 144 + 114 bp, and *S. kudriavzevii* 450 + 366 + 206 bp; *MspI*, *S. cerevisiae* 514 + 508 bp, and *S. kudriavzevii* 1022 bp; and *CfoI*, *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 colinear (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

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

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

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

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

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).

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).

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250 respectively being the first description of *S. cerevisiae* x *S. kudriavzevii* hybrids
251 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).

259 The clinical isolate MR25 exhibits the same haplotype K6 described in brewing
260 hybrids, which is related to haplotype K10 present in the wine hybrid PB7 (6
261 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).

269 In the case of reticulate evolution due to recombination, a better
270 representation of the phylogenetic relationships is obtained by a Neighbor-net
271 network analysis (Figure 4). Most wine hybrids (except PB7 and AMH) and two
272 Trappist beer hybrids (CECT 11003 and 11004) inherited their mitochondrial
273 genomes (haplotypes K2, K3 and K4) from *S. kudriavzevii*, AMH and IF6
274 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).

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10 302 independent losses in the three fractions of their hybrid genomes. Moreover, the
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12 303 wine triple hybrid inherited a *S. kudriavzevii* mitochondrial genome similar to
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14 304 that present in wine double hybrids, whilst the cider hybrid contains a
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16 305 mitochondrial *COX2* closely related to that present in most brewing, the clinical
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18 306 and a wine hybrid with similarities intermediate between *S. cerevisiae* and *S.*
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21 307 *kudriavzevii*.

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25 309 **Discussion**
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28 310 *New strains expanding the distribution range of Saccharomyces kudriavzevii*
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30 311 *hybrids*

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33 312 It is more than a decade since an unusual *S. bayanus* x *S. cerevisiae*
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35 313 hybrid, CID1, isolated from home-made Breton cider, was identified as bearing
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37 314 a mitochondrial genome coming from *S. kudriavzevii* (Masneuf *et al.* 1998;
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39 315 Groth *et al.* 1999). Later, a *S. kudriavzevii* contribution to a fraction of the
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41 316 chimerical nuclear genome of this strain was demonstrated (Naumova *et al.*
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43 317 2005; González *et al.* 2006).

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45 318 Some years later, a new type of natural hybrid strains between *S.*
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47 319 *cerevisiae* x *S. kudriavzevii* was described in wine fermentations (Bradbury *et*
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49 320 *al.*, 2006; González *et al.*, 2006; Lopandić *et al.*, 2007). and brewing
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51 321 environments (González *et al.*, 2008).

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53 322 In the present study, new *S. cerevisiae* x *S. kudriavzevii* hybrid yeasts are
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55 323 described and molecularly characterized. These hybrids contribute to expand
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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

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5 350 regions still allow *S. cerevisiae* to outcompete these hybrids.
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8 351 The present study also describes for the first time *S. cerevisiae* x *S.*
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10 352 *kudriavzevii* hybrids isolated from non-fermentative environments. Strain MR25
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12 353 is a human respiratory isolate from 'Hospital del Vall d'Hebron', Barcelona,
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14 354 Spain; and IF6, is commercialized as a dietary supplement. These hybrids are
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16 355 quite different at the genome level, particularly in their mitochondrial genomes.
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18 356 The clinical isolate MR25 shares a *COX2* sequence identical to that present in 4
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20 357 brewing hybrids, indicating that beer could likely be the source of infection, and
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22 358 the dietary supplement IF6 exhibits a *S. cerevisiae* mitochondrial DNA.
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29 360 *The high genetic diversity among Saccharomyces kudriavzevii hybrids suggests*
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31 361 *independent hybridization origins*
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34 362 The analysis of the nuclear and mitochondrial genome compositions of *S.*
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36 363 *kudriavzevii* double and triple hybrids unveiled a high diversity, which likely is
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38 364 indicative of independent primary, as well as secondary, hybridization events.
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41 365 The fact that hybrids inherited 3 types of mitochondrial genomes (*S.*
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43 366 *cerevisiae*-like, *S. kudriavzevii*-like and recombinant) from their parental
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45 367 ancestors is indicative of at least 3 different origins. Moreover, the important
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47 368 differences in their nuclear genome compositions could also be taken as
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49 369 evidences of independent primary hybridization events.
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53 370 The presence of recombinant mitochondrial genomes in hybrids can be
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55 371 explained by recombination events occurring after the fusion of mitochondria
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57 372 observed in conjugating *Saccharomyces* spores or cells. This kind of
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59 373 recombination events were already described in *S. cerevisiae* at the within-
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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

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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 colinear, however, gene orders differ for *S. bayanus* var. *uvorum* 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			
<i>S. kudriavzevii</i>	ZP542	Oak bark, Adagoi, Portugal	HQ414038		
	ZP591	Oak bark, Castelo de Vide, Portugal	HQ414037

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 colinear, 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.

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. *uvvarum* alleles

Chromo some	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
	MRC1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1	C1	C1K1	C1B1K1	C1B1K1
III	KIN82	C1K1	C1K1	C1K1	C1K1	C1K1	C1K2	C1	C1	C1	C1K1	C1B1K1	C1B1K1
	UGA3	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1B1	C1B1
IV	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
	NPR2	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1	C1K1	C1	C1K1	C1B1K1	C1B1K1
V	MET6	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1	C1K1	C1	C1K1	C1B1K1	C1B1K1
	EPL1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K2	C1K1	C1K1	C1	C1K1	K1	C1B1K1
VI	GSY1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	B1K1	C1B1K1
	GCN1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1B1K1	C1B1K1
VII	KEL2	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1	C1	C1	C1B1K1	C1B1K1
	CBP2	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1K1	C1K1	C1B1	C1B1K1
VIII	MNL1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1K1	C1K1	C1K1	C1B1	C1B1K1
	UBP7	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1K1	C1	C1	C1B1K1	B1K1
IX													

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

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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	<i>PEX2</i>	<i>Hae</i> III	330	210	120	40	B2
XI	<i>BAS1</i>	<i>Hae</i> III	690	380			B2
XI	<i>BAS1</i>	<i>Hae</i> III	485	485	110		C2
XI	<i>BAS1</i>	<i>Hae</i> III	700	180	140	110	K4

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