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Molecular Characterization of New Natural Hybrids of *Saccharomyces cerevisiae* and *S. kudriavzevii* in Brewing▽†

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We analyzed 24 beer strains from different origins by using PCR-restriction fragment length polymorphism analysis of different gene regions, and six new *Saccharomyces cerevisiae* × *Saccharomyces kudriavzevii* hybrid strains were found. This is the first time that the presence in brewing of this new type of hybrid has been demonstrated. From the comparative molecular analysis of these natural hybrids with respect to those described in wines, it can be concluded that these originated from at least two hybridization events and that some brewing hybrids share a common origin with wine hybrids. Finally, a reduction of the *S. kudriavzevii* fraction of the hybrid genomes was observed, but this reduction was found to vary among hybrids regardless of the source of isolation. The fact that 25% of the strains analyzed were discovered to be *S. cerevisiae* × *S. kudriavzevii* hybrids suggests that an important fraction of brewing strains classified as *S. cerevisiae* may correspond to hybrids, contributing to the complexity of *Saccharomyces* diversity in brewing environments. The present study raises new questions about the prevalence of these new hybrids in brewing as well as their contribution to the properties of the final product.

Beer has one of the highest rates of production and consumption among alcoholic beverages worldwide. Since its development in the Middle East during the Neolithic period (9, 24), brewing has been a traditional procedure based either on the spontaneous fermentation of wort or on its inoculation with spent yeast of a preceding fermentation.

Although a vast diversity of beers exists, most of beers can be classified into two major types, ale and lager, according to the yeasts involved and the fermentation conditions. On one hand, ales are produced by "top-fermenting" (ale) yeasts during fermentations at 20 to 25°C, followed in some cases by a short period of aging. On the other hand, lager beers are produced by "bottom-fermenting" (lager) yeasts at temperatures of 4 to 15°C and then subjected to a long, low-temperature period of maturation (lagering) (19). Historically, ale brewing developed first and was maintained and improved in Great Britain and Belgium whereas in Bohemia and Germany, ale brewing developed into lager brewing (15).

Lager and ale yeast exhibit different physiological traits, indicating that they belong to different species. Ale brewing strains constitute a broad variety of *Saccharomyces* strains, most of which seem to be closely related to *Saccharomyces cerevisiae*. In contrast, lager brewing yeasts are the best described examples of natural hybrid yeasts. These yeasts are partial allotetraploids coming from a hybridization event between *S. cerevisiae* and an *S. bayanus*-related yeast (5, 26, 33). Chromosome sets from both parental species are present in lager strains (2, 19).

Other *Saccharomyces* interspecific hybrids have been detected in different fermentation processes such as those involving wine and cider (14, 23, 25). Also, the type strain of *S. bayanus*, originally isolated from beer, has recently been suggested to be a hybrid between *S. cerevisiae* and *S. bayanus* due to the presence of subtelomeric repeated sequences and genes (10, 26, 27). However, the presence of certain introgressive subtelomeric sequences is not necessarily indicative of a hybrid genome (21, 25). This was corroborated by Rainieri et al. (33), who also demonstrated that the present *S. bayanus* taxon is a heterogeneous complex of two pure and one mixed (hybrid) genetic lines. These three lines contributed along with *S. cerevisiae* to the formation of different hybrids, including lager yeasts.

In a recent study (11), we described and characterized new hybrids of *S. cerevisiae* × *S. kudriavzevii* (including a triple hybrid of *S. bayanus* × *S. cerevisiae* × *S. kudriavzevii*) isolated from Swiss wine fermentations. New putative *S. cerevisiae* × *S. kudriavzevii* hybrids have been described among wine yeast isolated in Austria (22). Due to the fact that these hybrids are predominant in wine fermentations from several oceanic and continental climate regions of Europe and that they were originally misidentified as *S. cerevisiae* (35), we decided to study the possible incidence of *S. kudriavzevii* hybrids in brewing, the most common fermentation process in these European regions. Using a methodology described elsewhere (11), we analyzed beer strains from different origins deposited in the Spanish Type Culture Collection (Colección Española de Cultivos Tipo [CECT]) Valencia, Spain, and six new *S. cerevisiae* × *S. kudriavzevii* hybrid strains were found.

In the present study, we also compare the genomic diversity between beer and wine *S. kudriavzevii* × *S. cerevisiae* hybrids by

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TABLE 1. List of *Saccharomyces* strains from CECT analyzed in the present study[a]

CECT strain	Alternate name[b]	Original epithet	Isolation source	Country of origin	New characterization
1384	CBS 1636	Type of *S. diastaticus*	Brewer's wort	Ireland	*S. cerevisiae*
1387	CBS 7372	*S. cerevisiae*	Draft beer	United Kingdom	*S. cerevisiae*
1388	**NCYC 447**	*S. cerevisiae*	Draft beer	United Kingdom	***S. cerevisiae-S. kudriavzevii***
1462	NCYC 963	*S. cerevisiae*	Beer	United Kingdom	*S. cerevisiae*
1463	NCYC 102	*S. cerevisiae*	Beer	United Kingdom	*S. cerevisiae*
1971	CBS 1395	*S. ellipsoideus*	Beer	Unknown	*S. cerevisiae*
1990	**DSMZ 1848**	*S. cerevisiae*	Göttinger Brauhaus AG beer	Germany	***S. cerevisiae-S. kudriavzevii***
1991	DSMZ 70411	*S. bayanus*	Turbid bottled beer	Germany	*S. bayanus*
1995	NCYC 1001	*S. cerevisiae*	Ale beer	United Kingdom	*S. cerevisiae*
1996	NCYC 1296	*S. pastorianus*	Lager beer	France	*S. bayanus-S. cerevisiae*
1997	NCYC 1305	*S. pastorianus*	Lager beer	United Kingdom	*S. bayanus-S. cerevisiae*
1998	NCYC 1322	*S. cerevisiae*	Lager beer	Ireland	*S. bayanus-S. cerevisiae*
1999	NCYC 1323	*S. cerevisiae*	Lager beer	Australia	*S. bayanus-S. cerevisiae*
11000	MUCL 20463	*S. cerevisiae*	Beer	Unknown	*S. bayanus-S. cerevisiae*
11001	MUCL 20478	*S. cerevisiae*	Lager beer	Belgium	*S. cerevisiae*
11002	**MUCL 20488**	*S. cerevisiae*	Chimay Trappist beer	Belgium	***S. cerevisiae-S. kudriavzevii***
11003	**MUCL 20489**	*S. cerevisiae*	Orval Trappist beer	Belgium	***S. cerevisiae-S. kudriavzevii***
11004	**MUCL 20490**	*S. cerevisiae*	Westmalle Trappist beer	Belgium	***S. cerevisiae-S. kudriavzevii***
11008	NCYC 1025	*S. cerevisiae*	Ale beer	United Kingdom	*S: cerevisiae*
11009	NCYC 1140	*S. cerevisiae*	Stout beer	United Kingdom	*S. cerevisiae*
11010	NCYC 1309	*S. cerevisiae*	Ale beer	United Kingdom	*S. cerevisiae*
11011	**NCYC 1379**	*S. cerevisiae*	Wild yeast from brewery	New Zealand	***S. cerevisiae-S. kudriavzevii***
11035	CBS 380	Type of *S. bayanus*	Turbid beer	Denmark	*S. bayanus*
11036	CBS 381	Type of *S. willianus*	Spoiled beer	Japan	*S. bayanus*

[a] The new *S. cerevisiae* × *S. kudriavzevii* hybrids found in beer are shown in bold.
[b] Culture collections are abbreviated as follows: CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; MUCL, Mycotheque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; NCYC, National Collection of Yeast Cultures, Norwich, United Kingdom.

using PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of 35 nuclear gene regions located in the 16 *Saccharomyces* chromosomes and one mitochondrial gene to decipher the origin and evolution of these new natural hybrids.

MATERIALS AND METHODS

Yeasts strains and media. The yeast strains used in this study correspond to all brewing *Saccharomyces* yeasts deposited in the CECT database. Their references, original identifications, sources of isolation, and geographical origins are listed in Table 1. Strains were grown on YPD (1% of yeast extract, 2% peptone, 2% glucose) medium at 28°C and maintained on YPD medium supplemented with 2% agar.

PCR amplification. The characterization of hybrid *Saccharomyces* interspecific hybrids was performed by PCR amplification and subsequent RFLP analysis of 35 protein-encoding genes (see Table S1 in the supplemental material). As shown in Fig. 2, 32 gene regions are located more or less close to each end of the 16 *S. cerevisiae* chromosomes, and the other three genes are in central positions of chromosomes II, IV, and X. Oligonucleotide primers designed for the symmetrical amplification of the protein-coding gene regions are listed in Table S1 in the supplemental material. Primers were designed by comparing the available sequences of the strains of the species *S. bayanus* (NCYC 623; alternatively, CBS 7001), *S. cerevisiae* (S288C), and *S. kudriavzevii* (IFO 1802; CBS 8840) in the *Saccharomyces* Genome Database in the section of Budding Yeasts Genome Comparison (http://db.yeastgenome.org/cgi-bin/FUNGI/FungiMap). All of these primers are useful for the amplification of each gene from strains belonging to any *Saccharomyces* species, except primer *MET6*-K reverse, which is specific for *S. kudriavzevii* (11).

Yeast DNA was isolated according to standard procedures (32). PCR was performed in a mixture containing 10 μl of 10× *Taq* polymerase buffer, a 100 μM concentration of the deoxynucleotides, a 1 μM concentration of each primer, 2 units of *Taq* polymerase (BioTools; B & M Laboratories, Madrid, Spain), and 4 μl of DNA diluted to 1 to 50 ng/μl for a final volume of 100 μl.

PCR amplifications were carried out in Techgene or Touchgene thermocyclers (Techne, Cambridge, United Kingdom) as follows: initial denaturing at 95°C for 5 min and then 40 PCR cycles of the following program: denaturing at 95°C for 1 min, annealing at 56°C (for most genes), and extension at 72°C for 2 min, with a final extension at 72°C for 10 min. In the case of the genes *ATF1*, *DAL1*, *DAL5*, *EGT2*, *KIN82*, *MNT2*, *MRC1*, *RRI2*, and *UBP7*, annealing was performed at 50°C.

PCR products were run on 1.4% agarose (Pronadisa; Laboratorios Conda S.A., Madrid, Spain) gels in 0.5× TBE (Tris-borate-EDTA) buffer. After electrophoresis, gels were stained with a dilution of 0.5 μg/ml ethidium bromide (AppliChem, Darmstadt, Germany) and visualized under UV light. A 100-bp DNA ladder marker (Roche Molecular Biochemicals, Mannheim, Germany) served as a size standard.

Restriction analysis of nuclear gene regions. Simple digestions with one or two endonucleases were performed with 15 μl of amplified DNA to a final volume of 20 μl. Restriction endonucleases AccI, CfoI, EcoRI, HaeIII, HinfI, MspI, PstI, RsaI, and ScrFI (Roche Molecular Biochemicals) were used according to the supplier's instructions. Restriction fragments were separated on 3% agarose (Pronadisa) gel in 0.5× TBE buffer. A combination of 50-bp and 100-bp DNA ladder markers (Roche Molecular Biochemicals) served as size standards. Restriction endonucleases were selected to yield species-specific patterns to differentiate the gene copies in the hybrids coming from each parent species.

Mitochondrial *COX2* gene sequencing and phylogenetic analysis. The mitochondrial *COX2* genes from the strains identified as hybrids were sequenced to determine the parental donor of the mitochondrial genome. The *COX2* gene was amplified and sequenced as previously described (11). Additional *COX2* sequences from wine hybrids and reference strains (11) were also included in the analysis. These *COX2* sequences were aligned with MEGA3 (20).

The best tree was obtained under the optimality criterion of maximum parsimony (MP) by an exhaustive search among all possible trees. Tree reliability was assessed using nonparametric bootstrap resampling of 2,000 replicates. These phylogenetic analyses were performed using PAUP*, version 4.0b10 (36). Other phylogeny reconstruction methods, such as maximum-likelihood or distance-based procedures, gave very similar phylogenetic trees.

Pulsed-field gel electrophoresis. DNA for electrophoretic karyotyping was carried out in agarose plugs (4). Chromosomal profiles were determined by the contour-clamped homogenous electric field technique with DRIII equipment (Bio-Rad Laboratories), using as standard markers the chromosomes of the *S. cerevisiae* strain

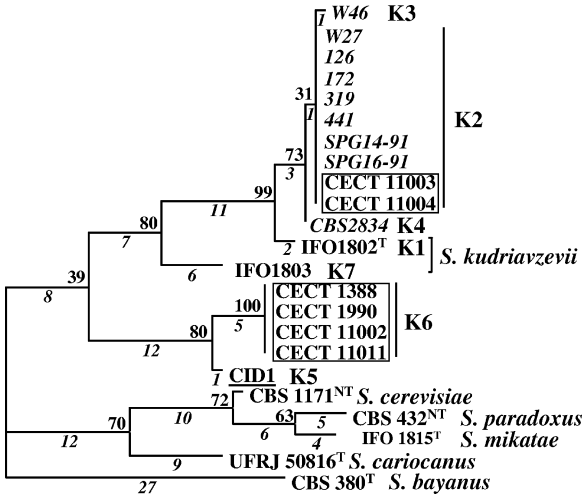

FIG. 1. MP tree that minimizes the number of nucleotide substitutions required to connect the mitochondrial *COX2* sequences from *S. cerevisiae* × *S. kudriavzevii* hybrids and type and reference strains of the *Saccharomyces* species. Brewing hybrids are included within squares, wine hybrids are indicated in italics, and the cider strain is underlined. The different *COX2* haplotypes are given in bold. Numbers in italics located under the branches correspond to branch lengths given in nucleotide substitutions. Numbers at the nodes correspond to BVs (percent) obtained from 2,000 pseudoreplicates.

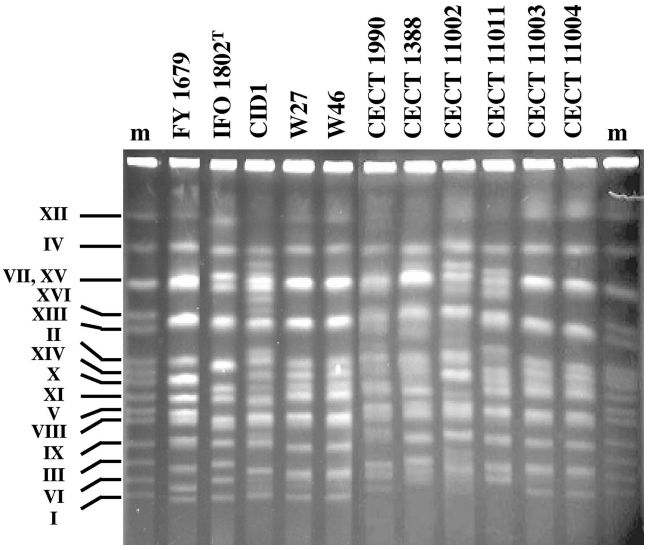

FIG. 2. Chromosomal profiles exhibited by the brewing *S. cerevisiae* × *S. kudriavzevii* hybrids under analysis. Some wine *S. cerevisiae* × *S. kudriavzevii* hybrids, W27 and W46, the triple hybrid CID1, and representatives of the parental species, *S. cerevisiae* FY 1679 and *S. kudriavzevii* IFO 1802^T, are also included. Lane m corresponds to the standard marker strain *S. cerevisiae* YNN295 (Bio-Rad); chromosomal numbers corresponding to each band are indicated on the left.

YNN295 (Bio-Rad Laboratories). Yeast chromosomes were separated on 1% agarose gels in two steps as follows: a 60-s pulse time for 14 h and then a 120-s pulse time for 10 h, both at 6 V cm^{-1} with an angle of 120°. The running buffer used was 0.5× TBE (45 mM Tris-borate, 1 mM EDTA) cooled at 14°C.

Nucleotide sequence accession numbers. Mitochondrial *COX2* sequences from the hybrids characterized in this work were deposited in the EMBL sequence database under accession numbers AJ966727 to AJ966733.

RESULTS

Identification of new *S. kudriavzevii* hybrids among brewing strains. Using a procedure based on the restriction analysis of different gene regions (19) to differentiate the species of the genus *Saccharomyces* and their hybrids, six new hybrids were identified among the beer strains from different origins deposited in the CECT.

These strains were reidentified (Table 1), and six of them, originally identified as *S. cerevisiae*, corresponded to new *S. cerevisiae* × *S. kudriavzevii* hybrids. Three strains, CECT 11002 (also called MUCL 20488), CECT 11003 (MUCL 20489), and CECT 11004 (MUCL 20490), were originally isolated from different Belgian Trappist ale beers: Chimay, Orval, and Westmalle, respectively. Strain CECT 1388 (NCYC 447) was originally isolated by J. S. Hough from a British brewery in 1955, CECT 1990 (DSMZ 1848) was isolated by O. Meyer from Göttinger Brauhaus lager beer (Germany), and CECT 11011 (NCYC 1379) corresponds to a wild yeast isolated from a New Zealand brewery. The hybrid origin of CECT 11011 was already postulated (21) on the basis of its hybridization patterns with *S. cerevisiae*-specific repetitive elements and the possession of a partial 26S RNA gene sequence identical to that of the *S. kudriavzevii* type strain.

Phylogenetic analysis of mitochondrial *COX2* sequences from *S. kudriavzevii* hybrids. The analysis of mitochondrial *COX2* gene sequences was useful for determining the parental species that contributed with their mitochondria to the hybrid strains (11). The new hybrid strains contain two *COX2* sequence types, and two of the Trappist beer strains (CECT 11003 and 11004) exhibited a *COX2* sequence identical to the one most frequently found in wine hybrid strains, type K2 according to González et al. (11). However, the remaining brewing hybrids contain a new *COX2* sequence (type K6) that is different from sequences described previously (11) but similar (only 6 nucleotides different) to type K5 present in the triple hybrid CID1, isolated from a homemade Breton cider.

To determine the phylogenetic relationships among *COX2* sequences from beer and wine hybrids and their parental species representatives, the MP tree depicted in Fig. 1 was obtained (see Materials and Methods). Other tree-making methods, such as maximum-likelihood or distance-based neighbor-joining, gave similar phylogenetic reconstructions (these are available upon request).

The MP tree shows a close relationship among the *COX2* genes from Trappist beer hybrid strains CECT 11003 and 11004 and wine hybrids with respect to the genes from the *S. kudriavzevii* type strain IFO 1802^T (bootstrap value [BV], 99%) and the reference strain IFO 1803 (BV, 80%), indicating that these hybrids strains contain a mitochondrial DNA coming from an *S. kudriavzevii* donor. The other brewing hybrids form a monophyletic group (BV, 80%) with the triple hybrid CID1, but the phylogenetic position of this group is unclear (BV, 39%), as it is located between the clusters where the parental species, *S. kudriavzevii* and *S. cerevisiae*, are included.

Comparative analysis of electrophoretic karyotypes of hybrids. Pulsed-field gel electrophoresis was performed to determine the electrophoretic karyotypes of the six new hybrid strains (Fig. 2). One strain of each parental species involved in

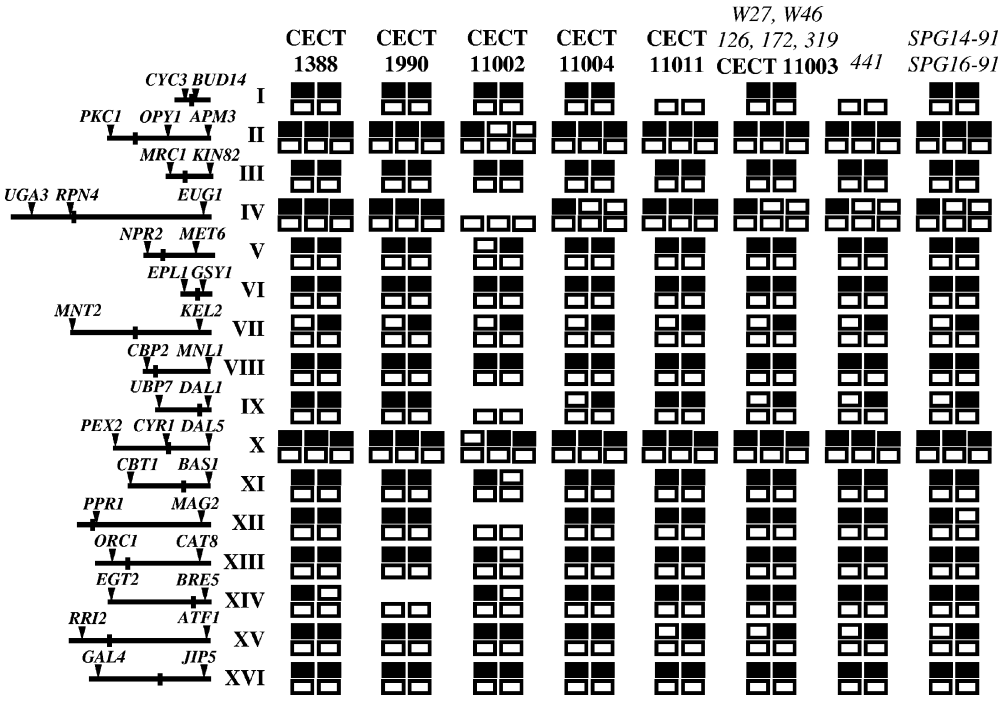

FIG. 3. Genotypes of the *S. cerevisiae* × *S. kudriavzevii* hybrids. Each square corresponds to a copy of each gene region according to its chromosome location, indicated at the left. White and black squares represent alleles of *S. cerevisiae* and *S. kudriavzevii* origin, respectively. Brewing and wine hybrids are indicated in bold and italics, respectively. The presence or absence of alleles coming from each parent species was determined by restriction analysis of the 35 gene regions amplified by PCR with general primers.

the formation of the hybrids as well as two representatives of the wine hybrid strains (*S. cerevisiae* × *S. kudriavzevii*) and the reference triple hybrid CID1 (*S. cerevisiae* × *S. bayanus* × *S. kudriavzevii*) were also included in this analysis to compare their chromosomal differences.

According to their karyotypes, brewing hybrids can be divided into the same groups that appear in the *COX2* phylogenetic analysis. The first group includes Trappist hybrids CECT 11003 and 11004, which exhibited chromosomal patterns identical to the pattern from wine hybrids. This group is characterized by their largely homogeneous karyotype, slightly more similar to the pattern exhibited by *S. cerevisiae* strains than to that of the *S. kudriavzevii* type strain because their chromosomes VII and XV form a single electrophoretic band (11).

The second group includes the other four brewing hybrids, which exhibit quite complex and heterogeneous chromosomal patterns, characterized by the presence of a larger number of bands, and also differ from each other in both the mobility and the intensity of the bands. Some of these patterns, especially those from strains CECT 11002 and 11011, show a certain resemblance to the pattern from the triple hybrid CID1 although the diagnostic bands of the translocated chromosomes from *S. bayanus* are not present.

Chromosomal composition in brewing and wine *S. cerevisiae* × *S. kudriavzevii* hybrids. To obtain better knowledge of the genetic and chromosomal structure of the *S. cerevisiae* × *S. kudriavzevii* hybrids, we have developed a method based on PCR amplification and restriction analysis of 35 gene regions. This analysis was performed with all *S. cerevisiae* × *S. kudriavzevii* hybrids isolated from beer and wine fermentations. As depicted in Fig. 3, 32 protein-coding genes are located near the ends of the 16 *S. cerevisiae* chromosomes, and three are in central positions of the large chromosomes II, IV, and X. The genome sequencing project of the *S. kudriavzevii* type strain demonstrated that the genome of this species is colineal (syntenic) with that of *S. cerevisiae*; therefore, these genes were expected to occupy similar positions in the chromosomes of the hybrid that come from the *S. kudriavzevii* parent. Genome rearrangements present in some beer hybrids, however, cannot be discarded since there are similarities in their electrophoretic karyotypes, described above.

As mentioned in Material and Methods, gene selection was performed by analyzing the available sequences from *Saccharomyces* species in the *Saccharomyces* Genome Database: *S. bayanus* MCYC 623 (CBS 7001), *S. cerevisiae* S288C, *S. kudriavzevii* IFO 1802^T (CBS 8840), *S. mikatae* IFO 1815^T (CBS 8839), and *S. paradoxus* NRRL Y-17217 (CBS 432). *Saccharomyces* general PCR primers to amplify the genes of interest were designed in conserved nucleotide stretches flanking variable regions, where the presence of variable restriction sites allows species differentiation. Restriction endonucleases yielding single or combined restriction patterns specific of species were selected for each gene region. The expected restriction patterns for the 35 PCR regions for the reference strains of the species *S. bayanus*, *S. cerevisiae*, and *S. kudriavzevii* that have been described as involved in interspecies hybridization are given in Table S2 of the supplemental material. These species-specific patterns were mostly conserved in the hybrids; however, new patterns, differing in one restriction site gain or loss,

were also found for some gene regions (see Table S3 of the supplemental material).

Figure 3 summarizes the conformation of the *S. cerevisiae* × *S. kudriavzevii* hybrid genotypes for each gene region according to the composite restriction patterns exhibited, which are given in more detail in Table S4 in the supplemental material. The hybrid strains exhibited a mixture of restriction patterns for most gene regions due to the presence of two different alleles of each region, one exhibiting the typical restriction pattern of *S. cerevisiae* and the other the same restriction pattern of *S. kudriavzevii* or a closely similar pattern. Each brewing strain exhibits a specific hybrid pattern of presence/absence of alleles, but several wine hybrids share the same genotype and have been grouped.

The presence of two alleles of different parental origin is not general for all gene regions; e.g., the *S. kudriavzevii MNT2* allele is absent in all hybrids and is also very variable depending on the strain, ranging between the beer strain CECT 1388, which contains the parental alleles for 33 genes but lacks the *S. kudriavzevii MNT2* and *BRE5* alleles, and the beer strain CECT 11002, which has lost the *S. kudriavzevii* alleles for 15 genes out of 35. In this way, for each gene hybrid strains contain the *S. cerevisiae* allele but may lack the *S. kudriavzevii* allele, indicating that in these hybrids there is a trend to maintain the *S. cerevisiae* genome but to lose part of the *S. kudriavzevii* genes; in fact, the *S. kudriavzevii* alleles of only 16 genes are present in all hybrids.

The comparison of genotypes between hybrids shows that there are certain similarities among strains. Thus, several groups of related genotypes can be obtained. Wine strain genotypes are very similar, with differences among strains of one to three genes. Strain CECT 11003, isolated from a Belgian Trappist beer, exhibits the same genotype as Swiss wine strains W27, W46, 126, 172, and 319, which differ in only one gene from the genotype exhibited by strain CECT 11004, also isolated from another Belgian Trappist beer. Something similar occurs among genotypes exhibited by beer strains CECT 1388, CECT 1990, and CECT 11011, with differences among these strains of two to three genes. Finally, the most different genotype is that from strain CECT 11002 due to the loss of many *S. kudriavzevii* alleles.

Since the *S. cerevisiae* and *S. kudriavzevii* genomes are considered to be colineal (16), the locations of the gene regions under analysis were chosen to obtain information about possible chromosomal rearrangements in the hybrid genomes. From Fig. 3, it is clear that the absence in the hybrids of *S. kudriavzevii* alleles for genes located in the same chromosome likely results from the loss of the whole chromosome from this parental species, as occurs in strains 441 and CECT 11011 for chromosome I, in beer strain CECT 1990 for chromosome XIV, and in beer strain CECT 11002 for chromosomes IV, IX, and XII.

By assuming that gross chromosomal rearrangements should be more frequent between "homoeologous" chromosomes than between different chromosomes from the same species (i.e., homologous recombination between homoeologous chromosomes is more frequent than heterologous recombination) and considering that these events are rare and can be considered irreversible, the relationships among hybrid genotypes can be deduced. In this way, Fig. 4 shows the MP diagram

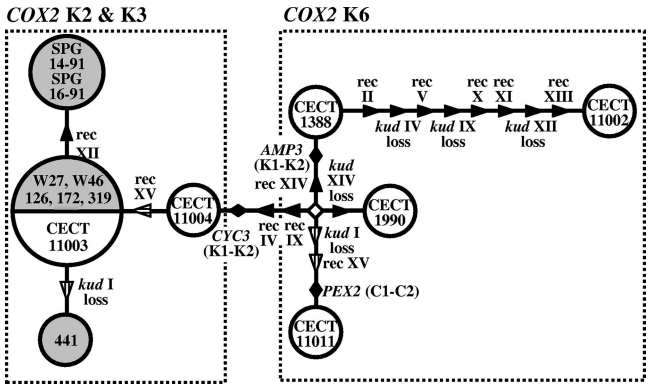

FIG. 4. Minimum number of chromosomal rearrangements and restriction site changes to connect the different genotypes exhibited by the *S. cerevisiae* × *S. kudriavzevii* hybrids (Fig. 3; see also Table S●●● in the supplemental material). Genotypes are represented by white and gray circles for wine and brewing hybrids, respectively. Rearrangements are indicated by arrows giving the direction of the irreversible change. Rearrangements were assumed to be caused by nonreciprocal recombination (rec) among homoeologous chromosomes (roman numbers) and whole chromosome losses (loss) of one of the parental chromosomes (*kud*, *S. kudriavzevii*). Restriction site changes can be reversible (gain/loss) and are represented by diamonds. The gene region and the restriction patterns involved are also indicated (for a description, see Tables S2 and S3 in the supplemental material). Dotted squares group genotypes of hybrids according to their mitochondrial *COX2* haplotypes.

indicating the minimum number of rearrangements necessary to connect the different genotypes depicted in Fig. 3. Considering segmental losses instead of recombination events would give the same number of minimum events.

As can be seen in Fig. 4, two events are convergent (*S. kudriavzevii* chromosome I loss and nonreciprocal recombination between homoeologous chromosome XV), which allows an alternative connection between genotypes 441 and CECT 11011; however, this connection is not possible due to the irreversibility of the rearrangements. Restriction pattern changes due to the gain/loss of one single restriction site were also considered in the diagram; however, these events are bidirectional (reversible). According to this diagram, wine strains and beer strains CECT 11003 and CECT 11004 are closely related; they share two recombinations at chromosomes IV and IX and the *S. kudriavzevii CYC3* K2 pattern. This close relationship is also supported by the phylogenetic analysis of the mitochondrial genome because all of the strains contain a *COX2* haplotype K2 gene or the derived K3 in the case of strain W46. The other hybrid genotypes show a higher number of differences, but these strains contain the same *COX2* haplotype K6, likely indicating a common origin.

Due to the irreversibility of the rearrangements, we can deduce the position in the diagram of a hypothetical ancestral genome with the smallest number of rearrangements. This hypothetical ancestor would contain a single rearrangement involving chromosome VII (present in all hybrids under analysis) and would be located at the white diamond. However, this hypothetical ancestor is connected to three lineages with strains having the same *COX2* haplotype K6 and one single lineage including hybrids with the *COX2* haplotype K2. Therefore, the most plausible explanation is that *S. cerevisiae* × *S.*

kudriavzevii hybrids originated from at least two hybridization events: one producing wine hybrid strains and beer strains CECT 11003 and 11004 and the other producing the remaining beer strains.

DISCUSSION

New hybrids contributing to *Saccharomyces* diversity in brewing. Lager and ale yeasts exhibit different physiological traits, indicating that they belong to different species. The lager yeast, originally assigned to the species *S. carlsbergensis*, was the first pure culture used for beer production, and most lager yeasts used today are closely related to this strain (19). Different molecular studies (19, 33) demonstrated that these strains, currently included in the *S. pastorianus* taxon, correspond to partial allotetraploids containing a rearranged hybrid genome coming from *S. cerevisiae* and other strains related to *S. bayanus*. In contrast, the less studied ale-brewing strains constitute a broad variety of *Saccharomyces* strains, most of which seem to be closely related to *S. cerevisiae*.

However, brewing yeast diversity seems to be much more complex. In a recent study, Rainieri et al. (33) evaluated the genetic variability of *S. bayanus* and *S. pastorianus* strains, most of which are brewing strains or contaminants, and could identify five types of strains. Two types correspond to "pure" lines, *S. bayanus* and *S. uvarum*, including strains with a single type of genome. The other types correspond to "hybrid" strains *S. cerevisiae*/*S. bayanus*/Lager, *S. bayanus*/*S. uvarum*/Lager, and *S. cerevisiae*/*S. bayanus*/*S. uvarum*/Lager, also containing alleles termed "Lager" that, according to these authors, correspond to an additional genome present in lager brewing strains.

The present study is a new contribution to decipher the complex diversity of *Saccharomyces*. We have clearly demonstrated the presence of the new type of hybrid, *S. cerevisiae* × *S. kudriavzevii*, in brewing. Our survey was limited to the few brewing strains deposited in the CECT and, hence, cannot be a good indication of the incidence of these hybrids in brewing. Nonetheless, the fact that 25% (6 out of 24) of the strains analyzed were determined to be *S. cerevisiae* × *S. kudriavzevii* hybrids originally misidentified as *S. cerevisiae* suggests that an important fraction of brewing strains classified as *S. cerevisiae* may correspond to hybrids that contribute to the complexity of the *Saccharomyces* diversity in brewing environments and to the properties of the beer produced.

This study confirms the presence of this new kind of *Saccharomyces* hybrid among brewing strains originally classified as *S. cerevisiae* according to conventional chemotaxonomy. An extensive reanalysis of the most important brewing yeast collections should be performed to determine the incidence of these hybrids. Moreover, some of these strains were isolated as predominant in Trappist beer bottles, where a secondary fermentation takes place, and, hence, the present study raises new questions about the prevalence of these new hybrids in brewing as well as their contribution to the properties of the final product.

Complex chromosome structure in the new hybrids. Both the intricate electrophoretic karyotypes exhibited by brewing hybrids and the molecular characterization of their genes by PCR-RFLP analysis are indicative of the presence of strain-specific gross chromosomal rearrangements in the *S. cerevisiae* × *S. kudriavzevii* hybrids.

The absence of *S. kudriavzevii* alleles in some genes and their presence in other genes of the same chromosome can be interpreted as due either to the loss of a chromosomal region or to the presence of a nonreciprocal recombination between homoeologous chromosomes (homologous from different parental species). However, the case of the *S. kudriavzevii* chromosome IV region between genes *RPN4* and *EUG1*, which is absent in wine strains and beer strains CECT 11003 and 11004, supports the recombination event because the loss of this region would generate a nonviable acentromeric chromosomal segment.

Preliminary results (data not shown) based on comparative genome hybridization (CGH) analysis with DNA chips, currently being performed in our laboratory, corroborate the presence of such mosaic recombinant chromosomes also in wine hybrids.

The presence of rearranged, mosaic chromosomes has already been demonstrated in lager *S. cerevisiae* × *S. bayanus* strains by classical genetic analysis (6, 7, 17, 28–30) and has recently been confirmed by CGH (1, 2, 19) and genome sequencing (19). These CGH analyses also showed that lager yeasts, as well as the former type strains of *S. pastorianus*, *S. carlsbergensis*, and *S. monacensis*, lack certain *S. cerevisiae* chromosomes (1, 2, 19).

In the present study, we deduced by restriction analysis of gene regions that certain chromosomes coming from the *S. kudriavzevii* parent are also completely absent in *S. cerevisiae* × *S. kudriavzevii* hybrids. However, there is a trend in these hybrids to maintain the *S. cerevisiae* genome and to reduce the non-*S. cerevisiae* (*S. kudriavzevii*-like) fraction, whereas lager strains exhibit an opposite tendency to preserve the non-*S. cerevisiae* (*S. bayanus*-like) genome and to reduce the *S. cerevisiae* fraction. Contrastingly, both types of natural hybrids contain the non-*S. cerevisiae* mitochondrial genomes (11, 31).

A possible mechanism to explain the generation of mosaic chromosomes by nonreciprocal recombination among homoeologous chromosomes and the loss of parental chromosomes was described by Chambers et al. (8) for *S. cerevisiae* strains containing additional, single *S. paradoxus* chromosomes introduced by cytoduction. These authors demonstrated that the mismatch repair system reduces meiotic homoeologous recombination, resulting in the aberrant segregation of chromosomes (meiosis II nondisjunction). Moreover, when recombination occurs, the mismatch repair system stimulates the loss of one partner of the recombination event, a phenomenon called recombinant-dependent chromosome loss in hybrids.

On the advantage of hybrids. Natural *S. cerevisiae* × *S. kudriavzevii* hybrids have been found thus far associated to fermentation processes in temperate areas of Europe, regions of oceanic and continental climate such as England (present study), Belgium (present study), Germany (3; also the present study), French Brittany (23) and Alsace (3) in France, Switzerland (11), and Austria (22). In these regions, hybrids can be predominant (11, 22, 35) due to a better adaptation than *S. cerevisiae* to lower temperatures (12, 18, 34). Although hybrids are generally less suited than the parents to specific environmental conditions, they can be better adapted to intermediate or fluctuating conditions. This is due to the acquisition of

physiological properties of both parents, which provide a mechanism for the selection of hybrids (12, 13, 23, 37). In this case, *S. cerevisiae* × *S. kudriavzevii* hybrids acquired the physiological properties of both parents, i.e., good alcohol and glucose tolerance and fast fermentation performance from *S. cerevisiae* plus better adaptation to low and intermediate temperatures as well as higher production of glycerol and aroma compounds from *S. kudriavzevii* (12).

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