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Natural Populations of *Saccharomyces kudriavzevii* in Portugal Are Associated with Oak Bark and Are Sympatric with *S. cerevisiae* and *S. paradoxus*[▽]

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Here we report the isolation of four *Saccharomyces* species (former *Saccharomyces* sensu stricto group) from tree bark. The employment of two temperatures (10°C in addition to the more commonly used 30°C) resulted in the isolation of *S. kudriavzevii* and *S. uvarum*, two species that grow at low temperatures, in addition to *S. cerevisiae* and *S. paradoxus*. A clear bias was found toward the bark of certain trees, particularly certain oak species. Very often, more than one *Saccharomyces* species was found in one locality and occasionally even in the same bark sample. Our evidence strongly suggests that (markedly) different growth temperature preferences play a fundamental role in the sympatric associations of *Saccharomyces* species uncovered in this survey. *S. kudriavzevii* was isolated at most of the sites sampled in Portugal, indicating that the geographic distribution of this species is wider than the distribution assumed thus far. However, the Portuguese *S. kudriavzevii* population exhibited important genetic differences compared to the type strain of the species that represents a Japanese population. In this study, *S. kudriavzevii* stands out as the species that copes better with low temperatures.

The yeasts belonging to the genus *Saccharomyces*, especially *S. cerevisiae*, play an important role in human activities. They are used as fermenting agents worldwide and stand out as model eukaryotic organisms in various fields of biological science ranging from biochemistry to genomics. The modern phylogenetic concept of *Saccharomyces* restricts this genus to eight species: *S. bayanus*, *S. cerevisiae*, *S. paradoxus*, *S. pastorianus*, *S. uvarum*, *S. cariocanus*, *S. kudriavzevii*, and *S. mikatae* (14, 24, 28, 33). Most of these species are delimited by postzygotic isolation, but *S. pastorianus* and *S. bayanus* are thought to be hybrid species, since their genomes have contributions from both *S. cerevisiae* and *S. uvarum* (formerly designated *S. bayanus* var. *uvarum*) (3, 10, 28). Despite the extensive use of *Saccharomyces* yeasts as model organisms and the fact that the genomes of five yeast species have been completely sequenced, relatively little is known about their natural habitats and population genetics. Most population biology studies of *Saccharomyces* deal with *S. paradoxus*, which has a global distribution and exhibits genetic differentiation over long distances (13, 22).

Traditionally, *S. paradoxus* has been regarded as a wild species associated mostly with natural habitats (18, 22). In contrast, several authors viewed *S. cerevisiae* as a domesticated organism chiefly adapted to man-made fermentations and normally absent in natural ecosystems (4, 16, 34). However, several lines of evidence suggest that *S. cerevisiae* existed in natural environments long before it was utilized in man-made fermentations. Nucleotide variation in more than 80 isolates collected worldwide supported the hypothesis that domesticated strains are derived from wild populations (5), and sympatric populations of *S. paradoxus* and *S. cerevisiae* were detected in a natural woodland site in North America (31). The implications of the study by Sniegowski et al. (31) for ecological research on *Saccharomyces* were several. First, it demonstrated that both *S. paradoxus* and *S. cerevisiae* could be obtained from uncultivated habitats by selective enrichment. In addition, it consolidated the view that *S. cerevisiae* can be found in natural environments because a substantial number of isolates was obtained from the same site, contrary to the occasional isolations previously reported (19, 20, 21, 23). Finally, it modified the concept that natural populations of *Saccharomyces* are mostly associated with tree fluxes because *S. cerevisiae* and *S. paradoxus* were consistently isolated from oak bark and soil in the vicinity of the trees.

Within *Saccharomyces*, *S. bayanus*, *S. pastorianus*, and *S. uvarum* are commonly considered cryophilic (or cryotolerant) because they are associated with low-temperature fermentation processes in the production of wines, beers, and ciders (6, 18, 25). The natural habitats of these species have not been identified. Besides fermentations conducted by humans, *S. bayanus*/*S. uvarum* have been sporadically isolated from insects (*Mesophylax adopersus* and *Drosophila* spp.), tree fluxes of *Ulmus*, *Carpinus*, and *Nothofagus*, and from a mushroom (26, 27). To date, both *S. kudriavzevii* and *S. mikatae* are represented by a few strains, all of which were isolated from decayed plant material or soil in Japan, leading to the tentative conclusion that these species may be endemic to this part of the globe (24).

The starting point of our investigation was to evaluate whether the findings by Sniegowski et al. (31) revealed a fundamental and general feature of the ecology of *Saccharomyces*

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yeasts, in which case one would expect the association with tree bark to exist at other geographic locations. Therefore, we aimed to compare *Saccharomyces* isolations previously performed from oaks in North America and Russia (22) with our own *Saccharomyces* isolations from the main types of Mediterranean oaks (*Quercus* spp.): *Q. faginea*, *Q. ilex*, *Q. pyrenaica*, and *Q. suber*. Our main question at the beginning of this study was, therefore, whether *Saccharomyces* yeasts colonize the Mediterranean oaks included in the survey and, if so, if the yeasts exhibit a clear preference for a tree or group of trees. A second fundamental issue we wanted to address was whether the temperature range usually used to incubate samples during *Saccharomyces* isolations could contribute to the fact that species more adapted to low temperatures have rarely been isolated from natural samples.

MATERIALS AND METHODS

Selective isolations and preliminary identification of *Saccharomyces* spp. Each bark sample (3 to 6 cm^2) was collected aseptically and subsequently cut in small pieces. Similar amounts (~3 g) were introduced into each one of two 20-ml sterile flasks to which 20 ml of selective enrichment medium was added. The selective medium consisted of YNB (yeast nitrogen base; Difco) supplemented with 1% (wt/vol) raffinose and 8% (vol/vol) ethanol. The flasks were subsequently tightly capped. One of the flasks was incubated at 30°C, while the other was incubated at 10°C without shaking. The flasks were surveyed periodically for turbidity and gas formation (indicative of fermentation) between the second and fifth weeks of incubation. These macroscopic signs of fermentation were observed usually after 1 week for the incubations at 30°C, while for the samples incubated at 10°C, evidence of fermentation was usually observed after the third week of incubation. Samples exhibiting yeast growth (checked microscopically) were plated onto low-pH (3.5) YMA (1% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract, 2% agar) with 8% ethanol. Cultures were subsequently purified on YMA and observed with a microscope. The preliminary identification and selection of *Saccharomyces* isolates were based on ascospore formation. Those cultures forming typical *Saccharomyces* asci and ascospores (nonquiescent asci containing two to four round ascospores) were selected for isolation. The cultures that failed to form ascospores on YMA were transferred to acetate agar (1% potassium acetate, 0.25% yeast extract, 0.1% glucose, and 1.5% agar), the usual sporulation medium for *Saccharomyces*.

HPLC analysis of bark samples. A total of 13 bark samples were analyzed. Approximately 3 g of each bark sample was first milled and subsequently mixed with 3 ml of distilled water. The mixture was incubated for 30 min at room temperature, filtered, concentrated to one-third of its original volume, and analyzed in a Dionex high-performance liquid chromatography (HPLC) system (Dionex, Sunnyvale, CA) equipped with a refractive index detector (LKB, Bromma, Sweden) and an Aminex HPX-87H column (Bio-Rad, Richmond, CA). The mobile phase consisted of 5 mM H_2SO_4, and the temperature was 65°C. Sugars were identified by comparing the retention times of the peaks obtained for the bark samples with those obtained with standard solutions of the different sugars.

Phenotypic characterization. For the estimation of maximum growth temperatures, 2-day-old inocula grown on YMA were used. The experiments were carried out in test tubes with 5 ml of YM medium. Incubations were carried out without shaking in a water bath. Readings were based on turbidity and were done 48 h after inoculation. The ability to assimilate different sugars was tested on YNB agar plates supplemented with 1% (wt/vol) of either melibiose, maltose, or galactose. Growth rates were determined in 100-ml batch cultures with YNB medium supplemented with 1% (wt/vol) of either glucose, fructose, or raffinose. The cultures were incubated at 6, 10, 25, or 30°C in an orbital shaker (150 rpm), and the optical density (at 640 nm) was measured at various time points during exponential growth.

Relative fitness tests. Separate liquid cultures of the two partner strains (*S. cerevisiae*-*S. kudriavzevii* or *S. paradoxus*-*S. uvarum*) were grown in liquid media similar to those used for growth rate measurements until mid-exponential phase. The cell concentration was then determined using a microscope counting chamber. Approximately 10^6 cells of each strain were inoculated simultaneously in 20 ml of the same medium. The mixture was divided in two equal portions, which were incubated at 10°C and 30°C without shaking. After the cultures had reached the late exponential phase of growth, samples were taken, plated onto YMA, and incubated at 21 to 23°C. After 24 h, a replica plate was made and incubated at 37°C. At 37°C, only *S. cerevisiae* (or *S. paradoxus*) was able to grow. Comparing the number of colonies in each plate yielded an estimate of the number of cells of each species present in a culture at the end of the mixed growth experiments.

PCR amplification, sequencing, and restriction analyses. Genomic DNA was isolated from cultures freshly grown in solid medium as previously described (30). DNA samples were diluted 1:100 in distilled water, and 5-μl portions of the diluted samples were used as template in 25-μl PCR mixtures. The ribosomal DNA (rDNA) fragments were amplified using primers ITS5 and LR6 (30). *MET2* gene fragments were amplified using primers based on the same regions as those previously described (9) as follows: 5′ CGA AAA CGC TCC AAG AGC TGG and 5′ GAC CAC GAT ATG CAC CAG GCA G for *S. cerevisiae*, *S. uvarum*, and *S. bayanus*; 5′ CGG AAA CGC TTC AAG AGC TGG and 5′ GAC CAC GAT ATA CAC CAA GCA G for *S. kudriavzevii*; and 5′ CGA AAA CAC TCC AAG AGC TGA and 5′ GAC CAT GAT ATG CAC CAG GCG G for *S. paradoxus*. The PCRs were performed as follows: 5 min at 94°C (denaturation), and 35 cycles of PCR, with 1 cycle consisting of the following steps: 30 s at 94°C, 30 s at 57°C, and 30 s at 72°C. For the amplification of *MET6* and *CYR1* gene fragments, primers CYR1-5(F), CYR1-3(R), MET6-5(F), MET6-3(R), and MET6-3K (*S. kudriavzevii* specific reverse primer) were used. Both the primer sequences and reaction conditions were as described by González et al. (8). The primers used to amplify the *S. kudriavzevii GAL1* gene fragment were as follows: 5′ GCT GAT (G/C)CA AA(G/A) TTT GCT C(G/A)A AG(A/G) AAG TTC GA (forward) and 5′CCG CA(T/A) ACA GAG GCA GC(T/C) TGA TCC ATA CC(A/G) CC (reverse).

Prior to sequencing and/or restriction, DNA fragments were purified using the GFX kit (GE Life Sciences). For restriction analyses, amplicons were digested with HinfI (*MET6*) and MspI and HaeIII (*CYR1*), and the restriction pattern was determined by electrophoresis in 3% agarose. Sequencing was performed at STABVIDA (Oeiras, Portugal), using primers F63 and LR3 for the D1/D2 region and primers ITS1 and ITS4 for the complete internal transcribed spacer (ITS) region (30).

RESULTS

Selective isolations. Our strategy to isolate yeasts of the genus *Saccharomyces* from bark samples was devised to allow us to address two main issues concerning the characterization of the natural habitats of these yeasts. The first aim was to investigate whether *Saccharomyces* spp. could be consistently isolated from bark from Mediterranean oaks and to clarify whether a clear bias could be established toward a tree species or type of tree. The second issue addressed was the hypothesis that the temperature usually employed for the incubations during isolation, 25 to 30°C, was decisive in narrowing the scope of *Saccharomyces* species that could be isolated from natural samples. To address these issues, we started by collecting bark samples at various locations in Portugal from the most common local representatives of the Fagaceae (e.g., *Quercus*, *Castanea*, and *Fagus*) and also from other trees at the same locations. Each sample was subsequently divided in two portions that were incubated at two different temperatures, 30°C and 10°C.

Our survey of bark samples in Portugal yielded a total of 34 *Saccharomyces* isolates. Due probably to the selective nature of the enrichment medium employed, non-*Saccharomyces* yeasts were rarely isolated. The non-*Saccharomyces* yeasts that were more frequently isolated were *Lachancea* (*Kluyveromyces*) *thermotolerans* and *Torulaspora* sp. For the vast majority of our *Saccharomyces* isolates, asci and ascospores could be observed after a few days of growth on YMA. Those strains that did not sporulate on YMA did so on acetate agar medium. The selection of *Saccharomyces* strains based on the microscopic examination of asci and ascospores did not yield false-positive results as revealed by sequence analyses of the D1/D2 domain of large-subunit (LSU) rDNA and complete ITS region (see be-

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TABLE 1. Geographic origin and number of bark samples from tree species belonging to the family Fagaceae investigated and number of *Saccharomyces* strains isolated from each tree species

Species	Country(ies)[a]	No. of bark samples	No. of yeast isolates
Quercus alba	C	5	1
Quercus coccifera	P	1	
Quercus dumosa	US	4	
Quercus faginea	P	14	10
Quercus garryana	C	24	11
Quercus ilex	P	10	6
Quercus palustris	P	1	
Quercus petraea	G	1	
Quercus pyrenaica	P	15	11
Quercus robur	C, G	26	2
Quercus rubra	C, G, P	11	
Quercus suber	P	15	
Castanea sativa	P	15	5
Fagus sylvatica	P, G	5	3

[a] C, Canada; G, Germany; P, Portugal; US, United States.

low). Most of these strains (27 isolates) were obtained from *Quercus pyrenaica*, *Q. faginea*, and *Q. ilex*, three of the oak species most commonly found in Portugal, with very high success rates (73%, 71%, and 60%, respectively). Surprisingly, one of the most common oaks in Portugal, *Q. suber*, the cork oak, did not yield any *Saccharomyces* isolates. A total of six strains could be recovered from other trees belonging to the Fagaceae family, namely, *Castanea sativa* and *Fagus sylvatica*. We subsequently broadened our survey to other locations in Canada and Germany and to a variety of oak trees other than those found in Portugal. Again, several *Saccharomyces* strains could be isolated at the two incubation temperatures (a total of 20 strains). On the whole, we obtained 53 *Saccharomyces* isolates from 191 bark samples collected in various localities in Portugal (76 samples), Baden Würtenberg, Germany (18 samples), British Columbia, Canada (49 samples), and Nevada, United States (4 samples) between March 2005 and January 2006. If the relative frequencies of isolation of *Saccharomyces* are considered, the samples obtained from trees that belong to the family Fagaceae yielded three times more *Saccharomyces* isolates (49 isolates from 147 bark samples, a success rate of 33%) than the samples from trees that do not belong to the Fagaceae (e.g., Betulaceae, Oleaceae, and Ulmaceae) (4 isolates from 44 bark samples, a success rate of 9%). The list of tree species of the Fagaceae that were studied as well as the number of samples examined and the number of *Saccharomyces* isolates obtained are summarized in Table 1. The most striking result from this broadened survey was the high frequency of isolation (11 strains from 24 samples, success rate of 45%) from a local *Quercus* species (*Q. garryana*) in Hornby Island, Canada. In summary, our results show that *Saccharomyces* can indeed be consistently isolated from bark samples of some of the most common Mediterranean oaks as well as from other oak species (Table 1). Two exceptions were particularly noted: *Quercus robur*, which was the most frequently sampled tree species, yielded very few isolates (two strains in 26 samples), while no *Saccharomyces* could be isolated from 15 bark samples from *Quercus suber*. Therefore, taken together, our results indicate that tree bark seems indeed to be a habitat for *Saccharomyces* spp., and there seems to be a clear bias toward certain species of trees, since some oak species exhibit a particularly high frequency of isolation, while others yielded no isolates (Table 1).

One of the intriguing issues arising when we consider the suitability of tree bark as a habitat for *Saccharomyces* concerns the availability of carbon and energy sources to support growth in this type of environment. It is well-known that *Saccharomyces* species exhibit a strong preference for hexoses (glucose, fructose, mannose, and galactose) or simple oligosaccharides, especially maltose, sucrose, and raffinose (1). This prompted us to investigate whether three of these sugars (glucose, fructose, and sucrose) could be detected in the bark samples from which *Saccharomyces* isolates were obtained. We used HPLC to evaluate qualitatively a set of bark samples with respect to the presence of these sugars. It was possible to detect the presence of at least one of the sugars in the bark from the four tree species that yielded the most isolates (*Q. ilex*, *Q. faginea*, *Q. garryana*, and *Q. pyrenaica*). In two cases (*Q. pyrenaica* and *Q. faginea*), all three sugars could be detected, while in *Q. garryana*, both glucose and sucrose were found, and in *Q. ilex*, only sucrose could be detected. We also investigated bark samples from trees that yielded very little or no *Saccharomyces* isolates (*Q. robur*, *Q. suber*, and *Betula pubescens*). Notably, no traces of the three sugars were found in these cases.

Species identification and distribution. Sequence analyses of the D1/D2 domain of LSU rDNA and of the complete ITS region indicated that our *Saccharomyces* isolates belong to four species: *S. cerevisiae*, *S. paradoxus*, *S. uvarum*, and *S. kudriavzevii* (Fig. 1a and b and Table 2). Although the sequences of D1/D2 and ITS regions were very similar among these species, they exhibited some differences that suffice for an unequivocal identification at the species level. For reference, in our alignment of the D1/D2 domains, we used the sequences of the type strains of all the species presently recognized in *Saccharomyces*. We ascribed some of our isolates to *S. uvarum* and not to *S. bayanus* (Fig. 1a and b) because the D1/D2 and ITS sequences of our isolates were identical to those of the type strain of *S. uvarum* (CBS 395), which differs from the sequences of the type strain of *S. bayanus* (CBS 380) by one substitution in the D1/D2 domain and two substitutions in the ITS region. Considering each of the four *Saccharomyces* species that we found, we observed very few (or no) intraspecific polymorphisms in the D1/D2 and ITS sequences. Therefore, only representative sequences of D1/D2 and ITS regions of isolates belonging to each of the four species were included in Fig. 1a and b. An exception to this trend was the D1/D2 sequence of NBRC 1803, one of the reference strains of *S. kudriavzevii*, which differed considerably from other sequences of this species (Fig. 1a). However, the ITS sequence of this strain was closely related to the other ITS sequences of *S. kudriavzevii* (Fig. 1b).

The distribution of the four *Saccharomyces* species according to the temperature of isolation and geographical origin is depicted in Table 2, and the occurrence of the different species in the 14 study sites investigated is shown in Table 3. The most striking result was the frequent isolation of *S. kudriavzevii* at most of the locations sampled in Portugal (Table 3), since up to now only four strains belonging to this species had been isolated in Japan. The isolation of *S. kudriavzevii* was strictly linked to the incubation of the samples at 10°C—no isolates

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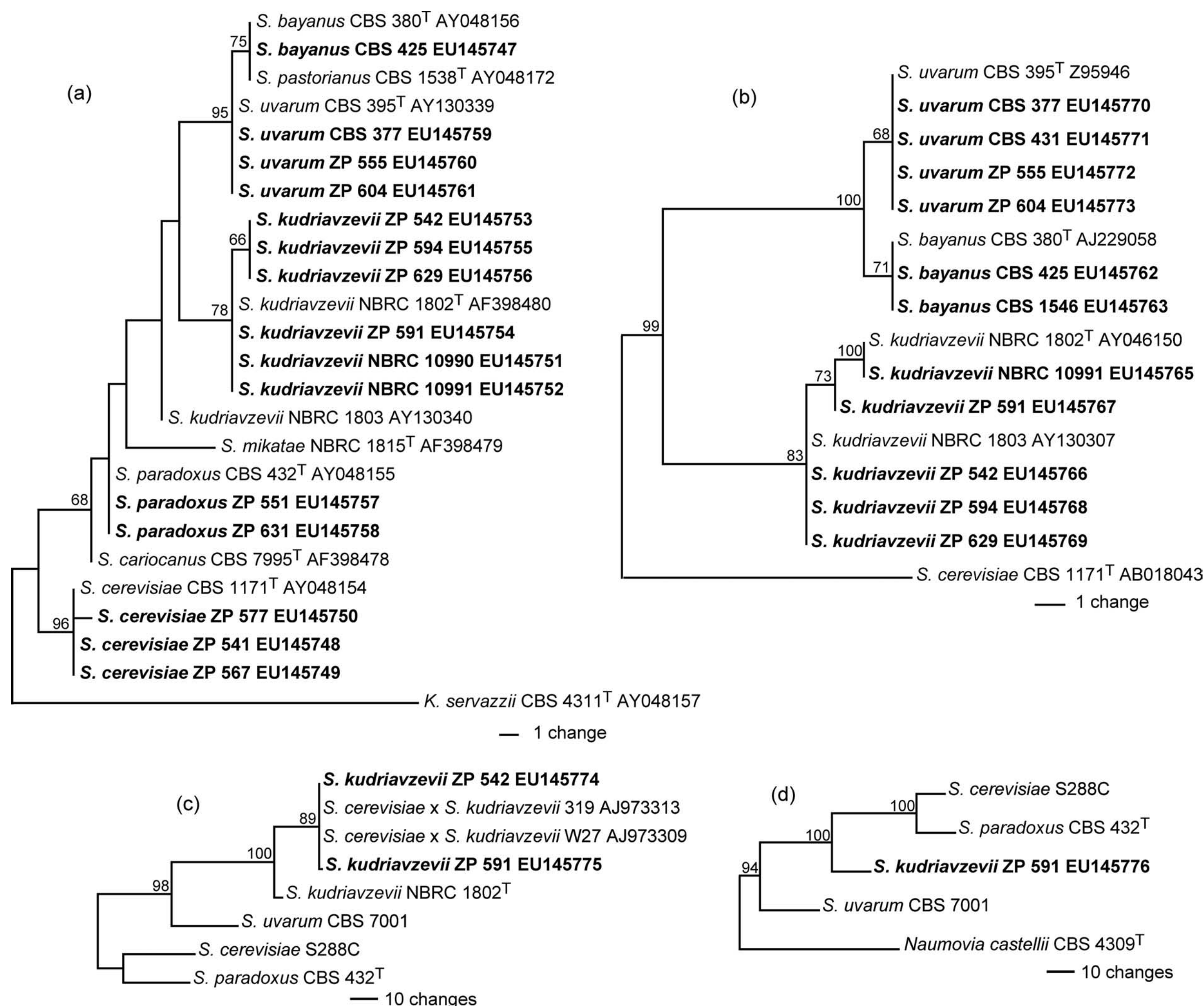

FIG. 1. Maximum-parsimony phylogenetic trees of *Saccharomyces* spp. obtained with sequences of D1/D2 domains of the 26S rDNA (a), complete ITS region (ITS1, 5.8S, and ITS2) (b), partial nuclear *MET6* gene (c), and partial *GAL1* gene (d). The organisms chosen to root the trees were *Kazachstania servazzii* (a), *S. cerevisiae* (b), *S. cerevisiae* and *S. paradoxus* (c), and *Naumovia castellii* (d). The D1/D2, ITS, and *GAL1* trees are the single most parsimonious trees, and the *MET6* tree is a consensus tree of the 15 most parsimonious trees. Numbers at the nodes are bootstrap values (1,000 replicates; values below 50% are not shown). GenBank accession numbers of the sequences are indicated after strain designations. The sequences without GenBank accession numbers were retrieved from the fungal sequence alignment section of the SGD (http://www.yeastgenome.org/). Sequences determined in this study are shown in boldface type. Type strains are indicated with a superscript T.

belonging to this species were obtained upon incubation at 30°C (Table 2). The same holds true for *S. uvarum*, a species already known to belong to the low-temperature subgroup within *Saccharomyces*. While *S. cerevisiae* showed a clear preference for 30°C (only one strain was isolated at 10°C), *S. paradoxus* was the only species that did not exhibit a strong bias toward an isolation temperature, since nearly the same number of isolates was obtained at each temperature (Table 2). *Saccharomyces paradoxus* and *S. cerevisiae* appear to be less specific to a particular tree or group of trees than the other species. In fact, whereas *S. paradoxus* and *S. cerevisiae* were isolated from eight and seven tree species, respectively, *S. kudriavzevi* and *S. uvarum* were isolated from four and three tree species, respectively (Table 2).

Phylogenetic position and phenotype of the new *S. kudriavzevii* isolates. Prior to this study, only four *S. kudriavzevii* strains were known, and all exhibited one important trait that distinguished them from other *Saccharomyces* species: they were unable to grow on galactose. Detailed analysis of the type strain of *S. kudriavzevii* of the genomic regions where the *GAL* genes are normally encoded revealed that all *GAL* genes had degenerated to a considerable extent (11). Therefore, we were surprised to find that our *S. kudriavzevii* isolates were all able to grow on galactose. The simplest explanation for this difference would be that our isolates were hybrids of *S. cerevisiae* and *S. kudriavzevii*. This hypothesis was pertinent because hybrids of these two species have been recently found in wine fermentations carried out at low temperatures (8, 15). Our approach to check whether our strains were hybrids followed that of González et al. (8) and was based on differences in the coding regions of the genes *MET2*, *MET6*, and *CYR1* among different *Saccharomyces* species. We started by using allele-specific

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TABLE 2. Distribution of the four *Saccharomyces* species according to the temperature of isolation, geographical origin, and type of tree bark employed in the isolations

Parameter	No. of isolates			
	S. cerevisiae	*S. paradoxus*	*S. uvarum*	*S. kudriavzevii*
Total no. of isolates	15	13	10	15
No. of isolates found at the following temp:				
10°C	1	6	10	15
30°C	14	7		
No. of isolates from the following geographic location:				
Portugal	14	5		15
British Columbia, Canada	1	5	10	
Baden-Wurtenberg, Germany		3		
No. of isolates from bark of the following tree species[a]:				
Q. alba (C)		1		
Q. faginea (P)	2	3		5
Q. garryana (C)		3	8	
Q. ilex (P)	1	1		4
Q. pyrenaica (P)	6			5
Q. robur (C, G)	1	1		
C. sativa (P)	3	1		1
F. sylvatica (G, P)	1	2		
Arbutus menziesii (C)		1	1	
Prunus sp. (C)			1	
Fraxinus sp. (P)	1			

[a] C, Canada; G, Germany; P, Portugal.

MET2 gene PCR primers that discriminate between different alleles found in three (groups of) species: *S. cerevisiae*/*S. bayanus*/*S. uvarum*, *S. paradoxus*, and *S. kudriavzevii*. In these experiments, several isolates of *S. cerevisiae* and *S. kudriavzevii* were used, and they all yielded an amplification product only when the primers used were those specific for the allele present in the species to which they were ascribed. This indicates that the Portuguese *S. kudriavzevii* isolates do not harbor an *S. cerevisiae*-like *MET2* allele. However, the *S. kudriavzevii* allele present in these isolates is probably slightly different from that present in the Japanese strains because when the primers designed for the amplification of the *MET2* gene fragment from the type strain of *S. kudriavzevii* were employed for the Portuguese isolates, amplification was consistently less efficient than that obtained for the four Japanese strains (data not shown). Subsequently, *MET6* and *CYR1* gene fragments were amplified from the 15 new *S. kudriavzevii* strains and also from the *S. cerevisiae* × *S. kudriavzevii* hybrid strains Anchor NT 50, AMH (Assmannshausen), W27, W46, SPG 14-91, and 319, previously characterized by Bradbury et al. (2) and González et al. (8). To this end, we used the same primers employed by González et al. (8) for characterization of the hybrids. Amplification of the *MET6* allele from *S. kudriavzevii* requires a species-specific reverse primer. Similar to what was observed for *MET2*, amplification of the *MET6* gene fragment using this same reverse primer was much less efficient both in the hybrid strains and in the new isolates, compared with the four Japanese strains under the same conditions (data not shown). For amplification of the *CYR1* gene fragments, a similar efficiency was observed for all strains. Restriction analysis of the *MET6* (using enzyme HinfI) and *CYR1* (using enzymes HaeIII and MspI) gene fragments was subsequently used to characterize the allele(s) present in the new isolates. Using the terminology employed previously by González et al. (8), we obtained for the new *S. kudriavzevii* isolates and for both *CYR1* and *MET6*, the restriction pattern K1, which corresponds to that of the type strain of *S. kudriavzevii*. In line with previous observations (8), the reference hybrid strains mentioned above originated restriction patterns that combine those of *S. cerevisiae* and *S. kudriavzevii* (pattern C1K1 or C1K2). These results strongly indicate that the new isolates are not hybrids of *S. cerevisiae* and *S. kudriavzevii*. Since our experiments suggested that there were some differences in the sequences of the *MET2* and *MET6* alleles between the new strains and the *S. kudriavzevii* type strain, we decided to sequence the *MET6* amplicon obtained from two of the new isolates (strains ZP 542 and ZP 591). The phylogenetic

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TABLE 3. Occurrence of four *Saccharomyces* spp. in the 14 study sites sampled in this study

Study site	Geographic location	Occurrence (temp of isolation [°C]) of the following *Saccharomyces* species[a]:			
		S. cerevisiae	*S. paradoxus*	*S. uvarum*	*S. kudriavzevii*
1	Adagoi, Portugal	+ (30)			+ (10)
2	Alvão, Olo, Portugal		+ (30)		
3	Aldeia das Dez, Serra da Estrela, Portugal	+ (30)	+ (30)		+ (10)
4	Marão, Campeã, Portugal	+ (30)			
5	Lisbon, Portugal				+ (10)
6	Lagoa de Albufeira, Portugal		+ (30)		+ (10)
7	Arrábida, Portugal				+ (10)
8	Sines, Portugal		+ (30/10)		
9	Castelo de Vide, Portugal	+ (30, 10)			+ (10)
10	Paul do Boquilobo, Portugal	+ (30)			
11	Tübingen, Germany		+ (10)		
12	Murrhardt, Germany		+ (10)		
13	Vancouver, Canada	+ (30)	+ (10)		
14	Hornby Island, Canada		+ (30/10)	+ (10)	

[a] +, species occurred.

TABLE 4. Phenotypic characteristics (growth on different carbon sources and at different temperatures) of the strains involved in sympatric associations

Species	Strain	Study site[a]	Growth on the following carbon source[b]:			Growth[c] at the following temp (°C):									
			MEL	MAL	GAL	32	33	35	36	37	38	39	40	41	42
S. cerevisiae	ZP 541	1	−	+	+	+	+	+	+	+	+	+	+	−	−
S. kudriavzevii	ZP 542	1	−	+	+	+	−	−	−	−	−	−	−	−	−
S. cerevisiae	ZP 577	3	−	+	+	+	+	+	+	+	+	+	+	+	+
S. paradoxus	ZP 600	3	−	+	+	+	+	+	+	+	+	+	−	−	−
S. kudriavzevii	ZP 594	3	−	+	+	+	−	−	−	−	−	−	−	−	−
S. paradoxus	ZP 631	6	−	−	+	+	+	+	+	+	+	−	−	−	−
S. kudriavzevii	ZP 629	6	−	+	+	+	+	−	−	−	−	−	−	−	−
S. cerevisiae	ZP 567	9	−	+	+	+	+	+	+	+	+	+	+	−	−
S. kudriavzevii	ZP 591	9	−	+	+	+	−	−	−	−	−	−	−	−	−
S. paradoxus	ZP 551	14	−	+	+	+	+	+	+	+	−	−	−	−	−
S. uvarum	ZP 555	14	+	+	+	+	+	+	−	−	−	−	−	−	−

[a] Study sites shown in Table 3.
[b] Growth (+) on melibiose (MEL), maltose (MAL), or galactose (GAL).
[c] +, growth; −, no growth.

comparison of the partial *MET6* sequences is shown in Fig. 1c. The new sequences were very closely related to each other, and the sequence of strain ZP 542 was identical to the *S. kudriavzevii* allele present on the *S. cerevisiae* × *S. kudriavzevii* hybrid strains W27 and 319, which were also isolated in Europe (Fig. 1c). Noteworthy, within *S. kudriavzevii*, the most divergent sequence was that of NBRC 1802, isolated in Japan. In our 645-bp alignment of partial *MET6* sequences, the sequence of the type strain of *S. kudriavzevii* (NBRC 1802) differed from the sequences of the new isolates by 13 to 14 nucleotide substitutions.

Having established that the new isolates of *S. kudriavzevii* were not likely to be hybrids, the question of growth on galactose remained to be elucidated. If our strains were not hybrids and were able to grow normally on galactose, then they should possess functional *GAL* gene alleles that were different from those already known in other *Saccharomyces* species (since the *S. kudriavzevii* type strain contains *GAL* pseudogenes only). To ascertain this, we amplified a fragment from the *GAL1* gene in strain ZP 591 using slightly degenerated primers based on all the other *Saccharomyces* sequences available. This fragment was shown to encode a novel sequence with approximately 80% identity to the *S. cerevisiae GAL1* gene fragment. A phylogenetic comparison of the partial sequences of *GAL1* is shown in Fig. 1d. We assume that this fragment sequenced from the Portuguese isolate represents a functional *S. kudriavzevii GAL1* allele because strain ZP 591 is able to grow on galactose and no interruption of the coding sequence was found.

Taken together, our results show that the Portuguese *S. kudriavzevii* population exhibits important genetic differences compared with the four strains of this species previously known but that they are not hybrids. In addition, *MET6* sequence data (Fig. 1c) suggest that the *S. kudriavzevii* population uncovered in this report seems to be genetically more related to the *S. kudriavzevii* parent of the hybrid strains isolated from wine fermentations in Europe (8) than to the Japanese population from which the type strain of *S. kudriavzevii* is derived. Finally, our results add further support to the view that *S. kudriavzevii* has considerable genetic heterogeneity as revealed by polymorphism in D1/D2, ITS, and *MET6* sequences (Fig. 1a to c).

Phenotypes of sympatric species. Our studies revealed that more than one *Saccharomyces* species can thrive in the same environment and that different populations (of different species) occupy the same area. These species are expected to exhibit phenotypic differences that allow them to exploit different ecological niches within the same habitat, thus avoiding the competitive exclusion principle. In order to initiate characterization of the sympatric associations between *Saccharomyces* species, we started by evaluating a few phenotypic traits of a group of 11 strains that represent the various sympatric associations detected (Table 4). We focused our attention on the ability to utilize galactose, maltose, and melibiose. Whereas growth on D-galactose was recorded for all isolates and maltose supported growth of all isolates except one strain of *S. paradoxus*, melibiose was utilized only by *S. uvarum* (Table 4).

In the sympatric associations, the species involved were almost always isolated at different temperatures. Therefore, we subsequently estimated the maximum growth temperature for the 11 representative strains as depicted in Table 4. In this case, striking differences were found between most of the partners in each sympatric association. The maximum growth temperatures of the members of the various sympatric associations were separated by two or more degrees Celsius. The maximum growth temperature of *S. cerevisiae* ZP 577 (42°C) was the highest among the *S. cerevisiae* strains considered in Table 4. Interestingly, *S. cerevisiae* ZP 577 coexisted with *S. paradoxus* ZP 600, which has a maximum growth temperature of 39°C, whereas the other two *S. cerevisiae* strains (ZP 541 and ZP 567) coexisted with *S. kudriavzevii*, which in those two cases was not able to grow at 33°C. The higher maximum growth temperature of strain ZP 577 might be a case of phenotypic displacement due to competition with *S. paradoxus*. For one pair of

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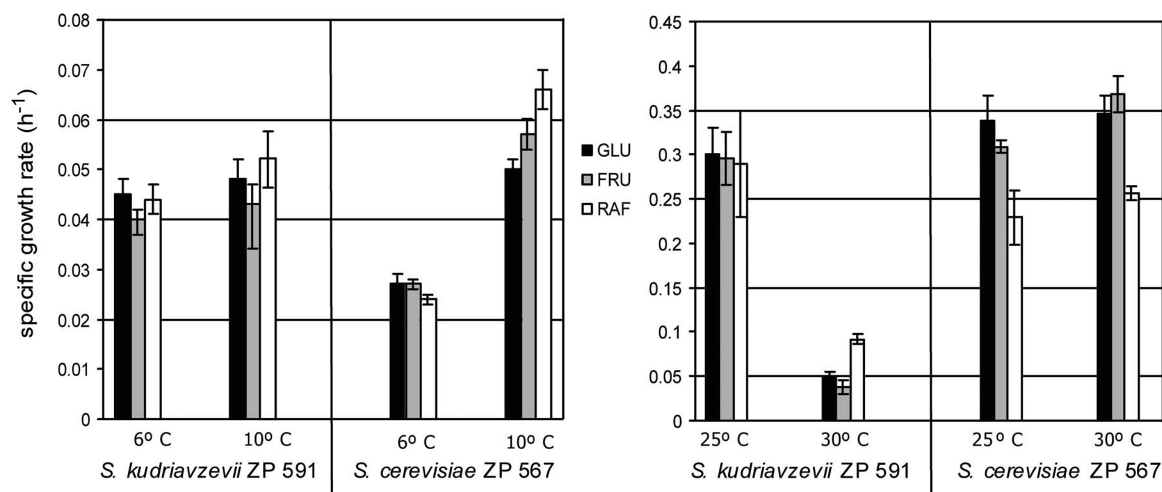

FIG. 2. Specific growth rates of *S. kudriavzevii* ZP 591 and *S. cerevisiae* ZP 567 measured at 6, 10, 25, and 30°C in media with glucose (GLU), fructose (FRU), and raffinose (RAF) as the sole carbon sources. Standard deviations (error bars) are shown.

sympatric strains (*S. cerevisiae* ZP 567 and *S. kudriavzevii* ZP 591), we determined the specific growth rates at four different temperatures and three different carbon sources as shown in Fig. 2. In most cases, growth rates were not markedly affected by the particular carbon source present in the culture medium. The effects caused by the growth temperatures were much more conspicuous. *Saccharomyces kudriavzevii* ZP 591 grew slowly at 30°C, but its specific growth rate at 6°C was nearly double that of *S. cerevisiae* ZP 567. At the intermediate temperatures of 10°C and 25°C, the two strains performed similarly.

We also performed preliminary relative fitness tests by coculturing the two partners of two of the sympatric associations (strains ZP 551 and ZP 555 in a sympatric association and strains ZP 567 and ZP 591 in another) in YNB medium supplemented with raffinose, a medium similar to that used during the isolation procedures. We were able to reproduce the isolation results by inoculating simultaneously equal numbers of cells of the two species and incubating the culture either at 30°C or at 10°C: if the culture was incubated at 10°C, at the end of the fermentation, only the species better adapted to low temperatures (*S. kudriavzevii* ZP 591 or *S. uvarum* ZP 555) was found, while the species better adapted to grow at high temperatures (*S. cerevisiae* ZP 567 or *S. paradoxus* ZP 551) dominated the fermentations at 30°C. These results indicate that the adaptation to different temperatures is the most likely basis for the sympatric associations between different *Saccharomyces* species detected in this study. Moreover, a compilation of our data concerning the maximum growth temperature of 35 Portuguese *Saccharomyces* isolates (Fig. 3) shows different maximum growth temperature profiles for the four species isolated in this study. Again, the most striking difference found concerns *S. kudriavzevii*, which clearly stands out as the species better adapted to low temperatures within the four species.

DISCUSSION

In this paper we report for the first time the isolation of four different species belonging to the group formerly known as *Saccharomyces* sensu stricto in a single survey of natural samples. The success rate in our study (33%) was higher than the rates previously observed in two similar studies that preceded this work which also report consistent isolation of *Saccharomyces* spp. from oak bark and soil beneath oak trees. In one of these studies, a success rate of 23% was reported for the isolation of *S. cerevisiae* and *S. paradoxus* from bark and soil associated with oaks in North America (31). A survey of mainly *Quercus robur* in England, in turn, yielded solely *S. paradoxus* isolates with a success rate of 8% (12). A comparison of the three studies suggests that the success rate increases with the number of different species recovered, as might be expected. Taken together, these three studies provide very strong evidence that tree bark, and particularly the bark from certain oaks, is a habitat for *Saccharomyces* yeasts. In particular, the frequencies of isolation from certain trees are strikingly high compared with those reported for any other natural sample, including grape berries. For example, it has been estimated that *S. cerevisiae* has a frequency of occurrence in undamaged

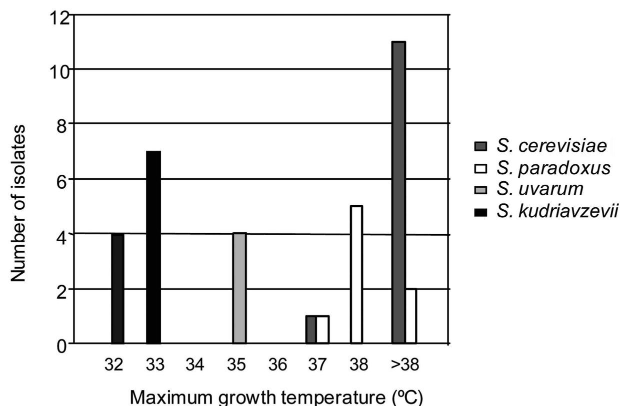

FIG. 3. Maximum growth temperatures of a set of 35 strains of *S. cerevisiae* (12 strains) *S. kudriavzevii* (11 strains), *S. paradoxus* (8 strains), and *S. uvarum* (4 strains). All strains were isolated during the present work.

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grapes lower than 0.1% and that its occurrence in berries damaged by birds or insects is about 24%, meaning that about 1 in 1,000 grapes contains *S. cerevisiae* (17). Some of the tree species in our survey were frequently sampled at various locations, allowing us to detect a very strong bias toward certain tree species irrespective of its location: for a similar number of samples, we found success rates for individual tree species that ranged from 0 to 73% (*Q. suber* and *Q. pyrenaica*, respectively). Notably, our survey of *Q. robur* resulted in a success rate of 8%, identical to that reported for the same tree species in England (12). As a corollary of these findings, we propose a new model for the ecological niche of *S. cerevisiae*, *S. kudriavzevii*, *S. paradoxus*, and *S. uvarum* in which tree bark constitutes the primary reservoir. Sugar-rich environments related to the maturation of different kinds of flowers and fruits typically have a seasonal occurrence and consequently would constitute the secondary habitat. This suggestion implies that besides the adaptations to thrive in sugar-rich environments, *Saccharomyces* yeasts have also evolved mechanisms to cope with quite distinct environmental conditions, such as those prevailing in tree bark.

We attribute the broad scope of species recovered and, in part, the high frequency of isolation to the employment of an isolation temperature of 10°C in addition to the more commonly used 30°C. In fact, our results suggest that the presence of the species better adapted to low temperatures may have been missed in previous surveys aiming at the characterization of natural populations of *Saccharomyces* because of the use of a high temperature of incubation during the enrichment step. The inability of the "thermosensitive" species to grow at higher temperatures may also be aggravated by difficulty coping with increased ethanol toxicity at these temperatures, since ethanol can be used as a selective agent in the enrichment medium used in *Saccharomyces* isolations.

Notably, we were able to detect the presence of simple sugars in bark samples of the trees exhibiting the highest frequencies of isolation, which could be an important factor for the maintenance of *Saccharomyces* in this environment. We did not attempt to determine the concentration of these sugars because we reason that they are not evenly distributed on the solid matrix of the tree bark. Instead, we hypothesize that if these sugars are important for the survival of *Saccharomyces* on tree bark, they have to achieve appreciable concentrations at the local (microscopic) level, since these yeasts are not well equipped to scavenge the environment for scarce amounts of sugars—in general, *Saccharomyces* yeasts do not possess active transport systems for hexoses, with the notable exception of the fructose symporter present only in *S. bayanus*, *S. pastorianus*, and *S. uvarum* (7, 29).

The most surprising consequence of our isolation strategy was the frequent isolation in Portugal of *S. kudriavzevii*, thus far thought to occur only in Japan (24). The existence of a European population of this species is in line with recent work that reports the presence of hybrids of *S. cerevisiae* and *S. kudriavzevii* in cider (France) (9) and wine fermentations in Switzerland and Austria (8, 15). Actually, we could show that our *S. kudriavzevii* strains are not hybrids but that the sequence of the *MET6* gene of two of our isolates is very closely related to the sequence found in the hybrid strains and somewhat different from the sequence determined for the type strain of *S. kudriavzevii*, which represents a Japanese population. This suggests that the hybrids may originate from the European stock of *S. kudriavzevii*, whose geographic distribution remains to be further elucidated, since so far, we were able to detect it only in Portugal. If the D1/D2 and ITS sequence data of the four Japanese isolates and the four Portuguese isolates depicted in Fig. 1a and b are combined and sequence polymorphisms are considered, a total of four genotypes—two Japanese genotypes and two Portuguese genotypes—is observed: genotype I (strains NBRC 1802 and NBRC 1991), genotype II (strain NBRC 1803), genotype III (strains ZP 542, ZP 594, and ZP 629), and genotype IV (strain ZP 591) (strain NBRC 10990 is excluded from this comparison because ITS data are not available). Further population genetic studies should elucidate the degree of genetic distance between the Japanese and Portuguese populations of *S. kudriavzevii*.

A more detailed characterization of the newly isolated *S. kudriavzevii* strains demonstrated that they share an important phenotypic trait with their Japanese counterparts: they have low maximum growth temperatures. However, several lines of evidence suggest that the Portuguese isolates are genetically distinct from the type strain. The most striking difference is undoubtedly the presence of functional *GAL* genes in the Portuguese population. It was shown that the inability of the type strain of *S. kudriavzevii* to grow on galactose was due to extensive and ancient gradual degeneration of the entire set of genes involved in galactose utilization, rather than from a discrete mutation event limited in time that could have taken place very recently (11). Calculations taking into account the neutral mutation rates in *S. kudriavzevii* date GAL pathway degeneration to a period immediately following separation of the lineage leading to *S. kudriavzevii*. This is difficult to reconcile with the fact that the Portuguese isolates have otherwise only modest sequence divergence with respect to the type strain: for example, all *S. kudriavzevii*-specific PCR primers worked on the new isolates, albeit sometimes less efficiently, and ITS, LSU, and *MET6* sequences show only minor differences compared with those of the Japanese population.

Our results strongly support the hypothesis that adaptation to different growth temperatures is a very important factor in the ecology of this group of microorganisms because it allows species that are otherwise phenotypically indistinguishable to occupy different ecological niches in the same habitats. It can be conceived that circadian temperature changes provide a range of temperatures that suit each of the two partners of a sympatric association involving a species more adapted to grow at high temperatures and another species more adapted to grow at low temperatures. In such scenario, along a 24-h period, the superior fitness of *S. cerevisiae* during the hours of higher temperatures would be compensated by a better fitness of *S. kudriavzevii* during those periods of lower temperatures. Although it was noted long ago that *S. bayanus* and *S. cerevisiae* exhibit markedly different maximum growth temperatures (29), the possible implications of these differences for the ecology of natural populations of *Saccharomyces* (*S. cerevisiae* and *S. paradoxus*) were only recently put forward (32). In the sympatric associations described in our study, in which the species involved typically exhibit strikingly different temperature adaptations, this factor is even more likely to play an important role, because the gap between the maximum growth temperatures of the strains involved is much larger than that between

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S. cerevisiae and *S. paradoxus*. We also show that at least for the *S. kudriavzevii* strain we studied in more detail, a trait that implies an apparent decrease in fitness, like a lower maximum growth temperature, is accompanied by the ability to grow much faster at a lower temperature. Our preliminary relative fitness tests using two pairs of sympatric strains replicated the results obtained during the isolations, i.e., the species more adapted to low temperatures dominates in the mixed culture if the incubation is performed at a low temperature. This is very likely to be a general feature of *S. kudriavzevii*, which allows it not to be outgrown by the *Saccharomyces* species adapted to higher temperatures that occupy the same habitats. Another argument pointing to a strong influence of temperature adaptation in the ecology of *Saccharomyces* is the apparent absence of overlap in the geographic distribution of *S. kudriavzevii* and *S. uvarum*, the two species more adapted to low temperatures. Therefore, we propose that adaptation to different growth temperatures drives speciation within this genus, although available data does not allow us to infer the phenotype of the common ancestor of this group with respect to growth temperature. We intend to use an integrated approach involving phylogenomic, physiological, and genetic studies to investigate this and other issues related to the role of the growth temperature in speciation in *Saccharomyces*.

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