

Analysis of the constitution of the beer yeast genome by PCR, sequencing and subtelomeric sequence hybridization

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The lager brewing yeasts, *Saccharomyces pastorianus* (synonym *Saccharomyces carlsbergensis*), are allopolyploid, containing parts of two divergent genomes. *Saccharomyces cerevisiae* contributed to the formation of these hybrids, although the identity of the other species is still unclear. The presence of alleles specific to *S. cerevisiae* and *S. pastorianus* was tested for by PCR/RFLP in brewing yeasts of various origins and in members of the *Saccharomyces sensu stricto* complex. *S. cerevisiae*-type alleles of two genes, *HIS4* and *YCL008c*, were identified in another brewing yeast, *S. pastorianus* CBS 1503 (*Saccharomyces monacensis*), thought to be the source of the other contributor to the lager hybrid. This is consistent with the hybridization of *S. cerevisiae* subtelomeric sequences X and Y' to the electrophoretic karyotype of this strain. *S. pastorianus* CBS 1503 (*S. monacensis*) is therefore probably not an ancestor of *S. pastorianus*, but a related hybrid. *Saccharomyces bayanus*, also thought to be one of the contributors to the lager yeast hybrid, is a heterogeneous taxon containing at least two subgroups, one close to the type strain, CBS 380^T, the other close to CBS 395 (*Saccharomyces uvarum*). The partial sequences of several genes (*HIS4*, *MET10*, *URA3*) were shown to be identical or very similar (over 99%) in *S. pastorianus* CBS 1513 (*S. carlsbergensis*), *S. bayanus* CBS 380^T and its close derivatives, showing that *S. pastorianus* and *S. bayanus* have a common ancestor. A distinction between two subgroups within *S. bayanus* was made on the basis of sequence analysis: the subgroup represented by *S. bayanus* CBS 395 (*S. uvarum*) has 6–8% sequence divergence within the genes *HIS4*, *MET10* and *MET2* from *S. bayanus* CBS 380^T, indicating that the two *S. bayanus* subgroups diverged recently. The detection of specific alleles by PCR/RFLP and hybridization with *S. cerevisiae* subtelomeric sequences X and Y' to electrophoretic karyotypes of brewing yeasts and related species confirmed our findings and revealed substantial heterogeneity in the genome constitution of Czech brewing yeasts used in production.

Keywords: brewing yeast, *Saccharomyces pastorianus*, *Saccharomyces bayanus*, hybrid genome, molecular taxonomy

INTRODUCTION

Brewing yeasts belong to two groups: top-fermenting yeasts, closely related to *Saccharomyces cerevisiae* and used for producing ale, and bottom-fermenting yeasts,

producing lager. The lager brewing yeasts form a heterogeneous species (Vaughan-Martini & Martini, 1998), renamed *Saccharomyces pastorianus* (Vaughan-Martini & Martini, 1987). *S. pastorianus* CBS 1513 (*Saccharomyces carlsbergensis*) was isolated by Hansen in the Carlsberg breweries in the 19th century; CBS 1513 and its close derivatives are the most studied lager brewing yeasts. These strains display features that have hampered genetic studies: they are non-maters,

The EMBL accession numbers for the sequences determined in this study are AJ250961, AJ251006–AJ251012, AJ251575 and AJ251576.

they sporulate poorly and they have very low spore viability. Several studies involving cytoduction combined with recombination and specific gene cloning and analysis have demonstrated that brewing yeasts are allopolyploid and that their genomes contain parts of two related but different genomes that have undergone numerous rearrangements (Nilsson-Tillgren *et al.*, 1981; Casey, 1986a, b; reviewed by Kielland-Brandt *et al.*, 1995). The identities of the species that contributed to *S. pastorianus* are still unclear. Vaughan-Martini & Kurtzman (1985) inferred from DNA–DNA reassociation tests with all members of the *Saccharomyces sensu stricto* group (*S. cerevisiae*, *Saccharomyces bayanus*, *S. pastorianus* and *Saccharomyces paradoxus*) that lager brewing yeasts are hybrids between *S. cerevisiae* and *S. bayanus*. Another hypothesis favours the formation of hybrids between *S. cerevisiae* and *S. pastorianus* CBS 1503 (*Saccharomyces monacensis*), a brewing yeast also isolated by Hansen in the Carlsberg breweries (Lodder & Kreger-van Rij, 1952) concomitantly with CBS 1513. The basis for this proposition is the presence of single alleles of particular genes such as *HIS4*, *LEU2*, *MET2* and *ACB1* in CBS 1503 (*S. monacensis*), whereas *S. pastorianus* CBS 1513 (*S. carlsbergensis*) has two alleles for each of these genes, one identical to that in *S. cerevisiae* and one identical to that in CBS 1503 (*S. monacensis*) (Pedersen, 1983, 1986; Hansen & Kielland-Brandt, 1994; Borsting *et al.*, 1997). *S. monacensis* is represented by only one strain, CBS 1503. More recently, Andersen *et al.* (1999) inferred from PCR amplification that CBS 1503 contained *S. cerevisiae* material and therefore was an incomplete hybrid, but primer specificity and PCR product sequences were not documented.

Fujii *et al.* (1996) cloned the *ATF1* gene of *S. pastorianus* and showed a positive hybridization signal in *S. bayanus* DNA with this gene used as a probe, indicating the presence of a related gene in the latter species. Tamai *et al.* (1998) deduced from DNA hybridization studies that two chromosomes (one originating from *S. cerevisiae* and one from *S. bayanus*) co-existed in Japanese bottom-fermenting yeasts. Yamagishi & Ogata (1999) showed further that chromosomes 8, 9, 12 and 14 of a derivative of *S. bayanus* CBS 380^T were largely conserved in various bottom-fermenting yeasts including *S. pastorianus* CBS 1513 (*S. carlsbergensis*). Recent proteomic analysis with 2D gel electrophoresis confirmed the hybrid nature of the bottom-fermenting yeast genome and inferred that the second parent of these strains was a strain isolated from beer, NRRL Y-1551^T, with an unclear taxonomic status but nevertheless assigned to *S. pastorianus* (Joubert *et al.*, 2000). However, no DNA sequence data supported these observations obtained with either DNA hybridization or protein electrophoresis.

In parallel with the debate over the origin of the brewing yeast hybrid, the composition of the *Saccharomyces sensu stricto* complex of species is con-

stantly being remodelled (Vaughan-Martini & Martini, 1987; Barnett *et al.*, 1990; Naumov, 1996; Vaughan-Martini & Martini, 1998) as molecular methods increasingly challenge traditional classification (Kurtzman & Robnett, 1998). Three new yeast species were recently added to this group (Naumov *et al.*, 2000). The *S. bayanus* species was also reassessed recently and it was proposed that this taxon was heterogeneous on the basis of its rDNA sequence (Nguyen & Gaillardin, 1997).

Few molecular methods are available that allow rapid characterization of the genome of the brewing yeasts (see Meaden, 1990) and, despite intensive work examining diversity within this species, very little variability has been found (Pedersen, 1983, 1985). The study of the brewing yeast genome can be laborious due to its complexity and the lack of information on its genetics. Nonetheless, analysis of genome composition is crucial to understanding the way that yeasts, domesticated by man over centuries, have evolved. We have undertaken the characterization of the genome of brewing yeasts related to *S. pastorianus* CBS 1513 (*S. carlsbergensis*) to improve the classification of the members of this species, to unravel the diversity within this species and to gain insights into the origin of these hybrids.

METHODS

Strains and plasmids. The yeast strains used in this study are listed in Table 1. The Y' telomeric sequence on pJY5 was obtained from R. Degrée (Lavallée *et al.*, 1994). The X telomeric sequence on pXRIRV was obtained from E. Louis (Dept of Biochemistry, Oxford University, UK).

Media. Cells were grown routinely in YPD (1% yeast extract, 1% peptone, 1% glucose) at 28 °C with shaking. Standard procedures and genetic techniques were performed according to Sherman *et al.* (1986).

DNA techniques. Common DNA manipulations were performed as described by Sambrook *et al.* (1989). Restriction enzymes were purchased from Gibco-BRL and BioLabs. For cloning, the *HIS4* fragment was purified from an agarose gel using a GeneClean kit (Bio 101). The protruding ends of the PCR-amplified fragment were filled in using a mixture of T4 DNA polymerase and Klenow polymerase (Stratagene), using the buffer provided and 0.25 µM dNTP. The fragments were then ligated into the *Sma*I site of pUC19 after dephosphorylation with alkaline phosphatase (Boehringer Mannheim). Blotting of DNA onto a GeneScreen nylon membrane (DuPont) was performed according to Zimmermann & Fournier (1996). DNA–DNA hybridization was performed according to Church & Gilbert (1984) with [α -³²P]dCTP-labelled DNA probes using the Megaprime labelling kit (Amersham) or in SSC buffer containing Denhardt's solution and 50% formamide at 42 °C. Final washes were done at 65 °C in 0.1 × SSC, 0.1% SDS.

Pulsed-field gel electrophoresis. Genomic DNA in agarose plugs was prepared according to Vézinhel *et al.* (1990) with slight modifications. Chromosomes were separated using a Bio-Rad CHEF DRII or MAPPER apparatus in 0.5 × TBE running buffer at 14 °C for 24 h with pulses from 40 to 120 s in 1% Seakem GTG agarose (FMC) gels at 6 V/cm.

Table 1. List of strains used in this study

Culture collections are abbreviated as: CBS (Centraalbureau voor Schimmelcultures, Delft, The Netherlands), CCY (Czechoslovak Collection of Yeasts, Bratislava, Slovakia), DBVPG (Industrial yeast collection of the Dipartimento di Biologia Vegetale, Perugia, Italy), MUCL (Mycothèque de l'Université Catholique de Louvains, Belgium), NCYC (National Collection of Yeast Cultures, Norwich, UK) and NRRL (Agricultural Research Service Culture Collection, Peoria, USA). CBS numbers are given when available.

| Strain | Other strain designation(s) |
|---|--|
| Strains from collections | |
| <i>S. bayanus</i> CLIB 181 ^T | NRRL Y-1262 ^T (CBS 380 ^T) |
| <i>S. bayanus</i> CLIB 534 | CBS 378 |
| <i>S. bayanus</i> CLIB 255 (syn. <i>S. heterogenicus</i>) | DBVPG 6294 (CBS 425) |
| <i>S. bayanus</i> CLIB 254 (syn. <i>S. intermedius</i>) | DBVPG 6260 (CBS 1505) |
| <i>S. bayanus</i> CLIB 251 (syn. <i>S. uvarum</i>) | DBVPG 6179 (CBS 395) |
| <i>S. cerevisiae</i> CLIB 227 ^{NT} | MUCL 31497 ^{NT} (CBS 1171 ^{NT}) |
| <i>S. cerevisiae</i> CLIB 112 | YNN295 |
| <i>S. pastorianus</i> CLIB 176 (syn. <i>S. carlsbergensis</i>) | NRRL Y-12693 (CBS 1513) |
| <i>S. pastorianus</i> CLIB 180 (syn. <i>S. monacensis</i>) | NRRL Y-1525 (CBS 1508) |
| <i>S. pastorianus</i> CLIB 271 | SpK17 (CCY 21-6-1) |
| <i>S. pastorianus</i> CLIB 276 | MUCL 28282 |
| <i>S. pastorianus</i> CLIB 277 | MUCL 28283 |
| <i>S. pastorianus</i> CLIB 278 | MUCL 28284 |
| <i>S. pastorianus</i> CLIB 279 | MUCL 28285 |
| <i>S. pastorianus</i> CLIB 537 ^T | CBS 1538 ^T |
| <i>S. pastorianus</i> CLIB 281 ^T | NRRL Y-1551 ^T |
| Czech brewing strains isolated as part of this work | |
| Top 75 | CLIB 220 |
| Top 76 | CLIB 221 |
| Branik bottom 1 | CLIB 223 |
| Smichov bottom 2 | CLIB 224 |
| Trebon bottom 96 | CLIB 225 |
| Budvar H 2 | CLIB 259 |
| Holosevice B 3 | CLIB 260, NCYC 680 |
| Holosevice C 4 | CLIB 261, NCYC 681 |
| Pilsen D | CLIB 262 |
| Pilsen H7 | CLIB 263 |
| Pilsen W | CLIB 264 |
| Smichow A9 | CLIB 265 |
| Smichow H10 | CLIB 266 |
| Weiden Stephen 95 | CLIB 267 |

PCR amplification. The primers used for PCR amplification are listed in Table 2. The OLIGO primer analysis software (National Biosciences Inc.) was used for the design of the primers with the MIPS yeast genome database and the EMBL database. PCR amplifications were performed with 50 ng genomic DNA, prepared according to Hoffman & Winston (1987), using a Crocodile III or a Perkin-Elmer 9600 thermocycler. Amplification conditions were: 4 min at 94 °C, 25 or 30 cycles of 30 s at 94 °C, 30 s at the T_m of the primers, and 1 min per kb to be amplified at 72 °C, followed by 7 min at 72 °C. Appligene *Taq* polymerase (2.5 U; Oncor) was used with the buffer supplied. The PCR products were run on a 0.8% agarose gel (ICN) in 1 × TAE buffer. For the amplification of the PCR products to be sequenced, 1 U *Pfu* polymerase (Stratagene) was added to each reaction.

Purification of PCR products. PCR products were purified for

sequencing by using GELase (Epicentre) essentially according to the manufacturer's recommendations. DNA separated on a 1% low-gelling Seaplaque agarose gel (FMC) was cut out and incubated in the buffer supplied (40 mM Bis-Tris/HCl pH 6, 40 mM NaCl, 1 mM EDTA) for 1 h at room temperature. The agarose was melted at 70 °C for 20 min, incubated for 10 min at 45 °C and GELase (1 U per 600 mg agarose) was added. After a 1 h incubation at 45 °C, DNA was precipitated with ethanol, resuspended in TE and used as the template for sequencing. *Escherichia coli* tRNA was sometimes added as a carrier (10 µg ml⁻¹ final concentration) but did not interfere with the sequencing reactions.

Sequencing. Sequencing was performed essentially as described by Maftahi *et al.* (1995). Sequences were compiled and analysed using the Staden package (Dear & Staden, 1991) and FASTA (Pearson & Lipman, 1988) in the GCG

Table 2. List of primers

| Primer | Sequence (5'–3') | Origin |
|-----------|---------------------------------|---|
| HIS4-U | ACT CTA ATA GTG ACT CCG | Consensus sequence, <i>S. pastorianus</i> and <i>S. cerevisiae</i> <i>HIS4</i> gene |
| HIS4-L | AAC TTG GGA GTC AAT ACC | Consensus sequence, <i>S. pastorianus</i> and <i>S. cerevisiae</i> <i>HIS4</i> gene |
| YCL008c-U | TTC GTT GGA TGT GCC ATC G | Consensus sequence, <i>S. pastorianus</i> and <i>S. cerevisiae</i> <i>YCL008c</i> gene |
| YCL008c-L | GGA GCC ACC AAG GGA TGG | Consensus sequence, <i>S. pastorianus</i> and <i>S. cerevisiae</i> <i>YCL008c</i> gene |
| URA3-U | GCA CAG AAC AAA AAC CT | Sequence specific to <i>S. cerevisiae</i> <i>URA3</i> gene |
| URA3-L | TCA TTA CGA CCG AGA TT | Sequence specific to <i>S. cerevisiae</i> <i>URA3</i> gene |
| URA3pa-U | TTG ACT GAC TTT TCC AT | Sequence specific to <i>S. pastorianus</i> <i>URA3</i> gene |
| URA3pa-L | CCT CTT CCC ACG ATA AT | Sequence specific to <i>S. pastorianus</i> <i>URA3</i> gene |
| MET2-U | CGA AAA CGC TCC AAG AGC TGG | Consensus sequence, <i>S. pastorianus</i> and <i>S. cerevisiae</i> (Hansen & Kielland-Brandt, 1994) |
| MET2-L | GAC CAC GAT ATG CAC CAG GCA G | Consensus sequence, <i>S. pastorianus</i> and <i>S. cerevisiae</i> (Hansen & Kielland-Brandt, 1994) |
| MET10-U | ATC ACT TAT GGG TCT TT | Sequence specific to <i>S. cerevisiae</i> <i>MET10</i> gene |
| MET10-L | TTC TTC CTT TAT TAT CC | Sequence specific to <i>S. cerevisiae</i> <i>MET10</i> gene |
| MET10pa-U | TAT GGG TCT TTG GAA TC | Sequence specific to <i>S. pastorianus</i> <i>MET10</i> gene |
| MET10pa-L | TCA GGT CTC AGT TTG TT | Sequence specific to <i>S. pastorianus</i> <i>MET10</i> gene |
| NL1 | GCA TAT CAA TAA GCG GAG GAA AAG | Universal primer for amplification of the D1/D2 region of rDNA (O'Donnell, 1993) |
| NL4 | GGT CCG TGT TTC AAG ACG G | Universal primer for amplification of the D1/D2 region of rDNA (O'Donnell, 1993) |

environment (Genetics Computer Group, Madison, WI, USA).

RESULTS AND DISCUSSION

Detection of specific alleles in brewing strains

We tested for the presence of alleles specific to *S. cerevisiae* and *S. pastorianus* in brewing yeasts of diverse origins and in the *Saccharomyces sensu stricto* species (list of strains in Table 1) using the available sequences of *S. pastorianus* CBS 1513 (*S. carlsbergensis*). In order to discriminate between brewing yeasts and to identify the contributing species, we assessed the distribution of markers of other species possibly involved in the creation of the brewing yeast hybrids. Initially, *S. bayanus* CBS 380^T and *S. pastorianus* CBS 1503 (*S. monacensis*) were tested, as several authors have suggested that these species, together with *S. cerevisiae*, gave rise to such hybrids. Regions of the genome were amplified by PCR (i) with primers designed using regions that are completely identical in *S. pastorianus* and *S. cerevisiae*, and PCR products were then analysed for RFLP, and (ii) with primers designed from divergent sequences of homologous genes from the two species, and the sizes of the PCR products were monitored.

Primers common to CBS 1513 and *S. cerevisiae* were designed from sequences within the *HIS4* (Porter *et al.*, 1996) and *YCL008c* genes (EMBL accession no.

Z86109). We also used the primers designed by Hansen & Kielland-Brandt (1994) to amplify the *MET2* gene and the primers designed by Nguyen & Gaillardin (1997) to amplify the rDNA non-transcribed spacer 2 (NTS2). For *MET10* and *URA3*, we designed pairs of primers specific for each of the species studied (Hansen *et al.*, 1994; Gjermansen, 1991). Pairs of primers derived from other gene sequences (*ILV2*, *YCL010c*) failed to give PCR products for at least one species. This could be due to sequencing errors or to strain sequence specificity. The primers are described in Table 2. Overall, we were able to test specific markers present on five chromosomes of *S. cerevisiae*: chromosomes III, V, VI, XIV and XII. Chromosome XII carries the rDNA repeats.

Fig. 1 shows examples of PCR amplification of part of the *URA3* gene (Fig. 1a) and RFLP restriction patterns of PCR products of part of the *HIS4* gene (Fig. 1b) for *S. cerevisiae*, *S. pastorianus* CBS 1513, *S. pastorianus* CBS 1503 and *S. bayanus* CBS 380^T. For *URA3*, a 0.9 kb PCR product was generated from *S. cerevisiae* and *S. pastorianus* CBS 1513 with primers specific for *S. cerevisiae*, whereas a 0.6 kb PCR product was obtained from *S. pastorianus* CBS 1513, *S. pastorianus* CBS 1503 and *S. bayanus* CBS 380^T with primers deduced from the *S. pastorianus* sequence (Fig. 1a). Thus, there is one allele of the *URA3* gene in all strains except *S. pastorianus* CBS 1513, in which there are two alleles, as expected (Gjermansen, 1991).

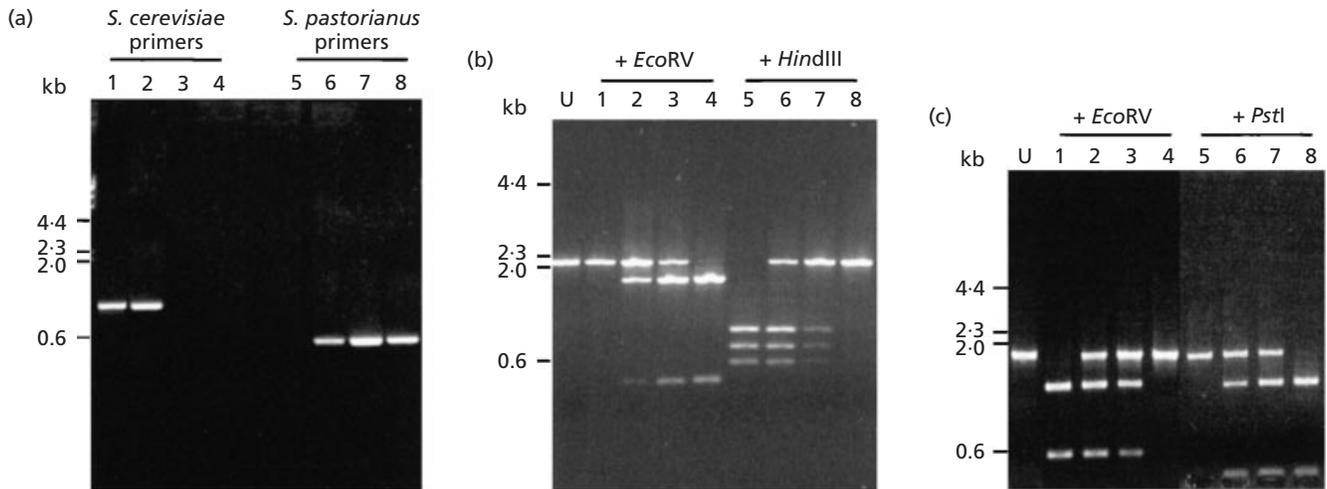


Fig. 1. PCR amplification of specific alleles of *HIS4*, *URA3* and *YCL008c* with genomic DNA from type strains as templates. Lanes: 1 and 5, *S. cerevisiae* CBS 1171^{NT}; 2 and 6, *S. pastorianus* CBS 1513 (*S. carlsbergensis*), 3 and 7, *S. pastorianus* CBS 1503 (*S. monacensis*); 4 and 8, *S. bayanus* CBS 380^T. (a) Lanes: 1–4, PCR amplification with primers URA3-U and URA3-L, specific for the *S. cerevisiae* *URA3* gene; 5–8, PCR amplification with primers URA3pa-U and URA3pa-L, specific for the *S. pastorianus* CBS 1513 *URA3* gene. (b) Lanes: PCR amplification with primers *HIS4*-U and *HIS4*-L followed by digestion of the PCR products with *EcoRV* (lanes 1–4) or *HindIII* (lanes 5–8). Lane U contains the undigested PCR product, included as a control. (c) PCR amplification with primers YCL008c-U and YCL008c-L followed by digestion of the PCR products with *EcoRV* (lanes 1–4) or *PstI* (lanes 5–8). Lane U contains the undigested PCR product, included as a control. Positions of molecular mass markers are indicated on the left.

Table 3. Distribution of specific alleles in various *Saccharomyces* species

Sc, Sp and Su correspond to sequences identical to those in *S. cerevisiae*, *S. pastorianus* (as *S. bayanus*) and *S. uvarum*, respectively. The reference sequences are that of *S. cerevisiae* (from servers SGD and MIPS) and the published sequence of *S. pastorianus* CBS 1513 (*S. carlsbergensis*) as described in the text and present in NCBI and EMBL databases except for the partial *S. pastorianus* CBS 1513 (*S. carlsbergensis*) *URA3* gene, which was resequenced in this work (accession no. AJ251006). Accession numbers of sequences determined during this work are given in parentheses. The presence of the X and Y' sequences is indicated as: –, absent; +, present; + + +, present in large numbers. n.a., Not available.

| Marker | <i>S. cerevisiae</i> chromosome | Comparison | Allele size (kb) | <i>S. cerevisiae</i> CBS 1171 ^{NT} | <i>S. pastorianus</i> CBS 1513 (<i>S. carlsbergensis</i>) | <i>S. pastorianus</i> CBS 1503 (<i>S. monacensis</i>) | <i>S. bayanus</i> CBS 380 ^T | <i>S. bayanus</i> CBS 395 (<i>S. uvarum</i>) |
|--------------------|---------------------------------|----------------------------------|----------------------------------|---|---|---|--|--|
| <i>ScHIS4</i> | III | Restriction profile and sequence | 2.1 | Sc | Sc | Sc (AJ250961) | | |
| <i>SpHIS4</i> | 2.1 | | | Sp | | Sp (AJ251575) | | |
| <i>SuHIS4</i> | 2.1 | | | | | | Su (AJ251576) | |
| <i>ScYCL008c</i> | III | Restriction profile and sequence | 1.6 | Sc | Sc | Sc | | |
| <i>SpYLC008c</i> | 1.6 | | | Sp | Sp | Sp (sequence n.a.) | | |
| <i>SuYLC008c</i> | 1.6 | | | | | | Su (AJ251012) | |
| <i>ScMET10</i> | VI | Amplification and sequence | 1.7 | Sc | | | | |
| <i>SpMET10</i> | 1.1 | | | Sp | Sp | Sp (AJ251008) | | |
| <i>SuMET10</i> | 1.7 | | | | | | Su (AJ251009) | |
| <i>ScURA3</i> | V | Amplification and sequence | 0.9 | Sc | Sc | | | |
| <i>SpURA3</i> | 0.5 | | | Sp | Sp (AJ251006) | Sp (AJ251007) | | |
| <i>ScMET2</i> | XIV | | Restriction profile and sequence | 0.6 | Sc | Sc | | |
| <i>SpMET2</i> | 0.6 | | | Sp | Sp | | | |
| <i>SuMET2</i> | 0.6 | | | | | | Su | |
| rDNA 26S D1/D2 | XII | Sequence | 0.6 | Sc | | | | Su |
| rDNA 26S D1/D2 | 0.6 | | | Sp | Sp* | Sp | Sp | |
| rDNA NTS2 type I | XII | Restriction profile† | 1.3 | Sc | | | | Su |
| rDNA NTS2 type II | 1.3 | | | | | | Sp | Su |
| rDNA NTS2 type III | 1.3 | | | | Sp | Sp | Sp | |
| X sequence | All | Hybridization | – | +++ | +++ | +++ | + | – |
| Y' sequence | Most | Hybridization | – | +++ | +++ | +++ | + | – |

* The sequences of CBS 1513 and CBS 380^T (this work, not shown) and the published sequence of *S. pastorianus* NRRL Y-1551^T (Kurtzman & Robnett, 1998) are identical. The CBS 1503 sequence displays a 1 bp mismatch relative to these sequences (this work, not shown).

† Data taken from Nguyen & Gaillardin (1997).

For *HIS4*, an identical 2.1 kb PCR fragment was obtained in all strains (Fig. 1b, lane U). In order to distinguish between the *HIS4* alleles in *S. pastorianus*, the 2.1 kb PCR product was digested with *EcoRV*. Two fragments, of around 1.7 and 0.5 kb, were expected from the *S. pastorianus HIS4* gene sequence, whilst the 2.1 kb PCR product amplified from the *S. cerevisiae* allele was expected to remain intact, since no *EcoRV* site exists in the published sequence. *HindIII* digestion was expected to cleave the *HIS4* PCR product amplified from the *S. cerevisiae* allele into three fragments, of about 0.8, 0.7 and 0.6 kb. The expected patterns were obtained for *S. cerevisiae* (Fig. 1b, lanes 1 and 5). In *S. bayanus* CBS 380^T, two fragments, of around 1.7 and 0.5 kb, were obtained after *EcoRV* digestion (lane 4), but no *HindIII* site was present in the 2.1 kb fragment (lane 8). The more complex patterns obtained with *S. pastorianus* CBS 1513 (lanes 2 and 6) and *S. pastorianus* CBS 1503 (lanes 3 and 7) can be explained as a combination of the restriction patterns of an *S. cerevisiae*-type allele and an *S. bayanus*-type allele.

These results indicate that, as in *S. pastorianus* CBS 1513, both the *S. cerevisiae*-type and *S. bayanus*-type alleles are present in *S. pastorianus* CBS 1503 (*S. monacensis*). This prompted us to test other markers and, in particular, the *YCL008c* gene located at kb 105–107 of chromosome III, in a 10 kb region sequenced by T. Andersen and T. Nilsson-Tillgren (accession no. Z86109). We obtained a result similar to that obtained with the *HIS4* alleles. A 1.6 kb fragment was generated from all strains by amplification with one pair of primers (Fig. 1c, lane U). In *S. cerevisiae*, the *EcoRV* digestion yielded two fragments, of 1.1 and 0.5 kb, whereas *PstI* digestion left the 1.6 kb DNA fragment intact, since it does not contain a *PstI* site (Fig. 1c, lanes 1 and 5). In *S. bayanus*, the expected *S. pastorianus EcoRV* restriction pattern was obtained: two fragments, of 1.2 and 0.3 kb, plus another fragment of 140 kb, too small to be visible under the gel migration conditions used in Fig. 1. As with *HIS4*, *S. pastorianus* CBS 1513 and CBS 1503 displayed restriction patterns corresponding to a combination of the *S. cerevisiae* and *S. bayanus* patterns.

The results concerning the distribution of alleles in the four strains are summarized in Table 3. We obtained the same results as Hansen & Kielland-Brandt (1994) with *MET2*. PCR products of 0.6 kb, distinguishable by their restriction patterns and specific to *S. cerevisiae* and *S. pastorianus* CBS 1503, were found in *S. pastorianus* CBS 1513, whereas *S. bayanus* CBS 380^T and *S. bayanus* CBS 395 (*Saccharomyces uvarum*) contained a third, different *MET2* allele (Table 3). For *MET10*, a PCR product of about 1.7 kb was obtained from *S. cerevisiae* with *S. cerevisiae*-specific primers, whereas a PCR product of about 1.1 kb was obtained with the *S. pastorianus*-specific primers. The sizes of the PCR products were as expected from the published sequence. Interestingly, a single 1.1 kb PCR product was obtained from *S. pastorianus* CBS 1513 with each

of the two pairs of primers used, suggesting that this strain harbours only the *S. pastorianus* allele.

Sequence analysis of specific alleles in *Saccharomyces* species

The presence of two different *HIS4* alleles in *S. pastorianus* CBS 1503 (*S. monacensis*) suggested that this strain is a hybrid. We decided to confirm that sequences originating from *S. cerevisiae* were present in *S. pastorianus* CBS 1503. The mixture of the two 2.1 kb PCR fragments obtained from *S. pastorianus* CBS 1503 using the *HIS4* primers (see Fig. 1b, lane U) was digested with *EcoRV*. This restriction enzyme cleaves the *S. pastorianus* allele but does not cleave the *S. cerevisiae* allele. The resulting fragments were separated on an agarose gel (Fig. 1b, lane 3). The remaining 2.1 kb PCR fragment was then purified from the gel, end-repaired and inserted into pBluescript. The sequence of 392 bp of one of the PCR products was determined (accession no. AJ250961) and compared to the published *S. cerevisiae* sequence. There were only two mismatches in the *S. pastorianus* CBS 1503 sequence: a C replaced a G in the promoter region at position –207 and a G replaced a T in the coding region at position +32 with respect to the start ATG. The replacement at +32 modifies the third base of the codon to give a conserved substitution: Asp in *S. cerevisiae* and Glu in CBS 1503. This 99.5% identity shows clearly that the sequenced *S. pastorianus* CBS 1503 (*S. monacensis*) fragment originated from *S. cerevisiae*, since the sequence identity of the same region between *S. cerevisiae* and *S. pastorianus* was only 81.5% (data not shown). Using PCR/RFLP, we showed that the *S. cerevisiae YCL008c* allele was present in *S. monacensis*. The *HIS4* and *YCL008c* genes are both on the left arm of *S. cerevisiae* chromosome III, at kb 65–68 and 105–107, respectively, suggesting that a stretch of *S. cerevisiae* chromosome spanning 40 kb is present in *S. monacensis*. Yamagishi & Ogata (1999) hinted that *S. cerevisiae* material was present in *S. pastorianus* CBS 1503 (*S. monacensis*). Andersen *et al.* (1999) also suggested that this strain was a hybrid. We show here by sequence analysis and PCR/RFLP that this clearly is the case.

We analysed the sequence of the *HIS4* gene in other related yeasts to gain insight into the origin of other contributors to the bottom-brewing yeasts, using the two primers defined for *S. cerevisiae* and *S. pastorianus* (Table 2). The species *S. bayanus* was shown to be heterogeneous (Nguyen & Gaillardin, 1997). Two subgroups were found in this taxon: one subgroup represented by *S. bayanus* CBS 395 (syn. *S. uvarum*) and the other represented by the type strain, *S. bayanus* CBS 380^T. Genomic DNA from two cultures of the *S. pastorianus* type strain, NRRL Y-1551^T, obtained from the ARS (Peoria, IL, USA), and CBS 1538^T, obtained from the CBS (Delft, The Netherlands), and from diverse *S. bayanus* strains, CBS 380^T, CBS 378, CBS 1505 (syn. *Saccharomyces intermedius*), CBS 425 (syn. *Saccharomyces heterogenicus*) and CBS 395 (*S.*

Table 4. Percentage DNA sequence identity between alleles in various *Saccharomyces* species

The reference sequences are described in the legend to Table 3. The accession numbers of each sequence determined in this study are indicated in Table 3 and in the text. NA, Not available.

| Allele | <i>S. pastorianus</i> | <i>S. bayanus</i> CBS 380 ^T | <i>S. bayanus</i> CBS 395 (<i>S. uvarum</i>) |
|--|-----------------------|--|---|
| <i>HIS4</i> | | | |
| <i>S. cerevisiae</i> | 79.2 | 79.2 | 78.4 |
| <i>S. pastorianus</i> | 100 | 100 | 93.7 |
| <i>S. bayanus</i> CBS 380 ^T | – | 100 | 93.7 |
| <i>MET10</i> | | | |
| <i>S. cerevisiae</i> | 77.7 | 77.7 | 79.2 |
| <i>S. pastorianus</i> | 100 | 100 | 92 |
| <i>S. bayanus</i> CBS 380 ^T | – | 100 | 92 |
| <i>YCL008c</i> | | | |
| <i>S. cerevisiae</i> | 77.2 | NA | 76.2 |
| <i>S. pastorianus</i> | 100 | NA | 93.4 |
| <i>S. bayanus</i> CBS 380 ^T | – | NA | NA |
| <i>URA3</i> | | | |
| <i>S. cerevisiae</i> | 82.2 | 82.2 | NA |
| <i>S. pastorianus</i> | 100 | 100 | NA |
| <i>S. bayanus</i> CBS 380 ^T | – | 100 | NA |
| <i>MET2</i> | | | |
| <i>S. cerevisiae</i> | 82 | 81.9 | 81.9 |
| <i>S. pastorianus</i> | 100 | 92.8 | 92.8 |
| <i>S. bayanus</i> CBS 380 ^T | – | 100 | 100 |

uvarum), was used as PCR templates with the *HIS4* primers. A PCR amplification product of a size similar to that obtained from *S. cerevisiae*, *S. pastorianus* and *S. bayanus*, i.e. 2.1 kb, was obtained for all of these strains. Some 375 bp of the 5' end of each of the PCR products was sequenced. The first strand was sequenced and a second primer, deduced from the 3' end sequence, was used to sequence the second strand. Except for CBS 395 (*S. uvarum*), which was found to be clearly divergent from *S. pastorianus*, and CBS 425, which had four bases deleted compared with *S. pastorianus*, the sequences of all the strains tested were 100% identical to the *S. pastorianus* sequence (see Table 3; CBS 380^T accession no. AJ251575 and data not shown). These results show that a group comprising *S. pastorianus* CBS 1513 (*S. carlsbergensis*), *S. pastorianus* CBS 1503 (*S. monacensis*), *S. bayanus* CBS 380^T and several *S. bayanus* strains is defined by an identical *HIS4* sequence. This indicates that the lager brewing yeasts, *S. pastorianus*, tested here share a sequence that is conserved in several *S. bayanus* strains and that they have a common ancestor.

Surprisingly, the sequence of *S. bayanus* CBS 395 (accession no. AJ251576) was 93.7% identical to that of *S. pastorianus* and 79.2% identical to that of *S. cerevisiae*. This clearly distinguished the *S. bayanus* subgroup represented by CBS 395 (*S. uvarum*) from the other *S. bayanus* strains. Thus, four groups of strains, defined by *S. cerevisiae*, *S. pastorianus*, *S.*

bayanus and *S. bayanus* CBS 395 (*S. uvarum*), can be differentiated on the basis of the *HIS4* sequence.

We amplified and sequenced further alleles from these strains (summarized in Table 4). Partial sequences of the *MET10* alleles were obtained from PCR products amplified from *S. bayanus* CBS 380^T (accession no. AJ251008) and *S. bayanus* CBS 395 (accession no. AJ251009). The sequence from *S. bayanus* CBS 380^T was identical to the published *S. pastorianus* sequence, whereas the CBS 395 sequence was 92% identical to that of *S. pastorianus* and 79.2% identical to that of *S. cerevisiae* over a 673 bp stretch, consistent with the result obtained for the *HIS4* gene. A total of 429 bp of the 5' end of the *URA3* gene PCR product amplified from *S. bayanus* CBS 380^T (accession no. AJ251007) was 100% identical to the *S. pastorianus* sequence. These sequences could not be compared to that of the CBS 395 *URA3* allele because no amplification product was obtained, suggesting that the CBS 395 sequence is divergent from that of *S. bayanus* CBS 380^T. For *YCL008c*, the allele from CBS 395 (*S. uvarum*) (accession no. AJ251012) displayed 93.4% identity to that of *S. pastorianus* and 76.2% identity to that of *S. cerevisiae* over a 440 bp stretch. We were unable to sequence the *S. bayanus* CBS 380^T allele directly. However, we were able to amplify and sequence the same fragment from another *S. bayanus* strain, CBS 1505 (*S. intermedius*), the sequence of which was identical to that of *S. pastorianus*. Thus, the *S.*

Table 5. Presence of alleles specific to *S. cerevisiae* and *S. pastorianus* in various *S. pastorianus* strains

The presence of an allele of each of the markers is indicated by Sc, Sp and Su for *S. cerevisiae*-type, *S. pastorianus*-type and *S. uvarum*-type with respect to the origin of the alleles, as defined in the text and Tables 3 and 4. ND, Not determined.

| Strain | <i>HIS4</i> | <i>YCL008c</i> | <i>MET2</i> | <i>URA3</i> | <i>MET10</i> | D1/D2 (rDNA) | NTS2 (rDNA) |
|--------------------------|-------------|----------------|-------------|-------------|--------------|--------------|-------------|
| MUCL 28282 | Sp | Sp | Sc/Sp | Sc/Sp | Sp | ND | Sp |
| MUCL 28283 | Sc/Sp | Sc/Sp | Sc/Sp | Sp | Sp | ND | Sp |
| MUCL 28284 | Sp | Sp | Sc/Sp | Sc/Sp | Sc/Sp | ND | Sc |
| MUCL 28285 | Sc/Sp | Sc/Sp | Sc/Sp | Sc/Sp | Sc/Sp | Sc | Sc |
| 14 Czech strains | Sc/Sp | Sc/Sp | Sc/Sp | Sc/Sp | Sc/Sp | ND | Sc |
| NRRL Y-1551 ^T | Sp | Sp | Su | ND | ND | Sp | Sp |
| CBS 1538 ^T | Sp | Sp | Sc/Sp | ND | ND | ND | Sp |

pastorianus-specific and *S. bayanus*-specific sequences tested are identical, whereas the sequences from *S. bayanus* CBS 395 (*S. uvarum*) are clearly different, systematically displaying 6–8% divergence from the sequences of the group composed of *S. bayanus* and *S. pastorianus* and 21–24% divergence from the *S. cerevisiae* group. This indicates that *S. bayanus* and *S. bayanus* CBS 395 (*S. uvarum*) diverged more recently.

These results nevertheless contradict both the sequence identity of the *MET2* genes in *S. bayanus* CBS 380^T and *S. bayanus* CBS 395 (*S. uvarum*) and their divergence from the *S. pastorianus* counterpart as demonstrated by Hansen & Kielland-Brandt (1994). We looked for *S. bayanus* strains with *S. pastorianus* characteristics by amplifying and sequencing the *MET2* gene. We found that *S. bayanus* CBS 1505 (*S. intermedius*) and *S. bayanus* CBS 425 (*S. heterogenicus*) carry an *S. pastorianus*-type *MET2* gene, with sequences identical to that of *S. pastorianus* over 447 and 430 bp, respectively (data not shown). Strains such as CBS 1505 and CBS 425 are therefore good candidates for having been involved in the formation of the modern lager hybrids. Transfer of DNA material must have occurred between *S. bayanus* CBS 380^T and *S. bayanus* CBS 395, as the *S. pastorianus*-type *MET2* allele can be found in some *S. bayanus* strains and in the hybrid lager strains; the allele found in *S. bayanus* CBS 1505 and CBS 425 and in *S. pastorianus* may have been replaced recently in some *S. bayanus* strains, including CBS 380^T, by the allele found in CBS 395 (*S. uvarum*). This correlates with the presence of two types of rDNA unit in *S. bayanus* CBS 380^T, proposed by Nguyen & Gaillardin (1997), one related to that of *S. pastorianus*, the other to that of CBS 395 (*S. uvarum*).

Distribution of *S. cerevisiae* and *S. pastorianus* alleles in brewing yeast strains

Several articles from the Carlsberg laboratory (Holmberg, 1982; Pedersen, 1983, 1985, 1986; Nilsson-Tillgren *et al.*, 1986) have described the analysis of the restriction patterns of markers in brewing yeasts of various origins. The distribution of *HIS4*, *LEU2* and rDNA markers seems to point towards very little variability within the group of bottom-fermenting

strains. We therefore used a combination of PCR and PCR/RFLP to test the distribution of alleles of *HIS4*, *YCL008c*, *MET2*, *URA3*, *MET10* and rDNA NTS2 in various yeasts isolated from beer. Fourteen strains originating from the Czech Republic, most of them being used in lager beer production, four lager yeast strains originating from Munich (Germany) obtained from the Mycothèque de l'Université Catholique de Louvains, Belgium (MUCL), and two *S. pastorianus* strains were tested.

The results of identification of different alleles are shown in Table 5. All of the 14 Czech strains contained both alleles of each marker tested as *S. pastorianus* with the exception of the rDNA marker (data not shown). These strains carry the *S. cerevisiae*-type allele of NTS2, as most of the strains originating from Germany (Pedersen, 1983, 1986) and the 14 brewing yeast strains from the Czech Republic cannot be distinguished in this way. This may be because they all have the same origin. On the other hand, the four MUCL strains and *S. pastorianus* CBS 1513 and CBS 1503 could be differentiated with all the markers tested. MUCL 28282 and MUCL 28283, isolated in German breweries, carry the *S. pastorianus*-type NTS2 sequence. This analysis, using the *HIS4*, *YCL008c*, *MET10* and *URA3* genes, therefore reveals an unexpected allele distribution within yeasts of the same origin. In addition, by analysing the RFLP patterns of rDNA NTS2 and the 26S rDNA sequence, we were able to classify these strains into two groups corresponding to *S. cerevisiae*-type units and *S. pastorianus*-type units (Table 5). Some methods rely only on rDNA polymorphism to differentiate brewing and non-brewing yeast strains (Yamagishi *et al.*, 1999). We consider, however, as exemplified here, that differentiation of hybrid strains based on rDNA polymorphism can be misleading when used alone.

Distribution of the *S. cerevisiae* subtelomeric sequences in brewing yeasts

In order to characterize the brewing yeast genome further, we applied rapid typing methods. The most useful typing methods devised for *S. cerevisiae* rely on chromosome length polymorphism assessed by electro-

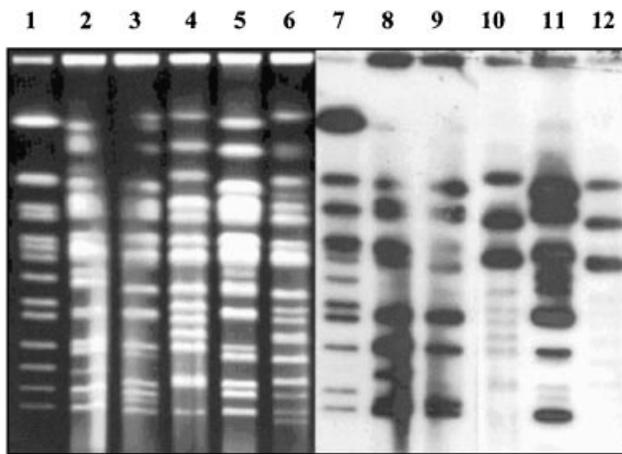


Fig. 2. Hybridization of the *S. cerevisiae* subtelomeric Y' sequence to electrophoretic karyotypes of various strains of *Saccharomyces* species. Lanes: 1–6, electrophoretic karyotypes; 7–12, hybridization of the *S. cerevisiae* subtelomeric Y' sequence. DNA from the following strains was used: *S. cerevisiae* YNN295 (lanes 1 and 7), *S. pastorianus* CBS 1513 (2 and 8), *S. monacensis* CBS 1503 (3 and 9), *S. pastorianus* NRRL Y-1551^T (4 and 10), *S. pastorianus* CBS 1538^T (5 and 11) and *S. bayanus* CBS 425 (6 and 12).

phoretic karyotypes and RFLP of mitochondrial (mt)DNA (Vézinhét *et al.*, 1990). Brewing yeast electrophoretic karyotypes can be quite complex and difficult to analyse (Fig. 2). Aigle *et al.* (1984) have shown that mtDNA of brewing yeast is poorly polymorphic and we confirmed this observation for the 14 Czech brewing strains: we detected only one polymorphic *EcoRV* fragment in the mtDNA of three Czech strains (CLIB 262, CLIB 263, CLIB 264) and MUCL 28282 and MUCL 28283 (data not shown).

Because of their repetitive nature, telomeric and subtelomeric sequences have been very useful for typing wine strains of *S. cerevisiae* by hybridization (Lavallée *et al.*, 1994). *S. cerevisiae* contains two types of repeated subtelomeric sequences: Y' sequences show sequence similarity to helicases and are present on most chromosome ends and X sequences of unknown function are present at the extremities of all chromosomes.

We used these repeated sequences to assess the presence of chromosomes or extremities of chromosomes originating from *S. cerevisiae*. The Y' sequence was hybridized to electrophoretic karyotypes of brewing yeast and various *S. bayanus* isolates. The patterns could be clearly separated in two groups: simple patterns with three bands and complex patterns with more than eight bands. The strains displaying a simple pattern, with three bands of 1200, 1000 and 750 kb, exemplified by CBS 425 (Fig. 2, lanes 6 and 12), all belonged to *S. bayanus* (Nguyen *et al.*, 2000). The same bands also hybridize to the X sequence, as a fourth band of about 600 kb was revealed by the X sequence probe on an electrophoretic karyotype of *S. bayanus*

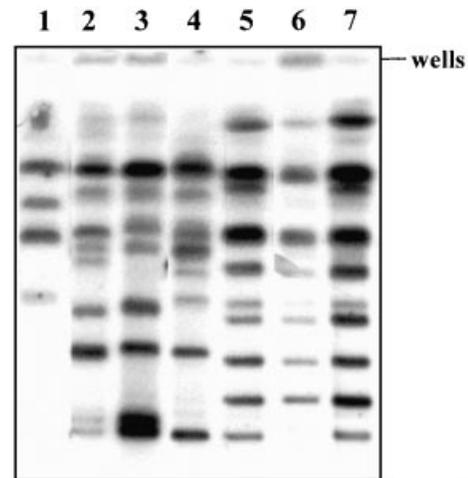


Fig. 3. Hybridization of the *S. cerevisiae* subtelomeric X sequence to electrophoretic karyotypes of various brewing strains. Lanes: 1, *S. bayanus* CBS 380^T; 2, *S. pastorianus* CBS 1513 (*S. carlsbergensis*); 3, *S. pastorianus* CBS 1503 (*S. monacensis*); 4, MUCL 28282; 5, MUCL 28284; 6, CLIB 220; 7, CLIB 221.

(Fig. 3). The detection of a larger number of chromosomal bands with the X sequence than with the Y' sequence was expected, since this type of sequence is present in all *S. cerevisiae* chromosomes, whereas Y' is not (Pryde & Louis, 1997).

S. cerevisiae and bottom-fermenting yeasts display the second hybridization pattern. As shown in Fig. 2, ten strong bands and two faint bands were detected in *S. cerevisiae* YNN 295 using the Y' sequence as a probe (lanes 1 and 7). *S. pastorianus* CBS 1513 (lanes 2 and 8) and CBS 1503 (lanes 3 and 9) were in this group of strains. The same results were obtained for the MUCL strains and the Czech strains (data not shown, but see Fig. 3). CBS 1503 displayed as many hybridizing bands as did CBS 1513. This is consistent with *S. pastorianus* CBS 1503 (*S. monacensis*) being a hybrid. It is assumed that rearrangements occur in hybrid genomes, and work in the Carlsberg laboratory has demonstrated that sequences from both contributors have been rearranged, deleted or duplicated (Kielland-Brandt *et al.*, 1995). *S. pastorianus* CBS 1503 (*S. monacensis*) has apparently lost *ScMET2*, *ScMET10* and *ScURA3*. As X and Y' sequences are present on all of the *S. cerevisiae* chromosome ends, the number of bands detected should reflect the number of chromosomes originating from that yeast in the hybrids. Possibly, subtelomeric sequences from *S. cerevisiae* have been retained preferentially in the hybrids, in contrast to other sequences, or reamplified subsequently on non-*S. cerevisiae* chromosomes through telomere–telomere recombination associated with gene conversion (Wang & Zakian, 1990; Teng & Zakian, 1999).

Our results therefore rule out the hypothesis that the *S.*

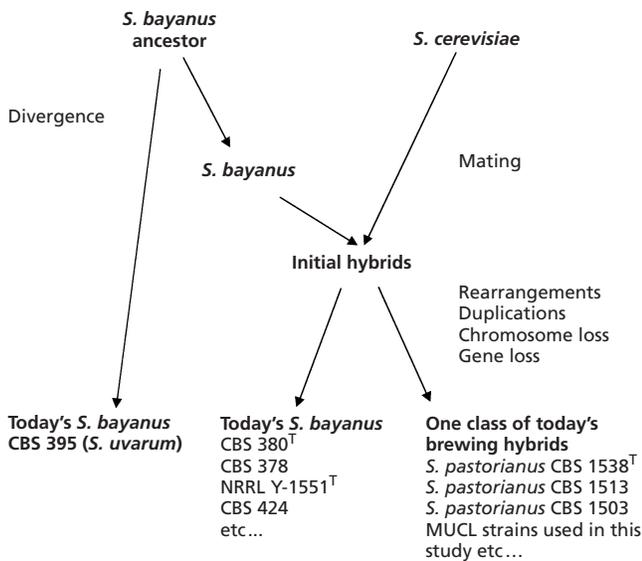


Fig. 4. Schematic representation of the formation of hybrids isolated from beer.

pastorianus hybrids arose from a single, rare mating between *S. cerevisiae* and *S. monacensis* (Pedersen, 1986; Hansen & Kielland-Brandt, 1994). Our analysis of the origin of several genes in brewing yeasts shows clearly that many strains isolated from beer are complex hybrids, like *S. pastorianus* strains. The complete identity of several DNA sequences from the *S. pastorianus* and *S. bayanus* strains tested, including *HIS4*, *YCL008c*, *URA3* and *MET10*, substantiates the idea that a large number of *S. bayanus* strains on the one hand and bottom-fermenting yeasts on the other have a common ancestor.

Recently, natural isolates, involved in wine making and cider making, were found to carry hybrid genomes containing *S. cerevisiae* and *S. bayanus* material (Masneuf *et al.*, 1998; Groth *et al.*, 1999), indicating that the formation of hybrids in the fermentation industry is not restricted to beer making. The use of the *MET2* gene sequence did not indicate to which subgroup within the *S. bayanus* species one of the non-*S. cerevisiae* parents belonged. It was shown recently that *S. bayanus* CBS 380^T and CBS 378 carry a sequence identical to that of *S. cerevisiae* Y' (Nguyen *et al.*, 2000). A large number of *S. bayanus* strains and all of the *S. pastorianus* strains tested here hybridized to Y' sequences and could be considered hybrids, although no *S. cerevisiae* sequences other than Y' were detected in the *S. bayanus* strains. Strains like CBS 1505 (*S. intermedius*) that do not hybridize to the Y' sequence were shown to display a mixed rDNA RFLP profile (Nguyen *et al.*, 2000). This, together with our results, suggests that a large number of hybrids might result from exchanges between *S. cerevisiae* and an ancestor common to the group of *S. bayanus* strains, followed by subsequent rearrangements and redistribution of both divergent genomes (Fig. 4).

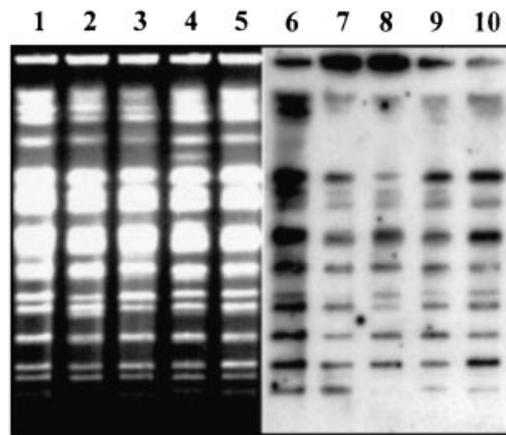


Fig. 5. Hybridization of the *S. cerevisiae* subtelomeric X sequence to electrophoretic karyotypes of various Czech brewing strains. Lanes: 1–5, electrophoretic karyotypes; 6–10, hybridization of the *S. cerevisiae* X sequence. DNA from the following strains was used: CLIB 261 (lanes 1 and 6), CLIB 224 (2 and 7), CLIB 225 (3 and 8), CLIB 260 (4 and 9) and CLIB 261 (5 and 10).

Surprisingly, the isolate *S. pastorianus* NRRL Y-1551^T, obtained from the ARS collection, possessed a simple three-band Y' pattern (Fig. 2, lanes 4 and 10) and an electrophoretic karyotype similar to that of CBS 380^T. This was unexpected, because this strain was chosen as the representative of the hybrid species when various brewing yeasts were gathered under the generic name *S. pastorianus* (Vaughan-Martini & Martini, 1987). We tested a second specimen of this strain, conserved at the CBS as CBS 1538^T. It displayed the complex Y' hybridization pattern expected for a brewing yeast (Fig. 2, lanes 5 and 12). This result shows that the two specimens of the type strain of *S. pastorianus*, NRRL Y-1551^T and CBS 1538^T, are different (compare lanes 10 and 11), consistent with the different proteomic patterns obtained by Joubert *et al.* (2000) for the two strains. The Y' pattern of NRRL Y-1551^T is undistinguishable from that of *S. bayanus* (compare lanes 10 and 12). NRRL Y-1551^T carries the *S. bayanus*-type *MET2* allele whereas CBS 1538^T has a typical *S. pastorianus* *MET2* pattern, showing the presence of both *S. cerevisiae* and *S. pastorianus* alleles. Previous results published for these two strains should clearly be reassessed in light of these findings.

Hybridization with the *S. cerevisiae* X sequence probe can differentiate lager strains of various origins (Fig. 3). We attempted to differentiate the strains originating from the Czech Republic, which appeared indistinguishable according to specific allele distribution. We observed few differences between the electrophoretic karyotypes of these strains (Fig. 5 lanes 1–5; compare lanes 2 and 3, for instance). The differences were clearly greater between the hybridization patterns obtained with the *S. cerevisiae* X sequence (Fig. 5, lanes 6–10). In addition, the relative intensity of the

signal for bands of the same size varied according to the strain. Among the 14 strains tested, we could define five groups on the basis of the presence or absence of a signal and its relative intensity. Note that karyotypic variations and hybridization variations were not correlated.

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