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Biological diversity of *Saccharomyces* yeasts of spontaneously fermenting wines in four wine regions: Comparative genotypic and phenotypic analysis

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ABSTRACT

Combination of molecular genetic analysis (karyotyping, PCR-RFLP of *MET2*, the ITS1-ITS2 region and the NTS region) and physiological examination (melibiose and mannitol utilization, sugar-, ethanol- and copper tolerance, killer activity, fermentation vigor and production of metabolites) of yeasts isolated from spontaneously fermenting wines in four wine regions revealed very high diversity in the *Saccharomyces cerevisiae* populations. Practically each *S. cerevisiae* isolate showed a unique pattern of properties. Although the strains originating from the same wine were quite similar in certain traits, they showed diversity in other properties. These results indicate that alcoholic fermentation in grape wines is performed by highly diverse yeast consortia rather than by one or two dominating strains. The less frequent *Saccharomyces uvarum* strains were less diverse, showed lower karyotype variability, were Mel^+, Man^+, more sensitive to 60% sugar, and ethanol or copper in the medium. They produced less acetic acid and fermented better at 14 °C than most of the *S. cerevisiae* isolates, but certain *S. cerevisiae* strains showed comparably high fermentation rates at this temperature, indicating that it is not a general rule that *S. uvarum* ferments better than *S. cerevisiae* at low temperatures. The segregation of certain traits (melibiose utilization, mannitol utilization and copper resistance) in both species indicates that the genomes can easily change during vegetative propagation. The higher diversity among the *S. cerevisiae* isolates suggests that the *S. cerevisiae* genome may be more flexible than the *S. uvarum* genome and may allow more efficient adaptation to the continuously changing environment in the fermenting wine.

1. Introduction

It has become generally accepted among wine scientists that the quality of wine is determined both by the quality of the grape juice and by the evolution and activity of the microbial communities during fermentation and vinification. In spontaneous fermentation there is an early and rapid succession of several yeast species which rarely belong to *Saccharomyces*; but later the rapidly increasing level of ethanol limits the growth and activity of almost all non-*Saccharomyces* yeasts (for a recent review see Fleet, 2008).

The conversion of the grape must into wine is almost exclusively performed by the more alcohol-tolerant species of *Saccharomyces*, especially by the principal wine yeast *Saccharomyces cerevisiae*. Another, but by far less common species is the cryotolerant *Saccharomyces uvarum* (synonym: *Saccharomyces bayanus* var. *uvarum*) characterized by its ability to ferment well at low temperatures, but also by its potential to release desirable flavor components (reviewed in Sipiczki, 2002).

Several investigations indicate that naturally fermenting *Saccharomyces* populations can vary from location to location and from year to year (e.g. Tini et al., 1979; Grando and Colato, 1994; Versavaud et al., 1995; Pramateftaki et al., 2000; Antunovics et al., 2005; Combina et al., 2005; Schuller et al., 2005; Blanco et al., 2006; Gonzalez et al., 2006; Lopandic et al., 2008).

Most reports on the composition of wine-fermenting yeast populations analysed single-cell colonies by using one or two of molecular methods such as RFLP of genomic DNA or mitochondrial DNA, microsatellite analysis, delta sequences or karyotyping to detect diversity (for a review see Schuller et al., 2004). These analyses lead to the conclusion that alcoholic fermentation is usually dominated by one (or a very few) *Saccharomyces* strains. In this study we apply a broader approach to characterize the composition of the *Saccharomyces* populations. It combines the examination of 10 physiological properties, measuring the production of 7 secondary metabolites during microfermentation in grape must, electrophoretic karyotyping and RFLP analysis of 3 chromosomal regions. In our previous works we focused on *Saccharomyces* yeasts of Tokaj, a wine region shared by Northeast Hungary and East Slovakia, where *S. uvarum* is a regular and

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Table 1
Yeast strains used in this study.

Strain designation	Description	Origin
10–157	*Saccharomyces cerevisiae* S.288c (YGSC X4005-11A)	YGS
10–198	*Saccharomyces bayanus* CBS 380^T	CBS
10–512	*Saccharomyces uvarum* CBS 395^T	CBS
11–183	*Saccharomyces cerevisiae* K1 killer strain NCYC 232	NCYC
11–182	*Saccharomyces cerevisiae* K2 killer strain NCYC 738	NCYC
10–562	*Saccharomyces cerevisiae* S1, S2 killer-sensitive strain NCYC 1006	NCYC
2006 isolates: 1 (I.BK10), 2 (IV.BK15), 3 (V.BK11), 4 (VI.BK19), 5 (I.BKR3), 6 (II.BKR1), 7 (IV.BKR6), 8(V.BKR2)	*Saccharomyces* isolates from spontaneously fermenting "Kéknyelű" must; Németh Winery, Badacsony wine region	This study
2007 isolates: 9 (IV.BK2.16), 10 (IV.BK2.21), 11 (V.BK2.1), 12 (V.BK2.2), 13 (V.BK2.24), 14 (VIII.BK2.11), 15 (VIII.BK2.19), 16 (XI.BK2.1), 17 (XI.BK2.12), 18 (XI.BK2.18), 19 (XI.BK2.20)		
2006 isolates: 20 (II.BO10), 21 (III.BO6), 22 (III.BO22), 23 (IV.BO1), 24 (IV.BO6), 25 (VII.BO9), 26 (XBO3)	*Saccharomyces* isolates from spontaneously fermenting "Olaszrizling" must; Németh Winery, Badacsony wine region	This study
2007 isolates: 27 (II.BO2.13), 28 (IV.BO2.6), 29 (IVBO2.12), 30 (IV.BO2.20), 31 (V.BO2.1), 32 (V.BO2.3), 33 (V.BO2.8), 34 (V.BO2.20), 35 (VIII.BO2.1), 36 (VIII.BO2.3), 37 (VIII.BO2.5), 38 (VIII.BO2.7), 39 (VIII.BO2.14), 40 (XI.BO2.24), 41 (XI.BO2.25)		
2006 isolates: 42 (I.FR7), 43 (I.FR11), 44 (III.FR8), 45 (III.FR24), 46 (IV.FR4), 47 (VI.FR40), 48 (VII.FR1), 49 (X.FR42)	*Saccharomyces* isolates from spontaneously fermenting "Ezerjó" must; Frittman Brothers Ltd, Soltvadkert, Kunság wine region	This study
2007 isolates: 50 (V.FR2.14), 51 (VI.FR2.1), 52 (VII.FR2.19), 53 (VIII.FR2.5), 54 (VIII.FR2.18)		
2006 isolates: 55 (I.CF1), 56 (I.CF14), 57 (II.CF9), 58 (III.CF9), 59 (III.CF18), 60 (IX.CF25), 61 (X.CF15), 62 (XI.CFr7)	*Saccharomyces* isolates from spontaneously fermenting "Cirfandli" must; Research Institute of Viticulture and Enology, Pécs, Mecsekalja wine region	This study
2007 isolates: 63 (III.CF8t.4), 64 (III.CF8t.7), 65 (X.CF8t.8), 66 (X.CF8t.17), 67 (X.CF8t.18), 68 (III.CF20t.2), 69 (III.CF20t.14), 70 (IX.CF20t.2), 71 (X.CF20t.3), 72 (X.CF20t.5), 73 (X.CF20t.16)		
2006 isolates: 74 (I.KA5), 75 (I.KA10), 76 (II.KA10), 77 (III.KA15), 78 (XIII.KA9)	*Saccharomyces* isolates from spontaneously fermenting "Kadarka" must; Palos Miklos Winery, Szekszárd wine region	This study
2007 isolates: 79 (IV.KA2.1), 80 (IV.KA2.4), 81 (V.KA2.11), 82 (V.KA2.25), 83 (VIII.KA2.3), 84 (XI.KA2.10), 85 (XI.KA2.19), 86 (XI.KA2.27)		

significant component of the fermenting yeast populations (Miklos et al., 1994; Sipiczki et al., 2001; Naumov et al., 2002; Antunovics et al., 2003, 2005). In this work we report on the analysis of *S. cerevisiae* and *S. uvarum* strains isolated during spontaneous fermentations of musts from four white grapevine varieties and one red grapevine variety in four traditional wine-growing regions located in Southern and Central Hungary that differ from Tokaj in climate, soil and wine-making technology. We show through the comparative characterization of 86 isolates that the *S. cerevisiae* populations of certain wines are quite homogeneous in certain traits but highly diverse in other properties. The diversity is so high that virtually each strain has a unique pattern of characteristics. This finding indicates that alcoholic fermentation in grape wines is performed by highly diverse yeast consortia rather than by one or two dominating strains.

2. Materials and methods

2.1. Strains and media

Yeast strains used in this study are listed in Table 1. The agar medium YPGA (1% yeast extract, 1% peptone, 3% glucose and 2% agar; w/v) was used for wine yeast isolation and testing killer activity. For karyotyping and DNA preparation, the cultures were grown in YPGL (YPGA without agar). Utilization of carbon sources and nitrogen sources was tested on the synthetic minimal medium MMA [1% glucose, 2% agar, 0.5% $(NH_4)_2SO_4$, 0.01% KH_2PO_4, $MgSO_4$ and vitamins; w/v] (Sipiczki et al., 1982) containing 1% (w/v) of the carbon source or 0.5% (w/v) of the nitrogen source to be tested instead of glucose or $(NH_4)_2SO_4$. Melibiose fermentation was examined in YPML (YPGL containing 2% melibiose instead of glucose; w/v). Sporulation was examined in cultures grown on acetate medium (1% potassium acetate, 0.1% yeast extract, 0.05% glucose, 2% agar; w/v).

2.2. Yeast isolation and taxonomy tests

1-ml samples were taken aseptically from the fresh grape juice and from the fermenting must throughout the fermentation process (six to eight times in two to three weeks). The samples were diluted and streaked on YPGA plates to obtain individual colonies. After 4 days of incubation at 25 °C, 25 to 100 colonies were isolated randomly from the plates for taxonomic identification. The taxonomic tests used included the examination of colony and cell morphology, sporulation, utilization of carbon and nitrogen sources, growth at various temperatures (14, 20 and 37 °C) and on vitamin-free medium (van der Walt, 1970; Antunovics et al., 2005). The carbon sources used in the assimilation tests were glucose, galactose, melibiose, glycerol and mannitol. The nitrogen source was lysine. Testing the isolates for growth on these nutrients was performed in two ways: by dropping 20-μl samples of suspensions of cells (10^6 cells/ml) or by streaking loop-full amounts of the suspensions onto agar plates.

2.3. Molecular methods

Electrophoretic karyotype analysis was carried out using the BIORAD CHEF-II system as described in Antunovics et al. (2005) or the CHEF-III system according to Sipiczki et al. (2001). CHE-III was used to

achieve a better resolution in the region of large chromosomes. The relatedness of the karyotypes was determined by analyzing the banding patterns with the UPGMA (Unweighted Pair Group Method with Arithmetic mean) algorithm available at (http://www.tinet.cat/~debb/UPGMA/) (Garcia-Vallve et al., 1999).

Isolation of genomic DNA and the PCR-RFLP analysis of the chromosomal *MET2* gene and the chromosomal region spanning ITS1, ITS2 and the intervening 5.8S tRNA gene were performed as described previously (Antunovics et al., 2005). The application of the method of NTS PCR-RFLP to differentiate between *S. cerevisiae* and *S. uvarum* was described by Nguyen and Gaillardin (1997). The D1/D2 domains of the 26S rDNA were amplified with the primers NL-1 and NL-4 (O'Donnell, 1993) and sequenced with an ABI PRISM 3700 (Applied Biosystems) sequencer using the PCR primers. Sequence similarity searches and pairwise sequence comparisons were performed using the BLAST service of the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4. Killer activity tests

Killer activity tests were performed by dropping 10 µl suspensions of the strains to be tested onto the surface of agar plates seeded with cells of the killer-sensitive strain 10-562. The *S. cerevisiae* killer strains to challenge isolated wine strains were the K1-type killer 11-183 and the K2-type killer 11-182 (Table 1). Suspensions (10 µl) of both killers were dropped on the surface of agar plates seeded with the wine isolate to be tested. The medium used was YPGA supplemented with 0.003 g/l methylene blue. Killer positive activity was registered when a halo of growth inhibition was produced on the lawn of the sensitive strain, including the presence of a dark blue zone of dead cells around the edge of the inhibition zone.

2.5. Physiological tests

Fermentation of melibiose was examined in Durham tubes filled with YPML (van der Walt, 1970). Sensitivity to high concentration of sugar was tested in YPML supplemented with 60% glucose. 50 ml of medium (in 100-ml flask) was inoculated with cells (optical density at 660 nm: 0.1) from an overnight culture (in YPML) and incubated on a gyratory shaker at 25 °C for 96 h. Growth was evaluated by measuring optical density (at 660 nm). Copper and ethanol sensitivity was tested by dropping 20 µl of suspensions (10^6 cells/ml) on MMA supplemented with various concentrations of $CuSO_4$ and ethanol. Growth was evaluated after 4 days (ethanol) or 5 days (copper) of incubation at 25 °C to determine the minimal inhibitory concentrations (MICs).

2.6. Microfermentation and chemical analysis

"Aglianico del Vulture" grape must characterized by 21% (w/v) fermentable sugars, 220 mg/l of yeast assimilable nitrogen, 0.70 titratable acidity and pH 3.15 was used to determine strain fermentation performance. Fermentations were carried out in 130-ml Erlenmeyer flasks filled with 100 ml of grape must, autoclaved at 100 °C for 20 min. Each sample was inoculated with 10^6 cells/ml from a pre-culture grown for 48 h in the same grape must. The fermentations were performed at three temperatures: 14, 18 and 26 °C. The weight loss caused by carbon dioxide (CO_2) evolution was measured at the end of fermentation (when CO_2 evolution seased) and used to express fermentation power. All experiments were performed in duplicate and standard deviation between two replicates ranged between 0.7% and 0.06%. Wine samples were cooled to 4 °C to clarify the wine and stored at −20 °C until required for chemical analysis.

By-products, such as higher alcohols (*n*-propanol, isobutanol and amyl alcohols), acetaldehyde, acetoin, ethyl acetate and acetic acid were analysed by a gas chromatograph as reported by Romano et al. (2003). The data obtained were standardized and converted into adimensional values, in order to elaborate a matrix, following the criteria reported by Mauriello et al. (2009). The matrix was used for a hierarchical agglomerative cluster analysis, taking the Euclidean distance as metric and the Ward's method as amalgamation rule, and standard hierarchical clustering dendograms were derived using the SAS software.

3. Results

3.1. Yeast isolation and identification of Saccharomyces isolates

Must was prepared in four wine-growing regions (Mecsekalja, Badacsony, Kunság and Szekszárd; see map in Fig. 1) from grapes of the white cultivars Kéknyelű ("blue-stalked"), Olaszrizling (Welschriesling), Cirfandli (Zierfandler), Ezerjó ("thousand good") and the red cultivar Kadarka (Cadarka) for spontaneous (non-inoculated) fermentation in years 2006 and 2007. In the samples taken in early phases of fermentation, yeasts were found that formed colonies of diverse size, color and morphology on the plates, but as fermentation progressed, the colony morphology gradually became more homogeneous, indicating that certain types of yeasts died off or were outcompeted by other types. 2480 randomly selected colonies were subjected to taxonomic examinations. The results of the tests separated most of the non-*Saccharomyces* yeasts from putative *Saccharomyces* isolates. For example the inability to utilize lysine as a nitrogen source is frequently used for the identification of *Saccharomyces* in fermenting yeast populations (e.g. Lin, 1975; Heard and Fleet, 1986).

In samples taken on the second day of fermentation or later, the majority of the isolates (60–100%) were unable to grow on the medium which contained lysine as nitrogen source. To confirm the taxonomic position of these isolates, 25 lys^- strains were randomly selected for amplification and sequencing of the D1/D2 domains of the 26S rDNA. Consistent with the lysine utilization deficiency, the D1/D2 sequences showed 99–100% identity with the corresponding sequences of the type strains of *S. cerevisiae*, *S. uvarum* or *S. bayanus*. The D1/D2 domain sequences separate *Saccharomyces* from other yeasts but are of no use within the *Saccharomyces* genus because the sequences of the *S. bayanus* and *S. uvarum* type strains do not differ significantly and sequencing can be expensive when large groups of isolates are to be examined. The conventional taxonomic tests based on physiological properties could not be used either for assigning the *Saccharomyces* isolates to species because of the variability of certain traits (e.g. Antunovics et al., 2005). Therefore we only used these tests for characterizing intraspecies biological diversity (see below) and applied molecular methods to species identification. For the molecular tests, we randomly selected 86 lys^- isolates (listed in Table 1) representing all wines from both years.

3.2. Species identification by molecular analysis

It has been shown that the unambiguous taxonomic identification of *Saccharomyces* wine strains at species level requires combinations of several methods (e.g. Naumov et al., 2002; Dellaglio et al., 2003; Antunovics et al., 2005). Therefore we examined the 86 randomly selected *Saccharomyces* isolates by PCR-RFLP analysis of three chromosomal regions and by electrophoretic karyotyping. For PCR-RFLP, the NTS2 segment, the ITS1-5.8S-ITS2 part of the ribosomal DNA region and the chromosomal *MET2* gene were amplified and digested with restriction endonucleases. Cutting of NTS2 with *Ban*I differentiates *S. uvarum* and *S. bayanus* from *S. cerevisiae* because *Ban*I only cleaves the *S. cerevisiae* DNA (Nguyen and Gaillardin, 1997). *Hae*III digestion of the ITS region also separates the *S. uvarum–S. bayanus* pair (three fragments) from *S. cerevisiae* (four fragments) (McCullough et al., 1998). The restriction endonuclease *Pst*I cuts the *MET2* gene of *S. bayanus* and *S. uvarum*, whereas *Eco*RI cleaves the *MET2* gene of

Fig. 1. Map of Hungary showing the geographical location of the wine-growing regions in which samples were collected.

S. cerevisiae (Masneuf et al., 1996).These tests divided the isolates into two groups. The prevailing majority proved to belong to *S. cerevisiae*, whereas 8 strains (3, 9, 17, 29, 46, 68, 70 and 71) showed RFLP patterns identical with those of the type strains of *S. uvarum* and *S. bayanus* (data not shown).

To complete the taxonomic identification, we karyotyped the isolates. Seven out of the 8 isolates (3, 9, 17, 29, 68, 70 and 71) which showed non-*cerevisiae* type RFLP patterns had electrophoretic karyotypes highly similar to the karyotypes of the type strains of both *S. uvarum* and *S. bayanus* (Fig. 2B and C). All contained the diagnostic group of 4–5 medium-size chromosomes. A group of five chromosomes in the region of 460–630 kb (marked with a bar in Fig. 2B) is characteristic of both *S. bayanus* and *S. uvarum* (e.g. Sipiczki et al., 2001; Antunovics et al., 2005). Thus, its presence in the seven isolates corroborated the conclusion drawn from the PCR-RFLP results that these strains were not *S. cerevisiae*. This group of chromosomal bands was less apparent in the eighth non-*cerevisiae* strain (46) but the overall layout of the pattern was more *uvarum*/*bayanus*-like than *cerevisiae*-like.

Careful inspection of chromosomal patterns also allows the separation of the *S. bayanus* strains from the *S. uvarum* strains because the type strains of these species differ in the number of bands in the size range 225–370 kb. *S. bayanus* has three bands in this region whereas *S. uvarum* has only two bands (Nguyen and Gaillardin, 1997). Since seven of the eight non-*cerevisiae* isolates did not have the small chromosome characteristic of *S. bayanus* (marked with an arrowhead in Fig. 2B), we concluded that they belonged to *S. uvarum*. Isolate 9 had a faint band in the position of the *bayanus*-specific small chromosome but this strain appeared an interspecific chimera (possibly a partial hybrid of *S. uvarum* with another *Saccharomyces* species) because its karyotype contained at least one supernumerary band (Fig. 2B). Since the *MET2* and *NTS* sequences showed *S. uvarum* patterns, additional markers located on different chromosomes need to be examined to verify this possibility.

3.3. Karyotype diversity

Comparison of the chromosomal patterns of the isolates revealed a high degree of chromosomal polymorphism in the *S. cerevisiae* isolates that affected almost all bands. 17 classes of patterns could be clearly distinguished (Fig. 2A) with only slight differences within the classes. In the region of the smallest chromosomes (225–370 kb), 8 strains had only two bands like the *S. uvarum* strains. The *S. uvarum* karyotypes were less heterogeneous. Four strains (9, 68, 70 and 71; branch U2 in Fig. 2A) had patterns similar to the pattern found previously in the overwhelming majority of Tokaj isolates (Naumov et al., 2002; Antunovics et al., 2003, 2005). The rest of the *S. uvarum* isolates were different and formed three branches in the dendogram.

3.4. Diversity of physiological properties of the Saccharomyces isolates

Previous studies proposed that melibiose and mannitol utilization and sensitivity to 37 °C distinguish *S. uvarum* from *S. cerevisiae* because the latter cannot utilize these compounds as carbon sources and grows at 37 °C (e.g. Yarrow, 1984;Vaughan-Martini and Martini, 1993, 1998). The type strain (CBS 395) of *S. uvarum* is Mel^+ and Man^+, whereas the neotype of *S. cerevisiae* (CBS 1171) is Mel^- and Man^- (http://www.cbs.knaw.nl/yeast/BioloMICS.aspx). To find out if these traits were species-specific and stable indeed, we tested our strain collection for them. All *S. uvarum* isolates were sensitive to 37 °C and could ferment melibiose (Mel^+, but two of them did not assimilate it). All *S. cerevisiae* isolates grew at 37 °C but not all were Mel^-. Isolates 69, 72 and 73 (all from Cirfandli fermentations) grew also on melibiose (Mel^+). Very few strains could utilize mannitol but, interestingly, Man^+ phenotype was found both among *S. uvarum* (3) and among *S. cerevisiae* isolates (1 and 25). About one third of the Man^- isolates and a few Mel^- isolates appeared unstable upon prolonged incubation. After 6 days of incubation on the mannitol and melibiose media, slowly developing colonies grew out from the non-growing lawns indicating that the inability to utilize mannitol and melibiose can be due to revertable loss-of-function genetic events.

Addition of 60% glucose to the medium retarded the growth of all isolates but not to the same extent. There was a great diversity in their ability to tolerate this condition (Fig. 3A). All *S. uvarum* isolates proved to be much more sensitive and less diverse than the *S. cerevisiae* isolates. The optical density of their cultures was only 1.23 to 2.5 times higher at the end of the 96-hour incubation period than at the beginning. Much higher growth rates characterized almost all *S. cerevisiae* isolates, although the most sensitive *S. cerevisiae* strain (21, 2.3 fold increase) was not better than the least sensitive *S. uvarum* strain (3, 2.5 fold increase). Whereas all groups were heterogeneous, the Kadarka and Kéknyelű strains were in general more tolerant than the isolates obtained from the other wines. Interestingly, the most tolerant strains of both species were isolated from Kéknyelű.

The *S. uvarum* isolates were also more sensitive to the presence of ethanol added to the medium (Fig. 3B). The *S. cerevisiae* strains

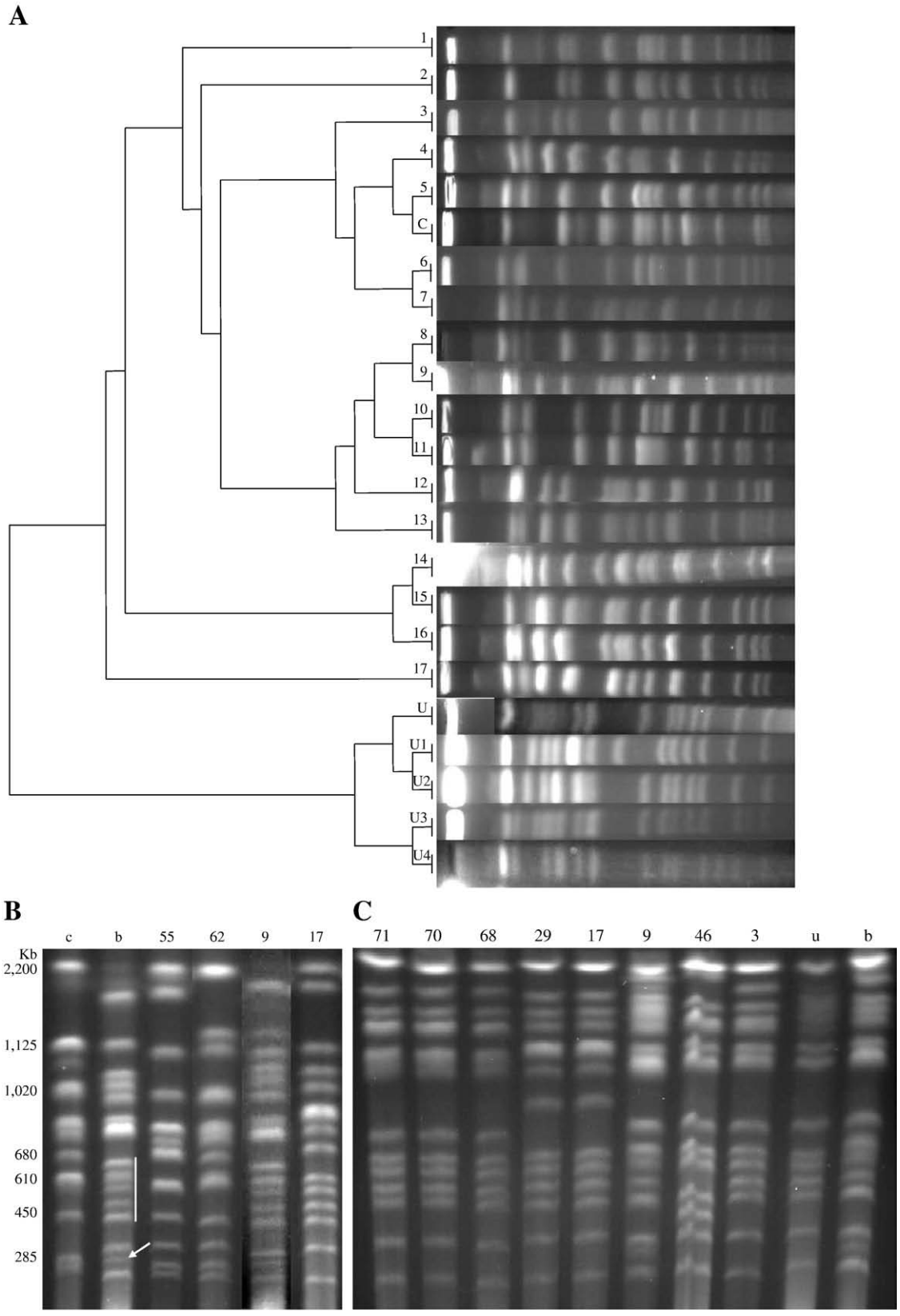

Fig. 2. Karyotype analysis. A. A dendrogram generated from the distance matrix of the major groups of electrophoretic karyotypes. 1 to 17: groups of *S. cerevisiae* isolates; U1 to U4: groups of *S. uvarum* isolates; c: *S. cerevisiae* 10–157; u: *S. uvarum* 10–512. B and C. Karyotypes of the *S. uvarum* isolates. c: *S. cerevisiae* 10–157; b: *S. bayanus* 10–198; u: *S. uvarum* 10–512. The *bayanus*/*uvarum*-specific group of chromosomal bands is marked with a vertical line. Arrowhead marks the small *S. bayanus* chromosome missing in the *S. uvarum* type strain. Band sizes of certain chromosomes of *S. cerevisiae* 10–157 are shown on the left size of panel B. Panel B shows CHEF-III karyotypes; panel C shows CHEF-II karyotypes.

were less sensitive and showed higher diversity again. The Kadarka isolates proved to be the most ethanol-tolerant group. Interestingly, they were the least tolerant and least diverse group in the copper-sensitivity tests (Fig. 3C). In general, the other *S. cerevisiae* groups showed bigger diversity than the *S. uvarum* group and many of their members were much more resistant than the *S. uvarum* strains. However, in certain isolates it was difficult to exactly determine the minimal inhibitory concentration because in the drop test they produced papillae or small colonies slowly growing out from the lawns of the non-growing cells at certain $CuSo_4$ concentrations.

Killer activity and sensitivity were also tested (Table 2). With the exception of three Cirfandli isolates and one Kéknyelű isolate all strains were sensitive to K1. The four non-sensitive strains were neutral. Five *S. uvarum* strains and 17*S. cerevisiae* strains were also sensitive to K2. Most of the Cirfandli isolates were double-sensitive, whereas only one Kadarka isolate was sensitive to both types of killer toxins. Only one *S. uvarum* isolate and one *S. cerevisiae* isolate proved to be killer; both showed K2 activity. However, even these strains were sensitive to K1. No isolate had K1-type killer activity and no isolate was sensitive to the K2 toxin.

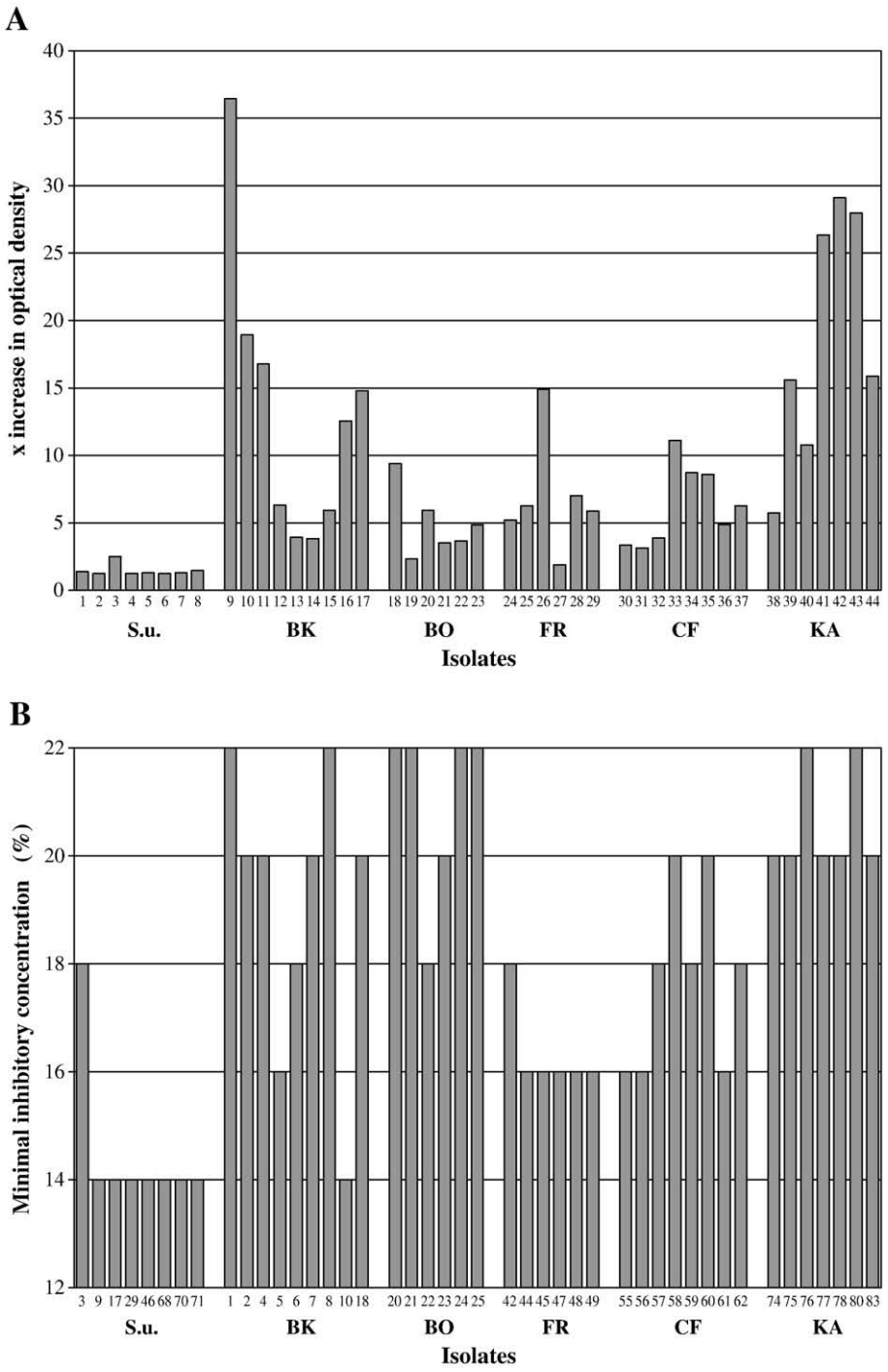

Fig. 3. Diversity of physiological properties of selected isolates. A. Growth at 60% glucose. B. Sensitivity to ethanol. C. Sensitivity to copper. Slowly growing papillae and/or small colonies were detected in 5, 7, 8, 22, 29, 42, 45, 46, 47, 48, 59 and 78. D. Fermentation efficiency. S.u., *S. uvarum* isolates. The rest of the isolates shown are non-*uvarum* strains. BO, Olaszrizling isolates. BK, Kéknyelű isolates. FR, Ezerjó isolates. CF, Cirfandli isolates. KA, Kadarka isolates. For strain list see Table 1.

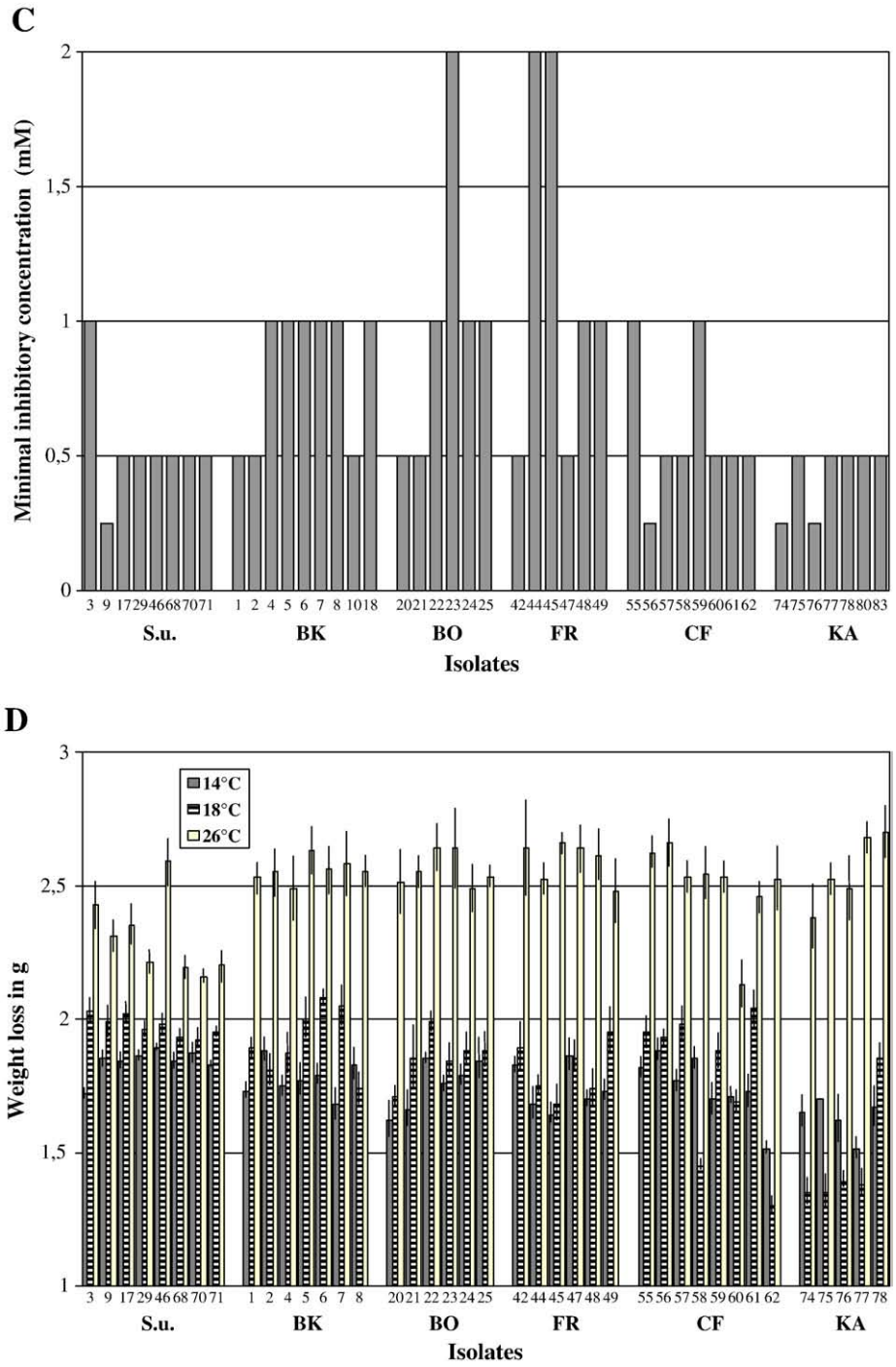

Fig. 3 (*continued*).

3.5. Diversity in fermentation efficiency

For the examination of diversity in fermentation efficiency and metabolite production under microvinification conditions, a smaller group of randomly chosen isolates was used. Thirty two *S. cerevisiae* and 8 *S. uvarum* strains representing all wines involved in this study were tested for fermentation at 14 °C, 18 °C and 26 °C (Fig. 3D). All of them had much higher rates at 26 °C than at the lower temperatures but varied significantly in the extent of difference. Particularly big differences were observed in the Kadarka (KA) group, which were the least psychrotolerant isolates. For example the *S. cerevisiae* isolate 77 (Fig. 3D) had the second highest rate at 26 °C (weight loss 2.68 g) and the lowest rate at 14 °C (weight loss 1.51 g). Interestingly, for certain *S. cerevisiae* isolates the latter temperature was not more inhibitory than 18 °C. All but one Ezerjo (49) isolates showed almost identical fermentation rates at 14 °C and 18 °C; four Kadarka strains (74 to 77), and two Cirfandli isolates (58 and 62) were even better at 14 °C than at 18 °C. The *S. uvarum* isolates slightly differed from the majority of the *S. cerevisiae* isolates. Their fermentation rates were somewhat lower at 26 °C and slightly higher at 14 °C. Another difference was that all of them fermented better at 18 °C than at 14 °C. The most psychrotolerant isolate was the *S. uvarum* isolate 46 (highest fermentation rate at 14º). Remarkably, it also had a high fermentation rate at 26 °C,

Table 2
Killer activity of the isolates.

Killer phenotype	Isolates					
	S. uvarum	*S. cerevisiae*[a]				
		BK	BO	FR	CF	KA
Sensitive to K1	3, 17, 70	2, 4 to 8, 11 to 16, 19	21 to 27, 30 to 40	43 to 45, 47, 48, 50 to 54	61, 64, 72, 73	74, 75, 77 to 86
Sensitive to K2	–	–	–	–	–	–
Sensitive to K1 and K2	9, 29, 46, 68, 71	1, 10	20, 28, 41	42, 49,	55, 56, 57, 59, 62, 63, 66, 67, 69	76
Neutral	–	18	–	–	58, 60, 65	–
K1-type killer	–	–	–	–	–	–
K2-type killer	70	–	–	–	–	74

[a] BO, Olaszrizling isolates. BK, Kéknyelű isolates. FR, Ezerjó isolates. CF, Cirfandli isolates. KA, Kadarka isolates.

which surpassed the rates of all *S. uvarum* isolates and most of the *S. cerevisiae* isolates.

3.6. Diverse metabolite profiles

The experimental wines produced by the selected 32 *S. cerevisiae* strains and 8 *S. uvarum* strains at 14 °C and 26 °C were analyzed for the content of certain fermentation compounds. After the completion of fermentation, the concentrations of four higher alcohols (isoamyl alcohol, isobutanol, active amyl alcohol and *n*-propanol), acetaldehyde, acetoin, acetic acid and ethyl acetate were determined. A broad range of production levels was found for all metabolites at both temperatures (Table 3). The majority of the isolates produced more ethylacetate, and isoamyl alcohol at 14 °C and more acetic acid, acetaldehyde, acetoin and isobuthanol at 26 °C. One Ezerjó isolate (49) was particularly active at the lowest temperature. It produced the highest concentrations of acetic acid, acetaldehyde and ethyl acetate at 14 °C but remained in the midrange at 26 °C for all metabolites. The Kadarka strain 78 was the poorest producer of the metabolites isoamyl alcohol, ethyl acetate and *n*-propanol at 14 °C. The *S. uvarum* strains produced much less acetic acid at both temperatures (ranging from 185.5 to 228.3 mg/l at 14 °C, and from 237.9 to 289.08 mg/l at 26 °C; compare with data in Table 3) than the *S. cerevisiae* isolates.

Cluster dendograms were constructed for the production of secondary compounds. Wines fermented at 14 °C (Fig. S1) gave three main clusters. Cluster A includes wines characterized by medium-to-low levels of ethyl acetate and *n*-propanol, low levels of acetic acid and acetoin, high levels of amyl alcohols and isobutanol. Group B includes wines with variable levels of all compounds and the highest levels of acetaldehyde and acetic acid. Wines containing low-to-medium levels of all compounds are included in group C.

Wines fermented at 26 °C (Fig. S2) gave four clusters. Wines grouped in cluster D have low levels of *n*-propanol, acetoin and acetaldehyde and medium-to-high levels of amyl alcohols. Cluster E includes wines containing high levels of ethyl acetate and acetic acid and variable content of the other secondary compounds. The peculiarity of wines grouping in cluster F is the very low level of acetic acid. Cluster G includes wines characterized by low levels of almost all by-products determined.

4. Discussion

In this study the genetic and physiological diversity of *Saccharomyces* yeasts isolated from spontaneously fermenting grape must was studied. Must was prepared from 5 grape varieties in 4 wine regions of Hungary, where similar studies had not been performed before. 86 isolates that did not grow on a medium containing lysine as nitrogen source were selected for testing diversity.

In grape-wine fermentation two lys^- *Saccharomyces* species play important roles: *S. cerevisiae* and *S. uvarum* (*S. bayanus* var. *uvarum*). The taxonomic differentiation of their wine strains can be a difficult task. To identify the *S. cerevisiae* and *S. uvarum* strains in our collection, we applied the combined approach published recently by Antunovics et al. (2005). We found that eight isolates displayed non-*cerevisiae* type PCR-RFLP patterns of three chromosomal sequences generally used for taxonomic identification. Seven of them had karyotypes with a group of medium-size chromosomes characteristic of *S. uvarum* and *S. bayanus* (e.g. Sipiczki et al., 2001). Since all of them lacked the small-size chromosome characteristic of the type strain (the taxonomic "standard") of *S. bayanus* (Nguyen and Gaillardin, 1997), we concluded that they all belonged to *S. uvarum*. However, the lack or presence of this chromosomal band (one of a group of three chromosomes in the size range 225–370 kb) alone would not be sufficient for assigning a strain to

Table 3
Metabolites produced by the selected *S. cerevisiae* and *S. uvarum* isolates at 14 °C and 26 °C.

Metabolite	Production (mg/l)				Comparison of production (number of strains)	
	At 14 °C		At 26 °C		26>14[b]	14>26[c]
	Range[a]	Av ± SD	Range[a]	Av ± SD		
Acetic acid	182.5 (46)–1489.5 (49)	612.8 ± 286.9	237.9 (70)–1098.5 (58)	722.4 ± 278.3	31	9
Acetaldehyde	14.9 (60)–107.2 (49)	52.2 ± 22.5	7.9 (4)–315.5 (75)	61.1 ± 44.9	24	16
Acetoin	4.8 (61)–141.0 (5)	20.1 ± 27.4	8.7 (23)–184.8 (75)	27.2 ± 30.1	36	4
Ethyl acetate	6.1 (78)–65.1 (49)	13.1 ± 9.5	4.0 (56)–11.9 (25)	7.1 ± 2.1	6	33
n-Propanol	8.0 (78)–153.9 (44)	25.3 ± 23.1	8.9 (3)–71.9 (44)	20.9 ± 12.2	20	20
Isobutanol	9.2 (23)–37.8 (3)	20.5 ± 10.1	10.2 (70)–64.8 (60)	22.9 ± 8.9	27	13
Amyl alcohol	1.7 (60)–43.9 (4)	19.2 ± 14.7	4.6 (60)–32.4 (62)	12.7 ± 6.4	17	23
Isoamyl alcohol	40.5 (78)–189.7 (1)	97.9 ± 42.2	44.2 (60)–134.3 (74)	74 ± 20.5	12	28

Av = average. SD = standard deviation.
[a] The serial numbers of strains (see Table 1) which produced the lowest and highest levels are given in brackets.
[b] Higher level at 26 °C than at 14 °C.
[c] Higher level at 14 °C than at 26 °C.

S. uvarum or to *S. bayanus* because it (or a size equivalent) can also occur in karyotypes of other *Saccharomyces* species. In this study, we found *S. cerevisiae* strains that had three bands and other strains that had two bands in this size region. When we compared the molecular data with the results of the physiological ("conventional taxonomy") tests, we encountered notable discrepancies. Although all isolates identified as *S. uvarum* by molecular tests were Mel^+, not all *S. cerevisiae* isolates were Mel^-. The ability to utilize mannitol (another trait characteristic of *S. uvarum* by definition) also occurred among *S. cerevisiae* isolates and many of the Man^- *S. cerevisiae* strains segregated, producing slowly growing papillae on the mannitol medium. Similarly, certain Mel^- isolates also produced slowly growing Mel^+ segregants. Thus, these physiological traits previously proposed for differentiation of *S. uvarum* from *S. cerevisiae* are too variable and cannot provide an unambiguous taxonomic identification without molecular tests.

These variable traits can be exploited as non-molecular markers in studies of intraspecific diversity. The presence of both plus and minus phenotypes and the segregation of the minus phenotypes during vegetative propagation of cells indicate that both species can occasionally silence and then reactivate the corresponding genes. It is worth mentioning here that similar instability was previously found in the melibiose deficiency of the type strain (CBS380) of *S. bayanus* which contains the silent sequence MEL^0 and can revert to the Mel^+ phenotype (Turakainen et al., 1993). We observed a similar alternation between less and more copper-resistant phenotypes. The instability (segregation) of these properties further increases the diversity in the fermenting yeast population.

Remarkable diversity was also revealed in certain properties of technological significance. When fermentation vigor was compared at three temperatures, the isolates were quite alike at 26 °C but differed markedly at the lower temperatures, particularly at 14 °C. Although *S. uvarum* is generally considered a psychrotolerant (cryotolerant) wine yeast (for a review see Sipiczki, 2002), its strains examined in this study were not superior to all *S. cerevisiae* isolates at 14 °C. In a previous study we even found *S. cerevisiae* strains that fermented better at low temperatures than the *S. uvarum* strains isolated from the same spontaneously fermenting grape must (Antunovics et al., 2005). Thus, it is not a general rule that *S. uvarum* ferments better than *S. cerevisiae* at low temperatures. Another interesting finding of this work was that the *S. uvarum* isolates were much more sensitive than the *S. cerevisiae* isolates to the presence of 60% glucose in the medium. This result is somewhat puzzling because *S. uvarum* is known to occur frequently in fermenting musts produced from dried and/or botrytized grapes (drying and noble rotting increases sugar content in the grape juice) (see Sipiczki, 2008 for references). These isolates were also more sensitive than the *S. cerevisiae* strains to exogenously added ethanol and most of them tolerated less copper in the medium than most of the *S. cerevisiae* strains. In a recent comparative study of commercial wine strains (Torija et al., 2003), it was hypothesized that the higher ethanol sensitivity of the *S. bayanus* (probably *S. uvarum*) strain examined might have been the consequence of the shorter chain length of the fatty acids in its plasma membrane.

The profiles of metabolite production were also very diverse. The *S. uvarum* isolates produced less acetic acid than the *S. cerevisiae* isolates. Since low levels of acetic acid in fermentations by *S. bayanus*/*uvarum* strains have also been reported by other authors (e.g. Delfini and Cervetti, 1991; Sipiczki et al., 2001;Vilela-Moura et al., 2008), low acetic acid production can be considered as a discriminating trait for *S. uvarum*. At 26 °C, the *S. uvarum* isolates remained within the range of the *S. cerevisiae* isolates in the production of other metabolites. Remarkable differences between them were found at 14 °C, where the *S. uvarum* strains produced higher amounts of isobutanol and amyl alcohols than the *S. cerevisiae* strains. These differences indicate that at low temperatures *S. uvarum* could be better at the transformation of certain grape must precursors into aroma compounds. Most strains (both *S. cerevisiae* and *S. uvarum*) tested in this study produced more ethyl acetate and isoamyl alcohol at 14 °C and more acetic acid, acetaldehyde, acetoin and isobutanol at 26 °C than at the other temperature. This observation is consistent with previous works that also reported on the effect of temperature on metabolite production of wine yeasts (e.g. Daudt and Ough, 1973; Ciolfi et al., 1985; Heard and Fleet, 1988; Torija et al., 2003; Molina et al., 2007). In a recent study of the influence of temperature on the synthesis of aroma compounds (Molina et al., 2007), a commercial wine yeast strain also produced more ethyl acetate and isoamylalcohol at 15 °C than at 28 °C but, in contrast to our findings, also more isobutanol and acetic acid. Taken all data together, the *S. cerevisiae* strains showed much higher diversity than the *S. uvarum* isolates. The profiles of the two species differed definitely and the *S. uvarum* strains formed distinct, well-separated clusters in the dendograms at both temperatures.

Significant diversity was also detected when killer phenotypes were tested. The isolate groups of all wines were mixtures of two or three phenotypes. All but four strains were K1-sensitive or double-sensitive but only one *S. cerevisiae* and one *S. uvarum* strain had killer activity, indicating that killer strains may be rare in the natural yeast populations of the four wine regions involved in this study.

The combination of molecular genetic analysis and physiological tests applied in this study revealed unexpectedly high diversity among the *S. cerevisiae* strains isolated from naturally fermenting wines. Although the isolates collected in the same region or from the same fermenting must showed considerable similarity in certain traits, they differed markedly in other properties. Practically each isolate showed an individual pattern. These results indicate that the yeast populations of spontaneously (naturally) fermenting wines can be much more diverse than previously thought. This finding is hard to interpret in view of the observations that *S. cerevisiae* is very rare on grapes and in context with the hypothesis that the fermenting *S. cerevisiae* comes mainly from yeasts that have survived on the winery equipment since the previous vintage season (Mortimer and Polsinelli, 1999; Ciani et al., 2004; Le Jeune et al., 2006). The surviving population can be very small, particularly when hygienic modern technology is used. When the founding population is small, its initial diversity is also small. In principle, its diversity can increase later, in course of growth in the must but this requires genetic changes. *S. cerevisiae* is supposed to have a genome prone to change easily e.g. by altering its chromosomal structures (karyotype variability) (e.g. Longo and Vezinhet, 1993; Miklos et al., 1997; Nadal et al., 1999) or as shown here, by segregating certain phenotypic traits at vegetative propagation. Interestingly, the *S. uvarum* isolates characterized in this study were less diverse. This result is consistent with our previous study in which we found almost no karyotype variability among *S. uvarum* strains isolated from Tokaj wines (Antunovics et al., 2005). Here we detected more chromosomal polymorphism but still less than among the *S. cerevisiae* isolates. The higher karyotype diversity in *S. cerevisiae* wine strains suggests that the *S. cerevisiae* genome may be more flexible than the *S. uvarum* genome and thus may allow faster and more efficient adaptation to the continuously changing environment during wine fermentation because its changes can result in diverse subpopulations. It is plausible that that concerted activity of these subpopulations can significantly contribute to the uniqueness of a wine. In view of recent reports on the occurrence of non-species-specific genes/alleles in *Saccharomyces* wine strains (supposed to have introgressed through mating with a different species) (reviewed in Sipiczki, 2008), it is conceivable that horizontal gene transfer can further enhances diversity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijfoodmicro.2010.03.024.

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