

Phenotypic and genotypic diversity of wine yeasts used for acidic musts

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Abstract The aim of this study was to examine the physiological and genetic stability of the industrial wine yeasts *Saccharomyces cerevisiae* and *Saccharomyces bayanus* var. *uvarum* under acidic stress during fermentation. The yeasts were sub-cultured in aerobic or fermentative conditions in media with or without L-malic acid. Changes in the biochemical profiles, karyotypes, and mitochondrial DNA profiles were assessed after minimum 50 generations. All yeast segregates showed a tendency to increase the range of compounds used as sole carbon sources. The wild strains and their segregates were aneuploidal or diploidal. One of the four strains of *S. cerevisiae* did not reveal any changes in the electrophoretic profiles of chromosomal and mitochondrial DNA, irrespective of culture conditions. The extent of genomic changes in the other yeasts was strain-dependent. In the karyotypes of the segregates, the loss of up to 2 and the appearance up to 3 bands was noted. The changes in their mtDNA patterns were much broader, reaching 5 missing and 10 additional bands. The only exception was *S. bayanus* var. *uvarum* Y.00779, characterized by significantly greater genome plasticity only under fermentative stress. Changes in karyotypes and mtDNA profiles prove that fermentative stress is the main driving force of the adaptive evolution of the yeasts. L-malic acid does not influence the extent of genomic changes and the resistance of wine yeasts exhibiting increased demalication activity to acidic stress is rather related to their ability to decompose this acid. The phenotypic changes in segregates, which were

found even in yeasts that did not reveal deviations in their DNA profiles, show that phenotypic characterization may be misleading in wine yeast identification. Because of yeast gross genomic diversity, karyotyping even though it does not seem to be a good discriminative tool, can be useful in determining the stability of wine yeasts. Restriction analysis of mitochondrial DNA appears to be a more sensitive method allowing for an early detection of genotypic changes in yeasts. Thus, if both of these methods are applied, it is possible to conduct the quick routine assessment of wine yeast stability in pure culture collections depositing industrial strains.

Keywords Wine yeasts · Stability · Karyotyping · mtDNA restriction analysis · Malic acid

Introduction

The majority of industrial strains of wine yeasts are classified as *Saccharomyces cerevisiae* however *Saccharomyces bayanus* are also used. They are closely related, and both belong to the *Saccharomyces sensu stricto* complex. Due to the development of molecular methods in yeast characterization, some species within this group have been reclassified (Kurtzman 2003; for a review see Rainieri et al. 2003). Also there is a debate about the species *S. bayanus*, which includes strains with very different physiological and genetic features. Despite the ongoing discussion concerning yeast classification and nomenclature (for a review see Sipiczki 2008), there is still widespread parallel use of the taxon names *S. bayanus*, *S. uvarum*, and *S. bayanus* var. *uvarum*. The names of industrial strains remain unchanged, mostly for the convenience of the users and nomenclature of strains deposited in collections and applied as starters in

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wineries are usually not verified. Moreover, in light of the latest research, many wine yeasts are interspecific hybrids between yeasts of the *Saccharomyces sensu stricto* complex (Bradbury et al. 2006; Gonzalez et al. 2006, 2007; Le Jeune et al. 2007; Lopandic et al. 2007; Lopes et al. 2010). At the same time, wine yeasts are known for their genome plasticity, which has been explained by the necessity to adapt to the changeable conditions of the environment during fermentation (Querol et al. 2003; Sipiczki 2011). Due to the large capacity of wine yeasts for genome reorganization, they are regarded as undergoing constant adaptive evolution. The model of fast adaptive genome evolution (FAGE), suggested for wine yeasts, indicates the possibility of inducing genotypic changes both during vegetative growth and at the sexual stage (Sipiczki 2011). From the technological point of view, it is important to determine the stability of commercially used wine yeasts and their sensitivity to environmental stresses occurring during fermentation. The main fermentative stresses include osmotic, hyperosmotic, ethanol, oxidative and ionic stresses as well as low pH, temperature shifts, nutrient limitation, and starvation (Cardona et al. 2007; Querol et al. 2003). Acidic stress adversely affects wine yeasts (Fleet and Heard 1993) and results in changes in the sensory properties of wines (Pretorius and Bauer 2002; Redzepovic et al. 2003). Acidic musts are one of the main problems in winery of cold regions countries, including Poland. Moreover, Polish wineries mostly rely on fruit musts rich in organic acids, so yeasts should be best suited for acidic environments. Biological deacidification with yeasts consuming organic acids leads to wines with the right balance between sugar, acid and aroma components (Volschenk et al. 2003). Tartaric and malic acids may constitute up to 90% of total organic acid content in must (Torija et al. 2003), but only L-malic acid is metabolized by yeasts during vinification, so L-malic acid decomposing yeasts are of the great value. Malate decomposition varies greatly and may reach 48%, depending on the strain (Pretorius and Bauer 2002; Redzepovic et al. 2003). We have previously selected and characterized industrial wine yeasts *S. cerevisiae* and *S. bayanus*, with a demalication activity reaching 68% (Rajkowska and Kunicka 2005), but their stability under acidic stress has not been examined. The objective of this paper was to investigate the physiological and genetic stability of selected *S. cerevisiae* and *S. bayanus* var. *uvarum* strains in the presence of malic acid. The yeasts were cultivated in standard media under aerobic and semi-anaerobic conditions, simultaneously being subjected to acidic stress. We assessed changes in the biochemical profiles of these industrial wine yeasts, their karyotypes and mitochondrial DNA (mtDNA) profiles after approximately 50–180 generations, depending on the strain and culture conditions. It is assumed that changes occurring

in the population for up to 20 generations result from physiological response to environmental conditions, while changes in later generations reflect evolutionary processes (Fox 1998). Furthermore, it is also possible to assess the influence of environmental stresses on yeast phenotypes and genomes. Therefore, the study presented in this paper gives a fair picture of changes in yeast populations leading to their adaptive evolution. To our knowledge, this report is the first one concerning phenotypic and genotypic stability of yeasts with extended demalication activity.

Materials and methods

Microorganisms

The following wine yeasts were used: four strains *S. cerevisiae* (Syrena, W-13, Y.00911, Y.00925) and two strains *S. bayanus* var. *uvarum* (Cz-2 and Y.00779). *S. cerevisiae* Syrena and W-13 are industrial strains commonly used in Poland and deposited in the Collection of Pure Cultures of the Institute of Fermentation Technology and Microbiology, Technical University of Lodz, ŁOCK 105. *S. cerevisiae* Y.00911, Y.00925 and *S. bayanus* var. *uvarum* Y.00779 are originated from National Collection of Agricultural and Industrial Microorganisms University of Horticulture and Food Science in Budapest Hungary. Strain Cz-2 was isolated from Italian dried wine yeast designated as *S. bayanus* and purchased from F.LLI MARESCALCHI S.p.A. (Casale Monferrato, Italy). Reference *S. cerevisiae* haploids Cm MAT α and Gm MAT α originated from the Collection of the Institute of Agricultural Sciences in Zamosc, Poland. The microorganisms were activated through double passaging in YGP liquid medium (yeast extract 10 g l⁻¹, glucose 20 g l⁻¹, peptone 10 g l⁻¹) at 28°C for 48 h.

To check homogeneity of industrial strains, yeasts from YGP liquid medium were restreaked on YGP agar plates (yeast extract 10 g l⁻¹, glucose 20 g l⁻¹, peptone 10 g l⁻¹, agar 20 g l⁻¹) and incubated at 28°C for 48 h. Subsequently, 20 representative colonies were picked randomly from the plates, subjected to macro-morphological and micro-morphological analysis and no differences in morphological features were observed.

Yeast segregates

Yeasts were sub-cultured 20 times in aerobic or semi-anaerobic conditions in both YGP and YG (yeast extract 4 g l⁻¹, glucose 100 g l⁻¹, L-malic acid 7 g l⁻¹, KH₂PO₄ 5 g l⁻¹, MgSO₄ 0.4 g l⁻¹, pH 3.0) media. Aerobic cultures were conducted in 50 ml liquid YGP or YG medium at 28°C for 48 h in 500 ml flat-bottomed flasks and constantly

shaken (200 rpm). Semi-anaerobically, yeasts were grown in 110 ml YGP or YG medium in conical 250 ml flasks for 7 days at 25°C. The media were inoculated by 1% yeast cell suspensions in saline solution (NaCl 8.5 g l⁻¹) standardized to a density of 10⁸ c.f.u. l⁻¹. Generation times were estimated for exponential growth phase according to Mesa et al. (1999). Numbers of generations were calculated independently for lag, exponential and stationary growth of yeasts and summarized.

After completing the last passage, yeast cells were centrifuged, resuspended in YGP medium and frozen at -80°C with glycerol added to 50%. Streaks from the evolved frozen samples on YGP were re-streaked on agar plates and incubated at 28°C for 48 h. Subsequently, 10 representative colonies were picked randomly from the plates, subjected to macro-morphological and micro-morphological analysis. Because no differences in morphological features were observed, for each experiment four colonies of segregates were restreaked on YGP agar slants and independently tested.

Nomenclature used for yeast segregates is presented in Table 1.

Biochemical profiles

The biochemical ability of the yeasts and their segregates to assimilate 19 substrates (glucose, glycerol, 2-keto-D-gluconate, L-arabinose, D-xylose, adonitol, xylitol, galactose, inositol, sorbitol, α -methyl-D-glucoside, N-acetyl-D-glucosamine, cellobiose, lactose, maltose, sucrose, trehalose, melezitose, raffinose) as sole carbon sources was checked with API 20C AUX tests (bioMérieux, Warsaw, Poland) according to the producer's guidelines. Glycerol, ethanol and nitrate assimilation as well as the fermentation of carbohydrates (glucose, galactose, maltose, lactose, sucrose, melibiose, raffinose, trehalose) were tested according to the procedure given by Barnett et al. (2000).

Table 1 Nomenclature used for segregates of yeasts

	Aerobic segregates		Semi-anaerobic segregates	
	YGP	YG + L-malic acid	YGP	YG + L-malic acid
<i>S. cerevisiae</i>				
Syrena	C1-A	C1-B	C1-C	C1-D
W-13	C2-A	C2-B	C2-C	C2-D
Y.00911	C3-A	C3-B	C3-C	C3-D
Y.00925	C4-A	C4-B	C4-C	C4-D
<i>S. bayanus</i> var. <i>uvarum</i>				
Cz-2	B1-A	B1-B	B1-C	B1-D
Y.00779	B2-A	B2-B	B2-C	B2-D

Sporulation

Sporulation abilities was checked on acetate agar (glucose 1 g l⁻¹, potassium chloride 1.8 g l⁻¹, sodium acetate trihydrate 8.2 g l⁻¹, yeast extract 2.5 g l⁻¹, agar 15 g l⁻¹), after incubation in 28°C for 7 and 14 days (Yarrow 1998).

Chromosomal DNA analysis

Chromosomal DNA isolation was conducted using a CHEF Genomic DNA Plug Kit (Bio-Rad, Warsaw, Poland) according to the methods described by Schwartz and Cantor (1984). Chromosomes were separated by pulsed field gel electrophoresis (PFGE) in 0.8% agarose gel by means of a CHEF-DR II apparatus (Bio-Rad, Warsaw, Poland). Electrophoresis was performed in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, and 10 mM EDTA; pH 8.2, at 10°C) for 28 h, at 6 V cm⁻¹ and linearly growing pulse duration from 110 to 220 s. Separated chromosomes were stained in ethidium bromide solution (0.5 μ g ml⁻¹). The gel was washed in distilled water and photographed.

The molecular weight of bands was estimated using SigmaGel software (Sigma-Aldrich, Gillingham, UK). The electrophoretic profiles of the segregates were compared to that of the *S. cerevisiae* YNN295 reference strain.

Ploidy assessment

Total DNA content was estimated by flow cytometry (Becton–Dickinson FACSCalibur cytometer, BD Biosciences, Erembodegem, Belgium) according to methods given by Hutter and Eipel (1979) and Nadal et al. (1999). Yeast cultures were grown in YGP at 28°C until cells reached the stationary phase. Cells were separated (3,500×g, 10 min), suspended in 70% ethanol, cooled down to 4°C and fixed for 30 min at -20°C. After centrifugation (3,500×g, 10 min), approximately 10⁶ cells were suspended in 500 μ l 50 mM sodium citrate (pH 7.5). RNA was removed by RNase A digestion (20 U per 500 μ l sample) for 2 h at 37°C. DNA was stained by propidium iodide (5 μ g per 500 μ l sample) for 16 h at 4°C in the dark. Propidium iodide fluorescence was recorded on a linear scale. The dominant cell population identified by its forward scatter/side scatter profile was gated and the median fluorescence of the G1 peak was recorded. The results were presented in fluorescence units and converted to relative DNA content. Yeast ploidy was calculated by comparison to the reference *S. cerevisiae* haploids Cm MAT a and Gm MAT α assuming that for reference strains 100 fluorescence units is equal to 1C and assigned as one set of chromosomes (n).

Mitochondrial DNA analysis

Restriction analysis of mtDNA was performed according to Querol et al. (1992), Querol and Ramon (1996). Restriction enzyme *Hinf*I (Roche Diagnostic, Basel, Switzerland) was used and separation was conducted in 0.7% agarose gel, in $0.5 \times$ TBE buffer, at 60 V for 5 h. Gels were stained in ethidium bromide solution ($0.5 \mu\text{g ml}^{-1}$), washed in distilled water, and photographed.

The molecular weight of bands was estimated using SigmaGel software (Sigma-Aldrich, Gillingham, UK). The mtDNA profiles of the segregates were compared to that of the Lambda DNA *Hind* III *Eco*R I digest (Sigma-Aldrich, Gillingham, UK).

Statistical analysis

Hierarchical cluster analysis was used to estimate the similarity of yeast segregates according to their biochemical, chromosomal DNA, and mtDNA profiles. Outcomes were displayed as binary data, 0 or 1 when the feature was absent or present in the yeast profile, respectively. Yeast segregate similarity was considered to be a function of the number of similar features versus the total number of features. Besides, yeasts revealing a high level of similarity formed clusters. The algorithm for hierarchical clustering was agglomerative and Manhattan distance was used as a measure of similarity between pairs of observations. Distances between clusters were calculated by the unweighted pair group method using averages (UPGMA) (Dąbkowski et al. 1997). In this method, the distance between two clusters is calculated as the average distance between all pairs of objects in the two different clusters. In calculation of percentage similarity values Demontax 1.2 program (written by Peter Halling, Strathclyde University and obtained by his courtesy) was used, and then the values obtained were applied on dendrograms. Cluster hierarchy was presented using a tree structure (dendrogram) with horizontal agglomeration distance. Agglomeration analysis was performed by means of Statistica 6.0 software (Tusla, Oklahoma, USA).

Generation times were presented as mean values of three separate experiments with standard deviation.

Total DNA content was given as mean values of three separate experiments with standard deviation. Data were analyzed by WinMDI 2.8 software.

Results and discussion

General characteristics of wild yeasts

The majority of the tested wine yeasts were classified as aneuploidal according to their DNA content, while only *S. cerevisiae* Syrena and *S. bayanus* var. *uvarum* Cz-2 were diploidal (Table 2). These results are consistent with the findings of Martinez et al. (1995), showing a predominance of aneuploidal strains among wine yeast populations. All the strains revealed sporulation ability, forming 2–4 spores. According to literature data, the ability of wine yeasts to sporulate varies, with aneuploidal strains revealing poor sporulation: if they sporulate at all, they usually produce 2 spores (Castrejon et al. 2004). It is believed that the limited reproduction ability is favored during natural selection, and the fermentative environment is dominated by aneuploidal, homothallic, and apomictic strains (Castrejon et al. 2004). However, data about the sterility of aneuploidal strains and the limited sporulation ability of wine yeasts are not fully consistent with the results of this study.

Saccharomyces cerevisiae strains significantly varied in terms of their biochemical profiles (from 2 to 8 different features), while *S. bayanus* var. *uvarum* differed in 4 features (data not presented). Surprisingly, in accordance with taxonomy (Barnett et al. 2000), only Y.00779 yeast classified as *S. bayanus* var. *uvarum* had a biochemical profile typical of *S. cerevisiae*. It may be misleading to assume that the characteristic feature of *S. bayanus* is its ability to ferment melibiose and inability to ferment galactose (Naumov et al. 1993). None of the examined strains fermented melibiose, while the Gal⁻ phenotype was found only in the strain *S. bayanus* var. *uvarum* Cz-2. Similar

Table 2 Genetic characterization of wine yeast

Features	<i>S. cerevisiae</i>				<i>S. bayanus</i> var. <i>uvarum</i>	
	Syrena	W-13	Y.00911	Y.00925	Cz-2	Y.00779
Total DNA (n)	1.36 ± 0.04	2.03 ± 0.06	2.15 ± 0.03	2.23 ± 0.03	2.06 ± 0.16	1.48 ± 0.06
Ploidy	Aneuploid	Diploid	Aneuploid	Aneuploid	Diploid	Aneuploid
Number of spores	2–4	2–4	2–4	2–4	2–4	2–4
Chromosomal DNA size (kb)	200–2,200	200–2,200	225–1,600	220–2,200	225–1,825	225–2,275
Chromosomal DNA bands (number)	14	13	11	13	12	16
Mitochondrial DNA size (bp)	694–5,205	564–5,148	564–1,584	564–6,590	564–5,200	564–8,426
Mitochondrial DNA bands (number)	17	16	5	12	14	16

results have been reported previously (Fernández-Espinar et al. 2001; Molnar et al. 1995; Sabaté et al. 1998) pointing out the possibility of misclassification of yeast strains mainly relying on their phenotypic characters. As proposed in other studies, melibiose and mannitol utilization could distinguish *S. cerevisiae* from *S. uvarum*/*S. bayanus* (Vaughan-Martini and Martini 1993) but this phenotypic pattern was also not expressed by every isolates of wine yeasts *S. cerevisiae* and *S. uvarum* (Csoma et al. 2010).

According to our previous study (Rajkowska and Kunicka 2005), the electrophoretic profiles of chromosomal DNA indicated genomic DNA polymorphism, while karyotyping was insufficient to unequivocally distinguish *S. cerevisiae* Syrena and W-13 as well as separate *S. bayanus* strains. At the same time, the karyotypes of *S. cerevisiae* Y.00911, Y.00925 and *S. bayanus* var. *uvarum* Y.00779 revealed three bands in the small-sized chromosome 225–365 kb region (Rajkowska and Kunicka 2005), which had been considered characteristic only of *S. cerevisiae* (Naumov et al. 2000; Tosi et al. 2009). Moreover, *S. bayanus* var. *uvarum* Y.00779 had a group of medium-size chromosomes (450–680 kb) recognized as characteristic of *S. bayanus* (Sipiczki et al. 2001; Csoma et al. 2010). Opinions on whether strains can be allocated to species based on karyotypes vary substantially, but most researchers believe that it is impossible to distinguish between yeast species within the *Saccharomyces sensu stricto* complex exclusively on the basis of the presence of a single band or set of bands (Fernández-Espinar et al. 2001; Rainieri et al. 2003; Csoma et al. 2010), which remains consistent with our data. Restriction analysis of wine yeast mtDNA has made it possible to distinguish between all the examined strains, being a better discriminative tool than karyotyping (Rajkowska and Kunicka 2005).

Generation times of yeast segregates

Generation times of the wine yeasts (data not presented) and their segregates changed depending on the culture conditions (Tables 3, 4) but generally were even up to three times longer for yeasts growing in the media with an elevated glucose concentration and L-malic acid presence. Literature data concerning the response of wine yeasts to the acidity of the environment show differences within the *Saccharomyces sensu stricto* complex. While some authors proved the sensitivity of *S. cerevisiae* wine strains to acidic stress (Fleet and Heard 1993), others showed that low pH did not significantly influence the growth or the fermentative activity of *S. bayanus* var. *uvarum* (Serra et al. 2005). These discrepant findings can be attributed to the fact that yeast resistance to stresses varies from strain to strain (Carrasco et al. 2001; de Melo et al. 2010) and it is also reflected in different responds of the tested strains *S.*

cerevisiae (e.g. Syrena versus Y.00925) and *S. bayanus* (Cz-2 versus Y.00779).

The generation times of segregates reached in YGP medium were about 29–62% longer in aerobic conditions than under fermentation (Tables 3, 4). On the contrary, the generation times estimated in YG medium with L-malic acid not differ very much between aerobic and semi-anaerobic conditions. In the course of fermentation, due to an excess of cytoplasmic NADH, *S. cerevisiae* are subjected to a reductive stress minimized by glycerol production (Albers et al. 1998, Valadi et al. 2004). Valadi et al. (2004) show that yeasts with deletions of *GPD2* and *TDH1* genes (encoding glycerol 3-phosphate-dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, respectively) improved their anaerobic growth by shortening the generation time by 1/9 comparing to aerobic conditions. The genetic manipulations of *GPD2* gene also decreased a glycerol production, so the excess carbon was redirected to biomass, resulting in significant increase in the specific growth rate in anaerobiosis (Hou and Vemuri 2010). Variable generation times of the wild and evolved yeasts tested by us may be a result of differences in expression of genes involved in glycerol metabolism, however multidirectional genome alterations under reductive stress cannot be excluded. Moreover, the combined glucose, acidic and reductive stresses (in YG medium) probably affected yeast growth more extensively than oxygen shortage. The karyotyping and mtDNA restriction analysis only show gross structural changes in the chromosomes and changes in mitochondrial DNA, respectively. Many other DNA changes that cannot be analyzed by those techniques may have occurred in the evolved strains (e.g. SNPs—single-nucleotide polymorphism) and could be related with their biochemical changes.

Biochemical profiles of yeasts and their segregates

Changes in the biochemical profiles of *S. cerevisiae* varied independently of culture conditions (Tables 3, 4). Yeast strains expressed a tendency to expand the range of compounds used as sole carbon sources. The highest stability of biochemical features was observed for glucose, galactose, maltose, sucrose and melibiose fermentation as well as for glycerol and nitrate assimilation. The most vivid changes concerned 9 compounds: lactose, inositol, N-acetyl-D-glucosamine, sorbitol, adonitol, 2-keto-D-gluconate, melezitose, trehalose and xylitol. Both *S. bayanus* var. *uvarum* strains revealed considerable changes in the biochemical profiles of their semi-anaerobic segregates under glucose and acidic stress (Table 4), which made them much more similar to *S. cerevisiae* strains than to their own wild ones. A dendrogram of biochemical profile similarity drawn for wild yeast and their segregates (Fig. 1) shows a clear division into two clusters: *S. cerevisiae* (22 strains) and *S.*

Table 3 Changes of wine yeast in aerobic conditions

Features	<i>S. cerevisiae</i>				<i>S. bayanus</i> var. <i>uvarum</i>	
	Syrena	W-13	Y.00911	Y.00925	Cz-2	Y.00779
YGP						
Generation time (min)	94.3 ± 2.5	88.2 ± 2.0	78.4 ± 1.6	77.2 ± 1.2	78.0 ± 1.8	147.8 ± 2.1
Number of generations	129 ± 4	138 ± 3	156 ± 3	158 ± 2	156 ± 4	83 ± 1
Assimilation differences (number)	2	3	6	0	0	2
Fermentation differences (number)	0	1	2	1	0	0
Total DNA (n)	1.42 ± 0.04	1.93 ± 0.08	1.88 ± 0.03	2.37 ± 0.05	2.08 ± 0.07	1.42 ± 0.04
Ploidy	Aneuploid	Diploid	Aneuploid	Aneuploid	Diploid	Aneuploid
Number of spores	2–4	2–4	2–4	2–4	2–4	2–4
Different bands ^a of chromosomal DNA size (kb)	–	–	2,200	2,200*, 1,300, 600	2,050	1,825*, 975
Different bands ^a of mitochondrial DNA size (bp)	–	–	4,557, 4,234, 3,328, 3,033, 2,581, 2,260, 1,880, 1,680, 1,117, 817	–	–	–
YG + L-malic acid						
Generation time (min)	96.5 ± 1.9	111.1 ± 1.7	93.6 ± 2.2	94.5 ± 0.8	127.8 ± 2.2	147.5 ± 2.6
Number of generations	124 ± 2	108 ± 2	128 ± 3	127 ± 1	94 ± 2	81 ± 2
Assimilation differences (number)	2	3	6	1	0	2
Fermentation differences (number)	0	0	2	1	0	0
Total DNA (n)	1.42 ± 0.05	1.79 ± 0.04	1.97 ± 0.04	2.24 ± 0.01	2.20 ± 0.04	1.35 ± 0.05
Ploidy	Aneuploid	Aneuploid	Diploid	Aneuploid	Aneuploid	Aneuploid
Number of spores	2–4	2–4	2–4	2–4	2–4	2–4
Different bands ^a of chromosomal DNA size (kb)	–	–	2,200	–	1,450	1,825*, 1,150*, 975
Different bands ^a of mitochondrial DNA size (bp)	–	–	4,557, 4,234, 3,328, 3,033, 2,581, 2,260, 1,880, 1,680, 1,117, 817	–	3,275, 1,965*, 947*	–

* Disappearing bands, bands without asterisk—additional bands

^a Different bands comparing to wild strains

bayanus (8 strains). Generally, malic acid did not influence changes in the biochemical profiles of *S. cerevisiae* grown under either aerobiosis or anaerobiosis, or *S. bayanus* grown under aerobiosis. Changes in the biochemical characteristics of *Saccharomyces sensu stricto* yeasts, concerning both the acquisition and loss of some characteristics, have been previously reported (Rosini et al. 1982). The appearance or loss of some features and the segregation of one during vegetative growth can be explained by gene polymorphism and occasional silencing and reactivation of the corresponding genes (Turakainen et al. 1993, Csoma et al. 2010). This behavior was previously observed for Mel[−] isolates of wine yeasts *S. cerevisiae* producing Mel⁺ segregates (Csoma et al. 2010) and *S. bayanus* strain

containing the silent sequence MEL⁰ reverting to the Mel⁺ segregates (Turakainen et al. 1993). Csoma et al. (2010) also mentioned similar diversity in copper-resistant phenotypes. Changes in morphological, physiological, and biochemical properties were also found during long-term storage of collection strains (Miklos et al. 1997). The significance of the impact of spontaneous mutations is disputable because of their very low rates (Querol et al. 2003), but their role in genetic alterations can be considered together with mitotic crossing over gene conversion (Puig et al. 2000). However *S. bayanus/uvarum* yeasts are considered to be more stable than most of the *S. cerevisiae* wine strains, the segregation of some traits in both species implying genome changes during vegetative propagation

Table 4 Changes of wine yeast in semi-anaerobic conditions

Features	<i>S. cerevisiae</i>			<i>S. bayanus</i> var. <i>uvanum</i>		
	Syrena	W-13	Y.00911	Y.00925	Cz-2	Y.00779
YGP						
Generation time (min)	55.6 ± 0.5	59.8 ± 0.4	51.8 ± 0.5	54.7 ± 1.0	57.7 ± 0.4	56.0 ± 0.7
Number of generations	173 ± 2	161 ± 1	185 ± 2	176 ± 3	166 ± 1	172 ± 2
Assimilation differences (number)	3	3	3	0	1	3
Fermentation differences (number)	1	1	1	1	0	2
Total DNA (n)	2.18 ± 0.06	1.98 ± 0.04	1.97 ± 0.05	2.38 ± 0.08	2.12 ± 0.15	2.18 ± 0.02
Ploidy	Aneuploid	Diploid	Diploid	Aneuploid	Diploid	Aneuploid
Number of spores	2–4	2–4	2–4	2–4	2–4	2–4
Different bands ^a of chromosomal DNA size (kb)	2,200*, 1,300, 725, 625, 300*	–	–	2,200*, 1,450, 600	1,450	1,825*, 1,550, 1,375*, 1,100*, 1,025*, 959, 900*, 800, 632*, 525*, 497, 350*, 325, 275
Different bands ^a of mitochondrial DNA size (bp)	3,790*, 2,976*, 2,400*, 709*	–	4,899, 4,234, 3,371, 3,076, 2,520, 2,260, 1,680, 1,229, 701	–	3,275, 1,965*, 947*	8,426*, 4,109, 3,447, 3,198*, 3,100*, 2,579*, 2,134*, 1,477*, 1,299, 1,230*
YG + L-malic acid						
Generation time (min)	101.8 ± 1.0	104.2 ± 2.1	108.7 ± 1.1	99.4 ± 0.9	99.3 ± 0.9	190.0 ± 0.6
Number of generations	94 ± 1	92 ± 2	88 ± 1	97 ± 1	97 ± 1	51 ± 1
Assimilation differences (number)	3	4	2	0	8	8
Fermentation differences (number)	1	1	2	1	2	2
Total DNA (n)	2.05 ± 0.12	1.95 ± 0.05	1.83 ± 0.03	2.25 ± 0.04	3.03 ± 0.03	2.09 ± 0.01
Ploidy	Diploid	Diploid	Aneuploid	Aneuploid	Triploid	Aneuploid
Number of spores	2–4	2–4	2–4	2–4	2–4	2–4
Different bands ^a of chromosomal DNA size (kb)	–	–	–	2,200*, 1,300, 600	2,200, 1,825*, 1,075	2,275*, 1,825*, 1,550, 1,375*, 1,350, 1,100*, 1,025*, 950, 900*, 800, 525*, 350*, 325
Different bands ^a of mitochondrial DNA size (bp)	5,208*, 3,790*, 2,976*, 2,400*, 709*	–	4,411, 3,233, 2,503, 2,242, 1,680	–	4,738, 1,965*, 1,303, 947*	8,426*, 6,144*, 4,850, 4,082, 3,342, 3,198*, 3,100*, 2,874*, 1,918*, 1,477*, 914*

* Disappearing bands, bands without asterisk—additional bands

^a Different bands comparing to wild strains

was previously noted (Csoma et al. 2010). The high diversity of *S. bayanus* var. *uvarum* strains tested by us was probably due to strain-specificity as it can be seen even comparing biochemical and genetic changes of Cz-2 and Y.00779. The changes in the phenotypes of *S. bayanus* segregates, observed in a medium similar to the natural fermentation environment, can be also explained by the selection of best-suited subpopulations.

Changes in chromosomal DNA and ploidy

Generally, *S. cerevisiae* Syrena and W-13 were characterized by stable chromosomal DNA irrespective of the acidity of the growth environment (Tables 3, 4). Some changes in band number and location have been observed for the Syrena segregate only after anaerobic passages in a medium without L-malic acid. The other two *S. cerevisiae* strains revealed karyotype changes that seem to be specific to the strains and independent of culture conditions (Fig. 2). The greatest changes in the number and intensity of bands were observed in the DNA profiles of *S. bayanus* var. *uvarum* Y.00779 semi-anaerobic segregates (differences in 14–15 bands), Table 4, Fig. 2. Hierarchical cluster analysis of yeast karyotypes led to defining one homogeneous group of strains, consisting of *S. cerevisiae* Syrena and W-13, *S. bayanus* var. *uvarum* Cz-2 and their segregates, as well as the *S. bayanus* var. *uvarum* Y.00779 semi-anaerobic segregate (Fig. 3). Our results are consistent with literature data, notifying significant variability in the number and size of chromosomes in wine yeast clones (Miklos et al. 1997). Wine yeasts tend to express a high level of chromosomal length polymorphism (Bidenne et al. 1992; Rachidi et al. 1999), which can contribute to the observed changes in karyotypes. Researches of wine yeast genome have reported both interchromosomal (translocation) and intrachromosomal (deletion and duplication) changes, or the presence of a variable number of chromosomes with high or low homology (Bidenne et al. 1992; Guerra et al. 2001; Vezinhet et al. 1990), which is reflected in chromosomal DNA profiles. Our results confirm that there occur considerable changes in the karyotypes of wine yeasts under semi-anaerobic conditions, which suggests greater genome plasticity under fermentative stress.

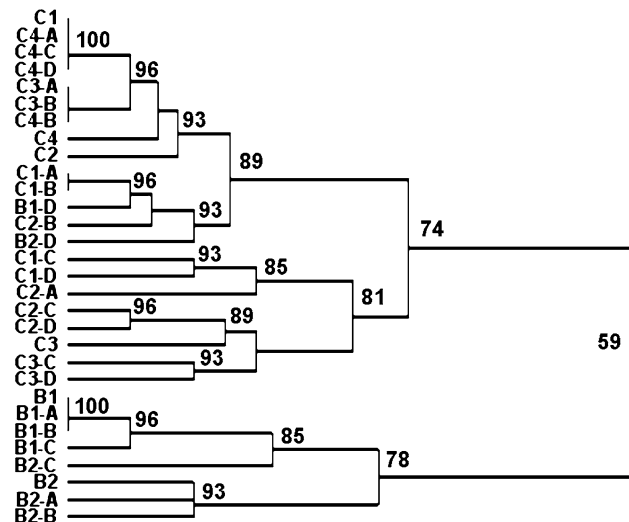
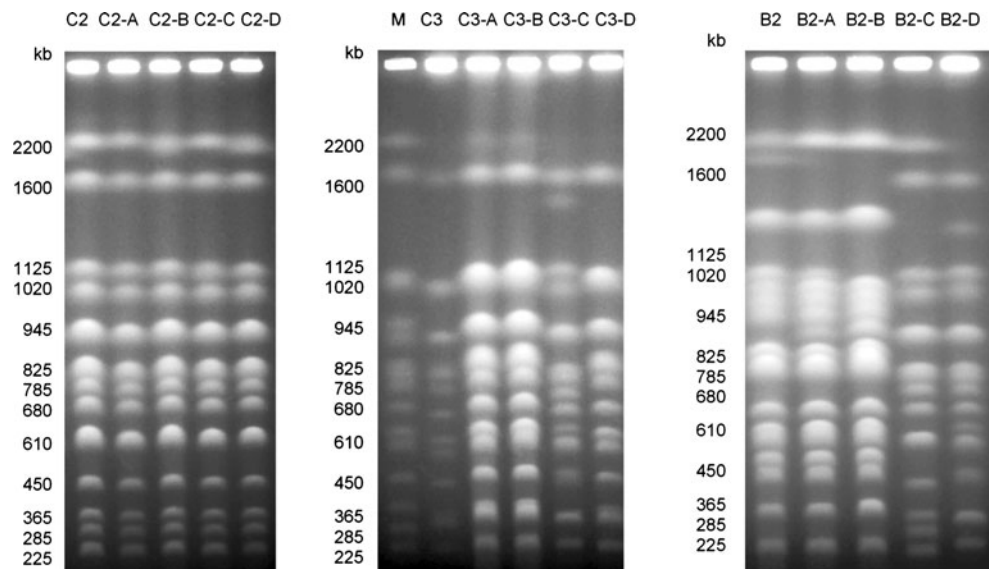


Fig. 1 Dendrogram of biochemical profiles similarity of wine yeasts: C1, *S. cerevisiae* Syrena; C1-A–C1-D, segregates of Syrena; C2, *S. cerevisiae* W-13; C2-A–C2-D, segregates of W-13; C3, *S. cerevisiae* Y.00911; C3-A–C3-D, segregates of Y.00911; C4, *S. cerevisiae* Y.00925; C4-A–C4-D, segregates of Y.00925; B1—*S. bayanus* var. *uvarum* Cz-2; B1-A–B1-D, segregates of Cz-2; B2, *S. bayanus* var. *uvarum* Y.00779; B2-A–B2-D, segregates of Y.00779; A—YGP, aerobic conditions; B—YG + L-malic acid, aerobic conditions; C—YGP, semi-anaerobic conditions; D—YG + L-malic acid, semi-anaerobic conditions

Fig. 2 Electrophoretic profiles of chromosomal DNA; C2, *S. cerevisiae* W-13; C2-A–C2-D, segregates of W-13; C3, *S. cerevisiae* Y.00911; C3-A–C3-D, segregates of Y.00911; B2, *S. bayanus* var. *uvarum* Y.00779; B2-A–B2-D, segregates of *S. bayanus* var. *uvarum* Y.00779; A—YGP, aerobic conditions; B—YG + L-malic acid, aerobic conditions; C—YGP, semi-anaerobic conditions; D—YG + L-malic acid, semi-anaerobic conditions; M—molecular marker *S. cerevisiae* YNN295



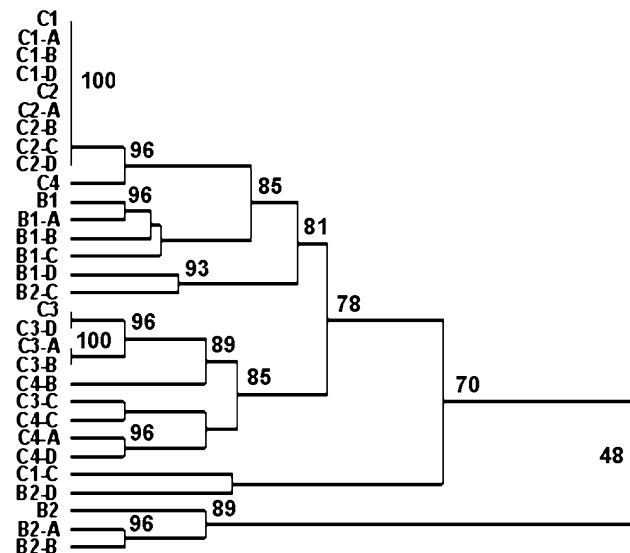


Fig. 3 Dendrogram of chromosomal DNA profiles similarity of wine yeasts: C1, *S. cerevisiae* Syrena; C1-A–C1-D, segregates of Syrena; C2, *S. cerevisiae* W-13; C2-A–C2-D, segregates of W-13; C3, *S. cerevisiae* Y.00911; C3-A–C3-D, segregates of Y.00911; C4, *S. cerevisiae* Y.00925; C4-A–C4-D, segregates of Y.00925; B1-*S. bayanus* var. *uvarum* Cz-2; B1-A–B1-D, segregates of Cz-2; B2, *S. bayanus* var. *uvarum* Y.00779; B2-A–B2-D, segregates of Y.00779; A—YGP, aerobic conditions; B—YG + L-malic acid, aerobic conditions; C—YGP, semi-anaerobic conditions; D—YG + L-malic acid, semi-anaerobic conditions

Considering the low rates of spontaneous mutations, recombination between homologous chromosomes as well as mitotic recombination during vegetative growth may have a greater impact on karyotype variability (Nadal et al. 1999; Puig et al. 2000).

Acidic stress has not been found to bear significantly on yeast karyotypes either under aerobic or semi-anaerobic conditions. Literature data concerning the effect of malate on wine yeasts was discussed in Generation times of yeasts segregates section. In our study, neither *S. cerevisiae* nor *S. bayanus* showed any substantial changes in chromosomal DNA that could be attributed to the presence of L-malic acid, which is consistent with the findings of Belloch et al. (2008) for the *Saccharomyces sensu stricto* group. Anyway, considering the changes in generation times of segregates, the effect of L-malic acid on yeast metabolism with the corresponding chromosomal DNA changes at the molecular level cannot be excluded (Redzepovic et al. 2003).

All segregates preserved their sporulation ability but changes in ploidy were found to reach 47%. However, the changes in ploidy were not strictly correlated with the extent of changes in chromosomal DNA, and reached about 12% even for the genotypically stable *S. cerevisiae* W-13 (Tables 2, 3, 4). Similarly to our study, differences in the ploidy of *Saccharomyces* yeasts have been detected in

strains with identical nuclear and mitochondrial DNA sequences (Spirek et al. 2003). At the same time, it should be remembered that DNA content in a cell may result from the monosomy, disomy or polysomy of single chromosomes (Ibeas and Jimenez 1996) and is not necessarily connected with the duplication of chromosome sets. Aneuploidy and polyploidy can ensure an advantage in adapting to the variable environment or increase the gene pool important for fermentation (Querol et al. 2003; Salmon 1997). Aneuploidal and polyploidal strains gain an advantage in natural selection conditions in respect of such characteristics as ethanol production, fast and efficient fermentation, and tolerance to high ethanol and sulfur dioxide concentrations (Guijo et al. 1997). Additionally, aneuploidy or polyploidy may protect the yeasts against spontaneous recessive mutations of lethal consequences (Tavares et al. 1988).

Changes in mitochondrial DNA

The mitochondrial DNA profiles of *S. cerevisiae* W-13 and Y.00925 segregates remained unchanged irrespective of growth conditions (Tables 3, 4). Substantial changes in the mtDNA of the other strains were observed mainly under fermentation. The only exceptions were *S. cerevisiae* Y.00911 segregates—from 5 to 10 additional bands appeared in their electrophoretic patterns after cultivation under any conditions tested (Fig. 4). The greatest changes in mtDNA were observed for the semi-anaerobic segregates of *S. bayanus* var. *uvarum* Y.0079. The dendrogram of mtDNA similarity revealed substantial heterogeneity of the yeasts, except for identical segregates of the same strains (Fig. 5). Restriction analysis of mitochondrial DNA is considered a good discriminative tool for estimating wine yeast differentiation (Fernández-Espinar et al. 2001; Muñoz et al. 2009; Nadal et al. 1996), which is consistent with our findings. In this study, segregates with stable chromosomal DNA reveal also an unchanged mtDNA electrophoretic profile, which indicates that the yeasts are stable under the particular test conditions. At the same time, karyotype changes have not always been correlated with mtDNA changes, which can be explained by differences in inheritance of mitochondrial and nuclear DNA (Groth et al. 2000). Generally, the greatest changes in mtDNA profiles were found under fermentation, which may be due to the fact that mitochondrial metabolism under aerobic and anaerobic conditions is quite different (Dejean et al. 2000). It may reflect the lesser role of respiration under anaerobiosis allowing mitochondrial changes to be maintained in the population. Rearrangements in mitochondrial DNA are not affected by elevated L-malic acid content.

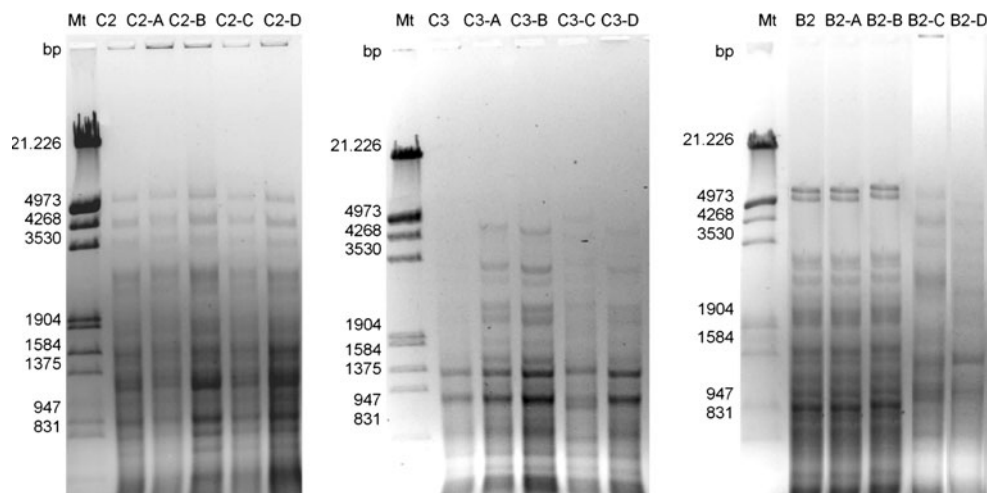


Fig. 4 Electrophoretic profiles of mitochondrial DNA; C2, *S. cerevisiae* W-13; C2-A–C2-D, segregates of W-13; C3, *S. cerevisiae* Y.00911; C3-A–C3-D, segregates of Y.00911; B2, *S. bayanus* var. *uvarum* Y.00779; B2-A–B2-D, segregates of *S. bayanus* var. *uvarum*

Y.00779; A—YGP, aerobic conditions; B—YG + L-malic acid, aerobic conditions; C—YGP, semi-anaerobic conditions; D—YG + L-malic acid, semi-anaerobic conditions; Mt—molecular marker Lambda DNA Lambda DNA *Hind* III *Eco* I digest

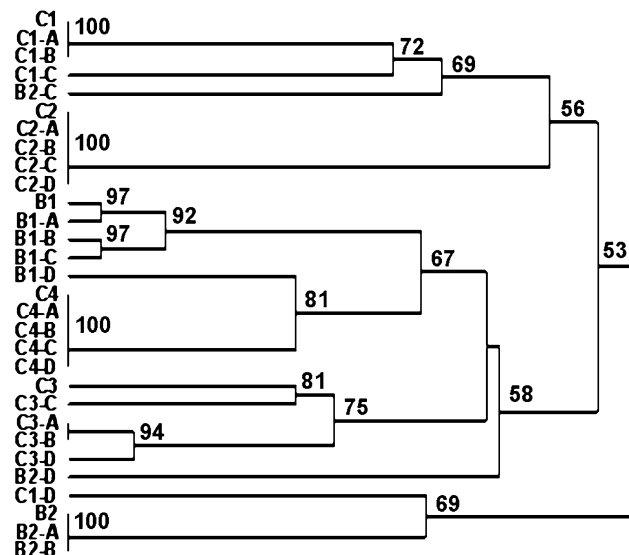


Fig. 5 Dendrogram of mtDNA profiles similarity of wine yeasts: C1, *S. cerevisiae* Syrena; C1-A–C1-D, segregates of Syrena; C2, *S. cerevisiae* W-13; C2-A–C2-D, segregates of W-13; C3, *S. cerevisiae* Y.00911; C3-A–C3-D, segregates of Y.00911; C4, *S. cerevisiae* Y.00925; C4-A–C4-D, segregates of Y.00925; B1-*S. bayanus* var. *uvarum* Cz-2; B1-A–B1-D, segregates of Cz-2; B2, *S. bayanus* var. *uvarum* Y.00779; B2-A–B2-D, segregates of Y.00779; A—YGP, aerobic conditions; B—YG + L-malic acid, aerobic conditions; C—YGP, semi-anaerobic conditions; D—YG + L-malic acid, semi-anaerobic conditions

Conclusions

Wine yeasts demonstrate considerable genome plasticity, which predisposes them for fast adaptive changes allowing them to survive in a constantly varying fermentation environment. Our study proves that among the yeasts

typically used in winemaking there are both strains expressing very high stability in both chromosomal and mitochondrial DNA (*S. cerevisiae* W-13) as well as labile strains, such as *S. cerevisiae* Y.00911 and *S. bayanus* var. *uvarum* Y.00779. Changes in karyotypes and mitochondrial DNA profiles, mostly observed under fermentation, confirm that fermentative stress is the main driving force in yeast adaptive evolution. The fact that L-malic acid does not influence the extent of genomic changes indicates that the resistance of wine yeasts exhibiting increased demalication activity to acidic stress may be related to their ability to decompose this acid. The same phenomenon has been found for the much more genotypically labile intraspecific and interspecific hybrids of these yeasts (Kunicka-Styczyńska and Rajkowska 2011). Phenotypic changes of segregates, detected even in those yeasts which do not reveal deviations in DNA profiles, prove that phenotypic characterization may be misleading in wine yeast identification. Significant changes in biochemical profiles are not always correlated with the genomic modifications detected with universally used methods such as karyotyping and mtDNA restriction analysis. Because of frequent length polymorphism and aneuploidy (gross genomic diversity), karyotyping even though it does not seem to be a good discriminative tool, can be successfully used to determine the stability of wine yeasts. Restriction analysis of mitochondrial DNA seems to be a more sensitive technique allowing for an early detection of genotypic changes in yeast. A combination of both methods makes it possible to conduct the quick routine assessment of wine yeast stability in pure culture collections depositing industrial strains. The segregates of wine yeasts obtained in our study provide suitable material for researching the adaptive

evolution of wine yeasts subjected to fermentative stress. The stability of their technological features during wine must fermentation will be explored in further studies.

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