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Research Article

Mitochondrial inheritance and fermentative : oxidative balance in hybrids between *Saccharomyces cerevisiae* and *Saccharomyces uvarum*

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Abstract

Breeding between *Saccharomyces* species is a useful tool for obtaining improved wine yeast strains, combining fermentative features of parental species. In this work, 25 artificial *Saccharomyces cerevisiae* × *Saccharomyces uvarum* hybrids were constructed by spore conjugation. A multi-locus PCR–restriction fragment length polymorphism (PCR–RFLP) analysis, targeting six nuclear gene markers and the ribosomal region including the 5.8S rRNA gene and the two internal transcribed spacers, showed that the hybrid genome is the result of two chromosome sets, one coming from *S. cerevisiae* and the other from *S. uvarum*. Mitochondrial DNA (mtDNA) typing showed uniparental inheritance in all hybrids. Furthermore, sibling hybrids, obtained by repeated crosses between the same parental strains, showed the same mtDNA, suggesting that the mitochondrial transmission is not stochastic or species-specific, but dependent on the parental strains. Finally four hybrids, two of which with *S. cerevisiae* mtDNA and two with *S. uvarum* mtDNA, were subjected to transcriptome analysis. Our results showed that the hybrids bearing *S. cerevisiae* mtDNA exhibited less expression of genes involved in glycolysis/fermentation pathways and in hexose transport compared to hybrids with *S. uvarum* mtDNA. Respiration assay confirmed the increased respiratory activity of hybrids with the *S. cerevisiae* mtDNA genome. These findings suggest that mtDNA type and fermentative : respiratory performances are correlated in *S. cerevisiae* × *S. uvarum* hybrids and the mtDNA type is an important trait for constructing new improved hybrids for winemaking. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: ***Saccharomyces cerevisiae*; *Saccharomyces uvarum*; yeast hybrid; gene expression; mitochondrial DNA**

Introduction

The species most commonly involved in wine fermentation belong to the genus *Saccharomyces*, including *Saccharomyces cerevisiae* and closely related taxa (Pretorius, 2000). *S. cerevisiae* is predominant at the end of alcoholic fermentation and it has been successfully used in winemaking as a starter culture. Another species frequently associated to winemaking at low temperature is *Saccharomyces bayanus* var. *uvarum* (Naumov *et al*., 2000), also called *S. uvarum* (Rainieri *et al*., 1999; Nguyen *et al*., 2000). The oenological importance of *S. uvarum* relies on its ability to ferment grape musts at very low temperatures, producing high amounts of glycerol and β-phenylethanol (Rainieri *et al*., 1998, 1999).

Breeding between *Saccharomyces* species is a useful tool for obtaining improved wine yeast strains, combining fermentative features of both

parents (Romano *et al*., 1985; Zambonelli *et al*., 1997; Rainieri *et al*., 1998; Giudici *et al*., 2005; Marullo *et al*., 2004, 2006). Interspecific hybrids between cryotolerant *S. uvarum* strains and non-cryotolerant *S. cerevisiae* strains have been obtained by spore conjugation and successfully employed in oenology (for review, see Giudici *et al*., 2005). These hybrids show a homogeneous phenotype (Solieri *et al*., 2005), including high fermentation competitiveness (heterosis) and both intermediate secondary metabolic compounds production and average optimal growth temperature compared to their parents (Rainieri *et al*., 1998).

S. cerevisiae × *S. uvarum* interspecific F1 hybrids are viable but sterile strains, producing only about 1% viable gametes that are generally highly aneuploid (Banno and Kaneko, 1989; Hawthorne and Philippsen, 1994; Giudici *et al*., 1998; Greig *et al*., 2002). However, some studies have also reported the occurrence of allotetraploid *S. cerevisiae* × *S. uvarum* hybrids with fertile gametes (Sebastiani *et al*., 2002; Antunovics *et al*., 2005). According to genome sequencing data (Souciet *et al*., 2000; Cliften *et al*., 2003; Kellis *et al*., 2003), *S. cerevisiae* and *S. uvarum* appear to be two closely related species within the *Saccharomyces sensu stricto* group, with highly similar genomes of 16 chromosomes, differing in five reciprocal translocations and three inversions (Kellis *et al*., 2003). Post-mating reproductive isolation in interspecific hybrids is primarily due to sequence divergence acted upon by the mismatch repair system, and not due to major gene differences or chromosomal rearrangements (Liti *et al*., 2006).

Differently from the nuclear genome, the mitochondrial DNAs (mtDNA) of *S. cerevisiae* and *S. uvarum* are quite different in both their size and their gene orders. In particular, *S. uvarum* was found to possess a smaller mtDNA than *S. cerevisiae* (57 kb vs. 70–85 kb), due to a lower number of *ori/rep* like sequences (four vs. eight) and GC-rich clusters (only 50–60 compared to 200 clusters in *S. cerevisiae*; Cardazzo *et al*., 1998). In *Saccharomyces* interspecific hybrids the mitochondrial genome shows a non-Mendelian inheritance mechanism: the hybrid cell zygote is heteroplasmic as soon as it is generated, but in the following hybrid offspring the homoplasmy is restored, so that only one type of mtDNA is present (Piskur, 1994; Berger and Yaffe, 2000). Several studies have reported homoplasmic inheritance in artificial *Saccharomyces* hybrids (Marinoni *et al*., 1999; Pulvirenti *et al*., 2000; De Vero *et al*., 2003), although with some differences. Marinoni *et al*. (1999) observed that *S. cerevisiae* mtDNA is preferentially transmitted to the progeny. In other studies, no preferential transmission of *S. cerevisiae* mtDNA type has been reported (Pulvirenti *et al*., 2000; DeVero *et al*., 2003).

In this study we attempt to explore the mitochondrial inheritance in interspecific *S. cerevisiae* × *S. uvarum* hybrids and to establish a correlation between the type of inherited mitochondrial genome and the fermentative phenotype of hybrids. For these purposes, we considered the two genomic sequences of *S. cerevisiae* and *S. uvarum* to evaluate the genome of 25 hybrids by multilocus PCR–restriction fragment length polymorphism (PCR–RFLP) analysis. Then a mitochondrial genome typing was performed. Furthermore, by means of DNA macroarray technology, we compared gene expression profiles between four interspecific hybrids, two of them with *S. cerevisiae* mtDNA type and two with *S. uvarum* mtDNA type. Our results suggest that mitochondria are specifically inherited in each strains combination and that they determine the fermentative : respiratory balance of the hybrids.

Materials and methods

Yeast strains

Yeast strains used in this study are listed in Table 1. Ten hybrids were obtained in our laboratory by spore conjugation, using gametes coming from wild-type strains or from their monosporic clones, as reported in Figure 1. Yeasts were grown at 28 °C on complete YPD medium (1% yeast extract, 2% peptone, 2% dextrose) solidified with 2% agar as necessary. Sporulation was induced by cells incubation at 28 °C for 4–6 days on agar acetate medium (1% sodium acetate, 2% agar). After digestion of the ascus walls with 0.2 mg/ml Zymolyase 20T (Seikagaku Corporation, Japan), tetrad analysis and crossing were performed, using a Singer micromanipulator (Singer Instruments).

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Table 1. Yeast parental strains and interspecific hybrids used in this work

Strain code	Species	Genetic characteristic	Isolation source*	Strain collection
3002	*S. cerevisiae*	HO/HO strain	Wine	Giudici *et al.* (1990)
7070	*S. cerevisiae*	HO/HO diploid strain	Wine	DIPROVAL
35G2	*S. cerevisiae*	HO/HO diploid strain	Wine	DIPROVAL
4003	*S. cerevisiae*	HO/HO diploid strain	Wine	Giudici *et al.* (1990)
7877	*S. uvaram*	HO/HO diploid strain	Refrigerated must	DIPROVAL
11 204	*S. uvarum*	HO/HO diploid strain	Refrigerated must	DIPROVAL
12 233	*S. uvarum*	HO/HO diploid strain	Refrigerated must	DIPROVAL
4003.1A	*S. cerevisiae*	HO/HO, monosporic clone of 4003	Wine	this work
4003.10B	*S. cerevisiae*	HO/HO, monosporic clone of 4003	Wine	this work
6167.3A	*S. cerevisiae*	HO/HO, monosporic clone of 6167	Wine	this work
6167.8C	*S. cerevisiae*	HO/HO, monosporic clone of 6167	Wine	this work
7877.10A	*S. uvarum*	HO/HO, monosporic clone of 7877	Refrigerated must	this work
7877.10B	*S. uvarum*	HO/HO, monosporic clone of 7877	Refrigerated must	this work
7877.9B	*S. uvarum*	HO/HO, monosporic clone of 7877	Refrigerated must	this work
7877.6C	*S. uvarum*	HO/HO, monosporic clone of 7877	Refrigerated must	this work
11052.1A	*S. cerevisiae*	HO/HO, monosporic clone of 11 052	Wine	DIPROVAL
11204.1A	*S. uvarum*	HO/HO, monosporic clone of 11 204	Refrigerated must	DIPROVAL
7070.1A	*S. cerevisiae*	HO/HO, monosporic clone of 7070	Wine	DIPROVAL
7877.3A	*S. uvarum*	HO/HO, monosporic clone of 7877	Refrigerated must	DIPROVAL
9109.10D	*S. cerevisiae*	HO/HO, monosporic clone of 9109	Wine	DIPROVAL
6213.1A	*S. cerevisiae*	HO/HO, monosporic clone of 6213	Wine	DIPROVAL
LS3**	Hybrid	Cross between 7877 and 3002	—	this work
LS4	Hybrid	Cross between 7877 and 3002	—	this work
LS6	Hybrid	Cross between 7877.10A and 4003.1A	—	this work
7877.10A × 4003.1A 2	Hybrid	Cross between 7877.10A and 4003.1A	—	this work
LS7	Hybrid	Cross between 7877.10B and 4003.1B	—	this work
7877.10B × 4003.1B 2	Hybrid	Cross between 7877.10B and 4003.1B	—	this work
LS8	Hybrid	Cross between 7877.9B and 6167.3A	—	this work
7877.9B × 6167.3A 2	Hybrid	Cross between 7877.9B and 6167.3A	—	this work
LS9	Hybrid	Cross between 7877.6C and 6167.8C	—	this work
7877.6C × 6167.8C 2	Hybrid	Cross between 7877.6C and 6167.8C	—	this work
11 204 × 11 052.1A 1**	Hybrid	Cross between 11 204 and 11 052.1A	—	DIPROVAL
11 204 × 11 052.1A 2	Hybrid	Cross between 11 204 and 11 052.1A	—	DIPROVAL
11 204 × 7070 1	Hybrid	Cross between 11 204 and 7070	—	DIPROVAL
11 204 × 7070 2	Hybrid	Cross between 11 204 and 7070	—	DIPROVAL
11 204.1A × 7070.1A 1	Hybrid	Cross between 11 204.1A and 7070.1A	—	DIPROVAL
11 204.1A × 7070.1A 2	Hybrid	Cross between 11 204.1A and 7070.1A	—	DIPROVAL
7877.3A × 7070.1A 1	Hybrid	Cross between 7877.3A and 7070.1A	—	DIPROVAL
7877.3A × 7070.1A 2	Hybrid	Cross between 7877.3A and 7070.1A	—	DIPROVAL
7877.3A × 7070.1A 3	Hybrid	Cross between 7877.3A and 7070.1A	—	DIPROVAL
11 204.1A × 9109.10D 1	Hybrid	Cross between 11 204.1A and 9109.10D	—	DIPROVAL
11 204.1A × 9109.10D 2	Hybrid	Cross between 11 204.1A and 9109.10D	—	DIPROVAL
11 204.1A × 9109.10D 3	Hybrid	Cross between 11 204.1A and 9109.10D	—	DIPROVAL
12 233 × 6213.1A 1	Hybrid	Cross between 12 233 and 6213.1A	—	DIPROVAL
12 233 × 6213.1A 2	Hybrid	Cross between 12 233 and 6213.1A	—	DIPROVAL
12 233 × 3562	Hybrid	Cross between 12 233 and 35G2	—	DIPROVAL

DIPROVAL, Dipartimento di Protezione e Valorizzazione Agroalimentare.

* Isolation source is referred to wild strains.

** Strains in bold were subjected to transcriptome analysis.

Genomic DNA extraction and multi-locus PCR–RFLP analysis

The genomic DNA extraction was performed as described by Querol *et al*. (1992). PCR amplification reactions were targeted to the 5.8S–*ITS* regions, comprising the 5.8S rDNA gene and the two flanking internal transcribed spacers, 1 and 2 (ITS), as well as to six nuclear-encoded gene regions (*CAT8, CYR1, GSY1, MET6, MET2* and *OPY1*). Each amplification mixture contained 1×

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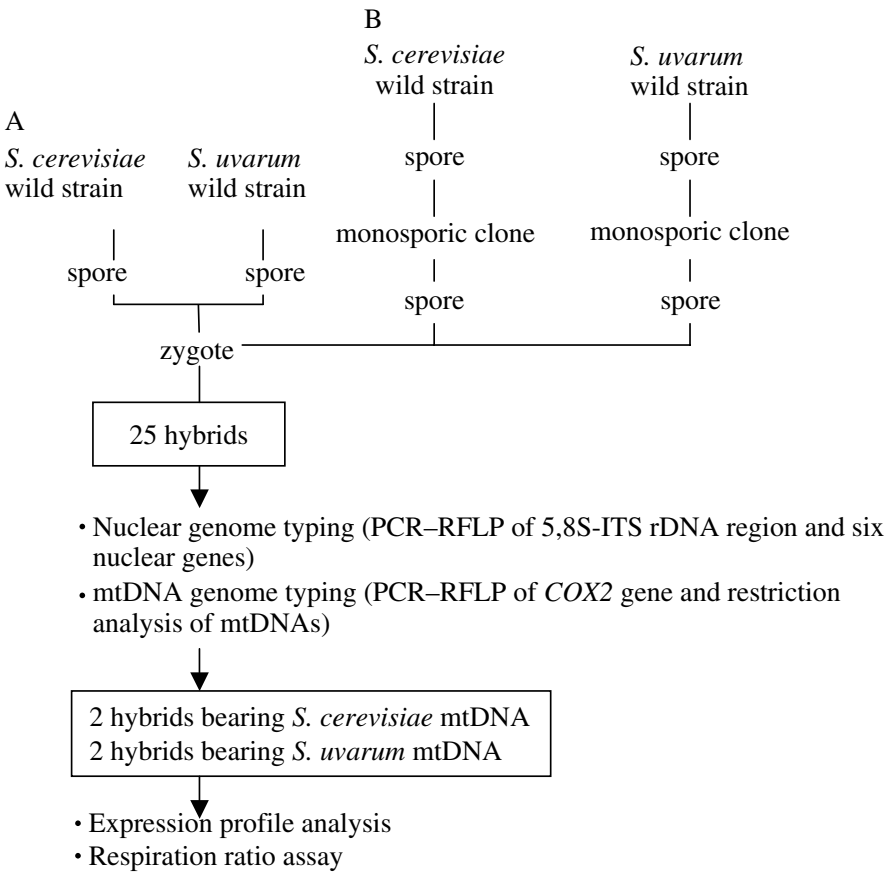

Figure 1. Experimental plan performed in this study

Taq polymerase buffer, 100 μM deoxynucleotides, 1 μM of each primer and 2 U Taq polymerase. A volume of 4 μl DNA (1–50 ng/μl) was transferred to a PCR tube before adding the reaction mixture to a final volume of 100 μl. The PCR amplification was carried out in a Techgene thermocycler (Techne, Cambridge, UK) as follows: initial denaturing at 95 °C for 5 min, then 40 PCR cycles of three steps (denaturing at 94 °C for 1 min, annealing at 55.5 °C for 2 min and extension at 72 °C for 2 min), followed by final extension at 72 °C for 10 min. PCR products were digested by different restriction enzymes (Roche Molecular Biochemicals, Mannheim, Germany) to differentiate the parental origin of the alleles present in hybrids. Oligonucleotide primers and endonucleases are listed in Table 2. The amplicons and restriction fragments were separated on 1.4 or 3% agarose gels with 0.5× TAE buffer, respectively. The gels were stained with ethidium bromide, destained in sterile water and photographed using an UV transilluminator. Restriction patterns of hybrids were compared to those obtained from the *S. cerevisiae* type strain CBS 1171^T and their parental strains. Estimations of fragment lengths were evaluated by comparison to a 100 bp DNA ladder marker (Gibco–BRL, Gaithersburg, MD, USA).

mtDNA typing

Mitochondria isolation, mtDNA extraction and *Eco*RV restriction analysis were carried out as described by De Vero *et al.* (2003). The mtDNA fragment sizes of hybrids were compared with those of parental strains using a 1 kb DNA ladder (Invitrogen, Cergy-Pontoise, France) as reference. PCR amplification of *COX2* mitochondrial gene was performed by using COII5 and COII3 primers and then digested, as described in the previous section (Table 2).

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Table 2. Primer sequences and restriction enzymes used in multi-locus PCR–RFLP analysis

Target	Chromosomal coordinates*	Primer sequence	Enzyme	References
5.8S–ITS	ChrVII, repeated 100–200 times	ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ ITS4 5′-TCCTCCGCTTATTGATATGC-3′	*Hae*IIII	White *et al.*, 1990
COX2	mitochondrial gene	COII-5 5′-GGTATTTTAGAATTACATGA-3′ COII-3 5′-ATTTATTGTTCRTTTAATCA-3′	*Hin*fI	Belloch *et al.*, 2000
MET2	ChrXIV: from 117 347 to 118807	MET2-5 5′-CGGCTCTAGACGAAAACGCTCCAAGAGCTGG-3′ MET″-3 5′-CGGCCTCTAGAGACCACGATATGCACCAGGCAG-3′	*Eco*RI/*Pst*I	Hansen and Kielland-Brandt, 1994
MET6	ChrV: from 342 163 to 339 860	MET6-5 5′-CTAGACCTGTCCTATTGGGTCCAGTTTCTTACTT-3′ MET6-3 5′-TTAGCTTCTAGGGCAGCAGCAACRTCTTGACC-3′	*Hae*III/*Hin*fI	Gonzalez *et al.*, 2006
OPY1	ChrII: from 495 295 to 494 309	OPY1-5 5′-CCGCGGACAACAGACCAYCATTAYTGGTGYGT-3′ OPY1-3 5′-CTCTTGAAATTTATTATCCARTCCACCATRTCYTG-3′	*Hae*III	Gonzalez *et al.*, 2006
GSY1	ChrVI: from 176 383 to 174 257	GSY1-5 5′-ATTGGAAAAAGAATTTTCGAGCAYACRATGAG-3′ GSY1-3 5′-AATTTCTTGCCACCGGCAAGGGTATTCATATT-3′	*Msp*I	Gonzalez *et al.*, 2006
CAT8	ChrXIII: from 831 328 to 827 027	CAT8-5 5′-TCCAATATTAGTATCAACAACTTTCTATAYCARAAYGA-3′ CAT8-3 5′-CTACTTGGCRTTTTGCCAYTGRAA-3	*Msp*I	Gonzalez *et al.*, 2006
CYR1	ChrX: from 424 851 to 430 931	CYR1-5 5′-CTACGAAGGAAAGTGTCCTCTTTRGTTCGTGG-3′ CYR1-3 5′-CCGTGTGTAGAATTTAGTGTAGAATTGACRGC-3′	*Msp*I	Gonzalez *et al.*, 2006

* Referred to *S. cerevisiae* genome.

Total RNA extraction, labelling and macroarray hybridization

The following four hybrids were subjected to macroarray-based transcriptome analysis: LS3, 11 204.1A × 7070.1A 1, 12 233 × 6213.1A 1 and 11 204 × 11 502.1A 1. 50 ml of each of three independent cultures of each hybrid strain were harvested at the middle logarithmic phase ($OD_{600} = 0.5–0.6$) by centrifugation after growing in YPD medium with orbital agitation, and the cell pellets were rapidly frozen and stored at −80 °C. Total RNA extraction and labelling by oligo-($dT_{15}VN$) priming, using [α^{33}P]dCTP (3.000 Ci/mM; 10 μCi/μl) were performed as described by Alberola *et al*. (2004). The labelled cDNAs were purified by using a MicroSpin S-300 HR column (Amersham Biosciences, NJ, USA). Around 3×10^6 dpm/ml labelled cDNA was used for filter hybridization. Pre-hybridization, hybridization and washing were carried out according to published protocols (Alberola *et al*., 2004). In addition, membranes were hybridized with total genomic DNA of *S. cerevisiae* strain 3002 labelled by random priming, as described by Alberola *et al*. (2004).

Signal acquisition and statistical analysis

Digital images of radioactive signals were acquired with a phosphorimager scanner FujiFilm FLA3000 and quantified using ArrayVision 7.0 software (Imaging Research Inc.), taking the artefact-removed median density (with the corresponding subtracted background) as signal. Poor or inconsistent signals were not considered for further analysis. Genomic hybridization signals of a *S. cerevisae* strain were used to normalize cDNA signals of hybrids in each corresponding filter.

The normalization process and the measure of the significance level for each ORF were performed using ArrayStat software (Imaging Research Inc.). Replicates reproducibility was tested considering the data as independent and allowing the program to take a minimum number of valid replicates of 2, in order to calculate the mean values for every gene. The Z-test ($p = 0.05$) was applied to estimate significant differentially expressed genes in all pairwise comparisons (hybrid vs. hybrid) and the correlation coefficient was calculated by iterative median and corrected by the false discovery rate test to estimate the statistical errors associated to each gene. Raw macroarray data were submitted to the GEO database, where they have the Accession No. GSE9888.

Computational treatment of gene expression profiles

Genes with a significantly altered expression by a factor of ≥2.5 (upregulated genes) or ≤0.4 (down-regulated genes), according to the Z-test, were chose for a subsequent functional analysis using the FuncAssociate tool (**http://llama.med.harvard.edu/cgi/func/funcassociate**), in order to find statistically significant over-represented functional classes. We considered significant categories when the adjusted p value cut-off was <0.05.

Respiration : fermentation ratio analysis

The respiration and fermentation activities of LS3, 11 204.1A × 7070.1A 1, 12 233 × 6213.1A 1 and 11 204 × 11 052.1A 1 hybrid strains, as well as those of the parental strains (*S. cerevisiae* strain 3002 and *S. uvarum* strain 7877, respectively), were measured with a Warburg constant volume respirometer instrument (B. Braun. Melsungen, Germany; Model V85), following the manufacturer's instructions. 1 ml each cell suspension was disposed in two respirometers for each tested yeast strain, then connected to a manometer. The cell suspensions were thermostated and agitated for 10 min before adding sugar solution. In the arms of the flasks, 0.6 ml sugar solution (20 g/l) were added and in the wells alternatively 0.4 ml concentrated NaOH or water. The manometer was regularly checked every 10 min. After 2 h the reaction was stopped and the data used for calculating the $Q_{O2} : Q_{CO2}$ ratio. This ratio was obtained for each strain, as a ratio between the volume differences observed at the two different conditions, with and without alkali.

Results

Multi-locus nuclear genome characterization

In this study interspecific crosses were performed between spores of two important oenological species, *S. cerevisiae* and *S. uvarum*, obtaining 10 new hybrid strains. In addition, other hybrids from the DIPROVAL collection were studied; in all, 25

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hybrid strains were subjected to nuclear genome typing. In particular we performed PCR–RFLP of the 5.8S–ITS rDNA region, as well as of six nuclear-encoded genes, including *CAT8, CYR1, GSY1, MET6* and *OPY1* (Gonzales *et al*., 2006) and *MET2* (Masneuf *et al*.,1998).

The *Hae*III restriction endonuclease allows differentiation of the 5.8S–ITS region of *S. cerevisae* from that of *S. uvarum* (Fernández-Espinar *et al*., 2000; Solieri *et al*., 2005). The parental strains belonging to *S. uvarum* species and their monosporic clones showed three bands of 495, 230 and 125 bp, respectively, whereas the restriction pattern of the *S. cerevisiae* parental strains or their monosporic clones showed four bands of 325, 230, 170 and 125 bp. All hybrids exhibited composite restriction profiles with five bands (Figure 2, line 2), which confirmed their hybrid nature.

Furthermore, six different nuclear genes, *CAT8, CYR1, GSY1, MET6, OPY1* and *MET2*, located on chromosomes XIII, X, VI, V, II and XIV, respectively, were amplified and subsequently digested using endonucleases suitable to distinguish the *S. cerevisiae* allele from the *S. uvarum* allele for each locus. All hybrid strains showed a complex restriction pattern determined by the presence of two different copies of each gene: one coming from the *S. cerevisiae* parental strain and the other from the *S. uvarum* parental strain, as reported in Table 3. Considering that 5.8S–ITS regions and the nuclear genes *MET2, MET6, CAT8, OPY1, CYR1* and *GSY1* are located on different chromosomes, these results confirmed that the hybrid genome contains two different chromosome sets, each from both parental strains, in agreement with the karyotype profile analysis performed with different

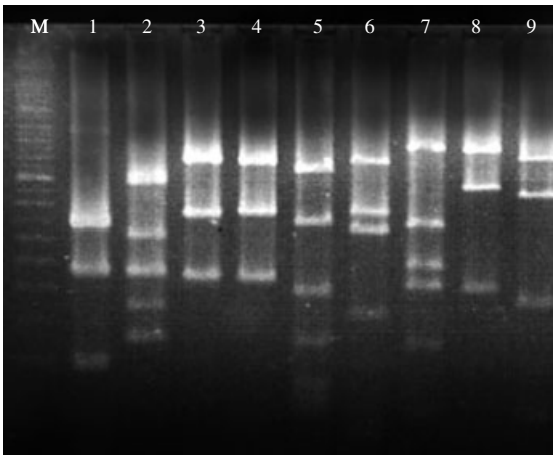

Figure 2. Endonuclease restriction pattern of different PCR products from hybrid LS3. M, marker 100 kb ladder; 1, *COX2* digested by *Hin*fI; 2, 5.8S-ITS region digested by *Hae*III; 3, *MET2* digested by *Pst*I; 4, *MET2* digested by *Eco*RI; 5, *OPY1* digested by *Hae*III; 6, *GSY1* digested by *Hpa*II; 7, *CAT8* digested by *Hpa*II; 8, *MET6* digested by *Hae*III; 9, *MET6* digested by *Hin*fI

S. cerevisiae × *S. uvarum* hybrids by other authors (Marinoni *et al*., 1999; Masneuf *et al*., 1998; Pulvirenti *et al*., 2000).

mtDNA typing

The hybrids and their parental strains were screened for the mtDNA type using both *Eco*RV digestion of the total mtDNA genome (Nguyen *et al*., 2000) and *COX2* PCR–RFLP analysis with *Hin*fI (Belloch *et al*., 2000).

S. cerevisiae and *S. uvarum* parental strains showed different strain-specific *Eco*RV restriction patterns, according to general assumption that mitochondrial genome exhibits sequence polymorphism

Table 3. PCR and restriction analysis of 5.8S–ITS rDNA region and six nuclear genes in *S. cerevisiae, S. uvarum* parental strains and their hybrids

Gene target	Bp*	Enzyme	*S. cerevisiae*	*S. uvarum*	Hybrids
5.8S–ITS	850	*Hae*III	325–230–170–125	495–230–125	495–325–230–170–125
MET2	580	*Eco*RI	369–211	580	580–369–211
		*Pst*I	580	369–211	580–369–211
MET6	682	*Hae*III	682	477–205	682–477–205
		*Hin*fI	450–160–57	625–57	625–450–160–57
OPY1	681	*Hae*III	554–127	355–175–127	554–335–175–127
GSY1	780	*Msp*I	608–161	381–338	608–338–381–161
CAT8	810	*Msp*I	688–122	360–250–200	688–360–250–200–122
CYR1	565	*Msp*I	397–168	561	397–168

* PCR product size; *S. cerevisiae, S. uvarum* and hybrids: all of *S. cerevisiae, S. uvarum* and hybrids indicated in Table 1.

Table 4. mtDNA typing by PCR–RFLP analysis of *COX2* mitochondrial gene and *Eco*RV restriction profiling of total mtDNA genome

Species	Strains	PCR–RFLP of COX2		*Eco*RV mtDNA profile
		Amp*	*Hin*fI profile	
S. cerevisiae	4003.1A, 3002, 4003.10B, 6167.3A, 6167.8C, 11 502.1A, 7070, 7070.1A, 9109.10D, 6213.1A, 35G2	630	327–200–100	*S. cerevisiae*
S. uvarum	7877, 7877.10A, 7877.10B, 7877.9B, 7877.6C, 11 204, 7877.3A, 7877.10A, 7877.10B, 7877.9B, 7877.6C, 11 204, 7877.3A, 12 233	630	327–300	*S. uvarum*
Hybrids with *Su* mtDNA	**11 204 × 11 502.1A 1****, 11 204 × 11 502.1A 2, 11 204.1A × 9109.10D 1, 11 204.1A × 9109.10D 2, 11 204.1A × 9109.10D 3, **12 233 × 6213.1A 1,** 12 233 × 6213.1A 2	630	327–300	*S. uvarum*
Hybrids with *Sc* mtDNA	**LS3**, LS4, LS6, 7877.10A × 4003.1A 2, LS7, 7877.10B × 4003.1B 2, LS8, 7877.9B × 6167.3A 2, LS9, 7877.6C × 6167.8C 2, 11 204 × 7070 1, 11 204 × 7070 2, **11 204.1A × 7070.1A 1**, 11 204.1A × 7070.1A 2, 7877.3A × 7070.1A 1, 7877.3A × 7070.1A 2, 7877.3A × 7070.1A 3	630	327–200–100	*S. cerevisiae*

* PCR product size.

** Strains in bold were subjected to transcriptome analyses.

Su, S. uvarum; Sc, S. cerevisiae; the terms *Sc* mtDNA and *Su* mtDNA were used to indicate the hybrid mtDNA restriction profiles identical to those of *S. cerevisiae* or *S. uvarum* parental strains.

within populations of same species (Clark, 1984). In all cases, the hybrids showed a single mitochondrial *Eco*RV-based pattern, identical to that of only one of the parental strains (Table 4).

*Hin*fI-based digestion analysis of the *COX2* mitochondrial gene confirmed the mtDNA typing obtained by mtDNA restriction analysis. *Hin*fI digestion of *S. cerevisiae COX2* amplicon yielded three bands of 327, 200 and 100 bp, respectively, whereas *S. uvarum COX2* digestion generated two bands of 327 and 300 bp. As reported in Table 4, all hybrids showed only one *COX2* restriction profile, belonging to either the *S. cerevisiae* or the *S. uvarum* parental strain. The results indicated a mtDNA uniparental inheritance in hybrids, but do not show any preferential transmission of the *S. cerevisiae* mtDNA genome. Moreover, repeated crosses between the same parental strains produced sibling hybrids with the same type of mtDNA genome, suggesting that mtDNA inheritance in hybrid progeny the might not be stochastic, but instead dependent on the specific parental strains involved.

Transcriptome analysis

To determine whether mitochondrial inheritance could cause differences in hybrids fitness, we performed a comparative transcriptome assay, using four hybrid strains, two of them bearing *S. cerevisiae* mtDNA (LS3 and 11 204.1A × 7070.1A 1) and the other two *S. uvarum* mtDNA (12 233 × 6213.1A 1 and 11 204 × 11 052.1A 1). In this study, three independent hybridizations of cDNA were performed for each hybrid, resulting in 12 datasets, normalized among replicates. Genomic DNA signal intensities obtained from *S. cerevisiae* strain 3002 were used to normalize the corresponding cDNA signals in each respective filter, in order to eliminate signal intensity changes related to sequence homology differences. Corrected cDNA signal values were then used for pairwise comparisons between hybrids. We performed six hybrid vs. hybrid comparisons.

Of the about 6049 gene probes contained in DNA macroarray, approximately 5000–5300 generated readable signals in both sets compared, only a few hundred showed significant changes of mRNA level in all six comparisons. The hybrids transcriptomes were similar, as confirmed by high Pearson correlation values comprised in a range 0.89–0.95 (data not shown). The number of up- or down-regulated genes for each pairwise comparison is reported in Table 5.

Most differentially expressed genes belong to the same significant functional categories (adjusted p value cut-off 0.05), as reported in Table 6.

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Table 5. Number of differentially expressed genes in six pairwise comparisons

Hybrid comparison	mtDNA	Differentially expressed ORFs
LS3 vs. 11 204 × 7070.1A 1	*Sc* vs. *Sc*	25(12; 13)*
11 204 × 11 502.1A 1 vs. 12 233 × 6213.1A 1	*Su* vs. *Su*	393(97; 268)
LS3 vs. 12 233 × 6213.1A 1	*Sc* vs. *Su*	606(175; 264)
LS3 vs. 11 204 × 11 502.1A 1	*Sc* vs. *Su*	408(123; 205)
11 204 × 7070.1A 1 vs. 11 204 × 11 502.1A 1	*Sc* vs. *Su*	286(170; 116)
11 204 × 7070.1A 1 vs. 12 233 × 6213.1A 1	*Sc* vs. *Su*	469(211; 253)

The number of more (ratio > 2.5) and less (ratio < 0.4) expressed genes are reported in parentheses.

Genes involved in fermentation/glycolysis pathways were less expressed in hybrids with *S. cerevisiae* mtDNA (LS3 and 11 204.1A × 7070.1A 1), compared to hybrids bearing *S. uvarum* mtDNA (11 204 × 11 052.1A 1 and 12 233 × 6213.1A 1). Hexose transport genes were less expressed in hybrids with *S. cerevisiae* mtDNA compared to the hybrid 11 204 × 11 052.1A 1. However, no significant expression differences were detected in the comparison between different strains containing both *S. cerevisiae* mtDNA.

Fermentation and glycolysis pathways

An important function for wine yeast is the ethanol biosynthesis. The last step of this pathway is catalysed by five isoforms of alcohol dehydrogenases, encoded by the genes *ADH1, ADH2, ADH3, ADH4* and *ADH5* (James *et al*., 2003; Thomoson *et al*., 2005). The *ADH* multi-gene family shows high sequence homology (up to 75%), with the exception of the *ADH4* gene, which appears to have a bacterial origin (Williamson and Paquin, 1987). Due to the high homology level, it is probable that *ADH* cDNAs cross-hybridize and thus it is difficult to distinguish different *ADH* isoforms by DNA chip assay. However, our results suggested a general lower expression levels of *ADH* genes in hybrids with *S. cerevisiae* mtDNA (LS3 and 11 204.1A × 7070.1A 1) compared to hybrids with *S. uvarum* mtDNA (11 204 × 11 052.1A 1 and 12 233 × 6213.1A 1; Table 7).

Genes encoding glycolytic enzymes were less expressed in hybrids with *S. cerevisiae* mtDNA (Table 7). In particular, some genes showed more than three times lower expression levels: two minor isoforms of pyruvate decarboxylase (*PDC5* and *PDC6*), which decarboxylate pyruvate to acetaldehyde; a pyruvate kinase (*PYK2*) that appears to be modulated by phosphorylation; and hexokinase I (*HXK1*), a cytosolic protein that catalyses glucose phosphorylation. Also genes encoding enzymes involved in the first stages of the hexose metabolism and glycolysis (glucokinase, hexokinase I, hexokinase II and phosphoglucose isomerase, respectively), as well as genes coding for glyceraldehyde 3-phosphate dehydrogenase isoform I (*TDH1*), pyruvate kinase (*CDC19*) and 3-phosphoglycerate kinase (*PGK1*), showed a lower expression level in hybrids with *S. cerevisiae* mtDNA compared to hybrids with *S. uvarum* mtDNA (Table 7).

Table 6. Main functional categories downregulated in four pairwise comparisons between hybrids with *S. cerevisiae* mtDNA (*Sc*) and hybrids with *S. uvarum* mtDNA (*Su*)

Hybrid *Sc* mtDNA	Hybrid *Su* mtDNA	Functional category	*p* Value	Number**
LS3	11 204 × 11 052.1A	Hexose transporter activity	1.3×10^{-9}	8 (17)
		Fermentation	8.6×10^{-7}	6 (16)
		Alcohol metabolism	4.7×10^{-7}	16 (157)
LS3	12 233 × 6213.1A	Oxidoreductase activity	2.7×10^{-6}	13 (68)
		Alcohol metabolism	6.2×10^{-5}	18 (157)
11 204.1A × 7070.1A 1	11 204 × 11 052.1A 1	Hexose transporter actvity	2.9×10^{-7}	6 (17)
		Glycolysis	1.3×10^{-5}	7 (22)
		Alcohol metabolism	1.1×10^{-4}	4 (16)
11 204.1A × 7070.1A 1	12 233 × 6213.1A 1	Glycolysis	1.3×10^{-5}	7 (22)
		Hexose catabolism	9.5×10^{-5}	7 (29)

* Functional categories with adjusted *p value* ≤0.05. ** The number of genes downregulated for each category is reported with number of genes overall belonging to functional category (in bracket).

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Table 7. Analysis of genes related to fermentation and glycolysis pathways in four comparisons (indicated in italic)

ORF	Gene	Function	Ratio	Homology (%)*
LS3 vs. 11 204 × *11 052.1A 1 (Sc* vs. *Su)*				
YMR145C	*NDE1*	Mitochondrial external NADH dehydrogenase	0.467	83
YBR084W	*MIS1*	Mitochondrial C1-tetrahydrofolate synthase	0.455	84
YGL253W	*HXK2*	Hexokinase isoenzyme II	0.454	90
YMR303C	*ADH2*	Glucose-repressible alcohol dehydrogenase II	0.301	87
YLR134W	*PDC5*	Minor isoform of pyruvate decarboxylase	0.299	90
YER062C	*HOR2*	DL-Glycerol-3-phosphatases	0.268	84
YMR083W	*ADH3*	Mitochondrial alcohol dehydrogenase isozyme III	0.268	86
YOR347C	*PYK2*	Pyruvate kinase II	0.235	81
YFR053C	*HXK1*	Hexokinase isoenzyme I	0.207	86
YGR087C	*PDC6*	Minor isoform of pyruvate decarboxylase	0.121	81
YBR145W	*ADH5*	Alcohol dehydrogenase isoenzyme V	0.115	82
LS3 vs. 12 233 × *6213.1A 1 (Sc* vs. *Su)*				
YBR196C	*PGI1*	Phosphoglucose isomerase	0.475	90
YMR083W	*ADH3*	Mitochondrial alcohol dehydrogenase isozyme III	0.450	86
YJL052W	*TDH1*	Glyceraldehyde-3-phosphate dehydrogenase	0.391	92
YLR134W	*PDC5*	Minor isoform of pyruvate decarboxylase	0.374	90
YMR303C	*ADH2*	Glucose-repressible alcohol dehydrogenase II	0.362	87
YAL038W	*CDC19*	Pyruvate kinase	0.360	95
YGL253W	*HXK2*	Hexokinase isoenzyme II	0.358	90
YOL086C	*ADH1*	Alcohol dehydrogenase I	0.321	94
YOR347C	*PYK2*	Pyruvate kinase	0.311	81
YBR145W	*ADH5*	Alcohol dehydrogenase isoenzyme V	0.307	82
YCR012W	*PGK1*	3-Phosphoglycerate kinase	0.302	95
YGR087C	*PDC6*	Minor isoform of pyruvate decarboxylase	0.270	81
YGL256W	*ADH4*	Alcohol dehydrogenase isoenzyme IV	0.249	82
YCL040W	*GLK1*	Glucokinase	0.247	80
YFR053C	*HXK1*	Hexokinase isoenzyme I	0.211	86
11 204.1A × *7070.1A 1* vs. 11 204 × *11 052.1A 1 (Sc* vs. *Su)*				
YFR053C	*HXK1*	Hexokinase isoenzyme I	0.295	86
YLR134W	*PDC5*	Minor isoform of pyruvate decarboxylase	0.275	90
YMR083W	*ADH3*	Mitochondrial alcohol dehydrogenase isozyme III	0.260	86
YOR347C	*PYK2*	Pyruvate kinase II	0.245	81
YMR303C	*ADH2*	Glucose-repressible alcohol dehydrogenase II	0.193	87
YBR145W	*ADH5*	Alcohol dehydrogenase isoenzyme V	0.112	82
YGR087C	*PDC6*	Minor isoform of pyruvate decarboxylase	0.110	86
11 204.1A × *7070.1A 1* vs. 12 233 × *6213.1A 1 (Sc* vs. *Su)*				
YLR134W	*PDC5*	Minor isoform of pyruvate decarboxylase	0.331	90
YAL038W	*CDC19*	Pyruvate kinase	0.329	95
YDL021W	*GPM2*	Homologue of Gpm1p phosphoglycerate mutase	0.328	83
YOR347C	*PYK2*	Pyruvate kinase	0.312	81
YJL052W	*TDH1*	Glyceraldehyde-3-phosphate dehydrogenase	0.306	92
YOL086C	*ADH1*	Alcohol dehydrogenase isoenzyme I	0.304	94
YGL253W	*HXK2*	Hexokinase isoenzyme II	0.300	89
YFR053C	*HXK1*	Hexokinase isoenzyme I	0.290	86
YBR145W	*ADH5*	Alcohol dehydrogenase isoenzyme V	0.288	82
YCR012W	*PGK1*	3-Phosphoglycerate kinase	0.266	95
YGR087C	*PDC6*	Minor isoform of pyruvate decarboxylase	0.238	86
YHR174W	*ENO2*	Enolase II (phosphopyruvate hydratase)	0.238	92
YMR303C	*ADH2*	Glucose-repressible alcohol dehydrogenase II	0.223	87
YGR254W	*ENO1*	Enolase I (phosphopyruvate hydratase)	0.199	92

* Homology evaluated by comparing the corresponding genome sequences of the reference strains of the species *S. cerevisiae* (S288c) and *S. bayanus* var. *uvarum* (MCYC 623 = CBS 7001) using the sequence alignment algorithm WU-BLAST2 (**http://blast.wustl.edu/**).

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Hexose transport

Hexose uptake is a critical step in sugar utilization and involves various hexose carriers (Luyten *et al*., 2002) encoded by *HXT* genes, that are differentially expressed in *S. cerevisiae* during wine fermentation (Pérez *et al*. 2005). Many hexose transport genes were differentially expressed in two comparisons (LS3 vs. 11 204 × 11 502.1A 1 and 11 204.1A × 7070.1A 1 vs. 11 204 × 11 502.1A 1, respectively; Table 8). *HXT1* and *HXT3* genes (coding for the main hexose low-affinity carriers during the logarithmic growth phase), as well as *HXT7* (coding for a high-affinity transporter activated at the end of the growth phase) were less expressed in hybrid LS3 compared to hybrid 11 204 × 11 502.1A 1. Five hexose transport genes were less expressed in hybrid 11 204.1A × 7070.1A 1, but their absolute expression levels were very low, according to that found in *S. cerevisiae* strains (Özcan *et al*., 1999). It is important to note that, similarly to the *ADH* gene family, the *HXT* multi-gene family has a high sequence homology, in the range 50–100%, and the expression level for each *HXT* gene is difficult to determine using DNA macroarrays.

Respiration : fermentation ratio assay

The results obtained by gene expression analysis suggested that hybrids with *S. uvarum* mtDNA had an increased fermentation : respiration balance with regard to those with *S. cerevisiae* mtDNA. In order to confirm this hypothesis, we evaluated the respiration : fermentation ratio of hybrids LS3, 11 204.1A × 7070.1A 1, 12 233 × 6213.1A 1 and 11 204 × 11 052.1A 1, as well as the parental strains *S. cerevisiae* 3002 and *S. uvarum* 7877. The respiration : fermentation ratio ($Q_{O2} : Q_{CO2}$) values were different among the tested strains, as shown in Table 9. In particular, the hybrid strains with *S. uvarum* mtDNA and the *S. uvarum* strain 7877 showed a lower respiration : fermentation ratio compared to hybrids with *S. cerevisiae* mtDNA and the *S. cerevisiae* parental strain 3002. This finding suggests that hybrids with *S. uvarum* mtDNA have a higher tendency to ferment and a lower tendency to respire than those with *S. cerevisiae* mtDNA.

Discussion

The genus *Saccharomyes* contains the main species for the fermentation industry. Meiosis and mating allow the mixing of two parental genomes for generating a unique and new allele combination and are exploited in yeast breeding to construct new strains with desired genetic features (Rainieri *et al*., 1998; Giudici *et al*., 2005). Artificial interspecific hybridization experiments (Marinoni *et al*.,

Table 8. Analysis of *HXT* genes differently expressed in two comparisons (indicated in italic)

ORF	Gene	Function	Ratio	Homology (%)*
LS3 vs. 11 204 × *11 052.1A 1 (Sc* vs. *Su)*				
YDR345C	*HXT3*	Low-affinity glucose transporter	0.436	90
YDR342C	*HXT7*	High-affinity glucose transporter	0.390	87
YHR094C	*HXT1*	Low-affinity glucose transporter	0.253	86
YHR092C	*HXT4*	High-affinity glucose transporter	0.184	87
YHR096C	*HXT5*	Hexose transporter with moderate affinity for glucose	0.173	82
YOL156W	*HXT11*	Putative hexose transporter	0.160	70
YJL219W	*HXT9*	Putative hexose transporter	0.157	81
YJL214W	*HXT8*	Protein of unknown function with similarity to hexose transporter family members	0.156	72
YFL011W	*HXT10*	Putative hexose transporter	0.110	81
11 204.1A × *7070.1A 1* vs. 11 204 × *11 052.1A 1 (Sc* vs. *Su)*				
YJL219W	*HXT9*	Putative hexose transporter	0.296	81
YHR096C	*HXT5*	Hexose transporter with moderate affinity for glucose	0.264	82
YJL214W	*HXT8*	Protein of unknown function with similarity to hexose transporter family	0.166	72
YFL011W	*HXT10*	Putative hexose transporter	0.149	81
YOL156W	*HXT11*	Putative hexose transporter	0.139	70

* Homology evaluated by comparing the corresponding genome sequences of the reference strains of the species *Saccharomyces cerevisiae* (S288c) and *Saccharomyces bayanus* var. *uvarum* (MCYC 623 = CBS 7001) using the sequence alignment algorithm WU-BLAST2 (**http://blast.wustl.edu/**).

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Table 9. Correlation between Q_{O2} : Q_{CO2} ratio, mtDNA type and gene expression results

				Expression analysis**	
Strain	**Species**	**Q_{O2} : Q_{CO2}***	**mtDNA**	**Fermentation**	**Hexose transport**
LS3	Hybrid	0.89 ± 0.06	*S. cerevisiae*	Down/down	Down/–
11 204.1A × 7070.1A 1	Hybrid	0.84 ± 0.13	*S. cerevisiae*	Down/down	Down/–
12 233 × 6213.1A 1	Hybrid	0.54 ± 0.08	*S. uvarum*	Up/up	Up/–
11 204 × 11 502.1A 1	Hybrid	0.68 ± 0.07	*S. uvarum*	Up/up	Up/–
3002	*S. cerevisiae*	1.57 ± 0.09	*S. cerevisiae*	nd	nd
7877	*S. uvarum*	0.66 ± 0.04	*S. uvarum*	nd	nd

* Two independent experimental replicates.

** Transcriptome results obtained by comparisons between two hybrids with *S. cerevisiae* mtDNA and two hybrids with *S. uvarum* mtDNA: up, more expressed; down, less expressed; –, not statistically significant; nd, not determined.

1999; de Barros Lopes *et al*., 2002; Sato *et al*., 2002; Antunovics *et al*., 2005) have also been performed extensively to delimit species of the genus *Saccharomyces*, according to the biological species concept (Naumov, 1996). Wild hybrids are employed in the brewing industry (*S. cerevisiae* × *S. bayanus*-like hybrids) and have recently being found also in other fermentation processes. Masneuf *et al*. (1998) characterized a putative *S. uvarum* × *S. cerevisiae* hybrid strain (S6U) isolated from Italian wines and a striking hybrid (CID1) from a home-made French cider that contains two copies of the nuclear gene *MET2*, one originating from *S. cerevisiae* and the other from *S. bayanus*, with the mitochondrial genome originating from a third species, *S. kudriavzevii* (Groth *et al*., 1999). Recently other putative *S. cerevisiae* × *S. bayanus* hybrids were detected in French wine (Le Juene *et al*., 2007), while hybrids between *S. cerevisiae* and *S. kudriavzevii* were isolated from Swiss wines and ale beers. A multi-locus genomic approach was proposed for their characterization, based on restriction analysis of 5.8S–ITS regions and of five nuclear markers (González *et al*., 2006, 2007).

By using a similar approach, we demonstrated that our hybrids showed two different alleles for every locus analysed, one coming from *S. cerevisiae* and the other from *S. uvarum*. These two *Saccharomyces sensu stricto* species have homologous chromosomes where the gene order is largely conserved, and for this reason both parental sets of chromosomes were compatible and could coexist in the hybrid offspring. However, in *S. cerevisiae* × *S. uvarum* hybrids the sequence divergence is wide enough to reduce rates of homologous recombination, resulting in their inability to efficiently segregate during meiosis. Marinoni *et al*. (1999) demonstrated that, after nuclei fusion, *S. cerevisiae* × *S. uvarum* hybrids are genetically stable and could propagate themselves through mitosis during many generations without undergoing any apparent rearrangements of their nuclear genome. Similarly, our hybrids have undergone a large number of mitotic divisions, but they appeared as genetically stable.

In the great majority of sexual eukaryotes, mitochondrial genomes are inherited almost exclusively from a single parent. Using two different methods to evaluate the mtDNA type in interspecific hybrid progeny, we confirmed the non-Mendelian mtDNA uniparental inheritance in interspecific *Saccharomyces* hybrids (Dujon, 1981). The mechanism involved in determining the homoplasmic state in yeast hybrids is still uncertain and different models were proposed (Berger and Yaffe, 2000). Recently, Yan *et al*. (2007) demonstrated that a sex-determining gene controls mitochondrial DNA inheritance in the basidiomycete yeast *Cryptococcus neoformans*: in crosses between strains of different mating types, progeny inherit mtDNA from the *MAT***a** parent. However, the mechanism could be different in *Saccharomyces* yeasts, as an early heteroplasmic state occurs immediately after zygote formation, but the hybrid buds become homozygous in a single mitotic generation (Piskur, 1994; Berger and Yaffe, 2000). Marinoni *et al*. (1999) found hybrids with only *S. cerevisiae* mtDNA after crosses between *S. cerevisiae* and *S. bayanus* strains, suggesting a preferential transmission of *S. cerevisiae* mtDNA type. Otherwise, mtDNA analysis of brewing hybrid strains

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highlighted that only *S. uvarum* mtDNA was inherited (Rainieri *et al*., 2005). In our crosses the parental strain transmitting the mtDNA can belong to any of the parental species, which seems to exclude the presence of a species-specific mechanism involved in the mtDNA transmission. However, it is remarkable that sibling hybrids, coming from the same type of crosses between two specific parental strains, showed the same kind of mtDNA genome. Although the number of replicated crosses per parental pair is low (no more than three), this result could suggest that the mtDNA inheritance in hybrids depends on the parental strains. This finding might support a competition during mitochondrial transmission rather than random transmission or a species-specific inheritance.

The mitochondrial genome is involved in expression of respiratory phenotype and, in general, in complex anterograde (from nucleus and cytoplasm to mitochondria) and retrograde (from mitochondria to nucleus) interactions with the nucleus affecting metabolic energy production, environmental adaptation, ageing and stress pathways. Parikh *et al*. (1987) showed that the mitochondrial genotype can influence nuclear gene expression in yeast. Zeyl *et al*. (2005) used *S. cerevisiae* × *S. cerevisiae* crosses to construct matched and unmatched pairings of nuclear and mitochondrial genomes to detect the fitness effects of nucleus–mitochondria epistasis. In this study transcriptome analysis of *S. cerevisiae* × *S. uvarum* hybrids was carried out to investigate functional differences between hybrids having different mtDNA. For this purpose we used a 'comparative method' by constructing hybrids having both different nuclear genome and different parental mtDNA. This choice was related to the low probability of obtaining sibling hybrids with different mtDNA from repeated crosses between the same parental strains. Transcriptome analysis of brewing natural strains (Cavalieri *et al*., 2000; Backhus *et al*., 2001; Higgins *et al*., 2001; Pérez-Ortín *et al*., 2002; James *et al*., 2003; Rossignol *et al*., 2003; Hirasawa *et al*., 2007) and *Z. rouxii* strains (Schoondermark-Stolk *et al*., 2002) have been carried out using *S. cerevisiae* micro- or macroarrays. In these cases, identification of gene expression levels depends on both homology to the spotted probe and the abundance of its mRNA. In a previous study we have demonstrated the potential use of yeast DNA macroarrays, with the gene content of the laboratory strain S288c (Alberola *et al*., 2004), to analyse the transcriptome in interspecific hybrids between *S. cerevisiae* and *S. uvarum* (Solieri *et al*., 2005). The high number of hybridization signals to *S. cerevisiae* probes using cDNA prepared from mRNA of *S. cerevisiae* × *S. uvarum* hybrids confirmed a close similarity of the genomes. However, the cDNAs from *S. uvarum* genome can cross-hybridize with the *S. cerevisiae* probes in the DNA macroarray in a complex way, because the homology between *S. uvarum* and *S. cerevisiae* genes is variable. Some of them could not hybridize (homology $<83\%$), others hybridize partially (homology in the range 83–90%) and others hybridize identically to *S. cerevisiae* cDNAs (homology $>90\%$) (Schoondermark-Stolk *et al*., 2002; see Tables 7 and 8). However, the genome constitution of our hybrids is similar because they were obtained by spore-to-spore crossing, and hence they likely are perfect diploids having one chromosome set from *S. cerevisiae* and another from *S. uvarum*. Therefore, the effect of cross-hybridization of the *S. uvarum* cDNA alleles is corrected in the pairwise comparisons as it applied for all hybrids.

Furthermore, in *S. cerevisiae* × *S. uvarum* hybrids the mRNA level for each gene is the balance of both parental alleles' expression. Therefore similar transcriptional levels can result from different balances in the expression of both parental genes. In this situation it is not possible to establish whether the transcriptional differences in each ORF are due to a low level of expression from *S. cerevisiae* allele alone, or the low levels of both *S. cerevisiae* and *S. uvarum* alleles.

On the basis of the comparative transcriptional analysis between two hybrids having *S. cerevisiae* mtDNA and two hybrids with *S. uvarum* mtDNA, the main differences were related to carbohydrate metabolism and hexose transport. The hybrids having a *S. cerevisiae* mtDNA profile showed lower mRNA levels of genes involved in glycolysis and fermentation pathways than the hybrids with *S. uvarum* mtDNA, whereas hexose transport function was downregulated in two of four comparisons. In particular, many alcohol dehydrogenase (*ADH*) coding genes are noticeably less expressed in hybrids with a *S. cerevisiae* mtDNA. The effects on glucose metabolism of low *ADH* transcription level could be complex and further biochemical studies are necessary. Nevertheless, we attempted to explain these results by considering the overflow

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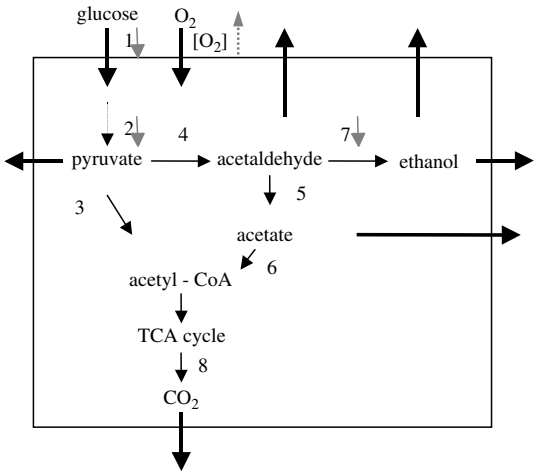

*Solid grey arrows indicate downregulated genes in hybrids with *S. cerevisiae* mtDNA compared to hybrids with *S. uvarum* mtDNA.

**Dotted grey arrow indicates a higher respiration activity of hybrids with *S. cerevisiae* mtDNA than that of hybrids with *S. uvarum*

Figure 3. Alternative routes of pyruvate catabolism in yeasts. The enzymes catalysing the various reactions are indicated as follows: 1, hexose transporters; 2, glycolysis pathway; 3, pyruvate dehydrogenase complex; 4, pyruvate decarboxylase; 5, acetaldehyde dehydrogenase; 6, acetyl-CoA synthetase; 7, alcohol dehydrogenase; 8, mitochondrial electron transport chain. TCA, tricarboxylic acid. Modified from Postma *et al.* (1989)

metabolism at two branching points in the glucose pathway (Figure 3). In high glucose concentrations, acetyl coenzyme A is a bottleneck because it is saturated, and pyruvate is preferentially converted via pyruvate decarboxylase and alcohol dehydrogenase to ethanol. The low *ADH* transcript levels of hybrids with *S. cerevisiae* mtDNA could trigger a cytoplasmatic enhancement of acetaldehyde, acetate and pyruvate concentrations, which in turn could determine the enhanced respiration of glucose, as well as a downregulation of hexose transport genes.

The increased respiratory activity of hybrids bearing the *S. cerevisiae* mtDNA genome with respect to hybrids with *S. uvarum* mtDNA was confirmed by a respiration assay. In agreement with our results, experiments in chemostat continuous culture showed that *S. uvarum* has a smaller respiratory capacity than *S. cerevisiae* (Serra *et al.*, 2003). The expression profile and respiration activity of our hybrids could indicate a correlation between mtDNA type and their fermentative : respiratory ability, according to the observation that different mtDNAs can have a significant influence on fitness. The comparative method used in this study highlighted that our strains, although having different hybrid nature, showed the same differential gene expression related to the type of the mitochondrial genome. The mechanisms involved in determining different respiration performances in hybrids with different mtDNA remain to be established, but one possibility could be a higher number of mitochondria of *S. cerevisiae* relative to *S. uvarum*, due to a higher number of *ori*/*rep* sequences in the *S. cerevisiae* mitochondrial genome (Cardazzo *et al.*, 1998) could result in a greater mitochondrial replication capacity.

Similarly, the molecular basis of cross-specific inheritance of mtDNA in *S. cerevisiae* × *S. uvarum* hybrids is unclear, but anterograde and retrograde intergenomic communications, which promote the maintenance of favourable mtDNA polymorphism within a population (Ballard and Rand, 2005), could be also involved. Further investigations are required to better understand the nuclear–mitochondrial epistasis for fitness in *S. cerevisiae* × *S. uvarum* hybrids. However, our findings suggest that the mtDNA type affects the respiration : fermentation capacity in hybrids and is an important parental strain-dependent trait in winemaking for constructing improved oenological hybrids.

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