Molecular genetic analysis of a fertile interspecific hybrid *Saccharomyces cerevisiae* and *Saccharomyces uvarum* and its progenies

Ph.D Thesis

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University of Debrecen
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1. Introduction

■ The majority of yeasts gets into the must from grapes and the equipments used in the winery (Pretorius, 2000; Mortimer and Polsinelli, 1999). At the initial phase of the fermentation the dominance of the species *Kloeckera*, *Hanseniaspora* and *Candida* are typically (Bison and Kunkee, 1993; Cocolin et al., 2000), however with the increase of the alcohol concentration species like *Cryptococcus*, *Kluyveromyces*, *Metscnikowia* and *Pichia* come into the limelight. By the last third period of the alcoholic fermentation the endured species are only the *Saccharomyces* ones (Fleet and Heard, 1993) due to them, must progressively becomes wine.

■ At present biotechnology is able to make the fermentation faster, the periods supervisabled and influenced by the musts inoculation with dry ”starter” yeasts. The different starter yeasts must have stable genetical materia in order to produce the same reliable savour, aroma, bouquet and not to change their character, which have in influence on the quality of wine, during the application. The most frequently applied starter cultures are made from the strains of *Saccharomyces cerevisiae* and *S. uvarum*.

■ Different yeast strains have been isolated from the wineries of Tokaj-hegyalja by the members of the Departments of Genetics and Molecular Biologics for years. We have succeeded in isolating a large number of *S. uvarum* strains, besides the *S. cerevisiae* ones at the late fermentation phase of the musts made by botrytised grapes. It is very likely, that this yeast (*S. uvarum*) contributes remarkably to the development of the characteristic „Tokaj bouquet”. In the course of sample isolation we succeeded in finding natural hybrids presumably of these two yeast strains (*Saccharomyces cerevisiae* and *S. uvarum*), yet the further genetical analysis came up against difficultes, due to the inability, or the very week spore formation.

■ The presence of the interspecific hybrids in nature is a very interesting phenomenon, because, with some rare exceptions, they are sterile: the developed combined parenthal genetical materia is handed down into only one generation. But, besides being a „luxury staff” of nature, the formation of the interspecific hybrids could lead to new strains arising, during some thousands years. These kinds of yeast hybrids came into the spotlight in also the modern winery’s area (Zambonelli et al., 1997; Masneuf at al., 2003). Their biotechnological importance is in the creation of a better fermenthor hybrid strain, which has stable genetical materia, by crossing selected, good qualited yeast strains, combining their advantaged properties (Caridi et al., 2002).

■ Through our crossing experiments, we tried to discover the possible molecular background of the hybrid formation between two evolutionary separated *Saccharomyces* yeasts. The piquancy of our

experiments is that our *S. cerevisiae* x *S. uvarum* hybrid produced fertile offsprings, which we managed to scrutinize even to the fourth generation.

1. Results

■ In the dissertation we will show the analysis of one hybrid in details, resulting from the cross between a leu$^-$ *S. cerevisiae* (10-170) and a ura$^-$ *S. uvarum* (m9) auxotrophic parental strains. We selected the hybrid from 21 colonies, grown on SMA medium. Its prototrophie is due to the succesfull cross of the auxotrophie parents. Moreover the hybrid was able to grow at 37°C, which is originally the *S. cerevisiae*'s propertie, and was able to ferment the melibiose, which is only the *S. uvarum* parent's stamp (Vaugham-Martini and Martini, 1987; Zambonelli et al., 1997).

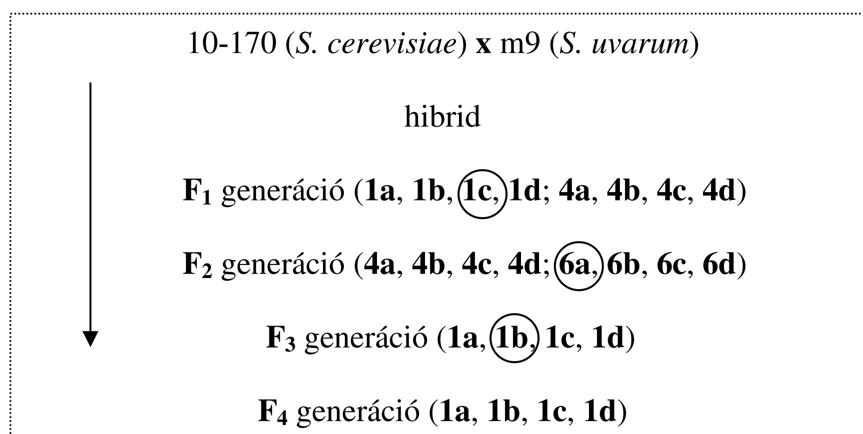

Figure 1. A simple illustration for the tetrads showed in the dissertation, originated from the hybrid

■ In contrast with the natural hybrids and the hybrids constructed by the spore-to spore crossing of Naumov, our constructed hybrid produced spores very well (50%), moreover, what is especially exeptional, the gained spores were highly viable (70%!). The spore clones caming from the spores of the hybrid's spores (F_1) also produced spores very well (F_2), and the follow generations came by the sporulations of the previous in this way into the fourth (F_4) generation. However, getting viable spores in such high proportion the crossed 10-170 *S. cerevisiae* and the m9 *S. uvarum* needed to do a genom duplication before or during the zygote formation, scil. the event of chromosome pairing, happened during the prophase, without the meiozis beeing complete, is explanable only this way. If the meiozis fails to happen or takes places incomplately, spores are not formed or formed with nonviable genome (deBarros Lopez et al., 2002; Delneri et al., 2003; Fisher et al., 2000; Marioni et al., 1999; Masneuf et al., 1998; Naumov, 1987; Zambonelli et al., 1997). In our case, spores were viable, which means that the zygote and the hybrid formed from it and the hybrid itself had a ploidity higher than 2n. It is likely, to be an allotetraploid. Our supposition was confirmed by the article of Sebastiani et al. (2002),

published in the meantime, about a fertile interspecific hybrid, producing viable spores. The hybrid was allotetraploid by their presumption.

■ Molecular genetical analysis of the hybrid's and its descendants came after the succesfull hybrid isolation. Firstly, we prepared the electrokaryogram of the hybrid by pulsed field gelelectrophoresis, on wich the chromosomes of the parents are well visible.

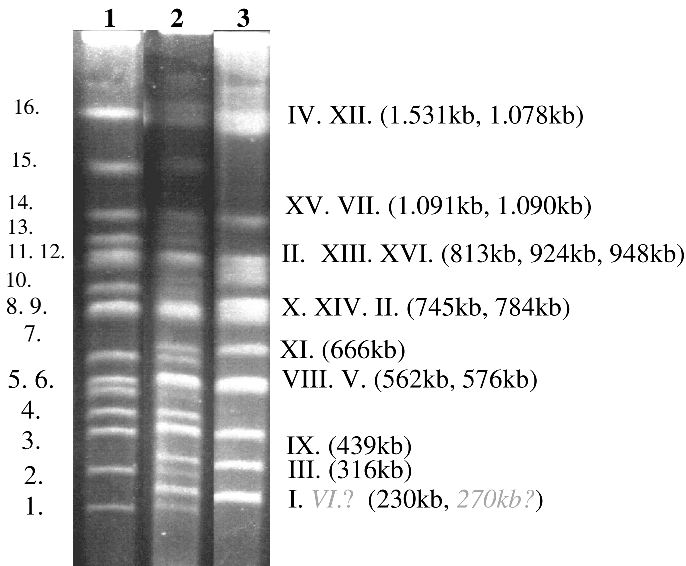

Figure 2. Chromosomal pattern of the crossed parents and their hybrid. 1.: m9, 2.: hybrid; 3.: 10-170.

■ The Y' Southern hybridisations on the electrokaryograms also correctly show the chromosomes, originating from the *S. cerevisiae* parent. Originally the *S. uvarum* has no sequences like this at all. We frequently found 2:2 segregations for the chromosome-lenght patterns between the members of the checked generations. The Y' Southern analysis does not show significant pattern-difference in the case of the third and fourth generations.

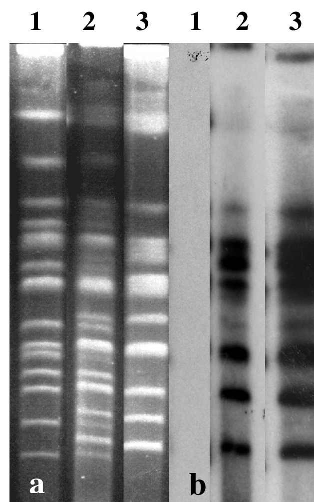

Figure 3. Chromosomal patterns, made by pulsed field gelelektrophoresis (a) and its Y' telomeric hybridisation (b). 1.: m9; 2.: hybrid; 3.: 10-170.

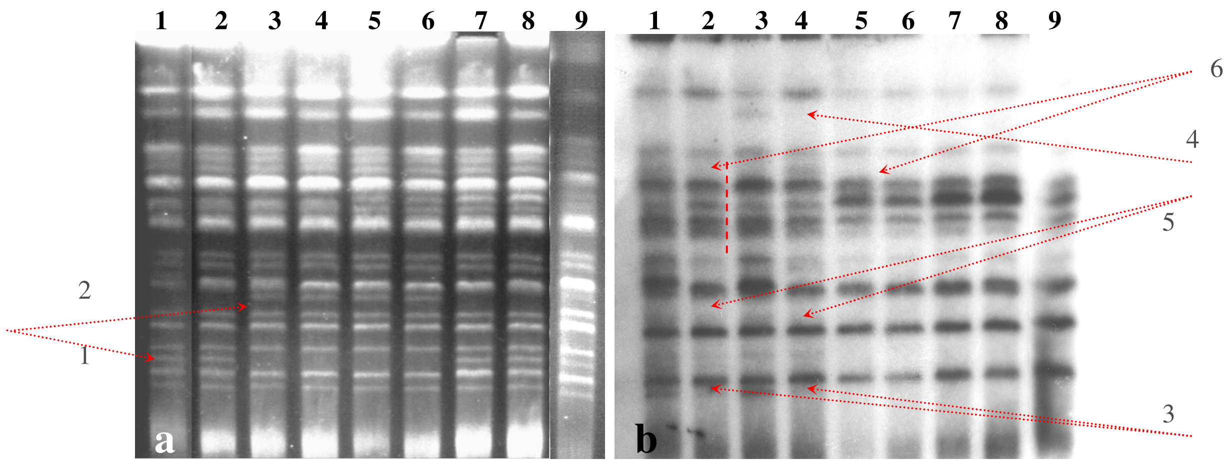

Figure 4. Chromosomal pattern of two full tetrads of the F_1 (a) and the Y' telomer hybridisation (b). 1.: F_1 1a; 2.: 1b; 3.: 1c; 4.: 1d; 5.: 4a; 6.: 4b; 7.: 4c; 8.: 4d; 9.: hybrid. The arrows number 1. and 2. show the chromosomal rearrangements in 2:2 regular segregation. The arrows number 3., 4., 5. and 6 signify newly appeared bands on the photo. The jagged line attent for the signal depth differences at the larger chromosomes' region.

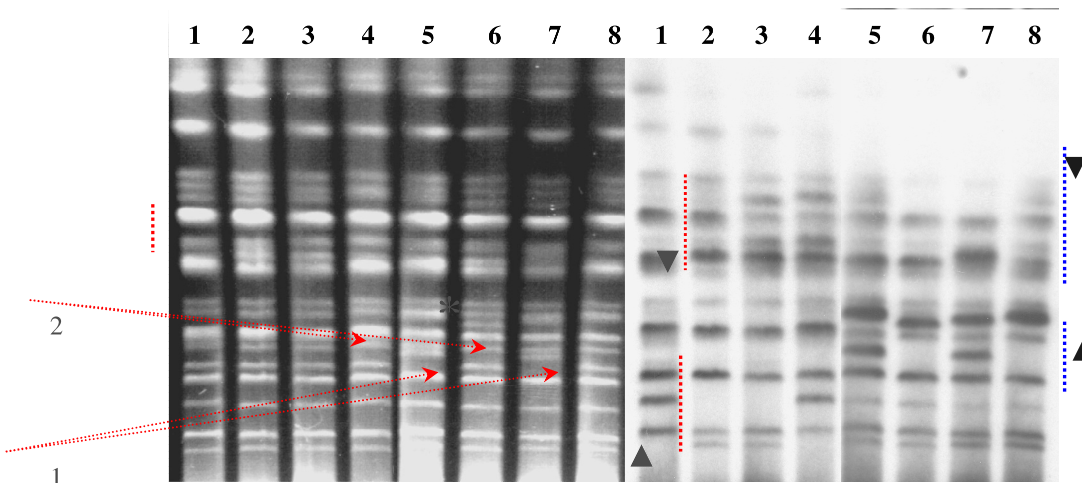

Figure 5. Chromosomal pattern of two full tetrads of the F_2 (a) and the Y' telomer hybridisation (b). 1.: F_2 4a; 2.: 4b; 3.: 4c; 4.: 4d; 5.: 6a; 6.: 6b; 7.: 6c; 8.: 6d. The arrows number 1. and 2. show the chromosomal rearrangements in 2:2 regular segregation on the electrokaryogram. The star shows a plus chromosome, the jagged line attent for the chromosome rearrangements at the larger chromosomes' region. On the membran, made from the electrokaryogram, the tringulars and the jagged lines mark newly appeared bands.

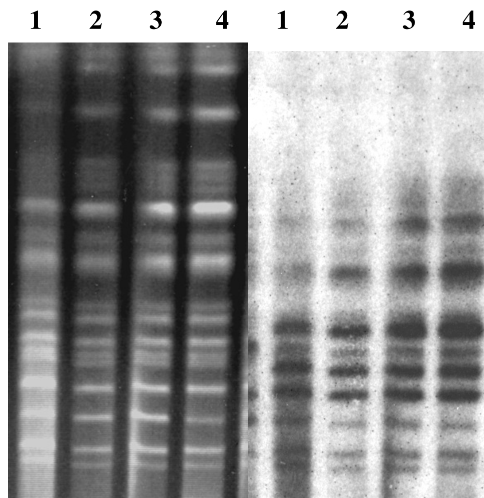

Figure 6. Chromosomal pattern of a full tetrad of the F_3 (a) and the Y' telomer hybridisation (b).
1.: F_3 1a; 2.: 1b; 3.: 1c; 4.: 1d.

Analysing further molecular markers, we always found the both parenthal genes or parts of genes in the hybrid, , while in the descendants we often found 2:2 segregations for *S. cerevisiae*: hybrid characters.

- The PCR-RFLP analysis of the the *MET2* gene (on the IV. chromosome) gave the same results in all checked generations.

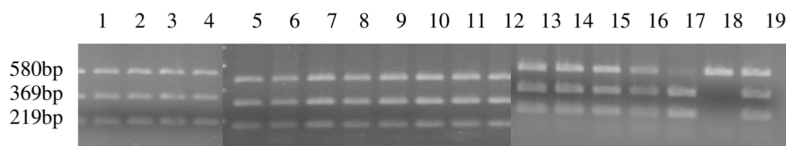

Figure 7. PCR-RFLP analysis of *MET2*. Digestion with *Eco*RI. Lanes 1 to 4: F1 tetrad No 1. Lanes 5 to 8: F2 tetrad No 6. Lanes 9 to 12: F3 tetrad. Lanes: 13 to 16: F4 tetrad. 17.: 10-170; 18.: 10-522; 19.: hybrid

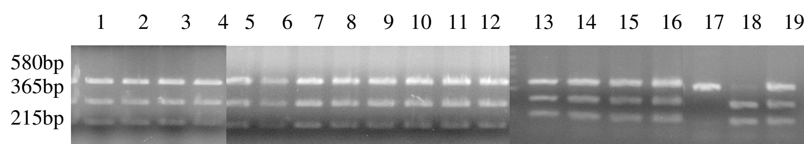

Figure 8. PCR-RFLP analysis of *MET2*. Digestion with *Pst*I. Lanes 1 to 4: F1 tetrad No 1. Lanes 5 to 8: F2 tetrad No 6. Lanes 9 to 12: F3 tetrad. Lanes: 13 to 16: F4 tetrad. 17.: 10-170; 18.: 10-522; 19.: hybrid

- The results of the PCR-RFLP designed on the *ITS1*-rDNS-*ITS2* (on the XII. chromosome) spacer gave mostly hybrid patterns, except the F_1 1a clone, which shows only *S. cerevisiae* pattern, and the recombinant-like F_2 6c spore clone.

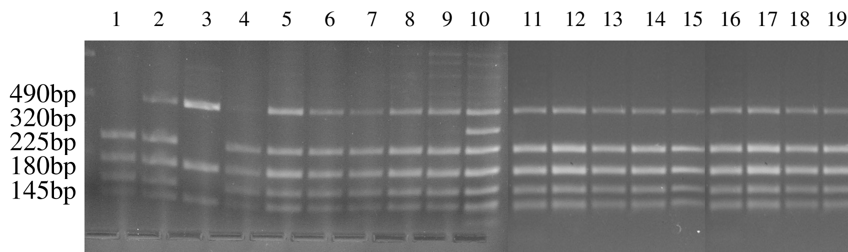

Figure 9. PCR-RFLP analysis of ITS1-5.8S rDNS-ITS2 and NTS2-ETS. Lanes 1 to 15: ITS1-5.8S rDNS-ITS2 digested with *Hae*III. Lane 1: 10-170. Lane 2: hybrid H1. Lane 3: 10-522. Lanes 4 to 7: F1 tetrad No 1. Lanes 8 to 11: F2 tetrad No 6. Lanes 12 to 15: F3 tetrad**.** Lanes 16 to 23: NTS2-ETS digested with BanI. Lane 16: 10-522. Lane 17: 10-170. Lane: hybrid H1. Lane: size marker. Lanes 20 to 23: F1 tetrad No 1.

- The PCR-RFLP analysis on the *HIS4* gene (on the III. chromosome) gave us only *S. cerevisiae* pattern from the first to the fourth generations, whereas the hybrid possessed both genes.

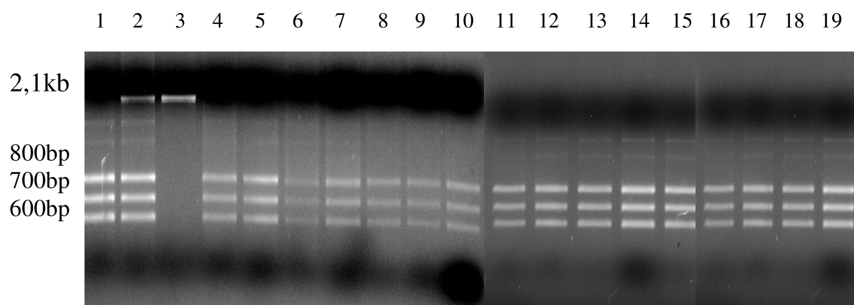

Figure 10. PCR-RFLP analysis of *HIS4*. Digestion with *Hind*III. Lane 1: 10-170. Lane 2: hybrid H1. Lane 3: 10-522. Lanes 4 to 7: F1 tetrad No 1. Lanes 8 to 11: F2 tetrad No 6. Lanes 12 to 15: F3 tetrad**.**

- The results of the PCR-RFLP analysis of the YCL008c gene (also on III. chromosome) show an interesting 2:2 segregation in the first generation: 1a, 1b have hybrid-like patterns, while members 1c, 1d have only *S. cerevisiae* patterns.

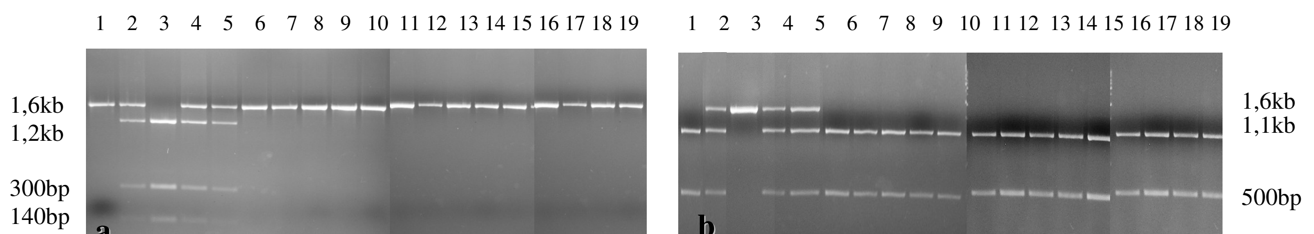

Figure 11. PCR-RFLP analysis of *YCL008c*. Digestion with *Pst*I (a) and *Eco*RV (b). Lane 1: 10-170. Lane 2: hybrid. Lane 3: 10-522. Lanes 4 to 7: F_1 tetrad No 1. Lanes 8 to 11: F_2 tetrad No 6. Lanes 12 to 15: F_3 tetrad**.** Lanes 16 to 19 F_4 tetrad

- The results of the *MET10* (on the IV. chromosome) PCR analysis gave the opposite: 1a, 1b have *S. cerevisiae*-like patterns, 1c, 1d have the hybrid-like patterns.

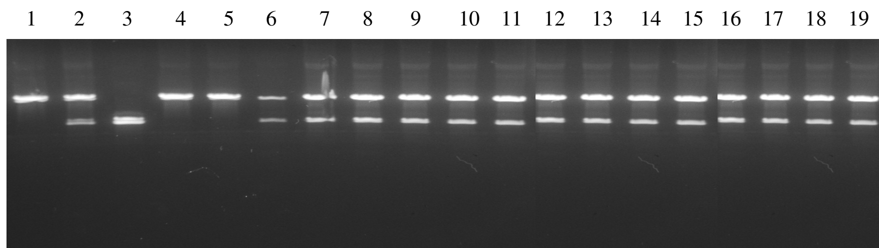

Figure 12. *MET10* PCR products obtained with mixed primers. Lane 1: 10-170. Lane 2: hybrid. Lane 3: 10-522. Lanes 4 to 7: F_1 tetrad No 1. Lanes 8 to 11: F_2 tetrad No 6. Lanes 12 to 15: F_3 tetrad, Lanes 16 to 19 F_4 tetrad

■ The mitochondrial DNA of the hybrid originates from the 10-170 parent on the basis of the RFLP analysis. It seems as if, there was no hybridisation on the part of the extrachromosomal genom. Naturally, another possibility is that, the zygote contained both species' mtDNA, but during the vegetative growth, a segregation event occured. But, in this latter case we also have to suppose that the segregants possessing the mtDNA of *S. cerevisiae* were more succesfull. The later generations also inherited the *S. cerevisiae* parent's mtDNA of corse.

*** 1 2 3 4 5 6 7 8 9 10 ***

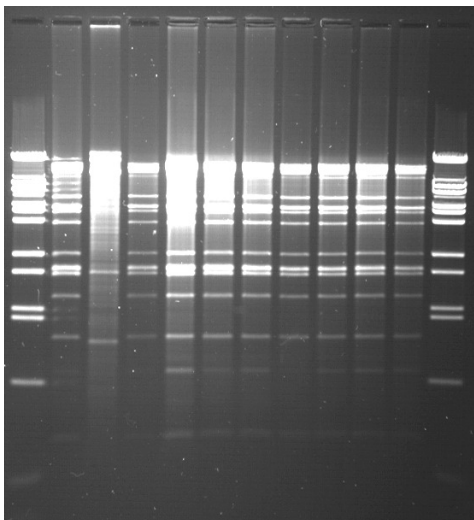

Figure 13. Restriction patterns of mitochondrial DNA. Digestion with *Eco*RV. Lane 1: 10-170. Lane 2: 10-522. Lane 3: hybrid H1. Lanes 4 to 7: F1 tetrad No 1. Lane 8.: F2 spore clone 6a. Lane 9: F3 spore clone 1b. *: λ DNA digested with *Bst*EII.

■ Analysing the sequence of the crossed m9 uracil auxotrophic strain we found that the base substitution caused the mutation. On the figure this is shown by underlining and framing. At the 460. position T**C**A (the original *S. uvarum* sequence) changed to T**T**A (m9 sequence), which means leucin instead of serin. The other, underlined base substitution is mute: the AC**C** and AC**G** at the position 355.both mean threonin.

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                                 360       370       380       390       400
 2 URAm9Url.gel     CATCACGAATGCGCACGGTGTGGTGGGTCCCGGTATCGTCAGCGGGCTAA
 4 URAhyUrl.gel     CATCACGAATGCGCACGGTGTGGTGGGTCCCGGTATCGTCAGCGGGCTAA
 1 URAUVARU.sdn     CATCACCAATGCGCACGGTGTGGTGGGTCCCGGTATCGTCAGCGGGCTAA
-3 URAm9Urr.gel     CATCACGAATGCGCACGGTGTGGTGGGTCCCGGTATCGTCAGCGGGCTAA
   CONSENSUS        CATCACGAATGCGCACGGTGTGGTGGGTCCCGGTATCGTCAGCGGGCTAA

                                 410       420       430       440       450
 2 URAm9Url.gel     AAGCAGCCGCAGAGGAGGTCACCAAGGAACCTAGAGGCCTTCTGATGCTA
 4 URAhyUrl.gel     AAGCAGCCGCAGAGGAGGTCACCAAGGAACCTAGAGGCCTTCTGATGCTA
 1 URAUVARU.sdn     AAGCAGCCGCAGAGGAGGTCACCAAGGAACCTAGAGGCCTTCTGATGCTA
-3 URAm9Urr.gel     AAGCAGCCGCAGAGGAGGTCACCAAGGAACCTAGAGGCCTTCTGATGCTA
   CONSENSUS        AAGCAGCCGCAGAGGAGGTCACCAAGGAACCTAGAGGCCTTCTGATGCTA

                                 460       470       480       490       500
 2 URAm9Url.gel     GCCGAATTATTTATGCAAGGGATCTTTAGCCACTGGGGAGTACACCAAGGG
 4 URAhyUrl.gel     GCCGAATTATTTATGCAAGGGATCTTTAGCCACTGGGGAGTACACCAAGGG
 1 URAUVARU.sdn     GCCGAATTATCATGCAAGGGATCTTTAGCCACTGGGGAGTACACCAAGGG
-3 URAm9Urr.gel     GCCGAATTATTTATGCAAGGGATCTTTAGCCACTGGGGAGTACACCAAGGG
   CONSENSUS        GCCGAATTATTTATGCAAGGGATCTTTAGCCACTGGGGAGTACACCAAGGG
```

Figure 14. The comparison of the ura$^-$ mutant parents' gene with the control one. URAm9Url: the m9 URA3 PCR-tproduct forward sequience, URAhyUrl: the 10-170 x m9 hybrid URA3 PCR products forward sequence, URAm9Urr: the m9 URA3 gene's reverse sequence, URA3UVARU: the controll *S. uvarum*'s URA3 sequence. Dates are from www.genelevures.fr database. Points of the mutations are labelled by sublined and framed.

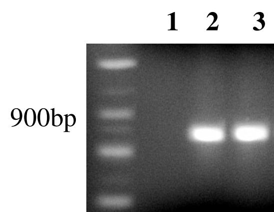

Figure 15. *URA3* PCR-reaction products made with *S. uvarum*-specific primers. 1.: 10-170; 2.: hibrid; 3.: m9.

We got PCR products in the case of the m9 S. uvarum parent, in the case of the hybrid and in every examined case of the descendants.

■ We have also done the analysis of the *S. cerevisiae* parent's *LEU2* gene, and the PCR reaction on the parents and the hybrid as well. We detected signals, by the primers, desired specificaly to the *S. cerevisiae,* in the case of the hybrid and the 10-170 (of corse) but not in the case of the m9, *S. uvarum* parent. Moreover we got PCR product in every examined case of the followers.

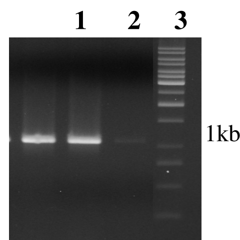

Figure 16. *leu2* PCR-reaction products made with *S. cerevisiae*-specific primers. 1.: 10-170; 2.: hibrid; 3.: m9.

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                                  283
10-170-U AGTGTTAGACCTGAACAAGGTTTACTATAAATCCGTAAAGAACTTCAATTGTACGCCAAC
Hyb-U    AGTGTTAGACCTGAACAAGGTTTACTATAAATCCGTAAAGAACTTCAATTGTACGCCAAC
M9-U     AGTGTTAGACCTGAACAAGGTTTACTAAAAATCCGTAAAGAACTTCAATTGTACGCCAAC
```

Figure 16. comparison of the 10-170 leu^{-} mutant parents' gene with the control one. 10-170-u is the forward sequence of the *S. cerevisiae* parent, Hyb-u is that of the hybrid one, m9-u is that of the *S. uvarum* ones. The frame shows the point of the mutation.

According to the analysis of the sequences, the base substitution AAA to TAA, which means stop codon instead of lysin, could cause the mutant leucin phenotype of the 10-170.

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