# Final report

The major objective of the project was an in-depth analysis of the postzygotic events that take place in alloploid hybrids of *Saccharomyces* species. These yeast species are isolated by hybrid sterility: they can produce viable hybrids capable of vegetative propagation but cannot produce viable gametes (ascospores) by meiosis. This deficiency prevents them from sexual reproduction but allows vegetative propagation. In spite of the sterility barrier, strains have been identified in natural yeast populations that had chimerical genomes consisting of genomic elements originating from two or three different species. Certain authors incorrectly call these strains hybrids, ignoring the fact that they possess only fractions of the partner genomes. Though they are not true hybrids, the use of this terminology is not fully unjustified in their case because they might have evolved from hybridisation by complex changes in the genome structure including recombination events between the partner genomes and genome-size reduction (loss of large parts of the partner gene pools). We proposed a model for these so-called „postzygotic" processes, largely based on the analysis of natural chimerical strains ("hybrids") (Sipiczki, FEMS Yeast Res. **8**: 996-1007, 2008). In this project we tested our rather hypothetical model experimentally.

We planned and performed two lines of research (I and II) devided in 11 work tasks. In the work tasks of line I, we identified and analysed strains in natural populations that had interspecies hybrid or chimerical genomes. In line II, we produced hybrids in the laboratory and monitored the changes of their genomes during mitotic and meiotic cell divisions. The synthesis of the results resulted in a model in which the species are reproductively isolated by two sterility barriers, but one of the barriers can be broken down in allotetraploids which then triggers genomic changes leading to chimerical genomes similar to those observed in natural "hybrid" strains.

***Work task I.1:*** Identification of putative interspecific hybrids in the yeast strain collections of our department and in that of the department of the collaborating Italian laboratory by PCR-RFLP analysis of molecular markers.
Results: We identified novel natural chimerical strains in a set of 78 strains of our wine yeast collection and 9 strains obtained from the laboratory of our collaborating Italian partner (Universita degli Studi della Basilicata). The identification was based on the PCR-RFLP analysis of three chromosomal genes. These genes differ in sequence in the three species (*S. cerevisiae*, *S. uvarum* and *S. kudriwzevii*) found to be represented in chimerical strains. In two strains, we found *S. cerevisiae* and *S. uvarum* alleles of theses genes. No *S. kudriavzevii* alleles were detected.

***Work task I.2*:** Isolation of *Saccharomyces* yeasts from natural resources and search for natural hybrids among the isolates by PCR-RFLP analysis of certain molecular markers.
Results: Samples were collected from various types of wines, grapes and bark of oak trees (Portuguese researchers recently found *S. kudriavzevii* on tree barks) in the wine growing region Tokaj. We also took advantage of a collaboration with Mexican researchers to collect natural samples in four Central-American countries. As expected, the Tokaj samples mainly contained *Saccharomyces cerevisiae* and *S. uvarum* strains. Karyotyping revealed high chromosomal polymorphism among *S. cerevisiae* isolates and much lower variability in *S. uvarum*. Only two strains had supernumerary chromosomes indicating alloploidy. PCR-RFLP of five chromosomal genes and RAPD analysis with two primer pairs confirmed that these isolates had *S. cerevisiae x S. uvarum* chimerical genomes. In the samples collected in Central America, we found four new yeast species but no *Saccharomyces* chimeras.

***Work task I.3:*** Comparative mapping of the genomes of the natural hybrids by molecular methods (PCR-RFLP of all markers, electrophoretic karyotyping, telomeric probes, retrotransposons Ty, etc.).

Results: The four "hybrid/chimerical" strains identified in the previous work tasks were subjected to molecular analysis. By electrophoretic karyotyping we could identify 1 to 4 chromosomal bands in these strains that differed in size from the bands of type strains of *S. cerevisiae* and *S. uvarum*. None of them had all chromosomes of both species. Size variability was observed among the shortest and longest chromosomes. Remarkably, the chromosomes that carry the mating-type determination (*MAT*) loci were not present from both species. The karyotype differences indicated that the four strains had chimerical genomes composed of incomplete sets of *S. cerevisiae* and *S. uvarum* chromosomes, rather than true allodiploid or allopolyploid genomes. Consistent with the chimerical genome structure, we detected both parental alleles (orthologues) of all five chromosomal marker genes tested for presence. In the alloaneuploid hybrids the *S. kudriavzevii* markers located on the chromosomes carrying the *MAT* locus were missing.

***Work task I-II.1:*** Extending the set of molecular markers.
Results: We managed to extend the list of molecular markers by seven new chromosomal genes and adapted the method of delta-PCR and RAPD to the investigation of hybrid and chimerical genomes. We selected markers located on chromosomes for which we had not had proper molecular markers in our previous hybridisation project. The markers were selected by comparing the number and location of potential recognition sites for restriction endonucleases in the sequences of *S. cerevisiae* genes with their *S. uvarum* and *S. kudriavzevii* orthologues available in the genome sequence databases. We also designed primers for their amplification.

***Work task II.1:*** Hybridisation of genetically markered (e.g. auxotrophic) strains of *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii*.
Results: Using the method of mass-mating of spores of auxotrophic strains, we constructed *S. cerevisiae x S. uvarum* and 3 *S. kudriavzevii x S. uvarum* hybrids. The putative hybrids were identified as prototrophic colonies growing on minimal medium. We isolated 50 prototrophic colonies for both species combinations.

***Task II.2:*** Verification of the hybrid genome structure.
Results: When examined with allele-specific PCR-RFLP, all prototrophic isolates were heterozygous for all chromosomal markers, indicating that they were true interspecies hybrids. Consistent with this, their karyotapes had all chromosomal bands of both parental strains. These results clearly demonstrated that we had true hybrids, but could not tell us, whether the hybrids were allodiploids or allotetraploids. To determine their ploidy level, we tested them for sporulation. Our previous work with *S. cerevisiae* and *S. uvarum* hybridisation had shown that allodiploids differ from allotetraploids in sporulation: allodiploids are sterile ( do not form viable spores) („hybrid sterility barrier"), whereas allodiplods sporulate normally and their spores are viable. We made use of this earlier observation for the determination of ploidy in this work. We tested our prototrophic isolates for sporulation and spore viability. Three major categories were found: non-sporulating clones, clones producing non-viable spores and clones producing viable spores. The first two categories were most probably allodiploids and the third category was allotetraploid. Occasionally, the spores in the third category were auxotrophic, indicating that the tetraploid genomes could segregate.

***Task II.3:*** Investigation of genome stability during vegetative propagation and selection of vegetative (mitotic) segregants.
Results: One „hybrid sterile" clone (incapable of sporulation and meiotic segregation) from both parental combinations was chosen for the investigation of genome stability during vegetative propagation of cells. The clones were inoculated into non-selective media and cultured at non-selective temperatures (*S. uvarum* is sensitive to 35 °C and higher temperatures). Samples were taken from the cultures after 10 and 50 generation times and tested for the presence of auxotrophic

segregants. No segregants were detected among 10,000 cells. Therefore we could not select vegetative segregants.

***Task II.4:*** Generation of meiotic progeny for investigating genome stability and segregation during meiosis.
Results: Asci of five presumably allotetraploid hybrids producing viable spores were dissected by micromanipulation, and the spores were separated on the surface of a complete medium. 70% of the spores germinated and produced colonies of cells propagating by budding. At least 40 spore clones (F1 generation) for each hybrid were tested for sporulation. Most clones either did not sporulate or their spores were dead („F1 sterility barrier"). Certain spores of four hybrids could also form viable spores (we called the process „loss or break-down of F2 sterility barrier"). We dissected asci of these „fertile" spore clones and separated the spores on the complete medium. 90 % of these spores germinated and produced vegetatively propagating clones (F2 generation). By repeating the process with the F2 clones we could also obtain F3, F4 and F5 generations. Altogether, we produced 160 F1, 88 F2, 76 F3, 44 F4 and 24 F5 clones.

***Task II.5:*** Comparative analysis of the genome structures of hybrids, mitotic (vegetative) and meiotic segregants.
Results: In this work task we compared the genomes of allotetraploid hybrids and their F1 to F5 progeny. The F1 clones were formed by spores produced by reductional (meiotic) division of allotetraploid cells and thus must have been allodiploids. Allodiploid cells can propagate by mitotic divisions but cannot divide by meiosis, so they can be considered sterile („F1 sterility barrier"). In spite of this certain F1 clones produced viable spores. We noticed that in these clones the cells could occasionally mate (fertilise each other). Fusion of two allodiploid cells results in an allotetraploid zygote, which then can perform a successful meiosis producing viable allodiploid spores. What makes certain allodiploid F1 clones able to mate (restore fertility, that is break down the F1 sterility barrier)? The RFLP analysis of the chromosomal markers, the comparison of the electrophoretic karyotypes and Southern hybridization of certain in-vitro labeled chromosomal markers to karyotypes revealed that these hybrids had lost one type of parental chromosomes carrying the *MAT* locus, the central regulator of the mating activities. As long as the hybrid was hetorygous for the parental *MAT* loci, its cells were unable to fuse with other cells (heterozygosity for *MAT* alleles suppresses the mating programme but allows the activation of the meiotic-sporulation programme). Remarkably, it was always the *S. cerevisiae* chromosome that was retained and the corresponding chromosome of the partner species had got lost. Once their *MAT* heterozygosity was broken, the cells became able to fuse with other spores to produce zygotes (restoration of higher ploidy: allotetraploid genome disomic for the chromosome carrying the *MAT* locus). Other chromosomes could also be lost, but not accidentally and not simultaneously but gradually. We noticed that one of the partner genomes was less stable than the other. Again, it was *S. cerevisiae*, whose chromosomes were usually retained. The gradual genome reduction eliminated most of the non-*cerevisiae* genome, resulting in chimerical genomes characteristic of the so-called „natural hybrids".

***Task II.6:*** Comparative fine mapping of certain hybrid genomes by array-CGH.
Results: This work task was conditional. At the time of writing the project proposal, no genome microarrays were available for the non-*cerevisiae* species, and the *S. kudriavzevii* genome was not completely sequenced. We put this task in the program because the *S. kudraivzevii* genome project was already close to completion. Now, all genome sequences are complete and available in databases. However, microarrays are still not available. The rather modest project budget did not allow to have such microarrays made by companies specialised in manufacturing microarrays. Fortunately, we have not encountered problems for which the array-CGH technique would have been the only solution. We do not expect principally new information from array-CGH results because the major trends of rearrangements in the hybrid genomes (postzygotic genome

rearrangement) can also be investigated with the methods used in the previous work tasks.

***Task II.7:*** Synthesis and model construction.
Results: Using a large number of synthetic alloploid hybrids, we have demonstrated that the nascent *S. cerevisiae* × *S. uvarum* hybrids are sterile or F1-sterile. The two types of infertility indicate that the postzygotic reproductive isolation of these species is ensured by double sterility barrier: by hybrid sterility (hybrid cells cannot produce viable spores) operating in allodiploids and by F1 sterility (F1 cells cannot produce viable spores) operating in allopolyploids. Hybrid sterility has been found (by other researchers) to be due to the inability of homoeologous chromosomes to pair (recombine) in meiosis. Here, we show that F1-sterility is ensured in a different way. It is ascribable to mating-type heterozygosity and can be circumvented by elimination of Chr. 2 of the *S. uvarum* subgenome. To the best of our knowledge, this is the first report on breaking down interspecies hybrid sterility by chromosome loss. As the fertility of the resulting aneuploids is heritable, series of filial generations can be produced by consecutive meiotic divisions at which additional, mostly directional genomic changes can take place.

Thus, the F1 sterility of the allotetraploid *S. cerevisiae* × *S. uvarum* hybrids is the consequence of mating-type heterozygosity and the hybrid becomes fertile if it abolishes this heterozygosity by eliminating the chromosome that carries the *S. uvarum MAT* locus (Fig. 1). Being nullisomic for the *S. uvarum* Chr. 2, the sporulation-proficient F1 clones are no longer *MAT*a/*MAT*α heterozygous because they have only one *MAT* allele (located on Chr. III in the *S. cerevisiae* subgenome). These nullisomes behave in sex determination as if they were haploid. They can switch their mating-type in haploid-like manner and can also activate the conjugation programme. The zygotes produced by the conjugating nullisomes of opposite mating types are also nullisomic for Chr. 2, as shown by karyotyping, but most probably heterozygous for mating-type in the *S. cerevisiae* subgenome. This heterozygosity prevents them from another mating and enables them to perform meiosis leading to viable alloaneuploid F2 ascospores nullisomic for Chr. 2 (Fig. 1).

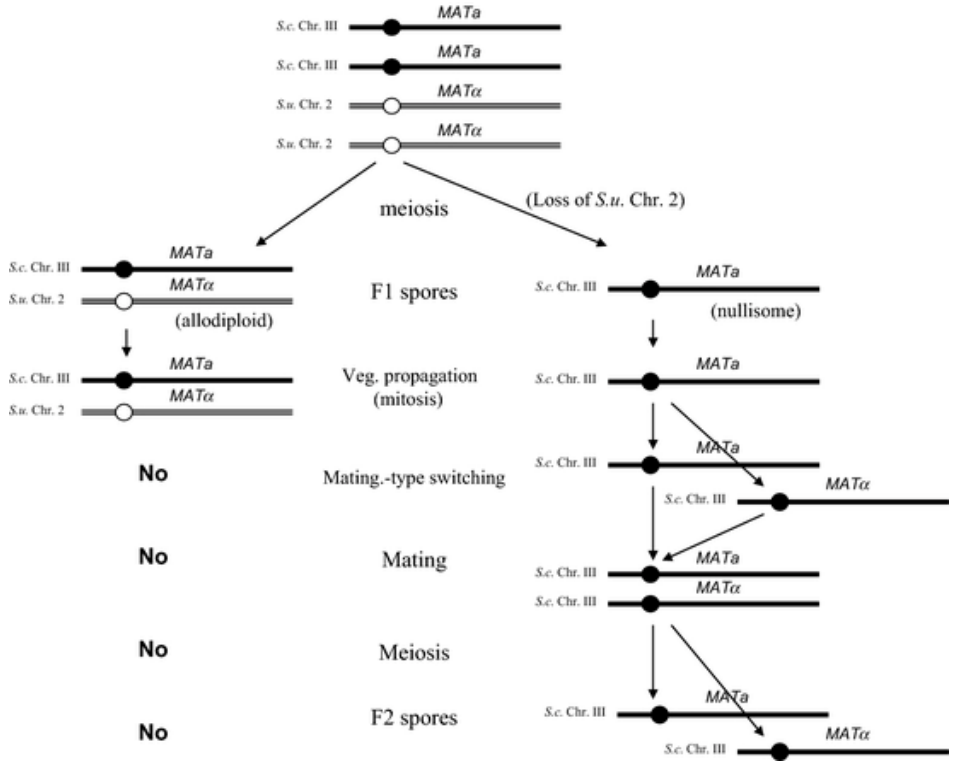


Fig. 1. A model of breaking F1 sterility by chromosome loss. A *MAT* -carrying chromosome can be lost either during meiosis (as shown) or prior to it, during vegetative propagation of the hybrid cells.

As spores are not only sexual products but also quiescent cells that ensure the survival of the yeast population under unfavourable conditions, the inability to produce viable spores can severely reduce the chances of the interspecies hybrids to survive stressful environmental changes and persist in sympatry with populations of their progenitors. This handicap may account for the rare occurrence of allodiploid and allotetraploid *Saccharomyces* hybrids in nature. Abolishing mating-type heterozygosity by elimination of one of the homoeologous chromosomes carrying the *MAT* loci seems to be a solution to this problem because it restores fertility. The fertile nullisomic segregants remain hybrid for the rest of the genome but undergo further genomic changes during sexual propagation that can gradually lead to allo(aneu)ploid and/or mosaic genomes identified in certain wine and brewing yeasts. Our preliminary results indicate that similar mechanisms may also operate in other combinations of *Saccharomyces* species.
The mode of fertility restoration revealed in this project may be one of the mechanisms that allow genomic evolution leading to domesticated yeast strains.

**Extension of the work:** When hunting for novel "natural hybrids", we made two unexpected observations which we found worth further investigation since both are relevant to the topic of this OTKA project. (1) It has been shown by several laboratories that although *S. kudriavzevii* genes occur in certain natural hybrids of wine yeasts, the species itself is not present in the fermenting population. Unexpectedly, we found traces of this yeast in high-sugar-content botrytised wines. We detected its genes by metagenomic methods in samples devoid of viable cells. We are now trying to find out whether the samples did contain "dormant" cells of this yeast or the genes which we found were from hybrid genomes. (2) In fermenting substrates, *Saccharomyces* yeasts are always accompanied by yeasts of other genera. We regularly found *Metschnikowia* in our samples but could not assign it to any known species. A detailed molecular analysis of the rDNA arrays of the isolates then revealed high intragenomic sequence heterogeneity due to the presence of various paralogs. This is a quite curious finding because the rRNA arrays of yeasts are assumed to be homogeneous due to gene homogenization. When we compared the type strains of certain *Metschnikowia* species, we observed that their arrays were also heterogeneous. The Bayesian and the Neighbour-net analyses of the sequences then revealed that the *Metschnikowia* genus has a group of species which are biologically not well isolated and their rDNA arrays evolve by reticulation, including interspecies hybridization and recombination.

**Project „by-products":**
While collecting yeasts from natural substrates in Central America, we found strains in Belize and Guatemala that represent four hitherto undescribed species. The paper describing one of them is accepted for publication in International Journal of Evolutionary and Systematic Microbiology.