Sex-determining regions as drivers for evolutionary potential and phenotypic plasticity in *Zygosaccharomyces rouxii* clade

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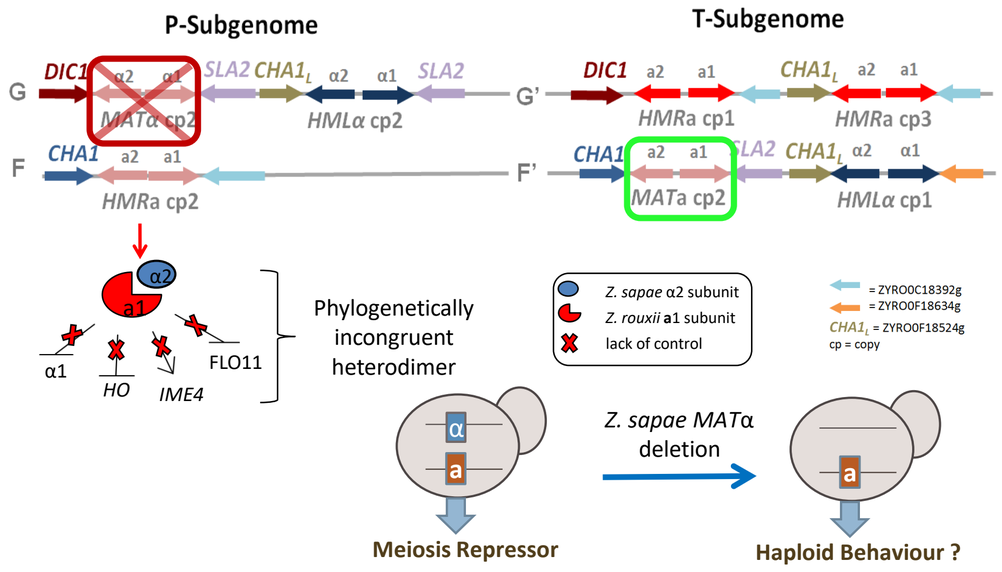
State of the Art and Aim of the Work

In the allodiploid ATCC 42981 genome, *MAT***a**1 and *MAT*α2 genes are from two different parents (*Z. rouxii* and *Z. sapae*, respectively). The different evolutionary history of **a**1 and α2 subunits could generate negative epistasis accounting for ATCC 42981 inability to repress *HO* gene and to enter into meiosis[1]. To verify that the chimeric **a**1-α2 heterodimer is responsible for ATCC 42981 sterility, we planned to selectively replace *Z. sapae MAT*α of ATCC 42981 with the orthologous *Z. rouxii MAT*α.

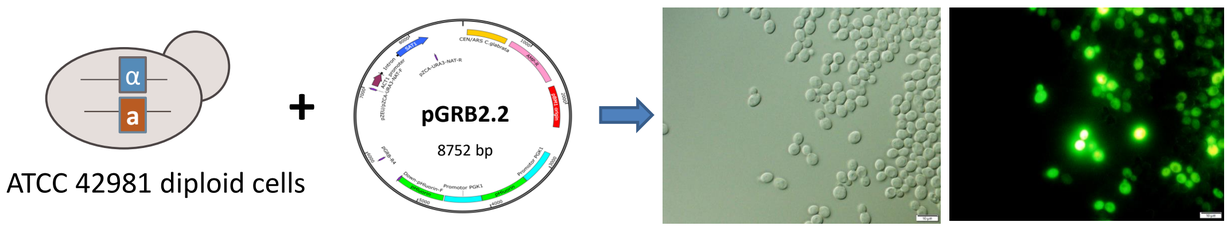
Main Activities

1. Development of genetic toolkits and optimization of the electroporation protocol for the allodiploid ATCC 42981. Targeted deletion of *Z. sapae MAT*α using a *loxP-kanMX-loxP* disruption system. Screening of deletion mutants for the ability to undergo meiosis and to survive salt stress.
2. Genome assemblies and functional genomics of ATCC 42981 and *Z. sapae* ABT301^{T}[2].
3. Discovering the α to **a** genotype switching in two different stocks of *Z. rouxii* CBS 732^{T}. Reconstruction of this mechanism that led to a new *MAT***a**2 gene. Investigation of the expression profile of *HO* endonuclease and analysis of the morphological and mating behavior of switched cultures.



1.1 Optimization of the Electroporation Protocol

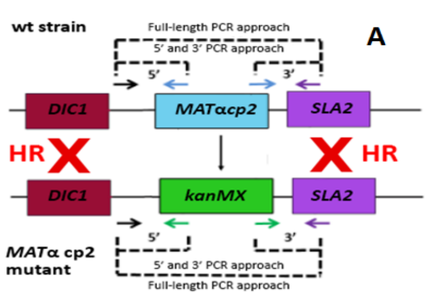
Protocol for *Z. rouxii* transformation from Prybilova and Sychorva (2003) was optimized to efficiently introduce plasmid DNA into ATCC 42981 by electroporation, using plasmid pGRB 2.2-pHluorin++ carrying *NAT*^{R} cassette and two pH-sensitive ratiometric pHluorins[3].



1.2 Targeted *MAT*α Disruption by *loxP-kanMX-loxP* Cassette and Construction of Δ*MAT*α Mutants

Targeted deletion of *Z. sapae* expressed *MAT*α was achieved by the integration of a *loxP-kanMX-loxP* cassette in ATCC 42981 genome.

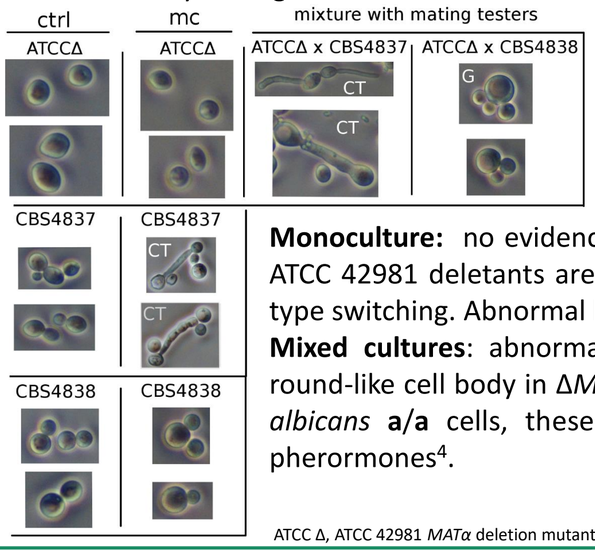
1. PCR amplification of the disruption cassette holding a Kanamycin selectable marker targeted to host genome by homologous recombination.
2. Four Δ*MAT*α ATCC 42981 mutants were constructed and confirmed by phenotypic analysis (growth on YPDA + G418) and PCR genotyping.



Panel A. Diagnostic PCR strategy to verify the targeted integration of *loxP-kanMX-loxP* disruption cassette in ATCC 42981 genome. wt, wild type; cp, copy; HR, homologous recombination; *kanMX*, kanamycin resistance gene; cp2, copy 2.

1.3 Phenotypic Effects

1. **Salt-stress tolerance**: drop tests on YNB5%Glu plates containing increasing amounts of NaCl. After 5 days no significant difference was observed in comparison to the wild-type strain.



2. **Self- and out-cross fertility assays**: wild type and deletion mutants were checked for self- and out-cross fertility as monoculture or in mixture with *Z. rouxii* CBS 4837 (**a**) or CBS 4838 (α) mating testers.

Monoculture: no evidence of conjugative bridge and/or conjugative asci. ATCC 42981 deletants are not functionally haploid and unable of mating-type switching. Abnormal long projections in CBS 4837 monoculture.

Mixed cultures: abnormal long projections (conjugation tubes) from a round-like cell body in Δ*MAT*α mutants x CBS 4837 cultures. Similarly to *C. albicans* **a**/**a** cells, these tubes could suggest a low production of α pherormones[4].

ATCC Δ, ATCC 42981 *MAT*α deletion mutant; ctrl, control; mc, monoculture; CT, conjugation tubes; G, giant cell.

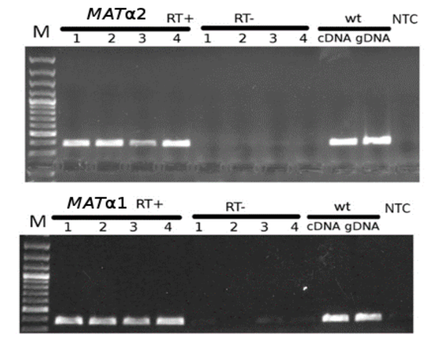
1.4 Cell-Identity Verification of Δ*MAT*α Mutants

- ***MAT* loci RT-PCR**: as expected Δ*MAT*α mutants transcribed *MAT***a**1 gene, but, surprisingly, they also actively transcribed *MAT*α copy 2 genes. **Why *MAT*α is still expressed?**

➢ **Possible explanations:**

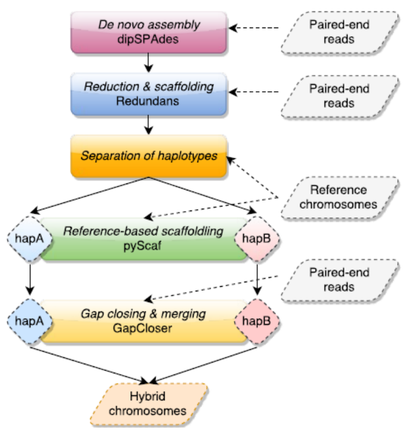
1. *DIC1-MAT*α*-SLA2* deletion could induce the linked *CHA1*$_{L}$*-HML*α*-SLA2* cassette de-silencing;
2. *HML*α transcription could be not completely repressed in the wild-type strain due to the recent acquisition of Sir1-driven silencing of *HML*/*HMR* transcription in *Z. rouxii*[5].

- ***HO*s RT-PCR**: the deletion mutants constitutively transcribe the endonuclease. Nevertheless, *HO* expression itself does not assure that ATCC 42981 switches mating-type .



Expression patterns of *MAT*α genes in ATCC 42981 mutants. +/- reverse transcription positive and negative controls. Numbers from 1 to 4 indicate deletion mutant clones. M, molecular weight marker; wt, wild type; NTC, no template control.

2. ATCC 42981 and ABT 301^{T} Genome Assemblies



REDUNDANS PIPELINE

- Genomes were assembled from Illumina paired-end reads combining de novo assembly (dipSPAdes), heterozygous contigs reduction and scaffolding (Redundans[6]).

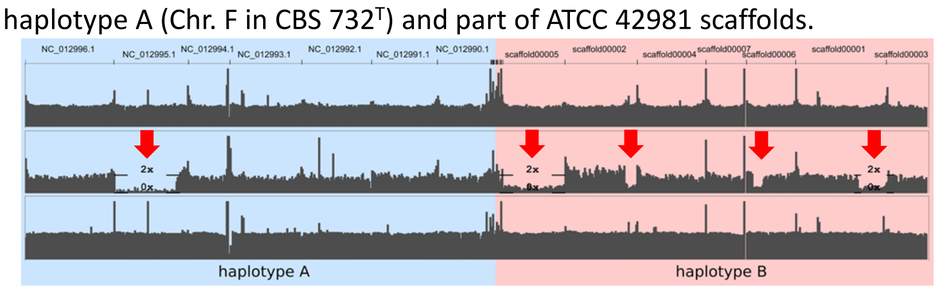
- Two haplotypes A and B (~85% identity) were separated using *Z. rouxii* CBS 732^{T} chromosomes as reference.

- Subsequently, contigs from both haplotypes were scaffolded independently based on synteny to reference chromosomes.

- Finally, the gaps were closed and both haplotypes were merged to give the full genome of each hybrid.

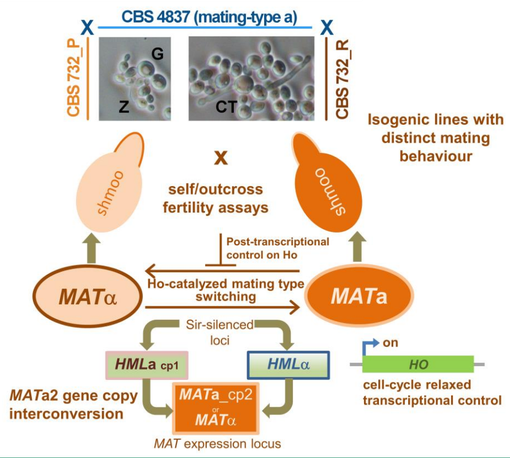
HAPLOTYPES DISSECTION

Genomes of *Z. rouxii* hybrids are composed of two haplotypes: one identical to CBS 372^{T} (blue) and one, not yet identified, ~15% divergent (red). ATCC 42981 and ABT301^{T} differ in chromosome content, suggesting an independent origin. The figure shows the sequencing coverage for all chromosomes of the three analyzed allodiploid strains. ATCC 42981 and CBS 4837 mostly share both haplotypes, while in ABT301^{T} a decrease in read coverage (red arrows) suggests the lack of one chromosome from haplotype A (Chr. F in CBS 732^{T}) and part of ATCC 42981 scaffolds.



3. Mating-type Switching: a Source of Genetic Instability and Phenotypic Novelties in *Z. rouxi* Haploid Cells

We reconstructed how two independent CBS 732^{T} stocks (namely CBS 732_R and CBS 732_P) underwent mating-type, generating a new *MAT***a**2 gene copy different from the silenced copy at the *HMR***a**. Both the *Z. rouxii* cells are haploids but, differently from *S. cerevisiae*, they bypassed the cell-cycle control and expressed *HO* at the stationary phase. Despite this, mating-type switching occurred rarely or belatedly during *Z. rouxii* colony formation. Despite being isogenic, the two cultures displayed distinct fertility response towards the opposite *Z. rouxii* mating testers.



Conclusions

ACTIVITY 1: i) *HO* expression does not assure mating-type switching; ii) with one copy of *MAT* disrupted, ATCC 42981 does not behave as a haploid; iii) *Z. sapae MAT*α deletion induces *HML*α loci de-silencing or, reveals the incomplete silencing of donor cassettes in the wild type strain.

ACTIVITY 2: ATCC 42981 genome assembly detected the an additional *MAT*α copy 2 cassette (table on the right). This could be co-expressed and explain why *Z. sapae MAT*α disruption does not cause ATCC 42981 behaving as a haploid .

ACTIVITY 3: Mating-type switching of two independent CBS 732^{T} stocks could be a plastic mechanism affecting genotype instability and phenotypic novelties in haploid homothallic yeasts.

	MAT	*HO*
ATCC 42981	6	2
ABT301^{T}	5	2
CBS 4837	3	2
CBS 732^{T}	3	1

References

1. Bizzarri, M., Giudici, P., Cassanelli, S., and Solieri, L. (2016). Chimeric sex-determining chromosomal regions and dysregulation of cell-type identity in a sterile *Zygosaccharomyces* allodiploid yeast. *PloS One*, 11(4), e0152558.
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3. Ullah, A., Lopes, M. I., Brul, S., and Smits, G. J. (2013). Intracellular pH homeostasis in *Candida glabrata* in infection-associated conditions. *Microbiology*, 159(4), 803-813.
4. Daniels, K. J., Lockhart, S. R., Staab, J. F., Sundstrom, P., and Soll, D. R. (2003). The adhesin Hwp1 and the first daughter cell localize to the **a**/**a** portion of the conjugation bridge during *Candida albicans* mating. *Molecular biology* of the cell, 14(12), 4920-4930.
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