

# Inheritance and organisation of the mitochondrial genome differ between two *Saccharomyces* yeasts

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Petite-positive *Saccharomyces* yeasts can be roughly divided into the sensu stricto, including *Saccharomyces cerevisiae*, and sensu lato group, including *Saccharomyces castellii*; the latter was recently studied for transmission and the organisation of its mitochondrial genome. *S. castellii* mitochondrial molecules (mtDNA) carrying point mutations, which confer antibiotic resistance, behaved in genetic crosses as the corresponding point mutants of *S. cerevisiae*. While *S. castellii* generated spontaneous petite mutants in a similar way as *S. cerevisiae*, the petites exhibited a different inheritance pattern. In crosses with the wild type strains a majority of *S. castellii* petites was neutral, and the suppressivity in suppressive petites was never over 50%. The two yeasts also differ in organisation of their mtDNA molecules. The 25,753 bp sequence of *S. castellii* mtDNA was determined and the coding potential of both yeasts is similar. However, the *S. castellii* intergenic sequences are much shorter and do not contain sequences homologous to the *S. cerevisiae* biologically active intergenic sequences, as *ori/rep/tra*, which are responsible for the hyper-suppressive petite phenotype found in *S. cerevisiae*. The structure of one suppressive *S. castellii* mutant, CA38, was also determined. Apparently, a short direct intergenic repeat was involved in the generation of this petite mtDNA molecule.

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## Introduction

The genetic material of *Saccharomyces cerevisiae* consists of 16 nuclear chromosomes and a separate circular mitochondrial genome. The multi-copy mitochondrial DNA (mtDNA) has the size of 85.8 kilobases (kb), a low gene density and extensive intergenic regions, which cover a majority of the mitochondrial genome.<sup>1</sup> These regions are composed of long adenosine and thymine (A + T) stretches, short guanosine and cytosine (G + C) clusters, and a special class of intergenic sequences, *ori/rep/tra*, which are present in eight copies.<sup>2,3</sup> A number of short repeats, direct or indirect, can be found within the *S. cerevisiae* mitochondrial sequence, especially within the intergenic regions.<sup>1,3</sup>

*S. cerevisiae* is a facultative anaerobe and can obtain energy purely by fermentation.<sup>4</sup> Several respiratory deficient mutants, which can grow only on fermentable carbon sources, can be isolated.<sup>5</sup> The largest class of these mutants is called petites, and is characterised by grossly rearranged and deleted mtDNA molecules (for review, see Bernardi<sup>6</sup>). Crosses of wild type cells with petite mutants exhibit a non-Mendelian segregation of the mutation, yielding only wild type progeny or both wild type and petite mutants present in different proportions. In the first case the petites entering the cross are called neutral and in the second one suppressive.<sup>7</sup> Among spontaneously arising petites, suppressive petites are the most commonly found class.<sup>8</sup> The degree of suppressiveness of petite clones is measured by the percentage of zygotes giving rise to petite clones. A special subclass, hyper-suppressive petites, is defined by the criterion that close to 100% of cells resulting from crosses are petites. mtDNA molecules of hyper-suppressive petites

Abbreviations used: mtDNA, mitochondrial DNA; ORFs, open reading frames.

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**Table 1.** Parental yeast strains

Strain	Yeast	Genotype or description	Origin or source
Y169	<i>S. cerevisiae</i>	<i>MATa, lys1</i>	Ref. 25
Y170	<i>S. cerevisiae</i>	<i>MAT<math>\alpha</math>, lys1</i>	Ref. 25
Y184	<i>S. cerevisiae</i>	<i>MAT<math>\alpha</math>, ade1, his1</i>	Ref. 25
Y185	<i>S. cerevisiae</i>	<i>MATa, ade1, his1</i>	Ref. 25
Y188	<i>S. castellii</i>	Diploid, homothallic	Ref. 25
Y235	<i>S. castellii</i>	Diploid, homothallic, <i>ura3</i>	Mutagenesis of Y188
Y239	<i>S. castellii</i>	Induced <i>ho, MATa</i>	Mutagenesis of Y188
Y252	<i>S. castellii</i>	<i>MATa, arg</i>	Mutagenesis of Y239
Y257	<i>S. castellii</i>	<i>MATa, met</i>	Mutagenesis of Y239
Y258	<i>S. castellii</i>	<i>MATa, arg</i>	Mutagenesis of Y239
Y319	<i>S. castellii</i>	<i>MAT<math>\alpha</math>, ura3</i>	From Y235 X Y258
Y320	<i>S. castellii</i>	<i>MAT<math>\alpha</math>, ura3</i>	From Y235 X Y258
Y321	<i>S. castellii</i>	<i>MAT<math>\alpha</math>, ura3</i>	From Y235 X Y258

reveal a common organisation: they consist of only a short multiplied intergenic segment carrying an *ori/rep/tra* sequence.<sup>9,10</sup> Upon a cross the petite mtDNA molecule out-competes the wild type mtDNA molecule in the zygote and gets almost exclusively transmitted to the progeny (reviews<sup>5,11,12</sup>). Similarly, respiratory competent, mitochondrial mutants lacking some of the *ori/rep/tra* sequences are out-competed by the wild type mtDNA molecules.<sup>13</sup> Apparently, the *ori/rep/tra* sequence embedded in a proper environment of neighbouring sequences, provides a structural basis for interactions between mtDNA and other elements involved in the process of selective transmission.<sup>14</sup> However, while *ori/rep/tra* sequences play a crucial role in transmission of the *S. cerevisiae* mtDNA molecule, these sequences seem to be present only in a limited number of yeast species.<sup>15</sup>

A majority of yeasts are petite-negative, including *Schizosaccharomyces pombe*, and only their mutator strains can yield mitochondrial respiratory mutants.<sup>16</sup> The genus *Saccharomyces* contains several species,<sup>17</sup> which can be divided into a group of petite-positive, including *S. cerevisiae*, and a group of petite-negative yeasts; the latter includes *Saccharomyces kluyveri*.<sup>18</sup> Apparently, the common progenitor of these two groups was a petite-negative yeast. Upon separation of the *S. kluyveri* and *S. cerevisiae* lineages the *S. kluyveri* lineage has remained petite negative, while the other lineage has developed the petite-positive characteristics.<sup>18</sup> These petite-positive *Saccharomyces* yeasts can be divided into sensu stricto yeasts, including *S. cerevisiae*, and sensu lato yeasts. The sensu stricto mtDNA molecules are phylo-

genetically very closely related, are larger than 60 kb in size and show similar gene order configurations.<sup>19</sup> On the other hand, the sensu lato mtDNA molecules are smaller in size and show a higher degree of phylogenetic and gene order diversity.<sup>19</sup> So far, sensu lato yeasts have not been studied for the inheritance of their mitochondrial genome.

In this project, the sensu lato yeast, *Saccharomyces castellii*, was studied for transmission and organisation of the mitochondrial genome and compared to *S. cerevisiae*.

## Results

### Heterothallic strains

*S. cerevisiae* mitochondrial inheritance has been studied using crosses between different mitochondrial mutants. Therefore, we intended to develop similar mutants in *S. castellii*. Upon mutagenesis a haploid heterothallic strain, Y239, was isolated from a diploid, well sporulating homothallic strain of *S. castellii* (Y188). Further on, haploid strains of the opposite mating type, carrying different auxotrophic markers, as Y252, Y257, Y258, Y319, Y320, and Y321, were developed using mutagenesis, crosses and spore dissection (Table 1). These strains were the parental strains of various strains (Tables 2 and 3) developed to be used in different crosses to study the inheritance rules of the *S. castellii* mitochondrial genome.

### Crosses involving point mutation mutants

Two drug resistance markers, conferring resistance to oligomycin and erythromycin, were

**Table 2.** *S. castellii* cross with mitochondrial respiratory competent mutants

Cross	Mitochondrial markers	Transmission (%)
Y258-ery <sup>R</sup> -1 X Y319-oli <sup>R</sup> -1 <sup>a</sup>	ery <sup>R</sup> oli <sup>S</sup>	40.8
	ery <sup>S</sup> oli <sup>R</sup>	35.4
	ery <sup>R</sup> oli <sup>R</sup>	14.1
	ery <sup>S</sup> oli <sup>S</sup>	9.7

<sup>a</sup> The progeny consisted of 319 daughter lines.

**Table 3.** Petites and their suppressivity

Petite strain	Origin	Suppressivity (%)	
<i>A. Spontaneous S. cerevisiae petites and their suppressivity</i>			
CE1	Y169	60.4	
CE2	Y169	95.1	
CE5	Y169	71.4	
CE6	Y169	16.4	
CE7	Y169	54.5	
CE8	Y169	69.4	
CE9	Y169	62.5	
CE10	Y169	64.6	
CE26	Y170	30.6	
CE27	Y170	45.4	
CE28	Y170	43.5	
CE29	Y170	69.3	
CE30	Y170	56.3	
CE31	Y170	60.4	
CE34	Y170	81.9	
CE35	Y170	70.6	
CE40	Y184	14.1	
CE41	Y184	16.1	
CE42	Y184	6.3	
CE43	Y184	60.9	
CE44	Y184	4.7	
CE46	Y184	3.1	
CE62	Y185	39.6	
CE63	Y185	31.3	
CE64	Y185	0	
CE65	Y185	6.3	
CE66	Y185	12.5	
CE67	Y185	4.2	
CE68	Y185	4.2	
CE69	Y185	20.8	
CE70	Y185	52.1	
CE71	Y185	60.4	
Petite strain	Origin	mtDNA type <sup>a</sup>	Suppressivity (%)
<i>B. Spontaneous S. castellii petites, their mtDNA-type and suppressivity</i>			
CA34	Y258	$\rho^-$	48.1
CA35	Y258	$\rho^0$	6.2
CA36	Y258	$\rho^0$	4.8
CA37	Y258	$\rho^-$	0
CA38	Y258	$\rho^-$	34.7
CA39	Y258	$\rho^-$	29.2
CA40	Y258	n.d.	1.4
CA41	Y258	$\rho^-$	1.7
CA42	Y258	$\rho^-$	0
CA43	Y258	$\rho^0$	0
CA44	Y258	n.d.	6.9
CA46	Y258	wt-like	11.0
CA47	Y258	$\rho^-$	47.1
CA54	Y319	wt-like	0
CA57	Y319	$\rho^-$	3.8
CA59	Y319	$\rho^-$	0
CA61	Y319	$\rho^-$	0
CA62	Y319	$\rho^-$	0
CA63	Y320	$\rho^-$	7.0
CA65	Y320	$\rho^-$	31.1
CA66	Y320	$\rho^-$	6.0
CA67	Y320	n.d.	0
CA68	Y320	$\rho^-$	13.0
CA69	Y320	n.d.	0
CA70	Y320	n.d.	16.7
CA71	Y320	$\rho^0$	0
CA72	Y320	$\rho^-$	5.9
CA74	Y321	wt-like	0
CA75	Y321	wt-like	4.2
CA76	Y321	wt-like	4.2

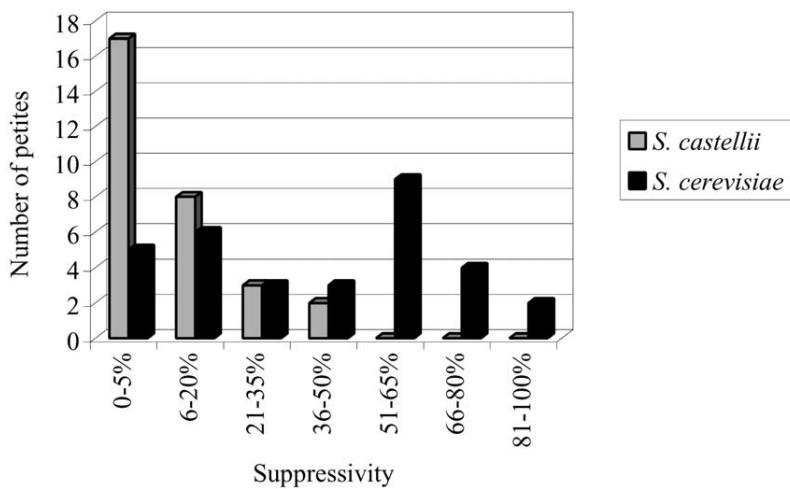
<sup>a</sup> The mtDNA-type was designated as wt-like if most of the wild type mtDNA fragments were present,  $\rho^-$  if only a few mtDNA restriction fragments were detected, and  $\rho^0$  if mtDNA fragments could not be detected at all. n.d., not determined.

introduced into the *S. castellii* mtDNA molecule. The obtained respiratory competent strains were used in a two-point genetic cross (Table 2). The diploid progeny was analysed for the antibiotic resistance and the level of recombination between the two markers was 23.8% (Table 2). The obtained results suggest that the inheritance rules, operating in crosses among respiratory competent *S. castellii* mutants, seem to be very similar to those reported for *S. cerevisiae*.<sup>20</sup> In contrast, the transmission pattern of respiratory deficient petite mutants in *S. castellii* was significantly different from *S. cerevisiae*.

### Transmission of petite mutants

In order to compare transmission of petites in mitochondrial crosses between *S. cerevisiae* and *S. castellii* spontaneous petites were isolated from different parental strains (Table 3). The parental strains of each species were isogenic for their mitochondrial genome. Each of the 32 *S. cerevisiae* petites was crossed with the two respiratory competent *S. cerevisiae* strains of the opposite mating type, and the progeny analysed for respiratory competence. The degree of petite suppressivity was presented as percentage of respiratory deficient colonies among the progeny (Table 3). Five *S. cerevisiae* petites, or approximately 15% of all tested petites, had the suppressivity lower than 5%, and were therefore considered as neutral. Over three quarters of petites were suppressive, and almost one third of all petite clones showed suppression between 51 and 65% (Figure 1). Two of the *S. cerevisiae* petite clones, CE2 and CE34, had suppressivity over 80%. The distribution of the degree of suppressivity among the analysed clones (Table 3, Figure 1) fits well with the previous observation made on other *S. cerevisiae* strains.<sup>8</sup>

When 30 spontaneous *S. castellii* petites were crossed with respiratory competent strains more than half of the petites exhibited the neutral phenotype. These neutral petites were analysed for the presence of mtDNA and surprisingly, a majority still contained at least a fragment of their mtDNA (Table 3). Only four clones, of which two exhibited low suppressivity (just above 5%), did not have detectable mtDNA. Therefore, the neutral phenotype is in general not a consequence of complete absence of mtDNA. Among neutral *S. castellii* petites a small portion exhibited a wild type-like restriction pattern of their mtDNA molecules. Presumably, these clones, which behaved like mitochondrial mutants (Table 3), carried mitochondrial point mutations or short deletions/rearrangements. A similar class of respiratory deficient mitochondrial mutants characterised by a low transmission capacity, but a wild type mtDNA restriction pattern has recently been described in another yeast, *S. kluyveri*.<sup>18</sup> Among suppressive petites 11 out of 13 showed suppression between 6 and 35%, and two, CA34 and CA 47, exhibited suppressivity close to 50% (Table 3, Figure 1). The



**Figure 1.** Suppressivity (%) of spontaneous *S. castellii* and *S. cerevisiae* petites determined in crosses with the wild type parental strains.

observed differences between *S. cerevisiae* and *S. castellii* could be due to nuclear or/and mitochondrial factors. In *S. cerevisiae* a crucial genetic factor behind the hyper-suppressive phenotype is the presence of the mitochondrial *ori/rep/tra* sequences (reviews<sup>6,14</sup>). Therefore, the total sequence of the *S. castellii* mtDNA molecule was determined and analysed for the presence of the biologically active mitochondrial *S. cerevisiae*-like sequences.

### Organisation of *S. castellii* mtDNA

The approximate size and gene order of the *S. castellii* mtDNA molecule has previously been determined by restriction analysis and gene mapping.<sup>15,19</sup> In this study the whole molecule was sequenced by direct sequencing on CsCl-purified mtDNA giving the size of 25,753 bp and the A + T content of 79.6% (accession number AF437291). The *S. castellii* mtDNA molecule is threefold smaller than the *S. cerevisiae* one and it is slightly less A + T-rich. When the sequence was analysed for the presence of protein-coding open reading frames (ORFs) the same eight as in *S. cerevisiae*<sup>1</sup> were found: *COX1*, *COX2*, *COX3*, *ATP6*, *ATP8*, *ATP9*, *COB* and *VAR1* (Figure 2). Several RNA genes were also found in *S. castellii*, two ribosomal RNA genes, *LSU* and *SSU*, and several transfer RNA (tRNA) genes (Figure 2). While in *S. cerevisiae*<sup>1</sup> and *H. wingei*,<sup>21</sup> 24 and 25, respectively, tRNAs have been found, only 23 were predicted in *S. castellii* (Figure 2). A detailed gene annotation can be found elsewhere (R.B.L. & J.P., unpublished results). In short, *S. castellii* mtDNA seems to have a very similar coding potential as *S. cerevisiae*, but the gene order is different (Figure 2). The whole mtDNA molecule is covered by global and local simple repeats, showing a moderate percentage of identity (Figure 2). The structural background for this organisation pattern is in general a high content of A + T. However, the loci around *tRNA<sup>Val</sup>*, *VAR1*, *COB* and *tRNA<sup>Met2</sup>* contain longer stretches of global direct

repeats with a very high nucleotide identity (Figure 2). In theory these regions could destabilise the mitochondrial genome.

In *S. cerevisiae*, the intergenic regions cover a majority of mtDNA. These sequences are important in generation of petite mtDNA, as well as they provide a selective advantage during transmission to the progeny.<sup>14</sup> In *S. castellii* the coding regions represent 52.7% of the total mtDNA molecule, the rest are introns and intergenic regions. The later are mainly composed of large A + T-rich stretches. However, also several G + C clusters were found. Nearly every G + C cluster contained at least one direct or indirect repeat also found at another G + C-rich locus. Often several direct and indirect repeats could be found (Supplementary Material).

The total *S. castellii* mtDNA was examined for the structural features of the putative origins of replication, and sequences responsible for the hyper-suppressive phenotype, i.e. the corresponding G + C clusters found in *S. cerevisiae*. Motifs, showing homology to the core of the *S. cerevisiae* *ori/rep/tra* cluster A, B and C,<sup>3</sup> were each found at a few places in the *S. castellii* mtDNA molecule but never were motifs from all three clusters located together.

### Petite mtDNA

Five suppressive *S. castellii* petite strains, CA34, CA38, CA47, CA65 and CA68, were further examined for their mtDNA molecules. Apparently, these strains contained the same amount of mtDNA as the wild type strain (data not shown). When CsCl-purified mtDNA was digested with different restriction enzymes, only a limited number of restriction fragments was observed, suggesting that only a part of mtDNA was preserved, and possibly rearranged and multiplied, in these strains. The repeated fragment varied in size from 0.2 kb in CA34 to 2.6 kb in CA38. When petite mtDNA was used as a probe against the wild type mtDNA molecule, it was found that the

analysed petite mtDNA molecules originated from at least two different loci, *SSU* and *COXI* (Table 4). The origin of CA34, containing only a 0.2 kb repeat, could not be determined, neither by Southern analysis, subcloning or direct sequencing.

To further understand the structure and origin of suppressive petites, the sequence of one of them, CA38, was fully determined. This petite mtDNA is circular and the multiplied fragment is 2570 bp in size (Figure 3, accession number AF437292). The petite mtDNA sequence starts to align to the wild type mtDNA sequence at position 17,205. Note that a G + C cluster maps to this position in the wild type mtDNA sequence. Only a few single base differences between the petite and wild type sequences could be found. The petite mtDNA molecule contains the whole *SSU* gene, three *tRNA* genes and a large G + C cluster (Figure 3). The CA38 petite region, from position 1 to 53, and containing a G + C cluster, represents almost a perfect direct repeat located at 17,205 and 19,782 of the wild type mtDNA molecule (Figure 3). Thus, it is likely that these two repeats were directly involved in generation of CA38. On the other hand, the analysed petite mtDNAs (Table 4) did not overlap with the longer stretches containing global direct repeats (Figure 2).

## Discussion

While *S. cerevisiae* is one of the best studied organisms, so far very little molecular biology research has been done on its closest relatives belonging to the genus *Saccharomyces*. For example, *S. cerevisiae* mitochondrial genetics has been studied for over five decades by a number of laboratories and has been presented in hundreds of publications (reviews<sup>5,11,14,22</sup>). It is still only poorly understood if the molecular mechanisms operating during transmission of the *S. cerevisiae* mitochondrial genome can also be found in other related *Saccharomyces* yeast species. Yeasts from other related genera, including yeasts as *Kluyveromyces lactis*, or far related genera, including *Candida albicans* and *Schizosaccharomyces pombe*, were shown to be petite negative (reviews<sup>11,16</sup>) and these yeasts apparently have fundamentally different inheritance of their mitochondrial genome than *S. cerevisiae*. A study of other *Saccharomyces* yeasts would therefore help to understand how "unique", regarding mitochondrial genetics, *S. cerevisiae* is, and also to elucidate the origin of the present molecular mechanisms behind the inheritance of the yeast mitochondrial genome. The basis of the present study was the isolation of *S. castellii* heterothallic strains (Table 1), which could be used in genetic studies. Different mitochondrial markers, including antibiotic resistance markers and petites, were isolated in these heterothallic strains, employed in mitochondrial crosses, and the resulting progeny analysed.

If the frequency of alleles in the progeny directly reflects the frequency in the parental cells, this is referred as non-biased transmission. In this case the parental and possible recombinant mtDNA molecules possess the same competitive advantage. Such crosses were described in *S. cerevisiae* for mitochondrial mutants resistant to various drugs.<sup>20</sup> Apparently, similar rules operate also in *S. castellii* crosses involving mutants conferring resistance to erythromycin and oligomycin (Table 2). The *S. castellii* mtDNA molecules actively recombined and the maximum level of recombination observed was close to 25% (Table 2), as reported previously also for *S. cerevisiae*.<sup>5,20</sup>

In *S. cerevisiae*, petite mutants carry mtDNA molecules characterised by large deletions and accompanied by reiterations and repetitions of the remaining segments.<sup>6,23</sup> Also *S. castellii* spontaneously generated petites at frequency similar to *S. cerevisiae*<sup>15</sup> and the resulting petite mtDNAs were significantly deleted and reiterated (Tables 3 and 4, Figure 3). In *S. cerevisiae* crosses involving petites, the output frequency does not reflect the frequency of alleles in the parental cells (reviews<sup>12,14</sup>). Among spontaneously isolated *S. cerevisiae* petites, suppressive petites, which after crossing with a wild type strain yield the progeny consisting predominantly of petites, were the most commonly found class (Table 3, Figure 1).<sup>8</sup> However, in *S. castellii*, neutral petites, which after crossing with a wild type strain yield the progeny consisting almost entirely of wild type cells, are the most commonly found class (Table 3, Figure 1). Most of these neutral petites contained a petite-like mtDNA (Table 3). Another interesting aspect is that among the tested *S. castellii* petites no highly suppressive clones, having frequency over 50%, were found (Table 3). On the other hand, approximately half of the *S. cerevisiae* petites had petite frequency over 50%. Apparently, spontaneous *S. castellii* petites exhibit a lower degree of suppressivity than *S. cerevisiae* petites.

The mitochondrial genome transmission process is influenced by *cis*-acting and *trans*-acting genetic elements. In *S. cerevisiae*, the *cis*-acting elements, which confer a transmission advantage to mtDNA, are represented by intergenic sequences, especially the *ori/rep/tra* sequences. While these sequences are dispensable for the normal functioning of mtDNA, respiratory competent mutant mtDNA lacking intergenic sequences are out competed from the *S. cerevisiae* cell.<sup>13</sup> Apparently, the *ori/rep/tra* sequences are also responsible, but not exclusively necessary, for the development of the suppressive phenotype.<sup>24</sup> On the other hand, *ori/rep/tra* are crucial for the hyper-suppressive phenotype.<sup>9,10</sup> The *S. castellii* mtDNA molecule contains almost the same coding potential as *S. cerevisiae* mtDNA, and interestingly all but one gene are encoded on the same strand (Figure 2). However, the intergenic regions are much shorter and several of the *S. cerevisiae*-like G + C clusters, as *ori/rep/tra*, could not be found. The intergenic



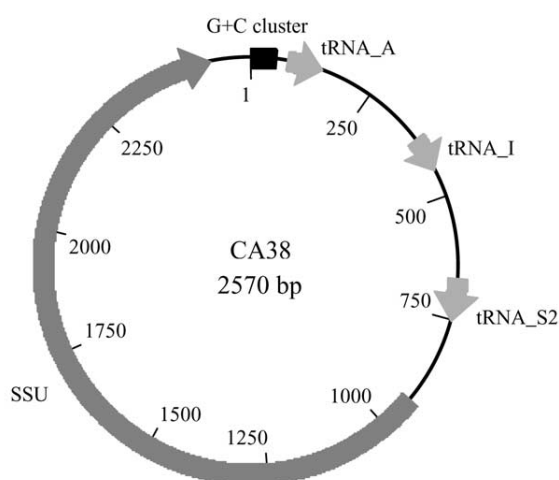
Figure 2 (legend opposite)

**Table 4.** *S. castellii* suppressive petite mutants

Strain	mtDNA size (kb) <sup>a</sup>	Restriction sites	Locus
CA34	0.2	1 × <i>Mse</i> I	n.d.
CA38	2.6	3 × <i>Hae</i> III, 3 <i>Msp</i> I	<i>SSU</i>
CA47	2.5	2 × <i>Hae</i> III, 2 × <i>Msp</i> I	<i>SSU</i>
CA65	2.0	3 × <i>Hae</i> III, 3 × <i>Msp</i> I	<i>SSU</i>
CA68	2.0	No <i>Hae</i> III, 1 × <i>Msp</i> I	<i>COXI</i>

n.d., not determined.  
<sup>a</sup> The size of the presumed repeated element is shown.

A



B

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*****  ***  *****
Upstream  17205  AAATATTAAGTAGATTTCGGGGCCCGAAGGGCCCCGGGTCGCCGTAGGAGAAAT 17257
CA38      1    AAATAAAAAGAAGATTTCGGGGCCCGAAGGGCCCCGGGTCGCCGTAGGAGAAAT 53
Downstream 19782 AAATAAAAAGAAGATTTCGGGGCCCGAA---CCCAGGGTCGCCGTAGGAGAAAT 19831
    
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**Figure 3.** The structure of the *S. castellii* petite CA38, showing the coding regions and the G + C cluster, presumably involved in generation of CA38 from the wild type mtDNA molecule (a). The CA38 G + C repeat was aligned with the homologous regions of wild type mtDNA sequence and the upstream and downstream sequences are shown (b). The numbers refer to the position of the G + C cluster and the stars indicate identical nucleotides among the three sequences. Generation of the petite CA38 could have occurred by recombination within the area marked in “gray”.

regions mainly consist of long A + T stretches sporadically interrupted by G + C-rich stretches. Both kinds of stretches contain several short direct and indirect repeats, which could potentially be involved in generation of petite mtDNA molecules (Figure 2, and Supplementary Material). Indeed,

one of the G + C-rich clusters was involved in generation of a suppressive, CA38, petite (Figure 3).

In conclusion, *S. cerevisiae* and *S. castellii* show several similarities and dissimilarities in the organisation and inheritance of their mtDNA molecules. The *S. castellii* intergenic sequences are

**Figure 2.** A DNA atlas of the circular *S. castellii* mtDNA genome. The outer ring shows the annotated genes. All genes, but one (*tRNA<sup>Thr</sup>*) marked with red, are encoded on one strand. Global direct and inverted repeats are indicated as blue and red bands, respectively, ranging from 50 to 80%. Simple repeats are shown in green, ranging from 50 to 80%, and the innermost circle is the genome A + T content, ranging from 59%, turquoise, to 100%, in red.

much shorter and contain only a few repeated G + C clusters. While *S. castellii* also frequently generates petites, these in general show a lower transmission advantage than the ones found among spontaneous *S. cerevisiae* petites. We propose that the differences in the organisation of the two mitochondrial genomes are responsible for the species characteristic petite phenotypes. One can speculate that after separation of the *S. cerevisiae* and *S. castellii* lineages, the *S. cerevisiae* lineage "acquired" a special class of G + C clusters, *ori/rep/tra*. These clusters enabled expansion of the intergenic sequences in the *S. cerevisiae* mtDNA genome, as well as they promoted development of a more suppressive petite phenotype. However, additional modifications in the nuclear genome influencing mitochondrial biogenesis might also have taken place during the evolutionary history of the *S. cerevisiae* lineage.

## Materials and Methods

### Yeast strains

The parental strains of *S. cerevisiae* and *S. castellii* used in this study are listed in Table 1. Previously, the diploid *S. castellii*, Y188, was sporulated and the mutagenised spores gave several haploid *ho* mutants.<sup>25</sup> One of these strains, Y239, was further mutagenised and screened for auxotrophic mutants,<sup>25</sup> thus giving Y252, Y257 and Y258. Haploid strains, with the opposite mating type, were obtained in the following way. Y258 was crossed with spores obtained from the diploid, *HO*<sup>+</sup>, strain Y235, the obtained diploid clones were sporulated and some of the asci dissected. The obtained lines were then tested for pheromone secretion and auxotrophic markers providing three haploid heterothallic strains, Y319, Y329 and Y321 (Table 1).

### Media and growth

YPD contained 1% yeast extract, 1% Bacto peptone and 2% glucose. GlyYP contained 0.1% yeast extract, 1% Bacto peptone and 2% glycerol. GGlyYP, for petite screening, was like GlyYP, but in addition it contained 0.1% glucose. SD was the minimal medium, and if necessary other supplements were added to this medium.<sup>26</sup> Solid media contained 1.5% agar. Antibiotics were added to solid GlyYP at the following concentrations: oligomycin, 1 mg/l; erythromycin, 0.5 g/l. Yeast strains were grown at 25 °C.

### Induction of antibiotic resistant strains

The overnight cultures of haploid *S. castellii* strains, Y258 and Y319, were diluted ten times in fresh YPD and 1 M MnCl<sub>2</sub> was added to a final concentration of 30 mM. After incubation at 25 °C for four hours, the cells were washed and plated onto selective antibiotic media. Resistant colonies, which appeared after several days, were named according to the resistant marker, *ery*<sup>R</sup> and *oli*<sup>R</sup>, tested for stability and used in crosses (Table 2).

### Respiration deficient petite strains

The parental strains of *S. castellii*, Y258, Y319, Y320, and Y321, and the parental strains of *S. cerevisiae*, Y169, Y170, Y184, and Y185, were grown overnight in YPD, diluted and approximately 100 cells plated on the petite screening plates, GGlyYP. After several days the plates were examined for the presence of small colonies; putative respiration-deficient mutants. These colonies were then tested for growth on GlyYP and for the respiratory potential using the tetrazolium method<sup>27</sup> as well as the obtained respiratory deficient strains (Table 3) were analysed for their mtDNA and employed in crosses.

### Crosses

A large population of diploids was obtained by random mass mating between the two parents.<sup>5,13</sup> Crosses were performed overnight in YPD at 25 °C. Cells were then washed and plated onto the minimal medium plates. After several days the generated diploid clones were tested by replica plating for antibiotic resistance or the respiratory phenotype.

### Petite suppressivity

Each spontaneous petite strain (Table 3) was crossed with at least two respiratory competent parental strains. At least 50 diploid colonies from each cross, which were preformed overnight, were then tested for the respiratory potential. Petite suppressivity was expressed as percentage of respiratory deficient colonies among the diploid progeny.

### *S. castellii* mtDNA sequencing

Mitochondrial DNA from the *S. castellii* type strain (NRRL Y-12630) was isolated using the CsCl based method as described.<sup>28</sup> The type strain is apparently isogenic for the mtDNA molecule with Y188 (data not shown). Initially, different restriction fragments of mtDNA were subcloned in the plasmid pTZ19U, and a few random clones were partially sequenced using the commercially available primers mapping to the vector part. In addition, a few primers were designed in mtDNA regions, which were conserved between *S. cerevisiae* (accession number M62622) and *Hansenula wingei* (accession number D31785). The obtained initial sequences were used to design further primers. Direct sequencing on CsCl purified mtDNA was carried out using the Thermo Sequenase kit (Amersham Pharmacia Biotech) as described.<sup>19</sup> The accession number of *S. castellii* mtDNA is AF437291.

### Genome analysis

Sequence homology to the already known and annotated mtDNA genomes of *S. cerevisiae*<sup>1</sup> and *H. wingei*<sup>21</sup> was carried out by using variations of BLAST, BLASTN and BLASTX,<sup>29</sup> available on the World Wide Web and the precise gene annotation can be found elsewhere (R.B.L. & J.P., unpublished results). The *S. castellii* mtDNA molecule is presented as a "DNA Atlas".<sup>30</sup> The outer ring in the *S. castellii* mtDNA atlas shows the annotated genes. Direct and inverted global repeats are indicated as blue and red band, respectively, simple repeats are in green, and the innermost circle is the calculated AT content. The DNA Atlases of *S. cerevisiae*

mtDNA can be found on <http://www.cbs.dtu.dk/services/GenomeAtlas/Eukaryotes/index.html>

### Repeats

For the global repeats the *S. castellii* mtDNA sequence was analysed using 100 nucleotide windows. A direct repeat is a 100 nucleotide sequence that is present in at least two copies defined as having at least 50% identity. Two copies of 100 nucleotides, having at least 50% identity, and where one is located on the opposite strand, represent global inverted repeats. Local repeats are occurrences of symmetry elements within a region of 100 bp.<sup>31</sup> The mtDNA sequence was also searched for G + C clusters, which were defined as stretches containing at least five G and/or C residues within a ten-nucleotide window. Stretches containing at least 16 nucleotides were compared to each other to deduce possible repeats. Short direct and inverted repeats within the analysed G + C clusters are defined as stretches having at least seven nucleotides of 100% identity (provided as Supplementary Material).

### Molecular characterisation of petite mtDNA

Total cellular DNA was isolated from *S. castellii* parental and petite strains,<sup>13</sup> and digested with *Hae*III or *Msp*I. The fragments were separated in agarose gels, transferred to Hybond N<sup>+</sup> membranes and the mtDNA fragments detected by hybridisation with labeled CsCl purified wild type *S. castellii* mtDNA probe. The obtained patterns were analysed for the presence of the wild type mtDNA bands. A few petite strains were selected for further molecular analysis. Their mtDNA was isolated on the CsCl gradient<sup>28</sup> and digested with different restriction enzymes to determine the size. Petite mtDNA molecules were labelled and used as probes against digested wild type mtDNA to find out, which mitochondrial loci were preserved in different petite strains. At last, one suppressive petite, CA38, was sequenced by direct sequencing on CsCl purified mtDNA<sup>19</sup> using the primers developed for sequencing of the wild type mtDNA molecule, and the accession number is AF437292. Afterwards the petite mtDNA was aligned with the wild type mtDNA to find the sequence(s) involved in generation and maintenance of petite mtDNA.

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