A natural chimeric yeast containing genetic material from three species

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The *Saccharomyces* sp. CID1 isolate (CBS 8614) and several other *Saccharomyces sensu stricto* yeasts were analysed for their mitochondrial and nuclear genes. The data show that *Saccharomyces* sp. CID1, found so far only in one location in Europe, is a natural hybrid between three different *Saccharomyces* yeast species. Two of them, *Saccharomyces cerevisiae*-like and *Saccharomyces bayanus*-like, are ubiquitous and contributed parts of the nuclear genome; the third, *Saccharomyces* sp. IFO 1802-like, which has been found only in Japan, contributed the mitochondrial DNA molecule. These data suggest that the yeast cell is able to accommodate, express and propagate genetic material that originates from different species, and the very existence of the resulting natural hybrids indicates that such hybrids are well adapted to their habitats.

Keywords: yeast, taxonomy, hybridization, mitochondrial DNA, *MET2*

INTRODUCTION

Horizontal transfer of genetic material is not as frequent among eukaryotes as it is among bacteria. However, hybridization between two species occurs occasionally in nature, and among plants the resulting hybrids are sometimes viable and can propagate. So far, there is only limited knowledge about horizontal transfer and resulting hybrids among yeasts and other fungi.

The best-described example of a fungal hybrid is the lager brewing yeast, *Saccharomyces pastorianus* (synonym *Saccharomyces carlsbergensis*). This cultured yeast is an allotetraploid hybrid between two yeast species belonging to the *Saccharomyces sensu stricto* group, the baker's yeast, *Saccharomyces cerevisiae*, and an unknown, *Saccharomyces bayanus*-related yeast. One possibility is *S. bayanus* CBS 1503 (syn. *Saccharomyces monacensis*). Almost complete parental chromosome sets are preserved in *S. pastorianus* (Hansen & Kielland-Brandt, 1994; Kielland-Brandt *et al.*, 1995; Pedersen, 1986), while the mitochondrial

genome (mtDNA) originates from the non-*S. cerevisiae* parent (Piškur *et al.*, 1998). Recently, several novel yeast isolates were analysed for the structure of their nuclear and mitochondrial genomes. When a cider yeast isolate from a home brewery in Brittany, *Saccharomyces* sp. CID1 (CBS 8614), was analysed, some peculiar features of its origin became apparent.

Saccharomyces sp. CID1 was shown by nucleotide sequencing to contain two versions of the nuclear *MET2* gene, an *S. cerevisiae*-like allele and an *S. bayanus*-like allele. Furthermore, karyotyping revealed *S. cerevisiae*-like as well as *S. bayanus*-like chromosomes (Masneuf *et al.*, 1998). However, sequencing of the mitochondrial *ATP9* gene revealed that the *Saccharomyces* sp. CID1 allele diverged equally from those of the type strains of *S. cerevisiae* and *S. bayanus* (Masneuf *et al.*, 1998). In this report, screening of several different isolates of the *Saccharomyces sensu stricto* group is described in order to find the origin of *Saccharomyces* sp. CID1.

METHODS

Yeast strains. The yeast strains used in this study were: *S. bayanus* CBS 380^T, *S. bayanus* CBS 395 (syn. *Saccharomyces uvarum*), *S. pastorianus* CBS 1538^T, *S. pastorianus* CBS 1513 (syn. *S. carlsbergensis*), *S. pastorianus* CBS 1503 (syn. *S. monacensis*), *Saccharomyces paradoxus* NRRL Y-17217^T, *S. paradoxus* CBS 2908 (syn. *Saccharomyces douglasii*), *Saccharomyces* sp. CID1 (CBS 8614) and *Saccharomyces* sp.

Abbreviation: mtDNA, mitochondrial DNA.

The GenBank/EMBL accession numbers for the nuclear *MET2* and mitochondrial *ATP8*, *ATP9* and *SSU* gene sequences reported in this study are AF112009, AF112010, AF114899, AF114901–AF114906, AF114908, AF114909, AF114911–AF114916, AF114918–AF114920, AF114922–AF114924, AF114927, AF114929, AF114930 and AF114933.

Table 1. Primers used for sequencing the mitochondrial small rRNA gene *SSU* and the mitochondrial ATP synthase subunit 8 and 9 genes *ATP8* and *ATP9*

Primer	Sequence
<i>SSU</i>	
SRNA YM-5 (forward)	5'-AAGAATATGATGTTGGTTCAGA
SRNA YM-9 (forward)	5'-CAGCAGTGAGGAATATTGCAGAAT
SRNA YM-8 (forward)	5'-TGGTTTAAAGGATCCGTAGAAT
SRNA YM-10 (forward)	5'-GACGGTTACAGACTTAAGCAGTG
SRNA YM-11 (forward)	5'-CTAGAGTAGCGAAACGGATTTCG
SRNA YM-12 (forward)	5'-GTTGTCTTTAGTTTCGTGCTG
SRNA YM-14 (forward)	5'-CGGTGAATATTCTAACTGTTTCGC
SRNA YM-16 (forward)	5'-TACAGTTACCGTAGGGGAACCTGC
SRNA YM-6 (reverse)	5'-TCTGAACCAACATCATATTCTT
SRNA YM-7 (reverse)	5'-CAATATTCCTCACTGCTGTATCTTATAG
SRNA YM-13 (reverse)	5'-ATTCTACGGATCCTTTAAACCA
SRNA YM-15 (reverse)	5'-CGAATCCGTTTCGCTACTCTAG
SRNA YM-17 (reverse)	5'-CAGCACGAACTAAAGACAAC
SRNA YM-20 (reverse)	5'-AGGATCATTATGATTTGTCTTAATTC
SRNA YM-18 (reverse)	5'-GCGAAACAGTTAGAATATTCACCG
SRNA YM-19 (reverse)	5'-GCAGGTTCCCTACGGTAACTGTA
<i>ATP8</i>	
AAP1 YM-1 (forward)	5'-ATGCCACAATTAGTTCCATTTTA
AAP1 YM-2 (reverse)	5'-TAATTTAGAAATAAATAATCTAGATAC
<i>ATP9</i>	
OLI1 YM-1 (forward)	5'-GCAATTAGTATTAGCAGCTAAATATATTGG
OLI1 YM-4 (reverse)	5'-AATAAGAATGAAACCATTAAACAGA

IFO 1802. The strains carrying the CBS designation were obtained from the Centraal Bureau voor Schimmelcultures, Delft, The Netherlands. Strain NRRL Y-17217^T originated from the National Center for Agricultural Utilization Research, Peoria, IL, USA. Strain IFO 1802 was obtained from the culture collection of the Institute of Fermentation, Osaka, Japan. CID1 is a cider yeast, isolated from a mixed culture, collected from the bottom of a bottle of home-made apple cider from Brittany, France.

Preparation and sequencing of *MET2* gene fragments. *MET2* fragments from *Saccharomyces* sp. IFO 1802 and *S. paradoxus* CBS 2908 were amplified by PCR using primers 5'-CGGCTCTAGACGAAACGCTCCAAGAGCTGG-3' and 5'-CGGCTCTAGAGACCACGATATG CACCAGG-CAG-3', which possess terminal *Xba*I restriction sites in addition to four arbitrary bases, thus allowing restriction digestion. Genomic DNA was prepared from liquid yeast cultures (Hoffman & Winston, 1987). For each DNA preparation, 10 µl of a 100-fold dilution was used as PCR template. The PCR was performed on a Stratagene Robocycler 40 for 25 cycles of 1 min at 94 °C, 2 min at 50 °C and 3 min at 72 °C, followed by 72 °C for 10 min (one cycle). Eight independent reactions with DNA template from each isolate were performed. Each series of identical reactions was pooled and the amplified DNA was precipitated, washed and redissolved in an appropriate volume of water before being used for direct sequencing or cloning. DNA fragments were isolated from agarose by using Bio-Rad Prep-A-Gene purification matrix. The sequencing reactions were performed on a Perkin Elmer DNA Thermocycler 480 and sequences were run on an Applied Biosystems Sequenator 310. Primers for direct sequencing were identical to those

used for PCR amplification, except that no restriction sites or additional arbitrary bases were included (Hansen & Kiehlbrandt, 1994). Sequencing of the cloned fragments was performed employing the same primers or standard M13 primers. Both strands of the DNA were sequenced in all cases.

Cloning of *MET2* DNA fragments. PCR-amplified *MET2* fragments were cloned into pUC19 as follows. Precipitated, redissolved DNA was restricted with *Xba*I and the resulting DNA fragments were purified from agarose using Bio-Rad Prep-A-Gene purification matrix. These fragments were ligated into pUC19 vectors that had been linearized with *Xba*I and treated with calf intestine alkaline phosphatase. The resulting plasmids used for sequencing were: pJH192, pJH193 and pJH194, containing IFO 1802 *MET2* inserts, and pJH195, pJH196 and pJH197, containing CBS 2908 *MET2* inserts.

Isolation and sequencing of mtDNA. For isolation of DNA, a pre-culture was grown overnight in YPD medium (2% glucose, 0.5% yeast extract, 1.0% peptone) and then the culture was grown overnight in GlyYP (2% glycerol, 0.5% yeast extract, 1.0% peptone) at 25 °C. Spheroplasts were prepared using zymolyase and lysed with SDS. mtDNA was separated from the other DNA using a bisbenzamide/CsCl gradient (Piškur, 1989).

The sequences of the mitochondrial *ATP8*, *ATP9* and *SSU* genes were obtained by direct sequencing of purified mtDNA (Groth, 1998). The primers used are listed in Table 1.

Sequence analysis. The partial nuclear *MET2* and the mitochondrial *ATP8*, *ATP9* and *SSU* gene sequences were aligned using the multiple-sequence alignment program

Table 2. Accession numbers of the nuclear *MET2* and mitochondrial *ATP8*, *ATP9* and *SSU* gene sequences

Sequences have been deposited in GenBank or EMBL. The *MET2* sequence from *S. pastorianus*, accession number L16688, originates from *S. pastorianus* production strain M204 (Hansen & Kielland-Brandt, 1994). The *ATP9* and *MET2* sequences from *S. bayanus* CBS 380 and *Saccharomyces* sp. CID1 are from Masneuf *et al.* (1998). The *ATP9* sequence from *S. bayanus* CBS 380 and *Saccharomyces* sp. CID1 were deposited in EMBL. ND, Not determined

Strain	<i>ATP8</i>	<i>ATP9</i>	<i>SSU</i>	<i>MET2</i>
<i>S. bayanus</i> CBS 380 ^T	AF114899	Y16965	AF114901	AF112004
<i>S. bayanus</i> CBS 395	AF114929	AF114930	AF114933	ND
<i>S. pastorianus</i> CBS 1538 ^T	AF114923	AF114924	AF114927	ND
<i>S. pastorianus</i> CBS 1513	AF114903	AF114902	AF114904	ND
<i>S. pastorianus</i> CBS 1503	AF114915	AF114916	AF114918	ND
<i>S. paradoxus</i> NRRL Y-17217 ^T	AF114919	AF114920	AF114922	ND
<i>S. paradoxus</i> CBS 2908	AF114909	AF114908	AF114911	AF112009
CID1 (CBS 8614)	AF114905	Y16964	AF114906	AF112005, AF112006
IFO 1802	AF114912	AF114913	AF114914	AF112010

CLUSTAL W (Thompson *et al.*, 1994). Phylogenetic analyses were performed by using the PHYLIP phylogeny inference package (Felsenstein, 1989). Distance matrices were obtained by using the DNADIST program and unrooted phylogenetic trees were constructed using the unweighted pair group method with averages (UPGMA) and the NEIGHBOR program. Subsequently, the trees were rooted and displayed by using the tree-drawing program TREEVIEW (Page, 1996). The stability of individual branches was assessed by using the bootstrap method with the SEQBOOT, DNADIST, NEIGHBOR and CONSENSE programs of the PHYLIP package. The nuclear *MET2* and mitochondrial *ATP8*, *ATP9* and *SSU* gene sequences that were determined in this study were deposited under the GenBank accession numbers shown in Table 2.

RESULTS AND DISCUSSION

Comparison of mitochondrial genes from several yeasts

The *Saccharomyces sensu stricto* group represents a closely related biological species complex that includes the currently recognized yeast species *S. bayanus*, *S. cerevisiae*, *S. paradoxus* and *S. pastorianus*, a number of subspecies and several new isolates that may represent new species (Masneuf *et al.*, 1998; Naumov, 1996; Naumov *et al.*, 1995a, b). The mitochondrial *ATP8*, *ATP9* and *SSU* genes have been sequenced from several of these yeasts. Note that the currently recognized type strains and their synonymous isolates showed identical or almost identical sequences in the coding and noncoding regions of the three mitochondrial genes analysed (data are accessible through the sequences deposited with GenBank as listed in Table 2; only the type strains are shown in Figs 1–4), thus confirming the presently accepted taxonomy of the genus (Barnett, 1992).

The *ATP8* gene is the shortest mitochondrial gene. In *S. cerevisiae*, the open reading frame consists of 144 bp,

	10	20	30	40	50	60
Cer	ATGCCACAAT	TAGTTCATT	TTATTTTATG	AATCAATTAA	TATATGGTTT	CTTATTAATG
Par					CT.....	
Pas					CT.....	A.....
Bay					CT.....	A.....
CID1					CT.....	T.....
IFO 1802					CT.....	

	70	80	90	100	110	120
Cer	ATTCTATTAT	TAATTTTATT	CTCACAATTC	TTTTTACCTA	TGATCTTAAG	ATTATATGTA
Par					A...T.....	
Pas						C.....
Bay						C.....
CID1		T.....			A...T.....	
IFO 1802					A...T.....	

	130	140	144
Cer	TCTAGATTAT	TTATTTCTAA	ATTATATA
Par			
Pas			
Bay			
CID1			
IFO 1802			

Fig. 1. The open reading frames of the mitochondrial *ATP8* genes from *S. bayanus* (Bay), *S. cerevisiae* (Cer) (Macreadie *et al.*, 1983), *S. paradoxus* (Par), *S. pastorianus* (Pas), IFO 1802 and CID1. The sequences begin with the start codon, ATG, and finish with the stop codon, TAA. Identical nucleotides are indicated by dots.

which corresponds to 48 amino acids (Macreadie *et al.*, 1983). The *ATP8* open reading frames in the yeasts examined were also 144 bp long (Fig. 1). Nucleotide substitutions were predominantly silent, with only three mutations leading to changes in the amino acid sequence. The mitochondrial *ATP8* gene sequence in *Saccharomyces* sp. IFO 1802 was identical to that from *S. paradoxus*, whereas the sequences of other *Saccharomyces* species, including *Saccharomyces* sp. CID1, were different (Fig. 1).

The *ATP9* gene is larger than *ATP8* and is one of the most conserved mitochondrial genes. In *S. cerevisiae* and *S. paradoxus* (syn. *S. douglasii*), the open reading frame consists of 228 bp, which corresponds to 76 amino acids. Only three silent substitutions were found

	10	20	30	40	50	60
Cer	ATGCAATTAG	TATTAGCAGC	TAAATATATT	GGAGCAGGTA	TCTCAACAAT	TGGTTTATTA
Par						
Pas						
Bay						
CID1						
IFO 1802						
	70	80	90	100	110	120
Cer	GGAGCAGGTA	TTGGTATTGC	TATCGTATTC	GCAGCTTTAA	TTAATGGTGT	ATCAAGAAAC
Par						
Pas						
Bay						
CID1						
IFO 1802						
	130	140	150	160	170	180
Cer	CCATCAATTA	AAGACCTAGT	ATTCCCTATGG	CTATTTTAG	GTTTCGCCTT	ATCAGAAGCT
Par						
Pas						
Bay						
CID1						
IFO 1802						
	190	200	210	220	228	
Cer	ACAGGTTTAT	TCTGTTTAAT	GTTTCATTCT	TATTATTAT	TCGGTGATA	A
Par						
Pas						
Bay						
CID1						
IFO 1802						

Fig. 2. The open reading frames of the mitochondrial *ATP9* genes from *S. bayanus* (Bay) (Masneuf *et al.*, 1998), *S. cerevisiae* (Cer) (Ooi *et al.*, 1985), *S. paradoxus* (Par) (this study), *S. pastorianus* (Pas) (Masneuf *et al.*, 1998), IFO 1802 (this study) and CID1 (Masneuf *et al.*, 1998). The sequences begin with the start codon, ATG, and finish with the stop codon, TAA. Identical nucleotides are indicated by dots.

between these two species (Nicoletti *et al.*, 1994; Ooi *et al.*, 1985). The *ATP9* genes in the isolates analysed were also found to be 228 bp long (Fig. 2). The amino acid sequences were identical in all cases, but several silent substitutions were observed. The *ATP9* gene sequences of *Saccharomyces* sp. CID1 and *Saccharomyces* sp. IFO 1802 were identical. Also, the *ATP9* sequences from *S. pastorianus* and *S. bayanus* were identical, while the sequences of other *Saccharomyces* species were different (Fig. 2). The data on the coding regions of the *ATP8* and *ATP9* genes suggested that a likely donor of the CID1 mitochondrial genome could be found among *S. paradoxus*-like and/or *Saccharomyces* sp. IFO 1802-like yeasts. However, the degree of polymorphism within these two genes was too low to obtain a more precise answer. To confirm further the origin of the CID1 mtDNA, the mitochondrial *SSU* gene was sequenced from several yeasts.

The complete *SSU* gene sequences each consisted of approximately 1600 nucleotides. The *SSU* sequence of *Saccharomyces* sp. CID1 was shown to be clearly divergent from *S. cerevisiae* and *S. bayanus*, but 99.4% identical to *Saccharomyces* sp. IFO 1802. Other species of the *Saccharomyces sensu stricto* complex showed 93.8–95.3% identity (Fig. 3). The *SSU* gene from *S. pastorianus* showed 94.5% sequence identity to *S. cerevisiae* *SSU* and 98.4% identity to *S. bayanus* *SSU* (Fig. 3), confirming the *S. bayanus*-like origin of its mitochondrial molecule. The number of data and the extent of polymorphism within the *SSU* gene were adequate to establish a rather precise phylogenetic tree. These results show that the mitochondrial genomes of *Saccharomyces* spp. CID1 and IFO 1802

Fig. 3. Phylogenetic tree based on the mitochondrial *SSU* genes from *S. bayanus*, *S. cerevisiae* (Li *et al.*, 1982), *S. paradoxus*, *S. pastorianus*, IFO 1802 and CID1. Note that the sequences can be obtained elsewhere (Table 2) and are not shown in this paper because of their extent. The bar represents 1% difference between sequences. *Hansenula wingei* was used as an outgroup (Sekito *et al.*, 1995). The stability of branches is represented by percentage bootstrap values (100 cycles were performed).

are closely related. However, this relationship could be interpreted in two ways: *Saccharomyces* sp. IFO 1802 may be a CID1-like hybrid, or may be the parental donor of the mitochondrial genome to the CID1 hybrid.

Nuclear genome of IFO 1802

Saccharomyces sp. IFO 1802 (Kaneko & Banno, 1991) was identified as a genetically isolated population of the *Saccharomyces sensu stricto* yeasts (Naumov *et al.*, 1995b). The nature of *Saccharomyces* sp. IFO 1802 was investigated by analysis of nuclear DNA at the *MET2* locus (Fig. 4). Note that CID1 contains two different *MET2* alleles (Masneuf *et al.*, 1998). RFLP analysis of IFO 1802 *MET2* PCR fragments indicated that this isolate only contained one *MET2* allele (data not shown) and thus it is not likely that this yeast is a hybrid. This finding was supported by Southern hybridization of genomic IFO 1802 DNA digested with six different restriction enzymes and probed with IFO 1802 *MET2*. Invariably, only one distinct signal was found (data not shown). The *MET2* DNA sequence of the IFO 1802 yeast was then determined. The nucleotide sequence of the 330 bp central region of the amplified IFO 1802 *MET2* DNA fragment was obtained through sequencing of the *MET2* PCR product, either by direct sequencing or by sequencing of subcloned PCR fragments in the plasmids pJH192, pJH193 and pJH194. This provided unambiguous sequences from the *Saccharomyces* sp. IFO 1802 *MET2*. The *S. paradoxus* CBS 2908 *MET2* region was also determined by sequencing the inserts of pJH195, pJH196 and pJH197.

The *MET2* allele of IFO 1802 varied from all previously sequenced *Saccharomyces* *MET2* alleles

Fig. 4. Partial nucleotide sequences of the *MET2* genes (a) and a phylogenetic tree based on these sequences (b) for *S. bayanus* (Bay) (Hansen & Kielland-Brandt, 1994), *S. cerevisiae* S288 (Cer) (Langin *et al.*, 1986), *S. paradoxus* (Par) (this study), IFO 1802 (this study), the *S. bayanus*-like allele from CID1 (CID1-1) (Masneuf *et al.*, 1998) and *S. pastorianus* production strain M204 (Pas-1) (Hansen & Kielland-Brandt, 1994) and the *S. cerevisiae*-like allele from CID1 (CID1-2) (Masneuf *et al.*, 1998). In (a), dots denote identity to the *S. cerevisiae* *MET2* sequence. In (b), the bar represents 10% difference between sequences. *Ascomobolus immersus* was used as an outgroup (Goyon *et al.*, 1988) and percentage bootstrap values are shown at individual branches (100 cycles were performed).

Fig. 5. The origin of a yeast with three parents. *S. bayanus*-like and *S. cerevisiae*-like yeasts contributed to the nuclear genome and a *Saccharomyces* sp. IFO 1802-like yeast contributed the mitochondrial genome to the triple hybrid yeast *Saccharomyces* sp. CID1 (CBS 8614).

(Hansen & Kielland-Brandt, 1994; Langin *et al.*, 1986; Masneuf *et al.*, 1998) as well as from *MET2* of *S. paradoxus*. The nucleotide sequence shows 88% identity to *S. cerevisiae* *MET2*, 85% identity to *S. paradoxus* *MET2* and 82% identity to the *S. bayanus* *MET2* and the *S. bayanus*-like *MET2* allele of the lager brewing yeast, and 83% identity to the *S. bayanus*-like *MET2* allele of the *Saccharomyces* sp. CID1 hybrid (Fig. 4). These data show that *Saccharomyces* sp. IFO 1802 is a distinct species belonging to the *Saccharomyces sensu stricto* complex.

Origin of *Saccharomyces* sp. CID1

Our data suggest that *Saccharomyces* sp. CID1, found so far only in one location in Europe, is a hybrid between three different *Saccharomyces* yeasts. Two of them, *S. cerevisiae*-like and *S. bayanus*-like, are ubiquitous and contributed parts of the nuclear genome (Masneuf *et al.*, 1998); the third, a *Saccharomyces* sp. IFO 1802-like yeast, which has been found only in Japan, contributed the mtDNA molecule (Fig. 5). However, the *Saccharomyces* sp. IFO 1802-like ancestor could also have contributed some of the nuclear genome of *Saccharomyces* sp. CID1. It is difficult to determine the nature of single steps in the origin of this triple hybrid and the geographical location of each event. However, it is likely that at least two interspecific mating events occurred and that parts of the parental genetic material, specifically mtDNA from two parents and the nuclear genome from a third parent, were lost from intermediate hybrids before the final and genetically stable triple hybrid arose.

Our results show that the fungal cell is able to accommodate, express and propagate genetic material that originates from different species, and the very existence of the resulting hybrids indicates that they are well adapted to their habitats. In this respect, fungi resemble members of the plant kingdom, rather than the animal kingdom. In addition, the existence of natural hybrids among yeasts suggests that horizontal transfer of genetic material is a significant additional source of genetic variation within the fungal kingdom.

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