Ribosomal DNA Restriction Analysis Reveals Genetic Heterogeneity in *Saccharomyces cerevisiae* Meyen ex Hansen

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A ribosomal DNA restriction analysis of 17 strains belonging to the *Saccharomyces cerevisiae* complex revealed two major groups, one of which corresponded to *Saccharomyces bayanus*. Our results generally correlate with previously reported genetic and molecular data and support the conclusion that *S. bayanus* should be reinstated as a separate taxon.

The schemes currently used for classifying yeasts in the genus *Saccharomyces* are based largely on physiological features, such as fermentation reactions and resistance to cycloheximide (3, 29). The use of these characteristics is necessitated by the paucity of morphological criteria. However, mounting evidence suggests that such characteristics may not always be adequate for species delimitation, and interstrain variability has been observed (3, 7, 18, 21, 29). The instability of distinguishing characteristics (22, 23) and the apparent absence of reproductive isolation (13) have led to the relegation of many previously recognized *Saccharomyces* species to physiological races of *Saccharomyces cerevisiae* Meyen ex Hansen (24, 29, 30). To resolve the taxonomic ambiguities that result from variable biochemical reactions, other criteria have been used for species delimitation within this genus. These criteria include serological properties (5), levels of DNA relatedness (4, 10, 21, 26, 30), restriction endonuclease patterns (17), and electrophoretic karyotyping (8). According to Vaughan-Martini and Martini (27), most *Saccharomyces* species can be differentiated only by using DNA-DNA hybridization data. On the basis of DNA reassociation results, these authors recognized *S. bayanus* and *S. pastorianus* as species that are distinct from *S. cerevisiae*, a view shared by Banno and Kaneko (2).

Because of the presence of both highly conserved and variable regions, rRNA sequences have been used as a tool for analyzing phylogenetic relationships (10, 11). In this study, we examined restriction fragment length polymorphisms in the gene that codes for the small-subunit ribosomal DNA (rDNA), and we obtained further evidence of genetic heterogeneity in *S. cerevisiae*. We also found that restriction patterns could be used to assign most of the strains which we examined to either *S. cerevisiae* or *S. bayanus*. The original specific epithets are used below.

We studied 17 strains (Table 1) of organisms that were considered to be either synonyms or physiological races of *S. cerevisiae* (3, 29, 30). *S. kluyveri*, a species that is thought to be an outlying member of the genus (10), was included for comparison. The procedure used for DNA isolation and the conditions used for the polymerase chain reaction (PCR) have been described previously (12). The primers which we used were those described by White et al. (28) and had the following sequences: primer NS1, 5'-GTAGTCATATGCTTGTCTC; and primer NS8, 5'-TCCGCAGGTTACCTACGGA. The PCR products were digested with the following enzymes: *AccI*, *Cfr13I*, *DdeI*, *HhaI*, *HinfI*, *MspI*, *PaiI*, *RsaI*,

Sau3AI, *ScrFI*, and *TaqAI*. The restriction fragments were electrophoresed on 2.6% NuSieve 3:1 agarose (FMC Bio-products) containing PGEM markers (ProMega) as external standards, stained with ethidium bromide, and photographed. The numbers of nucleotide substitutions per site (genetic distances) were estimated by using the method of Nei and Tajima (16). The values were arrayed in a matrix, and a cluster analysis was performed by using the SAHN feature of the NTSYS-pc program (19). A phenogram was then generated by using unweighted pair-group arithmetic average clustering (15). In order to test the goodness of fit of the clustering to the data set, a cophenetic value matrix was constructed (20), and the MXCOMP program was used to compare this matrix with the original matrix.

The PCR products from all of the strains were approximately 1,800 bp long. Selected restriction digests are shown in Fig. 1 through 4. *RsaI* (Fig. 1) and *TaqAI* (data not shown) could distinguish only between *S. inusitatus* ATCC 76517^T (T = type strain) and the other strains. *HinfI*, *HhaI*, and *ScrFI* (Fig. 2 through 4) yielded restriction patterns that separated *S. inusitatus* and *S. kluyveri* from the other strains and divided the remaining strains into two major groups. The other enzymes either did not cut the PCR products or yielded invariable patterns.

The genetic distances obtained by making pairwise comparisons of shared and unique fragments ranged from 0 to 0.059. *S. inusitatus* ATCC 76517^T exhibited the greatest small-subunit rDNA sequence divergence from all of the other strains; this strain was separated from its nearest neighbor (*S. kluyveri*) by a genetic distance of 0.026. Both *S. kluyveri* ATCC 76530 and *S. inusitatus* ATCC 76517^T occurred alone on separate branches of the phenogram (Fig. 5). The calculated cophenetic correlation coefficient (0.99) indicated that the fit for the cluster analysis was very good (19).

All of the other strains clustered into two groups (Fig. 5). Group 1 (*S. bayanus* cluster) contained *S. bayanus*, *S. uvarum*, *S. globosus*, *S. intermedius* var. *valdensis*, *S. abuliensis*, *S. intermedius* var. *turicensis*, and *S. mangini* var. *tetrasporus*. Group 2 (*S. cerevisiae* cluster) contained *S. tokyo*, *S. cerevisiae* subsp. *orsati*, *S. cerevisiae* subsp. *vetrozensis*, *S. ellipsoideus* subsp. *alpestris*, *S. ellipsoideus* subsp. *alpinus*, *S. ellipsoideus* subsp. *fulliensis*, *S. ellipsoideus* subsp. *thermophilus*, *S. italicus* var. *melibiosi*, and *S. carlsbergensis*. These two groups were separated by a distance of 0.017, which represented a level of sequence divergence in the small-subunit rDNAs of 1.7%. Within each cluster, the strains produced identical restriction patterns with all of the enzymes. The four subspecies of *S. ellipsoi-*

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FIG. 1 to 4. Comparison of small-subunit rDNA restriction digests. PCR products were digested with 11 endonucleases, and the resulting fragments were electrophoresed on 2.6% NuSieve 3:1 agarose. Fig. 1, *RsaI* digests; Fig. 2, *HinfI* digests; Fig. 3, *HhaI* digests; Fig. 4, *SbfI* digests. The PGEM molecular weight standards which we used were 2.64, 1.61, 1.20, 0.68, 0.52, 0.46, 0.40, 0.35, 0.22, 0.18, 0.13 kb long. Lanes pm, PGEM markers; lanes A, *S. bayanus* ATCC 76513^T; lanes B, *S. uvarum* var. *uvarum* ATCC 76514^T; lanes C, *S. globosus* ATCC 76515^T; lanes D, *S. intermedius* var. *valdensis* ATCC 76516^T; lanes E, *S. inusitatus* ATCC 76517^T; lanes F, *S. abuliensis* ATCC 76518^T; lanes G, *S. intermedius* var. *turicensis* ATCC 76519^T; lanes H, *S. tokyo* ATCC 76520^T; lanes I, *S. cerevisiae* subsp. *orsati* ATCC 76521^T; lanes J, *S. cerevisiae* subsp. *vetrozensis* ATCC 76522^T; lanes K, *S. ellipsoideus* subsp. *alpestris* ATCC 76523^T; lanes L, *S. ellipsoideus* subsp. *alpinus* ATCC 76524^T; lanes M, *S. ellipsoideus* subsp. *fulliensis* ATCC 76525^T; lanes N, *S. ellipsoideus* subsp. *thermophilus* ATCC 76526^T; lanes O, *S. italicus* var. *melibiosi* ATCC 76527^T; lanes P, *S. mangini* var. *tetrasporus* ATCC 76528^T; lanes Q, *S. carlsbergensis* ATCC 76529^T; lanes R, *S. kluyveri* ATCC 76530.

deus which we examined could not be distinguished from each other on the basis of the 11 digests studied.

Our analysis of restriction polymorphisms in enzymatically amplified small-subunit rDNAs provided further evidence that *S. cerevisiae* Meyen ex Hansen (29) is a genetically heterogeneous group that consists of at least two distinct clusters, one of which corresponds to *S. bayanus*. Similar results were obtained with cloned probes to the *RDN1* and *HIS4* genes of *S. cerevisiae* (17). Consistently low nuclear DNA relatedness values have been reported between strains belonging to the *S. bayanus* cluster and *S. cerevisiae* (21, 26, 27). Kaneko and Banno (9) obtained low DNA similarity values for the type strain of *S. bayanus* (strain ATCC 76513) and *S. cerevisiae* IFO 10217^T. The fact that these two species are genetically distinct is further indicated by the uniparental segregation of genetic markers in forced-mating studies, suggesting low levels of chromo-

some homology (2). These findings support the conclusion that *S. bayanus* should be reinstated as a distinct species (27).

None of the restriction patterns which we obtained permitted differentiation between *S. carlsbergensis* ATCC 76529^T and the other species. The occurrence of this strain in the *S. cerevisiae* cluster (this study) is inconsistent with reports of high levels of nucleic acid similarity with *S. bayanus* (9, 10, 27). However, high levels of DNA relatedness to both *S. cerevisiae* and *S. bayanus* have also been reported (26). *S. carlsbergensis* has been considered a synonym of both species (27, 29), and it has been suggested that this organism is a partial amphidiploid that resulted from a rare mating (26).

The occurrence of *S. kluyveri* on a branch of the phenogram that is separated from the *S. cerevisiae* cluster is consistent with previously reported differences in the chro-

TABLE 1. Strains of *S. cerevisiae* sensu Yarrow examined

Original epithet	ATCC no. ^a	CBS no. ^b	Substrate
<i>S. bayanus</i> Saccardo strains ^c			
<i>S. bayanus</i> Saccardo	76513 ^T	380 ^T	Turbid beer
<i>S. uvarum</i> Beijerinck var. <i>uvarum</i>	76514 ^T	395 ^T	Currant juice
<i>S. globosus</i> Osterwalder	76515 ^T	424 ^T	Pear juice
<i>S. intermedius</i> Hansen var. <i>valdensis</i> Osterwalder	76516 ^T	1505 ^T	Juice of Fendant grapes
<i>S. intermedius</i> Hansen var. <i>turicensis</i> Osterwalder ^d	76519 ^T	377 ^T	Pear wine
<i>S. inusitatus</i> van der Walt	76517 ^T	1546 ^T	"Spontaneous mutant" of 1546.1 (beer)
<i>S. abulensis</i> Santa Maria	76518 ^T	7001 ^T	<i>Mesophylax adopersus</i>
<i>S. cerevisiae</i> Meyen ex Hansen strains			
<i>S. tokyo</i> Nakazawa ^d	76520 ^T	435 ^T	Sake-moto
<i>S. cerevisiae</i> Hansen subsp. <i>orsati</i> Steiner	76521 ^T	1175 ^T	Wine
<i>S. cerevisiae</i> Hansen subsp. <i>vetrosensis</i> Steiner	76522 ^T	1176 ^T	Wine
<i>S. ellipsoideus</i> Hansen subsp. <i>alpestris</i> Steiner	76523 ^T	1191 ^T	Wine
<i>S. ellipsoideus</i> Hansen subsp. <i>alpinus</i> Steiner	76524 ^T	1192 ^T	Wine
<i>S. ellipsoideus</i> Hansen subsp. <i>fulliensis</i> Steiner	76525 ^T	1193 ^T	Wine
<i>S. ellipsoideus</i> Hansen subsp. <i>thermophilus</i> Steiner	76526 ^T	1194 ^T	Wine
<i>S. italicus</i> Castelli var. <i>melibiosi</i> van Uden	76527 ^T	2909 ^T	Human feces
<i>S. mangini</i> Guilliermond var. <i>tetrasporus</i> Dekker	76528 ^T	406 ^T	Oak tree exudate
<i>S. pastorianus</i> Reess ex Hansen strain			
<i>S. carlsbergensis</i> Hansen	76529 ^T	1513 ^T	Brewer's yeast
<i>S. kluyveri</i> Phaff et al.	76530	5828	Water-logged soil

^a ATCC, American Type Culture Collection.^b CBS, Centraalbureau voor Schimmelcultures.^c Based on the nomenclature of Vaughan-Martini and Martini (27) and Naumov (14).^d Affinities have not been determined previously by molecular methods.

mosome numbers of these organisms (5 in *S. kluyveri* versus 14 to 17 in *S. cerevisiae*) (8). This distinction also correlates well with the large rRNA sequence divergence between these species (10) and with differences in their cellular fatty acid profiles (1). Thus, there appears to be overwhelming support for the concept that *S. kluyveri* is an outlying member of the genus *Saccharomyces* (10).

We did not expect to find remarkably unique restriction patterns for *S. inusitatus* ATCC 76517^T (= CBS 1546^T) since a high level (94%) of nuclear DNA reassociation between this strain and the type strain of *S. bayanus* has been reported previously (26). Although strain ATCC 76517^T has been described as a spontaneous mutant (6), it is possible that this organism was actually an unrelated contaminant. Therefore, we examined its parent strain, *S. inusitatus* CBS

1546.1, and found that the resulting profiles were indeed identical to the restriction patterns of the strains belonging to the *S. bayanus* cluster and did not resemble the profile of the derived culture (strain ATCC 76517^T).

Correlation of our results with previous findings (9, 21, 26, 27) showed that *S. bayanus* is quite distinct from *S. cerevisiae*. We are presently investigating the extent of interstrain variation in restriction fragment length polymorphisms in this species complex. We are also examining the region that spans the two internal transcribed spacers and the 5.8S rDNA to determine whether we can obtain sufficient resolution to differentiate the two other distinct taxa in this species complex, *S. pastorianus* and *S. paradoxus*.

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FIG. 5. Phenogram generated from unweighted average pair group clustering of genetic distances between strains. The strain designations are American Type Culture Collection (ATCC) numbers.

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