Deoxyribonucleic Acid Relatedness among Species of the Genus *Saccharomyces* *Sensu Stricto*

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Twenty-four species assigned to the genus *Saccharomyces sensu stricto* were examined for deoxyribonucleic acid relatedness. Results with type strains demonstrated the presence of four distinct species: *S. cerevisiae*, *S. bayanus*, *S. carlsbergensis*, and *S. kluyveri*. *S. carlsbergensis* NRRL Y-12693^T showed intermediate relatedness between *S. cerevisiae* and *S. bayanus* and has a genome size approximately 1.5 times those of the last two species. These data suggest *S. carlsbergensis* to be a partial amphidiploid which may have arisen from natural hybridization between *S. cerevisiae* and *S. bayanus*.

Numerous studies in recent years have shown that the criteria used in traditional yeast taxonomy are not always satisfactory for delimitation of species. This is partly because many separations are based on the presence or absence of assimilative abilities which are often controlled by a single mutable gene (12, 14, 18, 23, 25). The species of *Saccharomyces sensu stricto* (34) provide a good example of this problem because of strain variability in standard carbon assimilation and fermentation tests (5, 16, 28, 34, 35, 36). As a result of the uncertainty of these tests, a number of other approaches have been attempted, such as comparisons of cell wall antigens (2, 10, 11, 27), proton magnetic resonance spectra of extracted mannans (11, 32), and attempted matings between haploid mating types of similar species (3, 21). Requirements for vitamins (9) and amino compounds (7) have also been used for taxonomic separations, as have differences in deoxyribonucleic acid (DNA) base composition (guanine plus cytosine [G + C] contents in moles percent) (36). Finally, numerical analyses (1, 8, 15, 17) by Adansonian principles have been used for species separations. None of these methods, however, seems to have resolved the status of the species investigated (25).

More significant information concerning taxonomic relationships among yeasts is obtainable by comparison of their nucleic acids (18, 25). In this light, some progress has been made with regard to *Saccharomyces* spp. Bicknell and Douglas (6) determined DNA relatedness among several species, whereas Rosini et al. (26) concluded from their DNA studies that common wine yeasts comprised at least three *Saccharomyces* species. In the present study, we measured DNA relatedness among all taxa assigned to *Saccharomyces sensu stricto* (34, 35, 36) in an effort to clarify their relationships.

MATERIALS AND METHODS

Microorganisms. Twenty-nine strains representing 24 species were examined. Strain designations are given in Table 1.

DNA purification, determination of G + C contents, and reannealing reactions. Extraction and purification of DNA was accomplished by a combination of the procedures of Marmur (20) and Bernardi et al. (4), as described by Price et al. (25). Ratios of absorbance at 260:280 and 230:260 nm

were used to assess DNA purity, as were thermal melting profiles and ultracentrifuge scans.

The G + C contents of nuclear DNAs were calculated from buoyant density values in cesium chloride (29, 33) based on three or four separate determinations made with a model E analytical ultracentrifuge (Beckman Instruments, Inc.) equipped with an electronic scanner. *Micrococcus luteus* (synonym, *M. lysodeikticus*) DNA was used as a reference; this DNA has a buoyant density of 1.7311 g/ml (25).

The extent of DNA reassociation was determined spectrophotometrically by using essentially the method reported by Seidler and Mandel (31) and Seidler et al. (30), as described by Kurtzman et al. (19).

Genome size. Estimates of genome size were determined by comparisons of $C_{0t_{0.5}}$ values (C_{0t} = initial nucleotide concentration in moles per liter $\times$ time in seconds) obtained from reassociation kinetics (31). The relative genome size of *S. cerevisiae* was defined as 1.00 for this comparison.

Single-cell isolates. Single vegetative cell isolates, used in certain comparisons, were obtained by micromanipulation.

RESULTS

Origins of the strains and G + C contents of the nuclear DNAs are listed in Table 1. Neither habitat nor DNA base composition provided evidence of taxonomic value. Consequently, more significant comparisons were sought from DNA reassociation studies.

Results obtained from DNA hybridization studies (Tables 2 and 3) suggest that the 24 taxa compared comprise four species. Of these taxa, 15 showed high relatedness with *S. cerevisiae* (Table 2), and 5 showed high relatedness with *S. bayanus* (Table 3). Little relatedness was detected between *S. kluyveri* and the other species included in this study.

Of particular interest were reassociations involving *S. carlsbergensis*. This species and *S. cerevisiae* showed 57% relatedness, whereas the extent of its complementarity with *S. bayanus* was 72%. To preclude the possibility that mixed-stock cultures were not responsible for these results, we made single-cell isolates from the type strains of all three species. Reassociations involving DNA from single-cell isolates gave the same results. Relative genome sizes were estimated from reassociation kinetics (31) and found to vary among the type strains of the three species: *S. cerevisiae*, 1.00; *S. bayanus*, 1.22; and *S. carlsbergensis*, 1.49.

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TABLE 1. Species of *Saccharomyces* sensu stricto examined

Organism	Strain designation		Isolation source	G + C (moles percent) ± SD ^a
	NRRL ^b	CBS ^c		
<i>S. abuliensis</i> Santa Maria 1978	Y-11845 ^T		<i>Mesophylax adoperus</i> (Tr.)	
<i>S. acetii</i> Santa Maria 1959	Y-12617 ^T	4054	Flor	41.1 ± 0.24
<i>S. bayanus</i> Saccardo 1895	Y-12624 ^T	380	Beer	41.8 ± 0.09
	Y-12624-1		Single-cell isolate from Y-12624	
<i>S. beticus</i> Marcilla ex Santa Maria 1970	Y-12625 ^T	6203	Grape must	
<i>S. capensis</i> van der Walt et Tscheuschner 1956	YB-4237 ^T	2247	Grape must	
<i>S. carlsbergensis</i> Hansen 1908	Y-12629	5112	Grape must	
	Y-12693 ^T	1513	Beer (bottom yeast)	41.1 ± 0.03
	Y-12693-1		Single-cell isolate from Y-12693	
<i>S. cerevisiae</i> Meyen ex Hansen 1883	Y-12632 ^T	1171	Beer (top yeast)	40.4 ± 0.13
	Y-12632-1		Single-cell isolate from Y-12632	
	Y-2034		Wine	
<i>S. chevalieri</i> Guilliermond 1914	Y-12633 ^T	400	Palm wine	40.3 ± 0.14
<i>S. cordubensis</i> Santa Maria 1970	Y-12636 ^T	6007	Wine	
<i>S. coreanus</i> Saito 1910	Y-12637 ^T	5635	Grape must	
<i>S. diastaticus</i> Andrews et Gilliland ex van der Walt 1965	Y-2416	1636	Highly infected gravity wort	40.2 ± 0.04
<i>S. gaditensis</i> Santa Maria 1970	Y-12644 ^T	6006	Wine	
<i>S. globosus</i> Osterwalder 1924	Y-12645 ^T	424	Pear juice	
	Y-12646	3008	Fruit must	
<i>S. heterogenicus</i> Osterwalder 1924	Y-1354 ^T	425	Fermenting apple juice	
<i>S. hienipiensis</i> Santa Maria 1962	Y-6677 ^T	4903	"Alpechin"	
<i>S. hispalensis</i> Santa Maria 1978	Y-11846 ^T		"Alpechin"	40.5 ± 0.12
<i>S. inusitatus</i> van der Walt 1965	Y-12648 ^T	1546	Beer	41.6 ± 0.06
<i>S. italicus</i> nom. nud., Castelli 1938	Y-12649 ^T	459	Grape must	40.1 ± 0.17
<i>S. kluyveri</i> Phaff, Miller, et Shifrine 1956	Y-12651 ^T	3082	<i>Drosophila pinicola</i>	41.9 ± 0.09
<i>S. norbensis</i> Santa Maria 1963	Y-12656 ^T	5378	"Alpechin"	
<i>S. oleaceus</i> Santa Maria 1958	Y-12657 ^T	3093	Olives	
<i>S. oleaginosus</i> Santa Maria 1958	Y-6679 ^T	3081	"Alpechin"	
	Y-12659	2910	Feces	
<i>S. prostoserdovii</i> Kudriavzev 1960	Y-6680 ^T	5155	Grape must	
	Y-12660	5835	Wine	
<i>S. uvarum</i> Beijerinck 1898	Y-12663 ^T	395	Currant juice	42.1 ± 0.11

^a Mean ± standard deviation. Standard deviations were calculated from three determinations.

^b NRRL, Northern Regional Research Center, Peoria, Ill.

^c CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

DISCUSSION

The controversies that have surrounded definition of species in *Saccharomyces* sensu stricto were caused, for the most part, by heavy reliance on fermentation and assimilation of carbon compounds, particularly sugars, as primary taxonomic criteria. This philosophy led to the description of many taxa that we have now shown to be synonyms of *S. cerevisiae* or *S. bayanus*. Although some conspecificity within *Saccharomyces* sensu stricto has been recognized in recent years after reevaluation of data from standard morphological and physiological tests (5, 35, 36), these tests appear incapable of discriminating all species. For example, *S. carlsbergensis* has been considered a synonym of *S. uvarum* (34) as well as of *S. cerevisiae* (35).

Confirmation of *S. kluyveri* as a distinct species was expected for a number of reasons. Its ecological isolation (in tree exudates) from the other species placed in *Saccharomyces* sensu stricto, as well as its inability to form "petite colonies" prompted van der Walt (34) to place it in his *Saccharomyces* group II. Meyer and Phaff (22) and Yarrow and Nakase (36) transferred it to group I; they considered it

closely allied although distinct from *S. cerevisiae*. Lastly, Barker and Miller (3) obtained only infertile hybrids in mating studies between *S. kluyveri* and *S. cerevisiae* despite physiological cross-reactions of their mating pheromones observed later by McCullough and Herskowitz (21).

Although our data clearly show *S. cerevisiae* and *S. bayanus* to be separate taxa, it is not evident why populations so similar in morphology, physiology, and habitat would have evolved into separate species. Further studies are needed to examine this interesting example of sympatry.

It was unexpected to find that *S. carlsbergensis* Y-12693^T showed relatively high DNA relatedness to both *S. cerevisiae* and *S. bayanus*. These observations can be explained by the proportionally larger genome size of this strain of *S. carlsbergensis*. We suggest that *S. carlsbergensis* Y-12693^T may represent a natural hybrid between these two species. This hybrid is probably a partial amphidiploid because its genome size is not quite double that of the proposed parents. Furthermore, the *S. bayanus* parent of this hybrid was probably a strain formerly classified as *S. uvarum* because both *S. carlsbergensis* and *S. uvarum* ferment melibiose, whereas strains originally classified as *S. bayanus* did not

TABLE 2. Extent of DNA reassociation between *S. cerevisiae* NRRL Y-12632^T and other species of *Saccharomyces* sensu stricto

Species	NRRL ^a no.	% DNA reassociation ^b
<i>Saccharomyces aceti</i>	Y-12617 ^T	96
<i>S. beticus</i>	Y-12625 ^T	99
<i>S. capensis</i>	YB-4237 ^T	94
	Y-12629	99
<i>S. cerevisiae</i>	Y-2034	91
<i>S. chevalieri</i>	Y-12633 ^T	96
<i>S. cordubensis</i>	Y-12636 ^T	100
<i>S. coreanus</i>	Y-12637 ^T	94
<i>S. diastaticus</i>	Y-2416	92
<i>S. gaditensis</i>	Y-12644 ^T	95
<i>S. hienipiensis</i>	Y-6677 ^T	95
<i>S. hispalensis</i>	Y-11846 ^T	94
<i>S. italicus</i>	Y-12649 ^T	96
<i>S. norbensis</i>	Y-12656 ^T	90
<i>S. oleaceus</i>	Y-12657 ^T	94
<i>S. oleaginosus</i>	Y-6679 ^T	89
	Y-12659	86
<i>S. prostoserodvii</i>	Y-6680 ^T	93
	Y-12660	101
<i>S. carlsbergensis</i>	Y-12693 ^T	57
<i>S. abuliensis</i>	Y-11845 ^T	15
<i>S. bayanus</i>	Y-12624 ^T	5
<i>S. globosus</i>	Y-12645 ^T	3
	Y-12646	8
<i>S. heterogenicus</i>	Y-1354 ^T	20
<i>S. inusitatus</i>	Y-12648 ^T	24
<i>S. kluyveri</i>	Y-12651 ^T	-3
<i>S. uvarum</i>	Y-12663 ^T	14
<i>Debaryomyces melissophilus</i>	Y-7585 ^T	6

^a NRRL, Northern Regional Research Laboratory, Peoria, Ill.^b Mean value of two to four determinations. Standard deviation, $\leq 5\%$.

ferment this sugar. Bicknell and Douglas (6) reported 96% DNA homology between *S. cerevisiae* and *S. carlsbergensis* and 40% homology between *S. cerevisiae* and *S. uvarum* (*S. bayanus*). The reason for the difference between their results and ours is unknown. We can only comment that their cultures were not the type strains of these species. In support of the idea that *S. cerevisiae* and *S. carlsbergensis* are separate taxa, Holmberg (13) noted that these two species had distinct differences in various zones of chromosome III. Additionally, Nilsson-Tillgren et al. (24) have demonstrated the possibility of single-chromosome transfers between *S. cerevisiae* and *S. carlsbergensis*. In the absence of contrary genetic evidence, we might expect that *S.*

TABLE 3. Extent of DNA reassociation between *S. bayanus* NRRL Y-12624^T and *Saccharomyces* sensu stricto species showing low DNA relatedness with *S. cerevisiae*

Species	NRRL ^a no.	% DNA reassociation ^b
<i>Saccharomyces abuliensis</i>	Y-11845 ^T	91
<i>S. globosus</i>	Y-12645 ^T	104
	Y-12646	87
<i>S. heterogenicus</i>	Y-1354 ^T	86
<i>S. inusitatus</i>	Y-12648 ^T	94
<i>S. uvarum</i>	Y-12663 ^T	98
<i>S. carlsbergensis</i>	Y-12693 ^T	72
<i>S. kluyveri</i>	Y-12651 ^T	24
<i>Debaryomyces melissophilus</i>	Y-7585 ^T	1

^a NRRL, National Regional Research Laboratory, Peoria, Ill.^b Mean value of two to four determinations. Standard deviation, $\leq 5\%$.

carlsbergensis would not form fully fertile hybrids with either *S. cerevisiae* or *S. bayanus*. If this prediction proves true, *S. carlsbergensis* will have to be considered a species distinct from its parents.

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