A Reconsideration of Species Related to *Saccharomyces dairensis* (Naganishi)

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A study of DNA reassociation kinetics and electrophoretic karyotypes of several strains classified as *Saccharomyces castellii*, *Saccharomyces dairensis*, and *Pachytichospora transvaalensis* strains revealed that the group included members of at least five distinct species. Two of the four strains classified as *P. transvaalensis* exhibited very high levels of DNA sequence homology with the type strain of *S. castellii*, while the type strain and another strain demonstrated intermediate levels of relatedness to the type strains of the unrelated taxa *S. castellii* and *S. dairensis*. Five strains that were classified as *S. dairensis* strains were not related to the type strains of the taxa studied. Two of these strains exhibited intermediate relatedness (65% base sequence homology), while another pair exhibited a level of DNA reassociation of 97%. The remaining strain was distinct from all of the other strains studied on the basis of both DNA base sequences and electrophoretic karyotype data.

Saccharomyces dairensis was originally described by Naganishi (13). While Stelling-Dekker (16) found many similarities between this species and both *Torulaspora delbrueckii* and *Saccharomyces unisporus*, the three taxa were distinguished on the basis of differences in cell morphology and relative number of spores per ascus. Later, these characteristics were considered less important when they were combined with a wider physiological profile; consequently, *S. dairensis* was reduced to synonymy with *Saccharomyces delbrueckii* after the genus *Torulaspora* was abolished (11).

van der Walt (18) reestablished the species *S. dairensis*. This followed a brief period during which it had been considered a synonym of *Saccharomyces globosus* (6).

During that time two new species that have physiological profiles very similar to that of *S. dairensis* were described: *Saccharomyces transvaalensis* (17) and *Saccharomyces castellii* (2). The first strains of *S. transvaalensis* were isolated from soil in Africa, and while a close phenotypic relationship to *S. delbrueckii* was immediately recognized, the two taxa were considered distinct because of morphological differences, such as the larger vegetative cells and very big, highly refringent ascospores produced by *S. transvaalensis* (17). These same characteristics were considered important by Capriotti (2) for distinguishing the new species *S. castellii* from *S. transvaalensis*, while a number of physiological differences apparently justified separation of *S. castellii* from *S. delbrueckii*.

A subsequent study revealed that the mating types of *S. transvaalensis* and the reinstated species *S. dairensis* are not interfertile (20). This finding prompted van der Walt (19) to create the genus *Pachytichospora* to accommodate *S. transvaalensis*, which has distinctive ascospores, which upon examination by transmission electron microscopy appeared to be quite different from those of the type strain of *S. dairensis* (20).

The most recent treatment of the genus *Saccharomyces*, in *The Yeasts: a Taxonomic Study*, 3rd ed. (27), considered *S. castellii* as a synonym of the older epithet *S. dairensis*, since the two taxa are indistinguishable on the basis of conventional taxonomic tests. This classification was later shown to be in-

correct, however, when it was found that these species are distinct because their DNA base sequences differ by 80% (24).

It has been amply demonstrated that species designations established solely on the basis of phenotypic profiles often are not consistent with actual genotypic relationships (7, 23). As a result, it was decided to reexamine the type strains of *S. castellii*, *S. dairensis*, and *Pachytichospora transvaalensis*, as well as several cultures classified over the years as members of these three taxa, by using molecular taxonomy techniques.

MATERIALS AND METHODS

Organisms. The strains studied are listed in Table 1. These organisms were kindly provided by the Yeast Division of Centraalbureau voor Schimmelcultures Collection, Delft, The Netherlands.

Preparation and analyses of nuclear DNA. High-molecular-weight nuclear DNA for reassociation experiments was obtained from 24- to 48-h cultures grown in YEPG (1% yeast extract, 1% peptone, 2% glucose) at 25°C. Cells were suspended in sucrose buffer (0.02 M Tris, 0.02 M EDTA, 15% sucrose) together with an equal volume of glass beads (diameter, 0.45 to 0.50 mm) and were disrupted mechanically with a Bead-Beater cell disrupter (Biospec Products, Inc., Bartlesville, Okla.). DNA was purified by the method of Bernardi et al. (1) by using modifications described previously (23). Levels of nucleotide sequence homology were determined optically by using the procedure of Kurtzman et al. (10) and a Gilford model 250 spectrophotometer equipped with a model 2527 thermoprogrammer (Gilford Instruments, Inc., Oberlin, Ohio).

Intact chromosomal DNA for pulsed-field gel electrophoresis was prepared as described previously (26). All analyses were performed with a Chef Mapper apparatus (Bio-Rad, Richmond, Calif.) by using gels composed of 1% agarose (type II-A; medium EEO; Sigma Chemical Co., St. Louis, Mo.) in 0.5× Tris-borate-EDTA (TBE) buffer. The temperature was maintained at 12 to 15°C during electrophoresis. Three separate modes of the electrical field were used: 8 to 15 min at 3 V for 50 h, a 90-s pulse at 6 V for 10 h, and a 60-s pulse at 6 V for 8 h.

RESULTS

DNA-DNA reassociation. The results of DNA reassociation kinetics experiments (Table 2) revealed a variety of relationships among the strains of the three species examined. In some cases the four strains classified as *S. transvaalensis* exhibited very high levels of relatedness (base sequence homology, 82 to 95%) (Table 2), while in other cases intermediate values were obtained (45 to 63% reassociation).

When all strains classified as either *S. castellii* or *S. dairensis* were analyzed (Table 2), mostly high levels of reassociation (over 79%) or low levels (less than 30%) were found. The

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TABLE 1. Strains studied

Species	Original name	Strain ^a	Isolation source
<i>P. transvaalensis</i> van der Walt	<i>S. transvaalensis</i>	DBVPG 6756 (= CBS 7662)	Soil
	<i>S. transvaalensis</i>	DBVPG 6757 ^T (= CBS 2186)	Soil
	<i>S. transvaalensis</i>	DBVPG 6758 (= CBS 2248)	Soil
	<i>S. transvaalensis</i>	DBVPG 6759 (= CBS 4906)	Cecal contents of a baboon
<i>S. castellii</i> Capriotti	<i>S. castellii</i>	DBVPG 6298 ^T (= CBS 4309 ^T)	Soil
	<i>S. dairensis</i>	DBVPG 6353 (= CBS 2913) ^b	Fermenting cucumber brine
	<i>S. castellii</i>	DBVPG 6361 (= CBS 4310)	Soil
	<i>S. dairensis</i>	DBVPG 6410 (= CBS 1579) ^c	Buttermilk
<i>S. dairensis</i> Naganishi	<i>S. dairensis</i>	DBVPG 6352 (= CBS 3007)	Fermenting cucumber brine
	<i>S. dairensis</i>	DBVPG 6356 (= CBS 3006)	Fermenting cucumber brine
	<i>S. dairensis</i>	DBVPG 6357 (= CBS 6904)	Soil
	<i>S. dairensis</i>	DBVPG 6359 (= CBS 6463)	Fermenting grapes
	<i>S. dairensis</i>	DBVPG 6366 ^T (= CBS 421 ^T)	Unknown
	<i>S. dairensis</i>	DBVPG 6747 (= CBS 7127)	Unknown
	<i>S. dairensis</i>	DBVPG 6750 (= CBS 7128)	Unknown
	<i>S. dairensis</i>	DBVPG 6751 (= CBS 7188)	Unknown
	<i>S. dairensis</i>	DBVPG 6752 (= CBS 6334)	Fermenting mushroom

^a DBVPG, Industrial Yeasts Collection of the Dipartimento di Biologia Vegetale, Università degli Studi, Perugia, Italy; CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands.

^b Level of DNA sequence relatedness with strain DBVPG 6298^T, 99% (26).

^c Level of DNA sequence relatedness with strain DBVPG 6298^T, 97% (26).

original type strain of *S. dairensis*, strain DBVPG 6366, was not homologous to any of the 12 *S. castellii* or *S. dairensis* strains with which it was crossed but exhibited intermediate levels of reassociation (60%) with the type strain and another strain of *P. transvaalensis* (strains DBVPG 6757 and DBVPG 6756). One of these strains (DBVPG 6757^T [T = type strain]) also exhibited an intermediate level of reassociation (54%) with the type strain of *S. castellii*, DBVPG 6298. The two remaining strains of *P. transvaalensis* (strains DBVPG 6758 and DBVPG 6759) had very high levels of DNA base sequence homology with the type strain of *S. castellii*, strain DBVPG 6298. This was also the case with several strains originally classified as *S. dairensis*: DBVPG 6352, DBVPG 6353, DBVPG 6356, DB

VPG 6410, and DBVPG 6751, all of which exhibited levels of reassociation with strain DBVPG 6298^T of well over 90%.

Five strains of *S. dairensis*, strains DBVPG 6357, DBVPG 6359, DBVPG 6747, DBVPG 6750, and DBVPG 6752, exhibited very low levels of reassociation with the type strains of *P. transvaalensis*, *S. castellii*, and *S. dairensis* (Table 2). Strains DBVPG 6747 and DBVPG 6750 showed a very high level of base sequence homology (97%). Two of the remaining strains, DBVPG 6357 and DBVPG 6359, had a moderately high level of relatedness (65%), while all other reassociation experiments performed with strain DBVPG 6357 or DBVPG 6359 never resulted in levels of homology of more than 41%. Strain DBVPG 6752, which was isolated from a fermenting mushroom,

TABLE 2. DNA base sequence homology between strains related to *S. dairensis*

Species	Strain	% DNA base sequence homology with:				
		<i>S. castellii</i> DBVPG 6298 ^T	<i>S. dairensis</i> DBVPG 6366 ^T	<i>P. transvaalensis</i> DBVPG 6757 ^T	<i>S. dairensis</i> DBVPG 6357	<i>S. dairensis</i> DBVPG 6747
<i>P. transvaalensis</i>	DBVPG 6756	7	60	45	ND ^a	23
	DBVPG 6757 ^T	54	59	(100)	ND	ND
	DBVPG 6758	95	ND	82	4	ND
	DBVPG 6759	79	29	63	ND	47
<i>S. castellii</i>	DBVPG 6298 ^T	(100)	35	ND	ND	30
	DBVPG 6353	95	27	ND	37	ND
	DBVPG 6361	96	30	ND	33	ND
	DBVPG 6410	97	37	ND	22	ND
<i>S. dairensis</i>	DBVPG 6352	100	30	ND	ND	ND
	DBVPG 6356	95	25	54	7	ND
	DBVPG 6357	38	9	ND	(100)	37
	DBVPG 6359	33	32	ND	65	40
	DBVPG 6366 ^T	35	(100)	59	9	6
	DBVPG 6747	30	6	ND	37	(100)
	DBVPG 6750	25	8	ND	40	97
	DBVPG 6751	98	ND	ND	ND	ND
	DBVPG 6752	28	34	ND	14	20

^a ND, not determined.

FIG. 1. Electrophoretic karyotypes of strains homologous to the type strain of *S. castellii*, strain DBVPG 6298. Lane 1, DBVPG 6759; lane 2, DBVPG 6758; lane 3, DBVPG 6751; lane 4, DBVPG 6410; lane 5, DBVPG 6361; lane 6, DBVPG 6356; lane 7, DBVPG 6353; lane 8, DBVPG 6352; lane 9, DBVPG 6298^T; lanes SD¹, *S. cerevisiae* standard; lanes SD², *Pichia canadensis* (referred to as *Hansenula wingei*) standard. Both standards were supplied by Bio-Rad, Richmond, Calif.

exhibited no base sequence homology with any of the strains with which it was crossed.

Electrophoretic karyotypes. The electrophoretic karyotypes of all of the strains were determined by arranging the gels on the basis of the relationships revealed by the results of the DNA-DNA reassociation experiments. In some cases (Fig. 1 and 2) the profiles were identical, as observed for homologous strains DBVPG 6747 and DBVPG 6750 (Fig. 2, lanes 10 and 11), which exhibited no DNA reassociation with the other strains studied. At the same time, noteworthy polymorphisms were observed even with closely related strains. This was particularly true for the strains homologous to the type strain of *S. castellii* (Fig. 1), which produced the same general karyotypic pattern (chromosomes in all three size classes) but significantly different numbers of bands: from a minimum of 7 in strain DBVPG 6751 (lane 3) to a maximum of 15 in strain DBVPG 6759 (lane 1) were seen.

The two strains which were unique as determined by DNA sequence homology, the type strain of *S. dairensis* (strain DBVPG 6366) and strain DBVPG 6752, had karyotypic profiles that were different from those of all of the other strains examined (Fig. 2, lanes 3 and 12, respectively). In both of these strains relatively low numbers (7 to 10) of only large and medium-sized chromosomes were observed. This is in contrast to the profiles found in the other strains, which were generally characterized by having more chromosomes, usually in all size classes, including small (<500 kb), medium (500 to 1,000 kb), and large (>1,000 kb).

DISCUSSION

Somewhat particular morphological features and a limited physiological profile have been the common denominators used to classify strains as *P. transvaalensis*, *S. castellii*, or *S. dairensis* strains (2, 11, 17–21, 27). The integration of these data with those based on cellular macromolecules has shown that the interspecific as well as the intraspecific relationships of these taxa are somewhat different from those established previously on the basis of the results of conventional taxonomic techniques.

The results of this study confirmed and augmented the results of a previous DNA reassociation study which demonstrated that *S. castellii* and *S. dairensis* are separate taxa (24). By extending the examination to more strains of these two

taxa, as well as to the phenotypically similar species *P. transvaalensis*, it was shown that results obtained by conventional techniques of yeast classification must be supplemented with molecular taxonomy data in order to clearly establish species boundaries.

By using levels of DNA base sequence homology (Table 2), two groups of highly related strains can be identified. The species *S. castellii* includes nine strains that exhibit levels of reassociation of more than 90%, while two strains (DBVPG 6747 and DBVPG 6750) that were previously classified as *S. dairensis* are distinct from all of the other strains examined, but exhibit a level of genome homology of 97%.

Intermediate levels of DNA sequence homology (54 to 60%), such as those found between the type strain and another strain of *P. transvaalensis* (strains DBVPG 6757 and DBVPG 6756) and the type strains of the two unrelated taxa *S. castellii* and *S. dairensis* (level of homology, <30%), ultimately raise more questions than they answer, as has been found previously in other investigations. Vaughan-Martini and Kurtzman (23) found that *Saccharomyces carlsbergensis* (now classified as *Saccharomyces pastorianus* [25]) occupies an intermediate position between the unrelated species *Saccharomyces cerevisiae* and *Saccharomyces bayanus*. On the basis of their results, which were later confirmed by extensive DNA base sequence homology studies performed with strains of *Saccharomyces sensu stricto* (5, 22, 25), the authors suggested that *S. pastorianus* could be a partial amphidiploid derived from a chance hybridization between *S. cerevisiae* and *S. bayanus*. Kurtzman and Robnett (8) later found, however, that while the rate of substitution in the species-specific 25S-625 rRNA regions of *S. cerevisiae* and *S. bayanus* was about 5% (indicating a clear separation), there were no differences between *S. pastorianus* and *S. bayanus*. From this they concluded that the actual mechanism of species differentiation is not clear and that it may involve different rates of chromosomal rearrangement and nucleotide substitution. In view of these observations and considering the fact that *Saccharomyces paradoxus*, which has been isolated only from natural habitats, is a separate species of *sensu stricto* *Saccharomyces* (22), a second hypothesis was proposed (26). It is possible that the hybrid *S. pastorianus* could be

FIG. 2. Electrophoretic karyotypes of *S. castellii*, *S. dairensis*, and *P. transvaalensis* strains. Lane 1, *S. dairensis* DBVPG 6357; lane 2, *S. dairensis* DBVPG 6359; lane 3, *S. dairensis* DBVPG 6366^T; lane 4, *P. transvaalensis* DBVPG 6756; lane 5, *P. transvaalensis* DBVPG 6757^T; lane 6, *P. transvaalensis* DBVPG 6758; lane 7, *P. transvaalensis* DBVPG 6759; lane 8, *S. castellii* DBVPG 6298^T; lane 9, *S. castellii* DBVPG 6361; lane 10, strain DBVPG 6747 (new species); lane 11, strain DBVPG 6750 (new species); lane 12, *S. dairensis* DBVPG 6752; lanes SD¹, *S. cerevisiae* standard; lane SD², *Pichia canadensis* (referred to as *Hansenula wingei*) standard. Both standards were supplied by Bio-Rad. The strains in lanes 6 through 9, as well as those in lanes 10 and 11, showed levels of nucleotide sequence homology of >85%; the strains in lanes 1 and 2 and 4 and 5 showed genomic interrelationships of 60 and 45%, respectively.

the result of a mating between *S. cerevisiae* and *S. paradoxus*, whose genomes are about 50% homologous (22). These organisms have also been shown to undergo a limited degree of spore-to-spore hybridization (14). Therefore, *S. bayanus* could have been derived from *S. pastorianus* by successive chromosomal doubling or substitution during an extraordinarily high number of vegetative reproductions in the highly favorable and selective alcoholic fermentation habitat, where these species are normally found. The relatively high level of affinity of these two taxa (base sequence homology, ca. 72%) (23, 25) may support this theory.

Meyer et al. (12) also found a mean level of DNA relatedness between *Hanseniaspora osmophila* and *Hanseniaspora vineae* of 47%. In a general discussion of the taxonomic interpretation of DNA sequence homology data, Kurtzman et al. (9) concluded that similar data suggest that two taxa are either sibling species or perhaps varieties of the same species. In the case of the results of this study, it appears appropriate that strains DBVPG 6757^T and DBVPG 6756 of *P. transvaalensis* should be classified as members of a distinct species since they occupy an intermediate position between two unrelated taxa, *S. castellii* and *S. dairensis*. This separation is also supported by the finding that the average level of interspecific rRNA sequence difference for the type strains of *P. transvaalensis*, *S. castellii*, and *S. dairensis* is 10% (6a). In addition, van der Walt and Liedenbergh (20) found that mating types of *S. transvaalensis* and mating types of two strains classified as *S. dairensis* (type strain DBVPG 6366 and strain CBS 1579 [DBVPG 6410]) were not infertile. It was later shown that DBVPG 6410 is 97% homologous to the type strain of *S. castellii* (26).

At this time the remaining three strains (DBVPG 6357, DBVPG 6359, and DBVPG 6752) will have to be classified as members of the general species complex *S. dairensis* until sufficient discriminating criteria can be found to separate them. This is despite the fact that their phenotypic similarities were not confirmed by the results of DNA base sequence analyses.

The electrophoretic karyotypes obtained from the analysis of all of the strains by pulsed-field gel electrophoresis (Fig. 1 and 2) confirmed that there are distinct taxa. At the same time, closely related strains were also distinguished, although in some cases polymorphism between two strains belonging to the same species was evident. *S. dairensis* DBVPG 6366^T and DBVPG 6752, which were not related to any of the other strains studied, were also unique on the basis of their electrophoretic karyotypes, which contained only large and medium-sized bands. Strain DBVPG 6366^T was also characterized by having a very low number of chromosomal bands (seven). On the other hand, strains related to *S. castellii*, as well as homologous strains DBVPG 6747 and DBVPG 6750, had electrophoretic karyotypes more similar to those generally found for species belonging to the genus *Saccharomyces*. In previous studies, especially those involving strains of sensu stricto *Saccharomyces* (3, 4, 15), workers found high numbers of bands (14 to 17 bands) belonging to all three chromosome size classes. Most other *Saccharomyces* species are characterized by this kind of karyotype (26); the only exception is *Saccharomyces kluyveri*, which has seven large and medium-sized chromosomes (4, 26).

In this study in which electrophoresis was performed for 68 h with three different modes of the electrical field, it was found that many strains have at least one chromosome that is larger than chromosome 12 (2,200 kb) of the strain of *S. cerevisiae* normally used as a standard in these analyses. By using another DNA standard (in this case the strain provided by Bio-Rad as *Hansenula wingei*), it is possible to produce a gel containing band sizes ranging from 3.13 to 0.225 Mb. Under these condi-

tions it was observed that most strains homologous to the type strain of *S. castellii* (strain DBVPG 6298) are characterized by the presence of chromosomes ranging in size from 3.1 to about 0.50 Mb. The only exceptions are the two strains previously classified as *P. transvaalensis* (strains DBVPG 6758 and DBVPG 6759), which produced the largest band of about 2,200 kb. One of these strains (strain DBVPG 6759) also contains many more chromosomes (15 chromosomes) than the other strains belonging to the *S. castellii* group, which are generally characterized by the presence of 8 to 12 bands. Nevertheless, many of these are probably doublets or triplets, as revealed by significantly thicker, more fluorescent chromosomal bands, and the analogous ones which are apparently clearly separated in strain DBVPG 6759.

While the results obtained in pulsed-field gel electrophoresis studies cannot be used alone in taxonomic studies, they can be useful when combined with other criteria. Since under standardized conditions fairly constant profiles can be obtained, the use of electrophoretic karyotypes for screening phenotypically similar strains has already proven to be useful for the control of strains in culture collections (22a). In addition, the differences in chromosome size and number sometimes observed with conspecific strains could explain why normal Mendelian processes of chromosome pairing and mating do not always occur.

In light of these results, an extensive reexamination of the physiology and morphology of the strains studied is warranted, even though the limited responses of these taxa in conventional taxonomic tests and the known limits of these types of analyses can be problematic. Nevertheless, it is possible that a combination of these results and data obtained from studies of cellular macromolecules will aid in clear differentiation of these species belonging to sensu lato *Saccharomyces*. This is important since while most laboratories are equipped for more traditional techniques of yeast classification, many still do not have the funding, equipment, or expertise necessary for the sometimes more precise methods of molecular analysis, such as RAPD-PCR or analysis of the restriction fragment length polymorphisms of ribosomal DNA ("ribotyping"). It must also be recognized that these techniques were found to be valid only after clear taxon boundaries were established by determining cellular morphology and physiology and subsequent nuclear DNA-DNA reassociation analyses of at least the type strains of species were performed. Consequently, if conventional criteria can be critically reevaluated after a molecular base has been established, it may be possible to verify whether some of these criteria can in fact be useful for taxon differentiation.

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