Deoxyribonucleic Acid Sequence Relatedness Among Some Members of the Yeast Genus *Hansenula*

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The evolutionary affinities among several morphologically and physiologically similar species of the ascogenous yeast genus *Hansenula* were studied by techniques of genome comparison. Among the organisms studied, two clusters of related strains were identified by deoxyribonucleic acid base composition and sequence relatedness. These clusters showed little relatedness to each other or to the other strains studied. Several strains of *H. wingei*, as well as the type strain of *H. canadensis*, exhibited 78 to 98% base sequence complementarity with the type strain of *H. wingei*. These species are all associated with coniferous trees. An additional strain of *H. wingei* shared 62% of its nucleotide sequences with the type strain. Three other yeasts resembling *H. wingei* but isolated from exudates of alder trees showed >98% complementarity among themselves but <10% relatedness to the *H. wingei* type. These strains are considered to represent a new species, *H. alni*. The results suggest the inadequacy of current taxonomic criteria and the need for new approaches to yeast systematics.

Genome comparison has elucidated the relationships among various groups of procaryotes, and it is now being employed increasingly in yeast systematics (cf. 10, 11). Recently we used this molecular approach to establish the evolutionary affinities among members of several ascogenous genera (14). In the present paper we apply this methodology to some members of the ascogenous genus *Hansenula*. Although this large and diverse genus has been extensively characterized by morphological, physiological, and ecological criteria (20), the natural relationships among the constituent species remain unclear. In addition, the taxonomic validity of the criteria that separate the species of *Hansenula* has not been satisfactorily demonstrated.

The current study focuses upon species placed by Wickerham (20) on lines three and four of his proposed evolutionary tree for *Hansenula*. Although these yeasts share substantial phenotypic similarity, they are presently classified in six separate species. They have been characterized, in addition to the conventional taxonomic system, by numerical analysis (4) and by cell wall structure (5). We became interested in these yeasts after isolating, in the Pacific Northwest, three strains that closely resembled *H. wingei*, one of the members of the group. This study was initiated to clarify relationships among the currently recognized species, and we provide evidence to confirm that the three new strains represent a novel species of *Hansenula*, *H. alni*,

which is described in an accompanying paper (12).

MATERIALS AND METHODS

Organisms. The yeasts used in this study are shown in Table 1.

DNA purification. Cultivation of cells and the extraction and purification of whole-cell yeast DNA were as previously described (14). Briefly, DNA purification involved cell breakage, chloroform extractions, ethanol precipitations, enzyme treatments, hydroxylapatite chromatography, and cesium chloride density equilibrium centrifugation.

Determination of base composition. The guanine-plus-cytosine (G+C) content of yeast nuclear DNA was calculated from the buoyant density in cesium chloride by using the equation of Schildkraut et al. (15), as previously described (14).

DNA reannealing reactions. Renaturation kinetics and sequence complementarity were determined by renaturation in 280 mM PB (an equimolar mixture of mono- and dibasic sodium phosphate, pH 6.8) at 25°C below the thermal denaturation point of native homologous DNA, as described in detail earlier (14). Before reannealing, rapidly renaturing minor DNA species were removed from the ³²P-labeled reference DNAs (14). After heterologous reannealing reactions, duplex molecules were separated from unreacted DNA with 140 mM PB at 60°C, using the hydroxylapatite batch technique of Brenner et al. (2). The same PB molarity was used for thermal elution studies of duplex molecules, essentially as described by Brenner et al. (2). The data are presented as percentages of actual binding to hydroxylapatite (experimentally determined) and as percentages of relative binding (normalized to the homologous reaction).

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TABLE 1. *Hansenula* strains used

Organism	Isolation source	Strain designation ^a		
		UCD	CBS	NRRL
<i>Hansenula alni</i> Phaff et al. 1978	Exudate of <i>Alnus rubra</i>	68-928A ^b	6986	
<i>H. alni</i>	Exudate of <i>A. rubra</i>	68-940	6987	
<i>H. alni</i>	Exudate of <i>A. rubra</i>	68-985	6988	
<i>H. beckii</i> Wickerham 1951 (syn. <i>Endomycopsis bisporus</i> (Beck) Dekker 1922)	Frass of <i>Picea</i> sp.	74-68 ^b	1890	Y-1482
<i>H. bimundalis</i> Wickerham et Santa Maria var. <i>bimundalis</i> 1965	Frass of <i>Pinus sylvestris</i>	71-33 ^b	5642	Y-5343
<i>H. bimundalis</i> Wickerham et Santa Maria var. <i>americana</i> Wickerham 1965	Frass of <i>P. jeffreyi</i>	74-69 ^b	5644	Y-2156
<i>H. canadensis</i> Wickerham 1951	Frass of <i>P. resinosa</i>	74-71 ^b	1922	Y-1888
<i>H. canadensis</i>	Frass of <i>P. strobus</i>	74-72	5676	YB-4170
<i>H. henricii</i> Wickerham 1969	Bird feces	74-73 ^b	5765	YB-2194
<i>H. nonfermentans</i> Wickerham 1969	Creek water	72-15 ^b	5764	YB-2203
<i>H. wingei</i> Wickerham 1956	Dead conifer	71-80 ^b	2431	Y-2340
<i>H. wingei</i>	Dead conifer	71-78	2432	Y-2340-5 ^c
<i>H. wingei</i>	Dead conifer	71-79	2433	Y-2340-21 ^c
<i>H. wingei</i>	<i>Ips</i> sp. from <i>Picea engelmannii</i>	74-76	2430	Y-1987
<i>H. wingei</i>	Dead conifer	74-77	5678	YB-4662

^a UCD, Department of Food Science and Technology Collection, University of California, Davis, Calif.; CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; NRRL, Northern Regional Research Laboratory, Peoria, Ill.

^b Type strain.

^c Haploid mating types derived from Y-2340.

RESULTS

Nuclear DNA base compositions of the organisms are shown in Table 2. Two clusters of strains were apparent from these data. The first cluster, ranging from 40.3 to 40.8 mol% G+C, included both strains of *H. canadensis* and four strains of *H. wingei*; a fifth strain of *H. wingei* had a slightly but significantly lower G+C content (39.7 mol%). The second cluster consisted of the three Pacific Northwest isolates of *H. alni*, with base compositions 1 to 1.5 mol% G+C higher than those of the yeasts of the *H. wingei*-*H. canadensis* subgroup. The two varieties of *H. bimundalis* differed by 2.5 mol% G+C; in our experience (14), a difference of this magnitude precludes significant sequence complementarity. By the same reasoning, we infer that *H. beckii* is not closely related to the other yeasts studied.

The type strain of *H. wingei* (71-80) and a representative strain (68-928A) of *H. alni* were chosen as reference organisms for DNA sequence comparisons. The renaturation kinetics of homologous reference DNA from *H. wingei* are shown in Fig. 1. The Britten-Kohne plot (3) of Fig. 1A shows that the reaction was essentially complete, with 76% reannealing at C_0t 80 (C_0 = initial nucleotide concentration in moles per liter $\times$ time in seconds [3, 14]). A modified Wetmur-Davidson plot (19) of the early data

TABLE 2. Base composition of nuclear DNA of 13 strains of the genus *Hansenula*

Organism	Strain designation ^a	G+C ^b (mol%)	$\pm$ SD ^c
<i>Hansenula beckii</i>	74-68 ^d	37.1	0.29
<i>H. wingei</i>	74-76	39.7	0.15
<i>H. wingei</i>	71-79	40.3	0.26
<i>H. wingei</i>	71-78	40.4	0.25
<i>H. canadensis</i>	74-72	40.4	0.26
<i>H. wingei</i>	71-80 ^d	40.5	0.25
<i>H. wingei</i>	74-77	40.6	0.21
<i>H. canadensis</i>	74-71 ^d	40.8	0.12
<i>H. bimundalis</i> var. <i>bimundalis</i>	71-33 ^d	41.1	0.10
<i>H. alni</i>	68-985	41.8	0.25
<i>H. alni</i>	68-940	41.9	0.09
<i>H. alni</i>	68-928A ^d	42.0	0.36
<i>H. bimundalis</i> var. <i>americana</i>	74-69 ^d	43.6	0.27
<i>H. nonfermentans</i>	72-15 ^d	45.3	0.26
<i>H. henricii</i>	74-73 ^d	49.6	0.11

^a University of California, Davis, Department of Food Science and Technology Collection strain number.

^b Calculated from the average of at least four buoyant density determinations. Braces indicate those values which are not separable at the 95% confidence level (Student-Newman-Keuls a posteriori range test [16]).

^c SD, Standard deviation.

^d Type strain.

points (Fig. 1B) indicates that only about 1% of the rapidly renaturing sequences remained in the radiolabeled preparation.

Table 3 shows the results of heterologous reannealing reactions with DNA from the type strain of *H. wingei* as a reference. Three strains of *H. wingei* shared at least 90% sequence complementarity with the reference *H. wingei* strain. The DNA sequences of the diploid *H. wingei* 74-76, which had a comparatively low G+C content, have undergone significant divergence from those of the type strain. Nevertheless, single ascospores of this strain, when crossed with spores of opposite mating type of the type strain, produced viable ascospore clones (20). However, no information on the interfertility of the ascospore clones was reported.

An unexpected finding was that the type strains of *H. wingei* and *H. canadensis* shared substantial relatedness. In another reannealing

experiment with *H. wingei* reference DNA (results not shown), the second strain of *H. canadensis* (74-72) was found to be related to the *H. wingei* type by essentially the same value as the type of *H. canadensis*. None of the other yeasts showed significant DNA sequence similarity to *H. wingei*.

Reannealing reactions with DNA from *H. alni* 68-928A as a reference are shown in Table 4. All three Pacific Northwest strains were virtually indistinguishable by our molecular criterion; only minimal complementarity was noted between these strains and the other yeasts.

DISCUSSION

Our data indicate that the current classification (20) of the yeasts used in this study is in some cases at variance with the relationships elucidated by molecular means. The various strains of *H. wingei* and *H. canadensis* share

FIG. 1. Reassociation kinetics of 0.2 µg of labeled DNA from *H. wingei* 71-80 with excess unlabeled homologous DNA, in 280 mM phosphate buffer at 64°C. At various times, 0.5-ml portions in sealed culture tubes were removed, and the degree of duplex formation was assayed by hydroxylapatite fractionation. (A) Data presented by the method of Britten and Kohne (3). (B) Early data points presented in modified second-order rate plot (19).

TABLE 3. Reannealing^a between labeled DNA from *H. wingei* 71-80 and DNA from nine strains of yeast

Organism or tissue	Strain designation ^b	Actual binding ± SD ^c (%)	Relative binding ^d (%)	Δ <i>T</i> _{m(e)} ^e
<i>Hansenula wingei</i>	71-80 ^f	76.2 ± 1.4	100	
<i>H. wingei</i>	71-78	74.3 ± 2.5	97.5	0.0
<i>H. wingei</i>	71-79	73.4 ± 1.8	96.3	0.2
<i>H. wingei</i>	74-77	69.6 ± 1.9	91.3	1.7
<i>H. canadensis</i>	74-71 ^e	59.3 ± 1.2	77.6	3.4
<i>H. wingei</i>	74-76	47.8 ± 2.6	62.2	5.2
<i>H. beckii</i>	74-68 ^e	7.6 ± 0.3	8.8	
<i>H. alni</i>	68-928A ^e	5.4 ± 0.3	5.9	
<i>H. bimundalis</i> var. <i>bimundalis</i>	71-33 ^e	4.1 ± 0.5	4.1	
<i>H. bimundalis</i> var. <i>americana</i>	74-69 ^e	3.4 ± 0.5	3.2	
Calf thymus		1.2 ± 0.2	0.3	

^a ³²P-labeled DNA (0.2 µg) and unlabeled DNA (200 µg) in 0.5 ml of 280 mM PB at 64°C.

^b University of California, Davis, Department of Food Science and Technology Collection strain number.

^c Average of triplicate samples corrected for zero-time binding = 0.23% ± 0.04%.

^d Corrected for self-renaturation = 1.02% ± 0.17%.

^e *T*_{m(e)}, Thermal elution midpoint, the temperature at which 50% of the adsorbed DNA is eluted from the hydroxylapatite. Δ*T*_{m(e)}, Decrease in *T*_{m(e)} between the heterologous reaction and the homologous (*H. wingei* 71-80) reaction.

^f Type strain.

TABLE 4. *Reannealing^a between labeled DNA from H. alni 68-928A and DNA from six strains of yeast*

Organism or tissue	Strain designation ^b	Actual binding $\pm$ SD ^c (%)	Relative binding ^d (%)	$\Delta T_{m(e)}$ ^e
<i>Hansenula alni</i>	68-928A ^f	82.4 $\pm$ 0.46	100	
<i>H. alni</i>	68-940	80.9 $\pm$ 0.41	98.1	0.4
<i>H. alni</i>	68-985	81.0 $\pm$ 1.06	98.3	1.0
<i>H. wingei</i>	71-80 ^e	9.2 $\pm$ 0.77	9.6	
<i>H. canadensis</i>	74-71 ^e	8.3 $\pm$ 0.68	8.4	
<i>H. bimundalis</i> var. <i>bimundalis</i>	71-33 ^e	5.2 $\pm$ 0.25	4.6	
<i>H. bimundalis</i> var. <i>americana</i>	74-69 ^e	5.2 $\pm$ 0.48	4.6	
Calf thymus		2.4 $\pm$ 0.04	1.0	

^a ³²P-labeled DNA (0.2 μ g) and unlabeled DNA (200 μ g) in 0.5 ml of 280 mM PB at 64°C.

^b University of California, Davis, Department of Food Science and Technology Collection strain number.

^c Average of triplicate samples, corrected for zero-time binding = 0.27 $\pm$ 0.03.

^d Corrected for self-renaturation = 1.55% $\pm$ 0.36%.

^e See footnote e of Table 3 for definition of $\Delta T_{m(e)}$.

^f Type strain.

substantial kinship, although the spectrum of relatedness values observed indicates that the group is somewhat heterogeneous (Table 3). Additional genome comparisons, using a larger number of strains as well as several reference strains, should define the boundaries of the cluster and possible subgroups within it. A comparison among *H. canadensis* strains and the atypical *H. wingei* 74-76 would be of particular interest. Exploratory experiments on electrophoretic mobilities of various enzymes (G. B. Fuson, C. W. Price, and H. J. Phaff, unpublished data) suggest that the electrophoretic patterns of the type strain of *H. canadensis* and *H. wingei* 74-76 resembled each other more closely than they did that of the type strain of *H. wingei*. For the present, it seems reasonable to combine *H. canadensis* and *H. wingei* into a single taxon, *H. canadensis*, with *H. wingei* as a synonym. Because *H. canadensis* was described 5 years earlier than *H. wingei* (Table 1), the name of the former has priority.

Our data (Table 4) show that the Pacific Northwest strains constitute a natural cluster not closely related to any of the other *Hansenula* strains studied; this supports the proposal (12) that these strains be recognized as a new species, *H. alni*.

A conventional classification (20) based on the then-available phenotypic data (Table 5) of the yeasts studied here does not accurately reflect the natural relationships within the group. For example, *H. wingei* 74-76 is phenotypically indistinguishable from the type strain of *H. wingei*, except that freshly isolated single ascospore clones of opposite sex isolated from strain 74-76 neither agglutinate nor conjugate. However, strain 74-76 has diverged further from the *H. wingei* type strain than has *H. canadensis*, currently classified as a separate species on the

basis of its apparently homothallic mating behavior, its ascospore number, and its lack of true hyphae. The phenotypic differences between the closely related *H. wingei* and *H. canadensis* appear no more significant than the differences between *H. wingei* and *H. alni*, yeasts which have little kinship.

The two varieties of *H. bimundalis* differ only in assimilation of D-arabinose and in growth at 37°C (Table 5); these properties do not adequately reflect the evolutionary distance between these two organisms. The 2.5 mol% G+C difference in DNA base composition between the two varieties of *H. bimundalis* strongly suggests that they are not members of the same species. Wickerham (20) showed that *H. bimundalis* var. *bimundalis* conjugates with *H. bimundalis* var. *americana* but that the zygotes do not produce ascospores. However, before *H. bimundalis* var. *americana* is accorded species status, its possible relationship to other *hansenulas* of similar base composition should be investigated. Our molecular data also indicate that *H. beckii* has little kinship with the other yeasts surveyed. Thus, the consistent application of classical taxonomic methods can result either in the separation of closely related organisms into different taxa or in the unwarranted combination of organisms which share little filiation.

Although many of the characters listed in Table 5 are considered significant species-specific traits (9, 18) and are often employed singly to delimit yeast species, their biological significance is uncertain. Several of the traits listed in Table 5 (e.g., the positive assimilation of D-arabinose) can be altered by single mutational events; for others (e.g., spore number, true hyphae formation), the genetic basis and selective forces involved are unknown. As a consequence of the sole dependence on the conventional sys-

TABLE 5. Salient phenotypic characteristics^a of the *Hansenula* strains used

Organism	Fermentation	Assimilation		Growth at 37°C	Spore no.	True hyphae ^b	Ploidy ^c	Mating system
		D-Arabinose	Ribitol					
<i>Hansenula beckii</i>	None	-	+	vw ^d	2	+++	D	Unknown
<i>H. bimundalis</i> var. <i>bimundalis</i>	Glucose only	+	-	+	4	+	H	Heterothallic
<i>H. bimundalis</i> var. <i>americana</i>	Glucose only	-	-	-	4	+	H	Heterothallic
<i>H. canadensis</i>	None	-	-	+	2 (rarely 3)	-	D	Homothallic ^e
<i>H. wingei</i>	None	-	±	+	4	+	H, D	Heterothallic
<i>H. alni</i>	None	-	-	-	4	-	H	Heterothallic

^a Data from Wickerham (20), except for *H. alni* (12).^b +, +++, Strongly developed; +, moderate to sparse; -, absent.^c D = diploid and H = haploid in the vegetative state.^d Very weak.^e According to Wickerham (20), but evidence not provided.

tem, Wickerham (20) separated the genus *Hansenula* into two major phylogenetic branches on the basis of homo- or heterothallic mating behavior. His classification placed the heterothallic *H. wingei* and *H. canadensis* (which he interpreted as homothallic) on different phylogenetic lines. These yeasts were shown by us to share substantial sequence complementarity. It has been demonstrated that mutation of a single locus in *Saccharomyces cerevisiae* (7) and *Schizosaccharomyces pombe* (8) is sufficient to effect a change from heterothallic to homothallic mating behavior. Thus, the distinction between homo- and heterothallism is not a sound basis for species separation.

With the exception of *H. alni*, all of the yeasts included in our survey have been characterized both by numerical analysis of the conventional phenotypic properties and by the proton magnetic resonance spectra and serology of their cell wall mannans. On the basis of Adansonian analysis, Campbell (4) proposed to reduce *H. wingei*, *H. canadensis*, and both varieties of *H. bimundalis* to a single species. Poncet (13), although not suggesting any changes in species assignment, nonetheless placed the same three species together with *H. beckii* into her group A1 as defined by factor analysis. By her criteria, *H. wingei* and *H. canadensis* had high overall phenotypic similarity but not significantly more than that found between the pairs *H. wingei*-*H. beckii* or *H. bimundalis* var. *bimundalis*-*H. bimundalis* var. *americana*. Thus, these two numerical analyses, while recognizing the commonality of *H. wingei* and *H. canadensis*, were not able to distinguish this level of kinship from the other, more distant relationships among the pairs mentioned above. As has been noted elsewhere (14), the lack of congruence between the molecular and numerical approaches probably results from the undue emphasis of numerical methods upon carbohydrate assimilation and fermentations.

Cell wall structures have been proposed as a reliable measure of common ancestry (17); these structures can be compared by several indirect techniques. Gorin and Spencer (5) examined the cell wall mannans of many of these yeasts by proton magnetic resonance spectroscopy. They found that *H. beckii* and *H. canadensis* had quite similar spectra, whereas *H. wingei* and both varieties of *H. bimundalis* shared another pattern. This grouping does not agree with our DNA-determined relationships. A similar disparity was noted earlier for members of the genera *Debaryomyces* and *Pichia* (14).

Tsuchiya et al. (17) found that *H. wingei*, *H. canadensis*, and *H. beckii* had indistinguishable cell surface antigens. The antigenic structures of

the two varieties of *H. bimundalis* were found to be similar to each other but different from those of the *H. wingei* group. The observed antigenic similarities between *H. beckii* and *H. canadensis* were not confirmed by our molecular data and are likely the result of evolutionary convergence. In addition, it has been demonstrated by Ballou (1) and co-workers that in *Saccharomyces* the syntheses of several immunochemical determinants in the mannan of the cell wall are determined by single gene products.

It is clear that the presently available methods of yeast systematics cannot reliably define the natural species of *Hansenula* that have been identified by genome comparison. Similar observations for other groups of yeasts (14) led us to propose several alternative approaches to provide more significant systematic criteria. Two of these suggestions, an expanded nutritional screening and ecological investigations, find further support here. It is suggestive that *H. alni*, although phenotypically similar to the other yeasts studied (Table 5), is the only member of this group which has its habitat associated with deciduous (12) rather than coniferous trees. The basis of this ecological specialization could prove to be taxonomically significant. Nutritional studies may also be useful to differentiate natural clusters within this group. Hedrick and Dupont (6) have shown that many *Hansenula* species can use single amino acids as sources of carbon and nitrogen. *H. bimundalis* var. *americana* was able to assimilate alanine, aspartate, glutamate, proline, and serine. *H. bimundalis* var. *bimundalis*, *H. canadensis*, and *H. wingei* assimilate all of these except serine, whereas *H. beckii* assimilates only glutamate and proline. These data suggest that a survey of the organic compounds metabolized by yeasts as sole sources of carbon or nitrogen, particularly those dissimilated via peripheral pathways, could reveal numerous meaningful phenotypic differences.

ACKNOWLEDGMENTS

This research was supported by Public Health Service Research grant GM-16307-08 from the National Institute of General Medical Sciences.

We gratefully acknowledge the capable assistance of Nancy Krauter in the DNA isolation and of Mary Miranda for diagnostic testing. We thank C. P. Kurtzman for supplying several of the yeast strains and for helpful discussions. We are indebted to Duane Brown for providing the Beckman model E ultracentrifuge.

REPRINT REQUESTS

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