Nuclear DNA Base Composition And Base Sequence Complementarity of Recently Described Candida Species And Strains Of Selected Species.

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ABSTRACT

As early as 1970, it was pointed out that conventional methods were not adquate to differentiate species of yeasts. However, species are still described without deoxyribonucleic acid (DNA) base complementarity studies. The definitions of yeast species and genera have been principally on the basis of morphological and physiological characters, and many of these appear to be unreliable indicators of kinship.

In this investigation, ten recently decribed Candida species were examined and strains of selected species were also evaluated. These strains were characterized by physiological and morphological studies. The base composition of DNA, expressed in molar percent of guanine plus cytosine (%G+C), of each strain was also determined. Nuclear DNA base sequence complementarity of these strains and physiologically similar strains were also examined in an effort to clarify the taxonomic status of the newly described species and strains of known Candida species. By using this method, it was found that:

(a) four new species have no significant relatedness with known species, although these strains have similar physiological characteristics and GC contents;

(b) one strain of C. norvegica showed insignificant DNA relatedness with the type strain of C. norvegica;

(c) C. amidevorans is a synonym of C. guilliermondii since it showed a high degree of DNA relatedness with one of the mating types of P. guilliermondii;

(This study was conducted at the Microbial Sciences Laboratory, Georgia State University, Atlanta, Georgia, U.S.A).

(d) two strains identified as C. milleri showed a high degree of DNA relatedness with the type strain of C. milleri, thus proving the status of these two strains are species of C. milleri.

(e) two strains identified as C. oleophila showed insignificant DNA relatedness with the type strain of C. oleophila and the type strain of C. sake, showing that these two strains do not belong to either species;

(f) one strain of Pichia stipitis and the three strains of C. shehatae showed a high degree of homol ogy with the type strain of each species;

(g) the proposition of P. stipitis being the perfect counterpart of C. shehatae has to be rejected since insignificant DNA relatedness was demonstrated between strains of C. shehatae and the type strain of P. stipitis.

In conclusion the determination of base composition is very helpful in obtaining further clues to, or supporting evidence to estimate the relatedness of morphologically and physiologically similar organisms; but it alone is insiginficant in assessing relatedness. DNA base complementarity provides the most useful means of evaluation and comparing yeast genomes.

INTRODUCTION

Morphological and physiological tests are the traditional methods most often used for differentiating and identifying yeasts. However, deoxyribonucleic acid (DNA) studies have shown that some yeasts have been incorrectly identified with these methods (Meyer, 19070; Meyer and Phaff, 1972, Meyer et al., 1975, 1978). This shows that the conventional morphological and physiological characteristics are not adequate for classifying yeast strains since these phenotypic characteristics represent a limited segment of the yeast genome (Meyer, 1970; Meyer and Phaff, 1972). Since the goal of taxonomy is to group organisms based on their evolutionary affinities, Meyer et al. (1975) recommended that the comparisons should be made at the molecular level. Strains possessing similar morphological and physiological properties should be evaluated for DNA base composition and DNA base complementarity.

DNA base composition is usually determined by two physical methods:

(a) the thermal denaturation method; and,
(b) the buoyant density method.

When the DNA undergoes a transition from the double-stranded structure to the denatured state as the temperature is increased, there is a sharp increase in its extinction coefficient. The Tm, the temperature that corresponds to the midpoint of the absorbance rise, is linearly related to the average DNA base composition (Marmur and Doty, 1962). For a solvent containing 0.195 M Na^+,

$$\%G+C = (Tm - 69.3)/0.41$$

where G+C represents the mole percentage of guanine plus cytosine and the Tm is in degrees centrigrade. This relation was derived from the measurements of 41 samples of known base composition. The accuracy of this method is 0.5° C.

The other method frequently employed is the determination of the buoyant density of the DNA by ultracentrifugation in a gradient of cesium chloride (Schildkraut et al., 1962). The buoyant density of DNA in cesium chloride (CsCL) is directly proportional to its guanine + cytosine (G+C) content. Based on the buoyant density value of 1.710 g cm^{-3} for DNA from Escherichia coli as a standard, these authors obtained the following relation:

$$\rho = 1.660 + 0.098\ (G+C)$$

where ρ is the buoyant density in g cm^{-3} and (G+C) represents the molar fraction of guanine plus cytosine. The formula was derived from the best fit of measurements on 51 different DNA samples. The density gradient centrifugation technique followed the method described by Meselson, Stahl, and Vinograd (1957).

The DNA base composition is very useful in obtaining further clues to or supporting evidence for the taxonomic position of yeasts. However, the taxonomic use of G+C values is mainly exclusionary. Meyer and Phaff (1970) reviewed the available data on the DNA base composition in yeasts of various genera. More than 600 yeast species ranged from approximately 30-70 mole percent in G+C contents and demonstrated overlapping between unrelated species. Other studies of %G+C in yeasts have appeared since that time.

Strains which differ in base composition by more that 1.5% (determined by the buoyant density) and 2.5% (by thermal denaturation method) are considered genetically unrelated (Kurtzman et al. 1983). However, strains with nearly identical values may or may not be related due to the sequence of the nucleotides in the DNA (Meyer and Phaff, 1970). Therefore, the G+C contents have limited use in assessing relationships, and it is necessary to determine the degree of DNA base sequence relatedness among morphologically and physiologically similar yeasts in order to evaluate the conventional taxonomic methods as to whether or not they do in fact reflect the evolutionary affinities within a group (Price et al., 1978). Studies of DNA relatedness have made many important contributions to the delimination of yeast species (Meyer and Phaff, 1970, 1972; Meyer et al., 1975, 1978; Price et al., 1978; Kurtzman et al., 1980).

There are several advantages to this approach since comparisons among species that either have no known sexual stage or differences in ploidy can be made (Kreger-van Rij, 1984). By determining the DNA relatedness between two organisms, incorrect identifications can be avoided.

There are three techniques that are usually employed to determine the DNA base sequence complementarity: the DNA filter method (Denhardt, 1966), the hydroxyapatite method (Bernardi et al., 1970) and the spectrophotometric method (Seidler and Mandel, 1971).

It has been noted in many cases that species are separated based on the organisms' ability or inability to assimilate a single compound (Meyer et al., 1972). Again, by using the technique of DNA-DNA reassociation, Meyer and Phaff (1972) were able to show the discrepancy of the present system of identification and classifcation.

Therefore, based on these findings, I haven chosen to employ methods to determine DNA base composition and DNA relatedness to investigate the status of ten recently described species and to evaluate new strains of known species.

MATERIALS AND METHODS

The strains examined in this study are listed in Table 1. The morphological and physiological characterizations of the yeasts were performed according to the methods described by van der Walt and Yarrow (1984) with modifications of the diazonium blue B (DBB) color test and assimilation tests. The inocula for assimilation test were grown for 48 hours at room temperature on a shaker in 0.1% glucose in 1X yeast nitrogen base (YNB) and adjusted to a density of 1+ (Wickerham, 1957) by diluting with sterile distilled water prior to inoculation (0.1 ml). Tubes were allowed to incubate for one month at room temperature on a rollerdrum or on a gyratory shaker at 140 rpm.

The technique used for the DBB test followed the procedure of van der Walt and Hopsu-Havu (1976) with the exception of the media used for growing the cultures. Yeast Morphological Agar (YMA), Difco and glucose-yeast extract-peptone agar (GYPA) were used instead of the suggested media (Mycological Agar or Sabouraud Agar). As positive controls, three strains of basidiomycetous yeasts (Candida amylolenta, Candida philya, and Cryptococcus neoformans) were used and the same procedure was employed. The DBB reagent was applied on days three, seven, fourteen and twenty-one.

The morphological test included:

1) growth on solid media [glucose-yeast-extract-peptone ager (GYPA = 2%(w/v) glucose, 1%(w/v) yeast extract, 0.5%(w/v) peptone, 2%(w/v) agar];
2) growth in liquid media (glucose-yeast extract-peptone) for the cell sizing;

Table 1

Origin of Strains[a]

Organism	Strain Designation	Source
C. amidevorans Balloni, Florenzano, Mazza et Polsinelli	CBS 7232[b]	Soil
C. bertae var. chiloensis Ramirez et Gonzalez	CBS 8169[b]	Rotten Nothofagus dombeyii
C. bertae var. chiloensis Ramirez et Gozalez	CBS 8168[b]	Rotten Eucryphia cordifolia
C. boleticola Nakase	CBS 6420[b]	Fruiting-bodies Astreus hygrometricus
C. diddensiae (Phaff, Mark et Williams) Fell et Meyer	CBS 2214[b]	Shrimps, Gulf of Mexico
C. diversa (van Uden et Buckley) Ohara, Nonomura et Yunome	CBS 4074[b]	Grape-must
C. drimysii Ramirez et Gonzalez	CBS 8185[b]	Rotten Drimys winteri
C. laureliae Ramirez et Gonzales	CBS 8180[b]	Rotten Laurelia philippiana
C. llanquihuensis Ramirez et Gonzalez	CBS 8182[b]	Rotten Nothofagus obliqua
C. milleri Yarrow	CBS 6897[b]	San Francisco sour dough
C. milleri Yarrow	CBS 2664	Alpechin
C. milleri Yarrow	CBS 8195	Sour dough

Table 1 continued

Organism	Strain Designation	Source
C. norvegica (Reiersol) Meyer et Yarrow	CBS 4239[b]	Sputum
C. norvegica (Reiersol) Meyer et Yarrow	CBS 1784	Apple-must
C. oleophila Montrocher	ATCC 28137[b]	Olives
C. oleophila Montrocher	CBS 8164	Rotten Nothofagus dombeyii
C. oleophila Montrocher	CBS 8165	Rotten Nothofagus obliqua
C. petrohuensis Ramirez et Gonzalez	CBS 8173[b]	Rotten Nothofagus dombeyii
C. ralunensis Ramirez et Gonzalez	CBS 8179[b]	Rotten Laurelia sempervirens
C. rancensis Ramirez et Gonzalez	CBS 8174[b]	Rotten Laurelia sempervirens
C. sake (Saito et Ota) Buckley et van Uden	CBS 159[b]	Sake-moto yeasts
C. sequanensis Saez et Miranda	CBS 8118[b]	oat grains, France
C. shehatae Buckley et van Uden	CBS 5813	Dead insect-invaded pine tree
C. shehatae Buckley et van Uden	CBS 2770	Soil
C. shehatae Buckley et van Uden	CBS 5712	Rose-hips
C. shehatae Buckley et van Uden	CBS 7261	Unknown origin

Table 1 continued

Organisme	Strain Designation	Source
C. sophiae-reginae Ramirez et Gonzales	CBS 8175[b]	Rotten Laurelia sempervirens
Metschnikowia bicuspidata (Kamienski) Metschnikoff var. chathamia Fell et Pitt	NRRL Y-7112[b]	Ocean water at Chathamia Island, New Zealand
Pichia guilliermondii Wickerham	NRRL Y-2075[b]	Frass of Ulmus american
P. stipitis Pignal	CBS 5773[b]	Insect larva
P. stipitis Pignal	CBS 6054	Uknown origin

[a]ATCC = American Type Culture Collection
CBS = Centraalbureau voor Schimmelculture
NRRL = Northern Regional Research Laboratory
[b]Type culture.

3) pseudo - and true mycelial production on corn meal agar;

4) growth on sporulation media of mycological agar (MY), YMA, Sabouraud, potato dextrose agar (PDA), corn meal agar (CMA), and 5% malt extract.

The tests that were performed in order to determine the physiological characteristics were:

1) the assimilation of twenty-eight carbon sources (glucose, galactose, L-sorbose, maltose, sucrose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, D-glucosamine, glycerol, i-erythritol, ribitol, dulcitol, D-mannitol, D-glucitol, alpha-methyl-D-glucoside, salicin, and inositol);

2) fermentation of six carbon sources (glucose, galactose, maltose, sucrose, lactose, and raffinose);

3) growth on vitamin-free medium;

4) utilization of nitrate;

5) splitting of arbutin;

6) growth at 37° C;

7) production of extracellular enzymes (urease and lipase) and starch-like compounds;

8) the diazonium Blue B test; and,

9) growth on media of high osmotic pressure [5%(w/v) and 10%(w/v) sodium chloride media and 50%(w/v) and 60%(w/v) glucose media].

Extraction and Purification of DNA

The procedure employed for the isolation and purification of DNA was based on the Marmur method (1961) as modified by Meyer and Phaff (1969) and Meyer (S. A. Meyer, Ph.D. Disertation, Univeristy of California, Davis, 1970). An additional modification included the use of diethyl pyrocarbonate (DEPC, Fisher) as noted below. The method used is briefly described as follows:

The cells were grown in 1.5 liters of glucose-yeast extract-peptone medium [GYP = 4%(w/v) glucose, 0.5%(w/v) yeast extract, 0.5%(w/v) peptone] in 2.8 liter Fernbach flasks for two days. Prior to harvesting the cells of some strains, DEPC [0.2ml DEPC in 0.8ml chloroform (Fisher) per liter of culture fluid] was added to each flask and returned to the shaker for an additional thirty minutes to one hour. After harvesting, the cells were washed twice with saline-ethylenediamine tetraacetate [saline-EDTA = 0.15M sodium chloride (Fisher) and 0.1M ethylenediamine tetraacetate (Fisher), pH 8.0]. The cells were resuspended in saline-EDTA. Sodium dodecyl sulfate [10g SDS (Fisher) in 100ml saline-EDTA], and 2-mercaptoethanol (Aldrich) were added to give a final concentration of 2%(w/v) and 1%(w/v) respectively, and incubated at 37° C overnight.

To the suspension, 5M sodium perchlorate (Fisher) was added to give a final concentration of 1M and a volume of chloroform-isoamyl alcohol (CIA, 24:1) equal to the aqueous suspension was added. The emulsion was allowed to shake on a gyratory shaker for one hour, after which it was centrifuged at 3700 x g for ten minutes. The nucleic acids were precipitated with two volumes of cold ethanol and collected by spooling around a glass rod or by centrifugation. The precipitate was dissolved in 0.1XSSC [1XSSC = 0.15M sodium chloride (Fisher) and 0.015M sodium citrate (Mallinckrodt), ph 7.0] and was treated sequentially with the following enzymes: alpha-amylase (40 mg/ml, Sigma B. subtilis) to give a final concentration of 2 mg/ml and incubated at 37° C for one hour; Pancreatic ribonuclease (RNase, Calbiochem) was added to give a final concentration of 100 ug/ml and incubation continued at 37° C for two hours; then, Pronase (2 mg/ml, Calbiochem) was added to give a final concentration of 100 ug/ml and incubation continued at 37° C for 4-16 hours, after which CIA (90% of the total volume) and phenol (10% of the total volume) was added. Centrifugation and precipitation followed as before. After three repetitions of the enzymatic treatments and precipitation with cold ethanol, the nucleic acids were precipitated with

one volume of cold isopropanol, allowed to dissolve in 0.1XSSC and reprecipitated with isopropanol. The final product was dialyzed against 1XSSC for 48 hours with several changes of 1XSSC and stored at -20° C in the presence of a few drops of chloroform.

If the slow lysis method [Meyer and Phaff (1969); Meyer (S. A. Meyer, Ph.D. Dissertation, University of California, Davis, 1970] was unsuccessful, the cells were broken in a Braun cell homogenizer (Bron-will Scientific, Inc.) using the technique described by Price et al., 1978. Enzymatic treatments, deproteinizations, and alcohol precipitations were performed as described above.

The quality of the DNA was determined by the absorbance ratios: A260/A280 and A230/A260. A DNA sample was considered of acceptable quality if the A260/A280 ratio was approximately 1.8 and A230/A280 less than 0.5 (Marmur, 1961). Another indicator of DNA quality was the hyperchromic shift (HS); that is, the difference in the absorbancy at 25° C and 100° C. A good sample usually had a hyperchromicity of 36 to 40%.

Determination of the DNA Base Composition

The DNA base composition, expressed as molar %GC, was calculated from the Tm (midpoint of the thermal transition) according to the technique of Marmur and Doty (1962).

DNA was denatured in 1XSSC at a concentration of 15-17 ug/ml using a Gilford Model 2400S recording spectrophotometer equipped with a Gilford 2527 thermoprogrammer and reference compensator.

Three determinations of each DNA preparation were averaged to obtain the final Tm value. The Tm value was converted to %GC according to the formula of Marmur and Doty (1962) :
%G+C = (Tm-69.3)/0.41

C. lusitaniae DNA, (ATCC 42720), Tm = 87.8° C, was employed as a standard which was standardized with DNA from C. parapsilosis type strain (ATCC 22019), Tm = 85.9° C (Graph 1).

DNA Reassociation

DNA reassociations were determined by the optical renaturation method of Seidler and Mandel (1971) as modified by Kurtzman et al. (1980). DNA preparations were fragmented by passage of a sample through a number 27 gauge needle 50 times with maximum pressure applied by hand. A Gilford 2400S spectrophotometer equipped with a Gilford 2527 thermoprogrammer and reference compensator was used.

The extent of DNA reassociation of the two strains was calculated from the formula of Seidler and Mandel (1971):

$1-[\text{obs. } Cot^{mix} + (Cot^{100} - Cot^{0})/Cot^{100}] \times 100$

where $Cot_{0.5}$ (mole second liter^{-1}) is the time at which a DNA sample of known concentration reaches 50% renaturation obs. Co_{tmix} of a renatured mixture; Cot^{100} is the $Cot_{0.5}$ of the mixture which would be expected if the two DNA molecules were identical in sequence; and Cot^{0} is the $Cot_{0.5}$ of the mixture which would be expected for no sequence similarity (Graph 2).

RESULTS

The type strains of ten Candida species described after the third edition of The Yeasts, A taxonomic Study (Kreger van-Rij, 1984) were characterized on the basis of morphological (Table 2 Appendix 1) and physiological properties. These properties were identical to the respective published description (Ramirez et Gonzalez, 1984 a, b, c, d, e, f: Saez et Rodriguez de Miranda, 1984). The DNA base composition was also determined and base on these values and the morphological and physiological properties, the species were compared to known species (Barnett et al., 1983; Kreger-van Rij, 1984).

If the characteristics were similar and the GC contents difference was less than 2.0 - 2.5% compared to any of the known species, the new species was then compared to the known species by DNA reassociation. The same criteria were undertaken in the evaluation of some strains of known species to obtain further clues to or supporting evidence for their taxonomic position.

The range of molar percent guanine plus cytosine (GC contents) for all yeasts examined extended from 32.7 to 48.5% (Table 3 Appendix 2). Again, the heterogeneity of the genus Candida was demonstrated here as expressed by the wide range of GC contents exhibited by these species. This range of GC contents coincided with the range of GC contents of the ascomycetous yeasts. The results form the DBB test and the urease test further verified that the strains under study were ascomycetes. The streak cultures of the strains tested with DBB at days 3, 5, 7, 14, and 21 days all revealed a negative DBB test except the control organisms. Positive results were observed only after fourteen days of incubation on both the YM agar and GYPA media. The urease test showed negative results for all the strains except the control organisms.

Candida amidevorans and Pichia guilliermondii

Pichia guilliermondii Wickerham, a heterothaliic hat-shaped spore perfect form of the species Candida guilliermondii (Castellani) Langeron et Guerra. Strain NRRL Y-2075, a mating type of P. guilliermondii, was employed in these studies. It demonstrated the ability to ferment numerous sugars (glucose, galactose, sucrose, raffinose, and trehalose) and to assimilate all but four (lactose, soluble starch, *i*-erythritol, and inositol) of the 28 tested carbon sources. Well-branched

pseudohyphae were formed on the corn meal agar. Growth on 5% and 10% sodium chloride medium and 60% glucose medium was also demonstrated. The GC content was 44.6%.

C. amidevorans, a new species isolated from soil by Balloni et al. (1987) is physiologically similar to P. guilliermondii. Morphologically, they were similar with the exception of the absence of pseudohyphae in the former. Mixtures of C. amidevorans and the two mating types of P. guilliermondii yielded no conjugating cells or ascospores. C. amidevorans CBS 7232 revealed that the same GC contents, 44.6% as P. guilliermondii NRRL Y-2075 and these two strains showed a high degree of DNA reassociation (102%).

Candida bertae var. bertae and C. bertae var chiloensis

Candida bertae var. chiloensis differed from the variety bertae only in the utilization of trehalose, and inability to grow on glycerol, ribitol, and salicin (Table 4); morphologivally these two varieties were identical (Table 2). A difference in Tm of 0.^{0}4 C was noted. This equates to 1.0 % GC. The DNA reassociation between the two varieties was 97.6%.

Table 4

Salient characteristics of Candida bertae var bertae (CBS 8169) and C. bertae var. chiloensis (CBS 8168)

Strains	Tre[a]	Gly[b]	Rib[c]	Sal[d]	Tm+SD	%G+C
CBS 8169	-	+	+	+	87.5+0.12	44.4
CBS 8168	+	-	-	-	87.1+0.12	43.4

[a]Tre = Trehalose,[b] Gly = Glycerol, [c]Rib = Ribitol, [d]Sal = Salicin

Candida laureliae

This species assimilated galactose, trehalose, D-xylose, D-glucosamine, i-erythritol, ribitol, D-mannitol, D-glucitol and salicin. It fermented glucose, split arbutin and grew on 50% glucose medium and 5% sodium chloride medium. Trué mycelium and pseudomycelium were both present.

C. laureliae is physiologically similar to C. boleticola differing only in the lack of utilization of L-sorbose, D-arabinose, D-ribose, and glycerol. The DNA base composition for these species is 44.9% for the former and 43.9% for the latter. An insignificant amount of DNA relatedness was revealed between these species (Table 5).

Candida llanquihuensis

Trehalose, D-xylose, D-ribose, i-erythritol, ribitol, D-mannitol, and D-glucitol were assimilated and glucose was fermented. Pseudohyphae were present.

The physiological properties of C. llanquihuensis and C. diversa (type strain CBS 4074) are very similar. They differed only in the utilization of trehalose, D-ribose, and i-erythritol in which C. llanquihuensis was positive and C.diversa was negative. As suspected from the large difference in GC contents (5.6%) between the two organisms, no significant DNA relatedness was demonstrated between these species (Table 5).

Candida rancensis

This species failed to assimilate lactose, melibiose, raffinose, inulin, soluble starch, L-arabinose, D-arabinose, D-ribose, L-rhamnose, glycerol, i-erythritol, alpha-methyl-D-glucoside, and inositol; and fermented only glucose. It grew in media with 5% sodium chloride and 50% glucose and split arbutin.

This species is physiologically similar to Metschnikowia bicuspidata (Metschnikoff) Kamienski. The varieties of M. bicuspidata, M. bicuspi-

Table 5

DNA Relatedness of Various Strains of New Species (A) and Known speices (B)

(A) New species[a]	(B) Known species[a]	% DNA relatedness
C. laureliae CBS 8180	C. boleticola CBS 6420	23.5
C. llanquihuensis CBS 8182	C. diversa CBS 4074	24.0
C. rancensis CBS 8174	Metschnikowia bicuspidata var. chathamia NRRL Y-7112	15.0
C. sophiae-reginae CBS 8175	C. sake CBS 159	15.0
C. sophiae-reginae CBS 8175	C. diddensiae CBS 2214	2.8

[a]type strain.

data var. chathamia Fell et Pitt. M. bicuspidata var. australis Fell et Hunter, M. bicuspidata var. bicuspidata (Metschnikoff) Kamienski, and M. bicuspidata var californica Pitt et Miller, showed variable utilization of L-sorbose, melezitose, D-xylose, glycerol, and alpha-methyl-D-glucoside, growth in 50% glucose media, and fermentation of galactose (Barnett et al., 1983; Miller and Phaff, 1984). The species C. rancensis differed from M. biscuspidata var. chathamia only in the lack of utilization of alpha-methyl-D-glucoside. Conventionally, these similarities suggest a possible relationship between these species. However, the GC contents were 48.3% for C. rancensis and 44.9% for M. bicuspidata var. chathamia (Table 3) and no significant DNA relatedness was demonstrated between these strains (Table 5).

Candida sequanensis

This species did not assimilate sucrose, lactose, melezitose, inulin, D-arabinose, L-rhamnose, D-glucosamine, dulcitol, alpha-methyl-D-glucoside, inositol, and nitrate. It fermented glucose and galactose and grew on vitamin-free medium, 50% and 60% glucose media, and 5% and 10% sodium chloride media. Pseudohyphae were present.

The only Candida species that resembled C. sequanensis was C. boleticola Nakase. Differences between C. sequanensis and C. boleticola were found with respect to the following physiological properties (positive in the former and negative in the latter species): fermentation of galactose, assimilation of L-arabinose, maltose, melibiose, raffinose, and starch, growth in vitamin-free medium, and 60% glucose media. Since the GC percentage between the type strains of these organisms differed by 11.4% (Table 3), the determination of DNA relatedness was unnecessary.

Candida sophiae-reginae

This species assimilated galactose, maltose, surcose, trehalose, melezitose, i-erythritol, ribitol, D-mannitol and D-glucitol. It fermented glucose and galactose, grew on vitamin-free medium, 5% sodium chloride and on 50% glucose medium and formed pseudomycelium on corn meal agar.

Many morphological and physiological similarities exist between C. sophiae-reginae and C. sake as well as between C. sophiae-reginae and C. diddensiae. Also, these organisms have similar GC contents (Table 3). However, no DNA relatedness (Table 5) was demonstrated between C. sophiae-reginae and the type strains of C. sake and C. diddensiae.

Candida ralunensis, Candida drimyssii and Candida petrohuensis

Since there were vast differences in physiological and morphological characteristics and the GC contents between C. ralunensis, C. drimysii, C. petrohuensis, and any other known species, DNA reassociation studies were not performed.

Candida milleri

Two strains, CBS 2664 and CBS 8195 were identified as C. milleri based on their physiological and morphological characteristics. These strains assimilated glucose, galactose, sucrose, trehalose, raffinose, and glycerol, and grew on 5% and 10% sodium chloride media. They also fermented glucose, galactose, sucrose and raffinose. True mycelium or pseudomycelium was not observed. The GC contents of CBS 8195 and the type strain were the same (47.1%) and that of the strain CBS 2664 was 0.3% lower at 46.8% (Table 3). The results in Table 6 show significant DNA relatedness between these strains.

Table 6

DNA Relatedness of Candida milleri Strains

Strains	% DNA relatedness
6897[a] x 2664	91.4
6897 x 8195	88.3
2664 x 8195	80.3

[a]type strain

Candida norvegica

The strain CBS 1784, isolated from apple-must, was identified as the species C. norvegica due to the identical morphological and physiological characteristics in comparison to the type strain of the species (CBS 4239). These strains assimilated glucose, cellobiose, D-xylose, glycerol, D-sorbitol, and salicin, and fermented glucose. The test for arbutin, nitrate, growth in 5% sodium chloride and 50% glucose media also were positive. Pseudohyphae were present. The GC value of strain 1784 was 47.8% and that of the type strain was 41.2% (Table 3). The great difference in the GC contents and lack of significant DNA relatedness (22.5%) between these two strains showed their distinctness.

Candida oleophila

The two strains identified as C. oleophila, strains CBS 8164 and 8165, had similar physiological and morphological characteristics to the type strain of C. oleophila, ATCC 28137. The strains utilized all tested carbon sources except lactose, melibiose, raffinose, inulin, soluble starch, L-arabinose, L-rhamnose, i-erythritol, dulcitol, and inositol; and fermented glucose, galactose, and sucrose.

Growth in 5% and 10% sodium chloride media and 50% glucose medium and splitting of arbutin were also demonstrated by these strains. A thick pellicle was formed in liquid medium and abundant true mycelium and pseudomycelium were produced on corn meal agar. The GC contents of these two strains were also similar (42.0%, CBS 8164; 41.5%, CBS 8165). These two strains are not representatives of the species, C. oleophila as demonstrated by the insignificant DNA relatedness between these and the type strain of the species (Table 7a). Meyer and Simione (unpublished data) studied two strains (including ATCC 2220) that were previously identified as C. oleophila to be considered as representatives of the species. The DNA for strain ATCC 2220 used in this investigation was obtained from the collection of Dr. Sally A. Meyer (Georgia State University). In order to make sure that the DNA of the type strain of C. oleophila (ATCC 28137) was correct and that there was no mix -up in the culture or the DNA, DNA reassociation between strain ATCC 2220 and strain ATCC 28137

Table 7a

DNA Relatedness of Candida oleophila Strains

Strains	% DNA relatedness
8164 x 8165	108.0
28137[a] x 8164	21.6
28137 x 8165	19.9
2220 x 8165	22.6
28137 x 2220	94.3

[a]type strain

Table 7b

DNA Relatedness of Two Candida oleophila Strains and Candida sake Type Strain CBS 159

Strains	% DNA relatedness
159 x 8164	0.0
159 x 8165	0.3

was performed. The result in Table 7a confirmed the identity of the DNA sample.

C. oleophila and C. sake are similar in their physiological and morphological characteristics. However, it has been shown by DNA reassociation experiments that they are distinct species (Meyer and Simione, unpublished). Therefore, DNA reassociations were done betweeen the type strain of C. sake (CBS 159) and these two strains. No significant DNA relatedness was observed as shown in Table 7b.

Candida shehatae

Four strains of C. shehatae were examined in this study. The type strain (CBS 5813), isolated from a dead insect-invaded pine tree, was compared with strains of similar morphological and physiological characteristics which were isolated from different sources. CBS 2779 was isolated from soil, CBS 5712 from rose hip and CBS 7261 from an unknown source. These strains fermented glucose, and maltose, split arbutin, and demonstrated growth on 10% sodium chloride media and at 37° C. Pseudohyphae were observed in all the strains. All four strains assimilated all carbon sources excep melibiose, raffinose, inulin, L-arabinose, D-arabinose, and inositol. The only difference which was noted was the inability of CBS 7261 to utilize lactose.

Three of the strains revealed similar GC values between 43.1-43.5% and the fourth strain (CBS 2779) was somewhat lower at 41.2% (Table 3). These strains showed signigicant DNA relatedness (82.9-102%), thus confirming the status of the three strains as members of the species, C. shehatae (Table 8).

Table 8

DNA Relatedness of the Candida shehatae Strains

Strains	% DNA relatedness
5813[a] x 2779	97.3
5813 x 7261	95.2
5712 x 2779	90.6
7261 x 5712	102.0
2779 x 7261	86.0
5813 x 5712	82.9

[a]type strain of C. shehatae

Pichia stipitis

Two strains of P. stipitis were examined. The type strain of P. stipitis utilized all of the tested carbon sources, lacking only in the utilization of raffinose, inositol, melibiose, and inulin. Arbutin was split. The strain was slow to ferment glucose, galactose, and maltose and had variable trehalose fermentation. Pseudomycelium was abundant, but no true mycelium was observed. Growth was demonstrated in the 10% sodium chloride media and at 37° C. The GC contents were 44.4%. Strain CBS 6054 shared similar morphological (Table 2) and physiological characteristics and GC contents (Table 3) with the type strain (CBS 5773). The high degree of DNA reassociation (102%) between these strains confirmed their taxonomic status.

C. shehatae and P. stipitis

The DNA reassociations between the four strains of C. shehatae and P. stipitis type strain revealed insignificant genetic relatedness between 0.8 - 19.4% (Table 9).

Table 9

DNA Relatedness of Various Strains of Candida shehatae and Pichia stipitis Type Strains CBS 57

Strains	% DNA relatedness
5773 x 5813[a]	19.4
5773 x 7261	16.0
5773 x 2779	5.8
5773 x 5712	0.8

[a]type strain of C. shehatae

Discussion

All twenty-one species examined in the study appeared to be ascomycetous types because no red, maroon or violet color was produced in the DBB test. Moreover, none of the isolates gave a positive urease reaction and none of the isolates had GC contents above 49%. These are general properties usually found among the ascomycetous yeasts (van der Walt and Hopsu-Havu, 1976; Ahearn and Hagler, 1981).

Candida amidevorans and Pichia guilliermondii

Wickerham and Burton (1952) reported that there are some yeasts classified in nonascosporogenous genera which are found to be haploid mating types. When sexually active strains of the opposite types are mixed together, ascospores are produced; provided that these mating types are brought together under conditions favoring sporulation. In the investigation of the relationship of Candida guilliermondii to other yeasts by studying their mating types, the strains NRRL Y-2075 and NRRL Y-2076 were found to be strains of opposite sex and the best sporulating pair. The ascospores were distinctly hat-shaped with a maximum of two spores per ascus and all asci consisted of two conjugated cells. These strains were grown on slants of malt extract-yeast-extract vegetation. medium (Wickerham, 1951) and the mixed culture was incubated at 25° C.

In the present study, although C. amidevorans was mixed with the mating types of P. guilliermondii, no conjugation was evident. The reason no conjugating cells or spores were formed may be because the conditions such as temperature, media, or the period of incubation were not favorable for sporulation.

The DNA relatedness of 102% clearly revealed the synonymy of C. amidevorans and C. guilliermondii.

Candida bertae variety bertae and C. bertae var. chiloensis

Based on the physiological properties, these two strains most resemble Debaryomyces vanrijae, except that D. vanrijae assimilates L-sorbose, maltose, trehalose, and melezitose and grows at 37° C (Barnett et al., 1983; Kreger-van Rij, 1984). The GC contents of these species are significantly different (C. bertae var. bertae: 43.4%); therefore, no DNA reassociations were needed. Obviously these are separate species.

Ramirez et Gonzalez (1984c) separated Candida bertae into two separate varieties based on the assimilation of trehalose, glycerol, ribitol, and salicin. The remaining physiological characteristics and morphological properties of the strains were identical. The results herein agreed with their findings.

Morphological and physiological characteristics may vary from strain to strain within a given species (Wickerham, 1957). Physiologically, variations are based on the ability or inability to assimilate or ferment carbohydrates. Variations may be found in both freshly isolated and old cultures which misled many investigators into misidentifying many of the strains. Wickerham believed that the limits for a species should be set wide enough for minor variations to be included. The strains are then separated into varieties only for those which are almost different enough to be set aside as separate species.

Therefore, it is necessary to determine the physiological reactions of many strains in each species in order to determine which reactions are common to all strains and which are variable. The GC difference between C. bertae var. bertae and C. bertae var. chiloensis was negligible (0.4° C) and the DNA relatedness

and C. bertae var. chiloensis was negligible (0.4° C) and the DNA relatedness between these two varieties was 97.6% confirming that these two strains belong to the same species.

More strains belonging to this species should be recovered in order to determine the value of the ability or inability to assimilate trehalose, salicin, ribitol and glycerol as key characteristics that would warrant the distinction of a variety. In my opinion, these differences are minor and merely denote variable characteristics within the species.

Candida laureliae

From a conventional viewpoint, C. laureliae and C. boleticola may be regarded as "closely related" on the basis of their similar physiological characteristics. Also, they have similar GC values (44.1% abd 44.9%, respectively). These results may be misleading unless one takes into consideration DNA complementarity. The results (Table 5) showed DNA relatedness (23.0%) between these two species was insignificant. This is an example of two organisms with similar GC contents, but whose nucleotides are not similarly aligned. They are separate and distinct species.

Candida llanquihuensis

The large difference in GC contents (5.6%) between the species C. llanquihuensis and C.diversa indicates that they are genetically unrelated. The insignificant DNA relatedness between these two organisms further emphasizes the distinctness of the two species.

Candida rancensis

This species differed from the Metschnikowia bicuspidata var. chathamia only in the lack of utilization of aplha-methyl-D-glucoside. Many species are separated based on the utilization of a single carbon. In this case, the results obtained from the DNA studies (GC difference 3.4% and DNA reassociation 15.0%) agree with the conventional methods of identification.

Candida sequanensis

In the case of the GC contents, the value determined in this study (32.7%) was not in line with the value (39.6%) previously reported (Saez and Miranda, 1984). The technique these investigators used to determine the GC contents which could account for some differences was not described. To ensure the validity of my results, two steps were undertaken:

First, the DNA was enzymatically-retreated, reprecipitated, redialyzed and melted again to determine if this difference in %GC was due to: (a) impurities of the sample or (b) a variation in ionic strength. The same melting temperature and GC percentage were obtained as previously determined. Second, the DNA was reisolated. As before, the DNA sample revealed similar ratios and hyperchromicity shift (Table 3). Tm values differed by 0.1° C, which equates to GC values of 32.7% and 32.9% respectively.

Candida sophiae-reginae

This species resembled C. sake and C. diddensiae physiologically but the insignificant DNA relatedness between these organisms confirmed that they are separate species. Both C. sake and C. diddensiae have numerous strains which vary physiologically. In order to differentiate C. sophiae-reginae from C. sake, the positive utilization of i-erythritol and growth on 50% glucose media should be used. Utilization of cellobiose and glycerol, and growth at 37° C should be used as criteria to separate C. diddensiae and C. sophiae-reginae, which is positive for the former, negative for the latter.

Candida milleri

Strain CBS 2664 was isolated from alpechin and the strain CBS 8195 was isolated from the same source as the type strain (sour dough). The high degree of DNA relatednessn agrees with the conventional method which confirms that these two strains do indeed belong to the species C. milleri.

Candida norvegica

The strain CBS, previously identified as C. norvegica, showed insignificant DNA relatedness with the type strain of the species C. norvegica CBS 4239. This is evidence of the inadequacy of the traditional morphological and physiological methodology used in making taxonomic decisions. Further investigation is needed to determine if this strain represents a new species.

Candida oleophila

Strains CBS 8165, previously identified as C. oleophila on the basis of morphological and physiological similarities, demonstrated insignificant DNA relatedness with the type strain of C. oleophila, ATCC 28137. Also, these strains showed low DNA relatedness with the type strain of C. sake (CBS 159). It is clear that these two strains are not representatives of C. oleophila or C. sake; this is another example of the failure of the conventional methods to identify strains correctly. The proper identification of these strains remains undetermined.

Candida shehatae and Pichia stipitis

The strains of CBS 2779, 5712 and 7261 showed significant DNA relatedness with the type strain (CBS 5813) and between strains, thus confirming the status of these three strains in the species of C. shehatae. The inability of the strain CBS 7261 to utilize lactose is considered a variable characteristic of the species.

Ramirez et al. (1985) gave and illustrated an emended description of C. shehatae which was originally described by Buckley and van Uden (1967). In this emendation, true mycelium was reported to be formed, as well as pseudomycelium on the corn meal agar.

In the present study, the initial examination of the Dalmau plate culture on corn meal agar for the type strain (CBS 5813), and strains CBS 5712, 2779, and

7261, revealed only pseudomycelium. Then, the Dalmau plate culture was repeated for every strain and again, no true mycelium was observed for three of the strains. For the type strain (CBS 5813), true mycelium was not very obvious. The septa looked more or less like constrictions of a typical pseudomycelium. In this case, the presence of the septa depends on the interpretation of the observer. In the original paper of the emendation, only drawings of the mycelium were presented. Higher magnification and higher resolution are necessary to determine the presence of the septa. It will be necessary to examine the growth on Dalmau plate by electronmicroscopy.

The results of DNA reassociation, correlated with fermentation and assimilation test and morphological characteristics, confirmed that the strain CBS 6054 is a representative of the species P. stipitis.

Based on the morphological and physiological characteristics, Kreger-van Rij (1970) proposed that these two taxa represent the perfect and imperfect form of one another. Bak and Stenderup (1969) and Meyer et al., (1978) showed that there exists a high degree of DNA homology between perfect and imperfect counterparts. Verification of this type of relationship is precluded, since the results in this study revealed insignificant DNA relatedness. This is in agreement with the results obtained by Martini (1984). Martini did the genome comparisons between Pichia stipitis and three Candida species, C. shehatae, C. terebra, and C. tenuis. The results showed low DNA relatedness (less than 25%) between P. stipitis and C. shehatae as well as between P. stipitis and C. terebra and C. tenuis. This is another example of two species with similar morphological and physiological properties and similar GC contents, but different base sequence alignment.

CONCLUSIONS

1. The recently described species of the genus Candida: Candida bertae, Candida drymisii, Candida laureliae, Candida llanquihuensis, Candida petrohuensis, Candida rancensis, Candida ralunensis, Candida sequanensis and Candida sophiae-reginae are distinct species.

2. Candida amidevorans is a synonym of Candida guilliermondii.

3. The two strains, CBS 8195 and 6897, are members of the species Candida milleri.

4. The strain CNS 1784, originally identified as Candida norvegica, is not a member of this species and may be a new species.

5. The two strains isolated from rotten wood in Chile (CBS 8164 and CBS 8165), which were originally identified as Candida oleophila, do not belong to Candida oleophila, or to Candida sake.

6. Candida shehatae is not the imperfect counterpart of Pichia stipitis.

7. The conventional methods for identifying species are very limited in differentiation of taxa; therefore, species which were identified based on these methods should be reevaluated using DNA reassociation techniques.

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Appendix 1

Table 2

Morphological Characteristics

Organism and Designation	Growth in Liquid Media	Growth on Dalmau Plate	Streak Culture
C. amidevorans CBS 7232[a]	After 3 days at 25° C, the cells are oval, (2.5-5.0) x (2.5-7.5) μ m, and occur singly, in pairs, and chains. A sediment is formed. After 30 days, a sediment is present.	No true mycelium or pseudomycelium present. Cells are oval to cylindrical.	White to cream-colored, glistening smooth, continuous margin.
C. bertae var. bertae CBS 8169[a]	After 3 days at 25° C, the cells are oval to ellipsoidal, (1.8-5.0), (2.5-10.5) μ m, and occur singly, in pairs, and chains. A sediment and pellicle formed. After 30 days, a sediment and a pellicle are present.	True mycelium and pseudomycelium present.	White-creamed color, waxy, smooth, dull with slightly serrated margin.

Organism and Designation	Growth in Liquid Media	Growth on Dalmau Plate	Streak Culture
C. bertae var. chiloensis CBS 8168[a]	After 3 days at 25° C, the cells are oval to ellip soidal, (1.9-5.0) x (2.5-10.8) μ m, and occur singly, in pairs, and chains. A sediment and pellicle formed. After 30 days, a sediment and a pellicle are present.	True mycelium and pseudomycelium present.	White-creamed colored, waxy, smooth, dull with slightly serrated margin.
C. drimysii CBS 8185[a]	After 3 days at 25° C, the cells are globose to oval, (2.5-6.0) x (2.8-6.9) μ m, and occur singly, in pairs, and chains. A sediment formed. After 30 days, sediment and ring are present.	No true mycelium or pseudomycelium present.	White, smooth, waxy, with an undulating margin.
C. laureliae CBS 8180[a]	After 3 days at 25° C, the cells are oval to ellip-soidal and elongate, (2.5-4.3) x (5.5-25.0) μ m, and occur singly, in pairs and chains.Some pseudomycelium present. A sediment and ring are present.	True mycelium and pseudomycelium consist of long, slender cells with ellipsoidal blastospores.	White, dull, wrinkled, with a lobulate margin.

Organism and Designation	Growth in Liquid Media	Growth on Dalmau Plate	Streak Culture
C. llanquihuensis CBS 8182[a]	After 3 days at 25° C, the cells are globose, (2.5-7.0) x (5.5-8.8) μ m, and occur singly, in pairs, and chains A sediment and incomplete ring are formed. After 30 days, a sediment and completed ring are present.	Pseudomycelium consists of long, slender, cylindrical cells with single or clusters of oval blastospores.	White, waxy, dull with a fimbriate margin.
C. milleri CBS 6897[a]	After 3 days at 25° C, the cells are oval to globose, (1.0-5.0) x (1.0-5.0) μ m, and occur singly, in pairs and some clusters. A sediment formed. After 30 days, a sediment is present.	True mycelium pseudomycelium absent.	Cream-colored, smooth, glistening; a lobed margin.
C. milleri CBS 2664	After 3 days at 25° C, the cells are oval to globose, (2.0-5.0) x (2.0-5.0) μ m, and occur singly, in pairs and some clusters. Sediment is formed. After 30 days, a sediment is present.	True mycelium and pseudomycelium absent.	Cream-colored, smooth, glistening; a lobed margin.

Organism and Designation	Growth in Liquid Media	Growth on Dalmau Plate	Streak Culture
C. milleri CBS 8195	After 3 days at 25° C, the cells are oval to globose, (2.0-5.0) x (2.0-5.0) μ m, and occur singly, in pairs and some clusters. Sediment is formed. After 30 days, a sediment is present.	True mycelium and pseudomycelium absent.	Cream-colored, smooth, glistening; a lobed margin.
C. norvegica CBS 4239[a]	After 3 days at 25° C the cells are globose, (2.5-6.3) x (2.5-6.3) μ m, and occur singly, in pairs and chains. A sediment is formed. After 30 days, a sediment is present.	Pesudomycelium consists of branched chains of cells with oval blastospores.	Cream-colored glossy, smooth; a continuous margin.
C. norvegica CBS 1784	After 3 days at 25° C the cells are globose, (1.9-6.3) x (1.9-6.3) μ m, and occur singly, in pairs and chains. A sediment is formed. After 30 days, a sediment is present.	Pesudomycelium consists of branched chains of cells with oval blastospores.	Cream-colored glossy, smooth; a continuous margin.

Organism and Designation	Growth in Liquid Media	Growth on Dalmau Plate	Streak Culture
C. oleophila CBS 8164	After 3 days at 25° C, the cells are oval to elongate and slender cylindrical, (1.5-4.5) x (4.5-9.5) μ m, and occur singly, in pairs and chains. Pseudomycelium present. Sediment and a thin, waxy pellicle are formed, After 30 days, a sediment and thick pellicle are present.	Pseudomycelium is present, with branched chains of cells with oval to elongated blasto-spores.	Cream-colored, dull, lacy, raised growth with lobed margin.
C. oleophila CBS 8165	After 3 days at 25° C, the cells are oval to elongate and slender cylindrical, (1.4-4.3) x (4.5-9.7) μ m, and occur singly, in pairs and chains. Pseudomycelium present. Sediment and a thin, waxy pellicle are formed. After 30 days, a sediment and thick pellicle are present.	Pseudomycelium is present, with branched chains of cells with oval to elongated blasto-spores.	Cream-colored, dull, lacy, raised growth with lobed margin.

Organism and Designation	Growth in Liquid Media	Growth on Dalmau Plate	Streak Culture
C. petrohuensis CBS 8173[a]	After 3 days at 25° C, cells are globose, (2.5-6.5) x (2.5-7.5) μ m, and occur singly, in pairs and short chains. Pseudomycelium present. A sediment formed. After 30 days, a sediment is present.	Pseudomycelium consists of short chains of elongate cells.	White, dull, smooth with continuous margin.
C. ralunensis CBS 8179[a]	After 3 days at 25° C, the cells are oval to cylindrical and elongated, (2.5-5.0) x (5.5-22.5) μ m, and occur singly, in pairs and short chains. Some pseudomycelium present. A sediment and an ellipsoidal blasto-incomplete ring are present. After 30 days, a sediment and completed ring are present.	True mycelium and pseudomycelium are present. The true mycelium is long and cylindrical with short chains of ellipsoidal blatospores.	White, dull, wrinkled, with lobulate margin.

Organism and Designation	Growth in Liquid Media	Growth on Dalmau Plate	Streak Culture
C. rancensis CBS 8174[a]	After 30 days at 25° C, cells are globose to oval, (3.0 6.5) x (5.5-8.8) μ m, and occur singly, in pairs, and chains and a sediment are formed. After 30 days, a sediment is present.	Mycelium and pseudo-mycelium absent.	White, waxy, smooth, serrated margin.
C. sequanensis CBS 8118[a]	After 3 days at 25° C, cells are globose, (2.5-7.5) x (2.5-7,5) μ m, and occur singly, cellile is formed. After 30 days, a sediment and a pellicle are present.	Pseudomycelium present, with oval blastospores occurring in chains or singly.	White, flat, glossy, lobulate margin.
C. shehatae CBS 5813[a]	After 3 days at 25° C, cells are globose to oval, (2.0-4.5) x (3.0-6.0) μ m, and occur singly in pairs, and chains. Pseudomycelium present. A sediment is formed. After 30 days, a sediment is present.	Pseudomycelium present, consisting of long cylindrical cells. Blastospores are round to oval.	Cream-colored, glistening, smooth, with slightly reticulate margin.

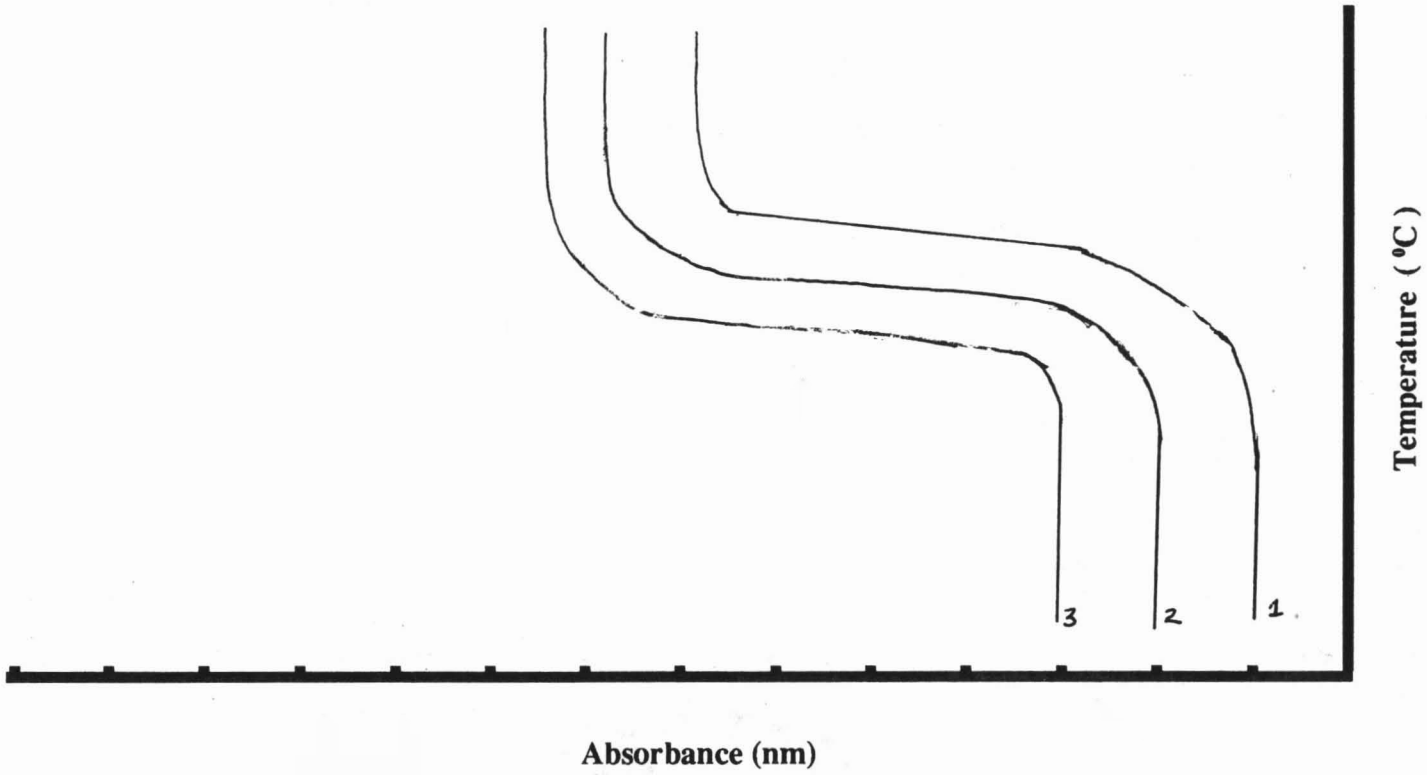
Organism and Designation	Growth in Liquid Media	Growth on Dalmau Plate	Streak Culture
C. shehatae CBS 2779	After 3 days at 25° C, cells are globose to oval; some cylindrical cells, (2.0-4.6) x (3.0-6.0) μ m, and occur singly, in pairs and short chains. Pseudomycelium present. A sediment is formed. After 30 days, a sediment is present.	Pseudomycelium present, consisting of long cylindrical cells. Blastospores are round to oval.	Cream-colored, glistening, smooth, with slightly reticulated margin.
C. shehatae CBS 5712	After 3 days at 25° C, cells are globose to oval and some cylindrical cells, (2.5-5.0) x (3.0-6.0) μ m, and occur chains. Pseudomycelium singly, in pairs and short chains. Pseudomycelium present. A sediment is formed. After 30 days, a sediment is present.	Pseudomycelium present, consisting of long cylindrical cells. Blastospores are round to oval.	Cream-colored, glistening, smooth, with slightly reticulated margin.

Organism and Designation	Growth in Liquid Media	Growth on Dalmau Plate	Streak Culture
C. shehatae CBS 7261	After 3 days at 25° C, cells are globose to oval and some cylindrical cells, (2.0-4.6) x (3.0-6.0) μ m, and occur chains. Pseudomycelium singly, in pairs and short chains. Pseudomycelium present. A sediment is formed. After 30 days, a sediment is present.	Pseudomycelium present, consisting of long cylindrical cells. Blastospores are round to oval.	Cream-colored, glistening, smooth, with slightly reticulated margin.
C. sophiae-reginae CBS 8175[a]	After 3 days at 25° C, cells are cylindrical to elongate, (2.5-4.2) x (3.5-25.5) μ m, and occur singly, in pairs, and short chains. A sediment is formed. After 30 days, a sediment is present.	Pseudomycelium consists of branched chains of cells with clusters of oval blastospores.	Creamy white in color, glistening, with fimbriate margin.
P. stipitis CBS 5773[a]	Cells are globose to oval, some cylindrical cells, (2.0-4.5) x (3.0-6.0) μ m, and occur singly, in pairs, and short chains. Pseudomycelium present. A sediment is formed. After 30 days, a sediment is present.	Pseudomycelium consists of branched chains of cells with clusters of oval blastospores.	Cream-colored, glistening, smooth, with slightly reticulated margin.

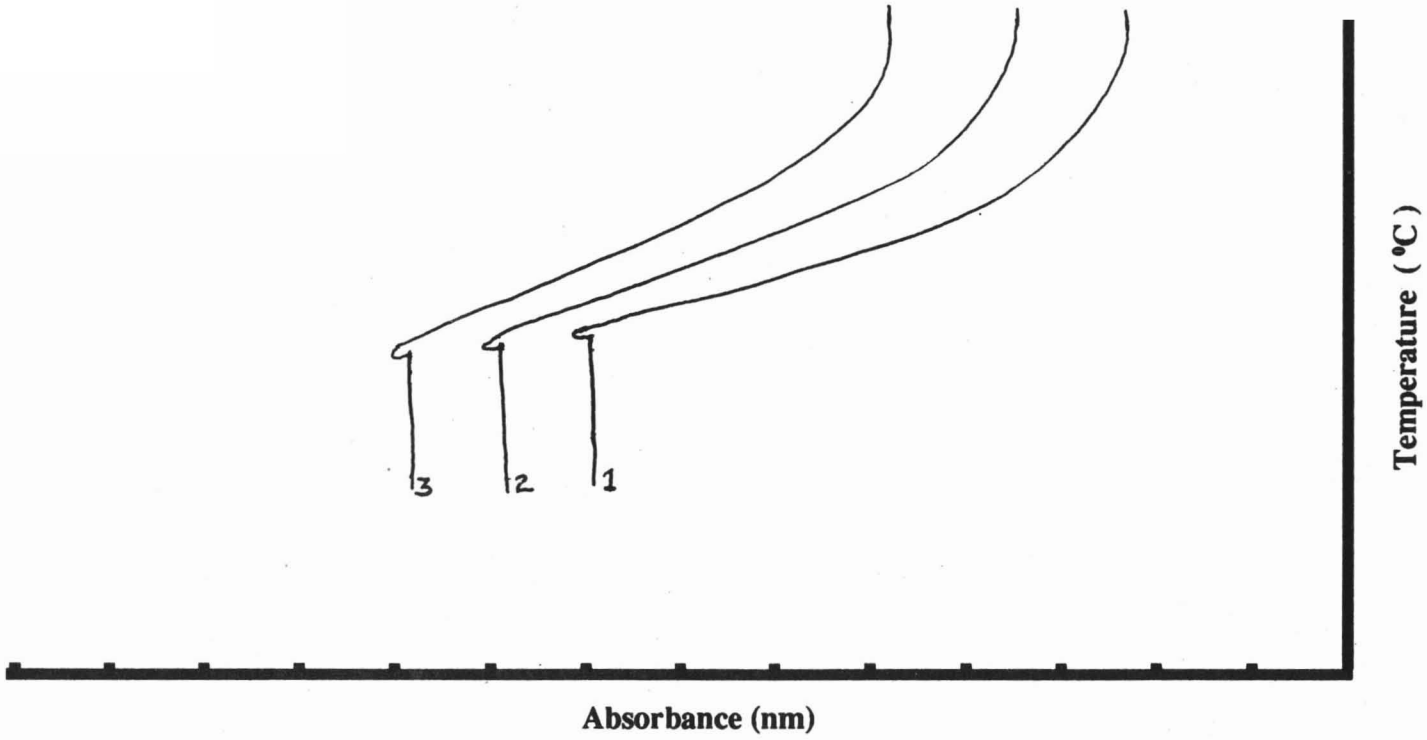
Organism and Designation	Growth in Liquid Media	Growth on Dalmau Plate	Streak Culture
P. stipitis CBS 6054	Cells are globose to oval some cylindrical cells, (2.0-4.5) x (3.0-6.0) μ m, and occur singly, in pairs, and short chains. Pseudomycelium are present. A sediment is formed. After 30 days, a sediment is present.	Pseudomycelium consists of branched chains of cells with clusters of oval blastospores.	Cream-colored, glistening, smooth, with slightly reticulated margin.

[a]Type culture

Graph 1 : Three determinations of each DNA preparation were averaged to obtain the final T_m Value



Graph 2 : Renaturation curves



Curve 1 : sample containing DNA of strain 1.
Curve 2 : sample containing DNA of strain 2.
Curve 3 : sample containing DNA of starin 1 and 2.

Appendix 2

Table 3

DNA Base Composition

Organism	Tm + SD	%GC (Experimental)	%GC (Literature)
Candida amidevorans CBS 7232[b]	87.6 ± 0.21	44.6	43.0[c]
C. bertae var. bertae CBS 8169[b]	87.5 ± 0.12	44.4	
C. bertae var. chiloensis CBS 8168[b]	87.1 ± 0.12	43.4	
C. boleticola CBS 6420[b]	87.3 ± 0.07	43.9	43.2-44.1[d]
C. diddensiae CBS 2214[b]	84.7 ± 0.00	37.6	37.3-38.8[d]
C. diversa CBS 4074[b]	83.6 ± 0.12	34.9	34.4-36.6[d]
C. drimysii CBS 8185[b]	87.1 ± 0.07	43.4	
C. laureliae CBS 8180[b]	87.7 ± 0.00	44.9	
C. llanquihuensis CBS 8182[b]	85.9 ± 0.07	40.5	
C. milleri CBS 6897[b]	88.6 ± 0.07	47.1	46.1-48.0[d]
C. milleri CBS 2664	88.5 ± 0.12	46.8	
C. milleri CBS 8195	88.6 ± 0.00	47.1	
C. norvegica CBS 4239[b]	86.2 ± 0.16	41.2	40.7[d]

Organism	Tm + SD	%GC (Experimental)	%GC (Literature)
C. norvegica CBS 1784	88.9 ± 0.21	47.8	
C. oleophila ATCC 28137b	86.1 ± 0.00	41.0	40.7-42.2[d]
C. oleophila ATCC 2220	86.0 ± 0.00	40.7	
C. oleophila CBS 8164	86.5 ± 0.17	42.0	
C. oleophila CBS 8165	86.3 ± 0.12	41.5	
C. petrohuensis CBS 8173[b]	88.2 ± 0.78	46.1	
C. ralunensis CBS 8179[b]	89.2 ± 0.16	48.5	
C. rancensis CBS 8174[b]	89.1 ± 0.12	48.3	
C. sake CBS 159[b]	85.7 ± 0.16	40.0	37.8-41.0[d]
C. sequanensis CBS 8118[b]	82.7 ± 0.16 82.8 ± 0.00	32.7 32.9	39.6[e]
C. shehatae CBS 5813	87.1 ± 0.07	43.4	41.2[f]
C. shehatae CBS 2779	86.2 ± 0.00	41.2	
C. shehatae CBS 5712	87.1 ± 0.10	43.4	
C. shehatae CBS 7261	87.0 ± 0.12	43.2	

Organism	Tm + SD	%GC (Experimental)	%GC (Literature)
C. sophiae-reginae CBS 8175b	85.4 ± 0.10	39.3	
Metschnikowia bicuspidata var. chathamia NRRL Y-7112[b]	87.7 ± 0.00	44.9	44.69
Pichia guilliermondii NRRL Y - 2075[b]	87.6 ± 0.10	44.6	44.1-49.7[d]
P. stipitis CBS 5773[b]	87.2 ± 0.10	43.7	41.2-42.5[d]
P. stipitis CBS 6054	86.1 ± 0.10	41.0	

[a]Expressed as %GC (mean molar percent of guanine plus cytosine).
Average Tm value and standard deviation based on at least 3 determinations.

[b]Type culture of species.

[c]Balloni et al. (1978).

[d]Barnett et al. (1983).

[e]Saez and Rodriguez de Miranda (1985).

[f]Meyer et al. (1984).

[g]Meyer and Phaff (1972).