

THE EVOLUTION OF ANTARCTIC YEASTS

BY

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PREFACE

This thesis is composed of six manuscripts which are presented as chapters, each complete in itself without additional supporting material. The manuscripts constituting the first four chapters have been published in the following refereed national or international journals: " Budding morphology of a psychrophilic *Cryptococcus* and related species compared with *Leucosporidium scottii*" (Chapter I) in *Mycologia* (73: 618-633, 1981), " A fixation method for visualization of yeast ultrastructure in the electron microscope" (Chapter II) in *Mycopathologia* (77: 19-22, 1982), " The evolution of Antarctic yeasts: DNA base composition and DNA-DNA homology" (Chapter III) on *Canadian Journal of Microbiology* (28: 406-413, 1982), and "*Cryptococcus lupi* sp. nov., an Antarctic Basidioblastomycete" (Chapter IV) in *International Journal of Systematic Bacteriology* (32: 229-232, 1982). The manuscript entitled "Five new basidioblastomycetous yeast species segregated from *Cryptococcus vishniacii* emend. auct., an Antarctic yeast species comprising four new varieties" (Chapter V) has been accepted for publication in *International Journal of Systematic Bacteriology*. The data presented in Chapter VI, "Phylogenetic relationships in the basidiomycetous yeasts: complementary DNA-25S ribosomal RNA homology, have not yet been submitted for publication.

Approval for presenting the thesis in this manner is based upon the Graduate College's policy of accepting a thesis written in

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INTRODUCTION

The present day fungi are the result of a complex evolutionary process. They constitute a world of extraordinary variety, far more than the microscope reveals. For about two centuries, mycologists have tried in vain to understand the natural relationships among fungi and to impose some order on the bewildering array of forms, physiologies, and ecologies. Variety amongst fungi is mostly variety within simplicity, and so it provides little information about phylogenetic relationships.

Among fungi, we have been interested in the evolution of yeasts. Our interest in this problem originated from our studies in the microbial ecology of extreme environments. The fact that the only isolated heterotrophic organisms demonstrably indigenous to the Dry Valleys of South Victoria Land, Antarctica (the most extreme cold desert on earth) consisted entirely of a group of anamorphic yeasts, *Cryptococcus vishniacii* (Vishniac and Hempfling, 1979b), necessitated their characterization as fully as possible for comparing them with related yeasts of other habitats and correlating their traits with the requirements imposed by this multiply stressed and variable environment.

Because yeasts occur in more than one division within the kingdom Fungi (Ascomycetes, Basidiomycetes, Deuteromycetes, and Zygomycetes), a prime objective of their systematics must certainly be the

establishment of an evolutionary relationship that spans this kingdom. However, classification by traditional techniques has been difficult reflecting the relative simplicity and antiquity of these organisms. The most commonly used system in yeast classification (van der Walt, 1970) has given greatest weight to developmental characters (such as the mode of vegetative reproduction) which involve a series of complex genetic and biochemical events rather than a few enzymatic steps.

For a great majority of higher organisms as well as some eukaryotic microbes (e.g. protozoa and sexually reproducing fungi), the readily observable distinctiveness of most species results from the fact that individuals belonging to different species seldom mate with one another. This definition of species however, cannot be applied to those organisms in which mating rarely or never occurs. In an operational sense, this includes all prokaryotes and many eukaryotes including the anamorphic yeasts and filamentous fungi. For these organisms, the most defensible approach in constructing a reliable taxonomic system based on evolutionary affinities would be a comparison of the informational macromolecules - if two organisms are closely related, they must retain in their genome base sequences which are descendant from a common ancestral base sequence.

Determination of mean base composition of the nuclear DNA constitutes the coarsest genetic probe available to indicate possible evolutionary relationships between two yeasts. Accurate base composition values can serve the same exclusionary function in yeast systematics as in bacterial taxonomy. Additional forms of comparisons (DNA-DNA homology) are required to determine whether or not yeasts

which exhibit similar base compositions are related (Price et al., 1978).

The determinations of DNA base composition and DNA-DNA homology, however, are inadequate for evaluating relationships at taxonomic levels above species. A reasonable approach to this problem is the use of phylogenetic indicators which have undergone less evolutionary change than the whole genome. The primary structure of ribosomal RNA is a good candidate for such studies since rRNA cistrons are known to be much more conserved than the total genome (Doi and Igarashi, 1965; Moore and McCarthy, 1967). The small rRNA classes can be sequenced; comparison of these sequences has been the basis of grand evolutionary schemes (see Hori, 1975). The 18S and 25S rRNA molecules are too large to allow their sequencing from a large number of organisms for evolutionary studies. Comparative cataloging of 16S rRNA has been used for phylogenetic studies of bacteria (Fox et al., 1977). Although sequence homology of the large rRNA molecules has not been widely used in evolutionary studies, the work of several bacteriologists (Johnson and Francis, 1975; Pace and Campbell, 1971; Palleroni et al., 1973) has demonstrated the potential of this technique.

The present study was undertaken to establish phylogenetic relationships and the extent of evolutionary divergence which may have occurred within the *C. vishniacii* biotypes and to determine their phylogenetic relations to other yeasts on the basis of genetic similarities. Base composition of nuclear DNA and DNA-DNA homology within the *C. vishniacii* complex and related yeasts, and complementary

DNA-25S rRNA homology between this group and other basidiomycetous yeasts were determined. The probable generic affinity of the group was determined by scanning and transmission electron microscopic studies of budding (the only developmental process in anamorphic yeasts) thus augmenting the physiological similarity index used when this complex was first described (Vishniac and Hempfling, 1979a).

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CHAPTER I

BUDDING MORPHOLOGY OF A PSYCHROPHILIC *CRYPTOCOCCUS*
AND RELATED SPECIES COMPARED WITH
LEUCOSPORIDIUM SCOTTII

BUDDING MORPHOLOGY OF A PSYCHROPHILIC *CRYPTOCOCCUS* AND
RELATED SPECIES COMPARED WITH *LEUCOSPORIDIUM SCOTTII*

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SUMMARY

Cryptococcus bhutanensis, *C. himalayensis*, and *C. vishniacii* are anamorphic basidiomycotinous yeasts. *Cryptococcus bhutanensis* and *C. vishniacii* are the first such reported to lack extracellular urease. The budding characters of these three species occur in an association here described for the first time in yeasts. Budding is monopolar and repetitive through the site of the birth scar. The primary bud is holoblastic, secondary buds enteroblastic and continuous with inner wall layers of the entire parental cell. Septum formation occurs at or slightly above the level of any previously formed collar (bud scar). The *Cryptococcus* species, budding monopolarly, have a higher width: length ratio (0.70 to 0.83) than the bipolarly budding *Leucosporidium scottii* (0.55), in which primary buds are distal to the birth scar. Surface topography reflects the degree of cell encapsulation, but appears to result in part from artifacts of capsule collapse during drying in thinly encapsulated cells.

Size, shape, surface topography and the details of budding observable with scanning and transmission electron microscopy are major descriptors of yeast cells, particularly valuable for yeasts which fail to reproduce sexually. A list of the budding characteristics which have been useful at various hierarchical levels in systematic schemes as well as in ontological studies (see 13, 21) should include the relative age of primiparous cells, the site of primary and successive buds, the relation of budding sites to cell shape, the origin of the bud cell wall, septum formation and partitioning, and the resultant appearance of bud and birth scars. Despite this, size is frequently not described in statistical terms, shape is rarely described quantitatively, and there are few yeasts other than *Saccharomyces cerevisiae* Hansen for which available evidence yields an inclusive picture of the budding process.

We here report morphological investigations of an ecologically interesting group of yeasts, psychrophiles or psychrotrophs isolated from frigid habitats: *Cryptococcus bhutanensis* Goto and Saito, *C. himalayensis* Goto and Saito, *C. vishniacii* Vishniac and Hempfling, and *Leucosporidium scottii* Fell, Statzel, Hunter, and Phaff. *Cryptococcus vishniacii* is the only heterotroph known to be indigenous to the Dry Valleys of Antarctica (28); *L. scottii* is the yeast most commonly reported from Antarctic sites outside of the Dry Valleys. It became evident that the three *Cryptococcus* species were not only morphologically very similar, but that they displayed a constellation of budding characters not previously reported in yeasts.

MATERIALS AND METHODS

Cryptococcus bhutanensis ATCC 22461, *C. himalayensis* IAM 4963, *C. vishniacii* isolates representing each biotype (TABLE I), and *Leucosporidium scottii* a ATCC 22182 were examined. (*Leucosporidium scottii* was not examined by transmission electron microscopy). Stock cultures were maintained by methods previously reported (27). Populations described were grown for 5 to 6 generations in GPYPi (27) at 10 C (unless otherwise specified) on a New Brunswick Scientific Co. Model G-76 gyrotory water bath shaker, inoculated (to ca. $OD_{650} = 0.2$) from exponentially growing cultures similarly grown, examined directly for cell measurements, and harvested by centrifugation at 5,000 x g for 5 minutes in a Sorvall RC-2 refrigerated centrifuge at 4 C for electron microscopic studies.

Cell measurements.- Approximately 30-100 mature (budding) cells of each population were measured in calibrated prints of photographs taken at 1,000 x using a Nikon S-U phase contrast microscope. Ratios of width:length were calculated for individual cells. Significance was determined by the one-tailed t test.

Electron micrography.- Harvested cells were washed and resuspended in cold, sterile dilute (1:10) mineral base (27) to $OD_{650} = 40$ before fixation for scanning electron microscopy (SEM), fixed by addition to an equal volume of 4% glutaraldehyde in cacodylate buffer (pH 7.3, 0.2 M), held on ice for 2 hours and allowed to settle at room temperature for 30 minutes onto coverslips coated with 1% polylysine hydrobromide

Type VII B (Sigma) before completing fixation in 2% glutaraldehyde buffer at 4 C overnight. Coverslip preparations were dehydrated with a graded series of ethanol solutions, dried in a Polaron E-3000 critical point dryer, coated with gold-palladium alloy (to 100 Å thickness) in a Technics Model Hummer II sputtering coater, and examined in a JOEL Model JSM-35 scanning electron microscope at an accelerating voltage of 25 KV.

In preparing cells for transmission electron microscopy (TEM), sodium cacodylate buffer (pH 7.2, 0.2M) was used for washing and suspension. Cells were fixed on ice for 2 hours in an equal volume of glutaraldehyde (3%)-acrolein (1.5%)-paraformaldehyde (1.5%) in cacodylate buffer (0.05 M), post-fixed at room temperature for 1 hour in 6% potassium permanganate, dehydrated in a graded series of ethanol solutions, placed in propylene oxide transition fluid and embedded in Epon 812 mixture. Sections were cut with a DuPont diamond knife in a Sorvall Porter-Blum ultramicrotome Model MT-2, stained with lead citrate for 15 minutes and examined in an RCA Model EMU-3G electron microscope operating at an accelerating voltage of 100 KV.

RESULTS

Cell dimensions.- The vegetative cells of *Cryptococcus* species are typically more or less extended ovoids. The width, length, and width: length ratios of mature (budding) cells of *C. bhutanensis* and *C. vishniacii* are given in TABLE I, significant differences in TABLE II. While some biotypes of *C. vishniacii* which did not differ in any of these parameters formed a discrete cluster (2,4,5,7), other such

TABLE I
DIMENSIONS OF *CRYPTOCOCCUS VISHNIACII* AND *C. BHUTANENSIS*

<i>C. vishniacii</i> biotypes	Strain designation	Width \pm SD μm	Length \pm SD μm	W:L \pm SD
1	302-Y-216	5.07 \pm 0.69	6.70 \pm 0.83	0.76 \pm 0.06
2	303-Y-365	4.03 \pm 0.42	5.61 \pm 0.45	0.72 \pm 0.08
3	303-Y-200	3.82 \pm 0.45	5.39 \pm 0.31	0.70 \pm 0.06
4	309-Y-215	4.24 \pm 0.49	5.60 \pm 0.46	0.76 \pm 0.07
5	202-Y-312	3.95 \pm 0.45	5.47 \pm 0.62	0.72 \pm 0.06
6	306-Y-212	6.45 \pm 0.93	8.11 \pm 1.05	0.80 \pm 0.07
7	304-Y-268	4.09 \pm 0.47	5.54 \pm 0.55	0.74 \pm 0.07
8	303-Y-338	3.48 \pm 0.44	4.70 \pm 0.70	0.74 \pm 0.05
9	303-Y-206	4.77 \pm 0.56	6.70 \pm 0.78	0.72 \pm 0.08
10	302-Y-265	5.57 \pm 0.85	7.15 \pm 0.98	0.78 \pm 0.06
11	202-Y-349	4.87 \pm 0.59	6.42 \pm 0.69	0.76 \pm 0.08
12	202-Y-252	5.06 \pm 0.71	6.11 \pm 0.71	0.83 \pm 0.07
13	202-Y-375	4.94 \pm 0.72	6.15 \pm 0.76	0.80 \pm 0.07
14	302-Y-259	4.85 \pm 0.66	6.62 \pm 0.87	0.74 \pm 0.09
15	303-Y-336	4.62 \pm 0.58	5.76 \pm 0.70	0.81 \pm 0.08
16	302-Y-310	5.78 \pm 0.94	7.26 \pm 1.22	0.80 \pm 0.07
<i>C. bhutanensis</i>	ATCC 22461	4.52 \pm 0.62	5.95 \pm 0.94	0.77 \pm 0.10

TABLE II

SIGNIFICANT DIFFERENCES IN WIDTH (W), LENGTH (L), AND WIDTH-LENGTH RATIO (R) OF

CRYPTOCOCCUS VISHNIACII BIOTYPES AND *C. BHUTANENSIS*

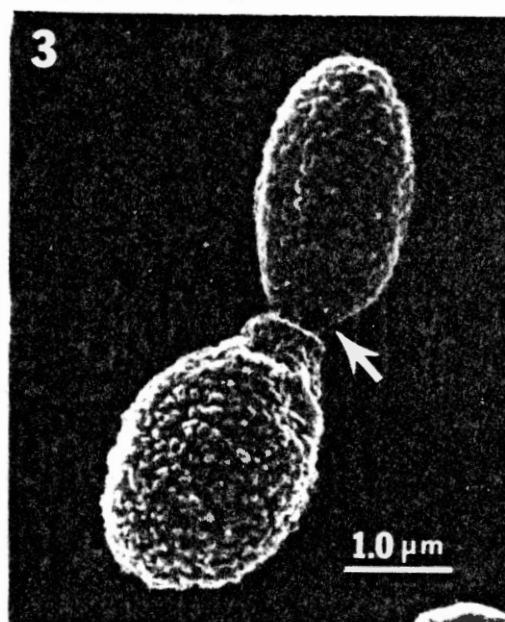
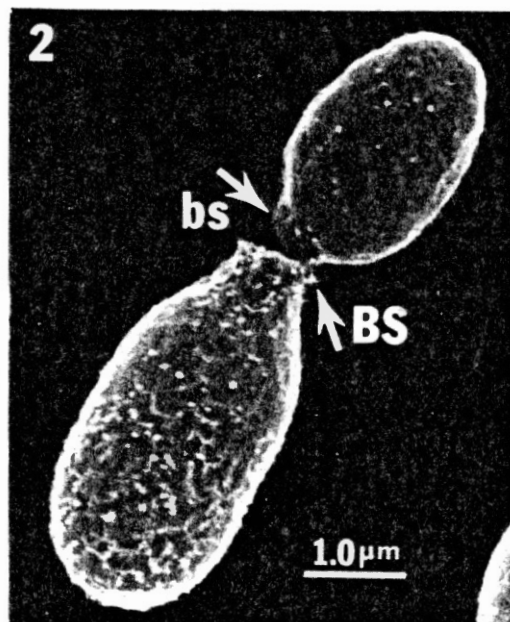
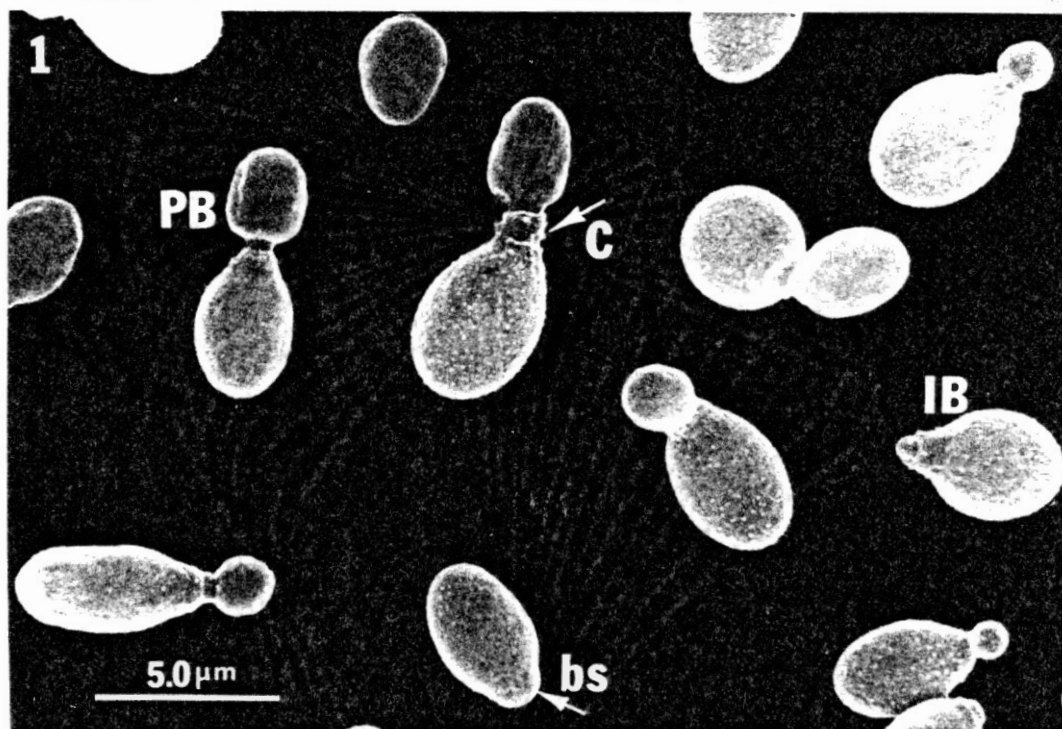
<i>C. vishniacii</i>																
biotypes	WLR															
2	**N															
3	***	***														
4	**N	NNN	*N*													
5	***	NNN	NN*	*N*												
6	***	***	**N	**N	***											
7	**N	NNN	*N*	NNN	NNN	***										
8	**N	**N	***	**N	**N	***	**N									
9	*N*	**N	***	**N	**N	***	**N	**N								
10	**N	***	***	**N	***	**N	***	***	***							
11	NNN	***	***	**N	**N	**N	**N	**N	NN*	**N						
12	N**	***	**N	***	***	**N	***	***	***	**N	NN*					
13	N**	***	**N	***	***	**N	***	***	N**	**N	NNN	NNN				
14	*NN	**N	***	**N	**N	***	**N	**N	NNN	***	NNN	***	N*N			
15	***	*N*	**N	*N*	***	**N	*N*	***	N**	***	N**	**N	**N	N**		
16	***	***	**N	**N	***	**N	***	***	***	NN*	**N	***	**N	***	**N	
<i>C. bhutan.</i>	**N	***	***	**N	***	**N	**N	**N	*N*	**N	**N	N**	N*N	**N	NN*	**N
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
<i>C. vishniacii</i> biotypes																

* = significant *t*, *p* < 0.01N = non-significant *t*, *p* > 0.05

clusters overlapped {(1,11), (11,13,14), (12,13), and (9,14)}, demonstrating the continuous nature of variation in size and shape. The ratio of cell width to length was less variable than either of the component measurements. The measurements of *C. himalayensis* are not given since the heavy capsule did not permit comparison with measurements of other yeasts; the width:length ratio of 0.81 ± 0.09 fell within the range of those listed for other *Cryptococcus* isolates. The distinctly elongated shape of *Leucosporidium scottii* was reflected in a very different ratio: 0.55 ± 0.09 . While the shape of the majority of cells appeared, on cursory inspection, to be non-varying under other conditions of cultivation, populations grown at 15 C sometimes displayed asymmetrical crescentic buds (*C. vishniacii* biotype 8 to less than 5% of the population, biotype 9 ca. 15-20% of budding cells), buds resembling the figured cells of *Sporopachydermia cereana* (20) and *Selenozyma peltata* (33).

Surface topography.- Since all of the isolates examined were enveloped by a capsule (India Ink test), one would expect the surface material seen in SEM to be capsular. The cell surface of thinly encapsulated *C. vishniacii* biotypes appeared smoothly granular in biotype 16, more often showed few (biotypes 3-5) to many (biotypes 1,2,7-9,12) more or less prominent nodules. Less prominent nodules are seen in Fig. 1, more prominent nodules against a somewhat reticulate background in Fig. 2, representing the appearance of biotypes 10, 11, 13-15. *Cryptococcus bhutanensis* (Fig. 3) typically appeared heavily nodulated as did *L. scottii* (Fig. 4). In no case were the nodules as prominent as those figured by Watson and Arthur (30) on *L. frigidum*. Surface decorations

Figs. 1-3. *Cryptococcus* species. SEM micrographs of budding cells.
bs = birth scar, BS = bud scar, C = collar (bud scar), IB = incipient
bud, PB = primary bud. 1. *Cryptococcus vishniacii* biotype 12,
202Y252, cells at various stages of cell cycle. 2. *C. vishniacii*
biotype 14, 30ZY259, bud separating from parental cell. Note the
reticulate-nodular surface of parent cell. 3. *C. bhutanensis*
budding cell with heavily nodulated surface. Neck of attached bud
is clearly visible (arrow).

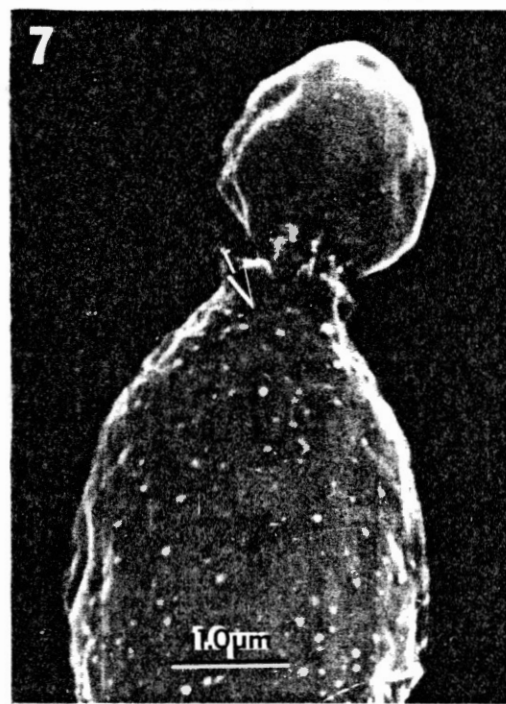
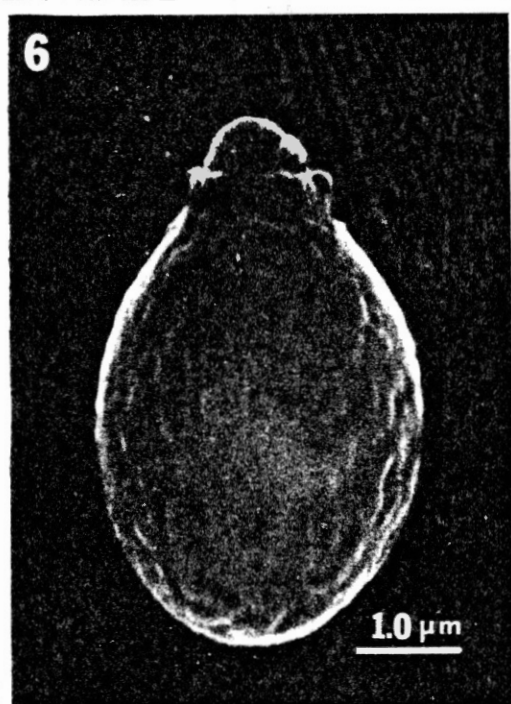
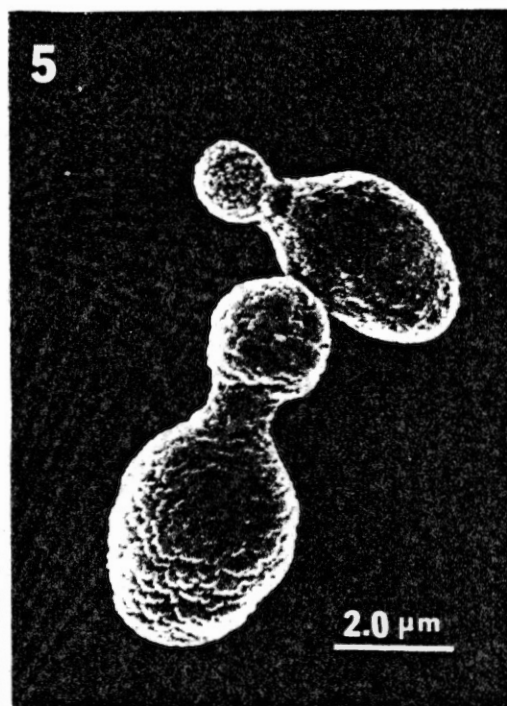
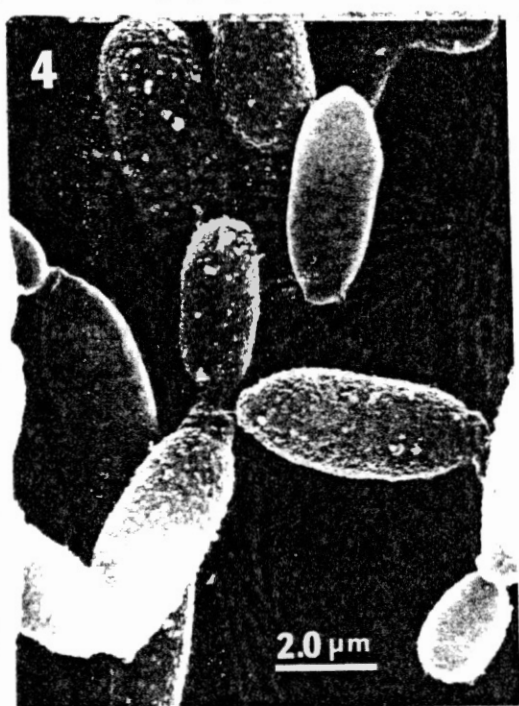


were always more fully developed on parental cells than on the buds. The broad nodules seen on the small bud of *C. himalayensis* (Fig. 5) develop into wide foliations on the parent cell, obscuring the details of budding, birth scar and bud scars. *Cryptococcus himalayensis* resembled earlier described species of *Cryptococcus* in being heavily encapsulated. *Cryptococcus vishniacii* biotypes 6 (Fig. 6) and 8 (which sometimes appeared nodular) had a soft, billowy appearance.

The surface of mature cells was somewhat variable within a biotype. Biotype 8 varied in a single preparation. Additional preparations (grown at 15 C) produced cells of biotype 8 which were more often nodular, of biotype 12 which were nodular-reticulate, of biotype 14 which were nodular. While surface appearance varied with degree of encapsulation, it is suggested that artifacts of capsule collapse during critical point drying are primarily responsible for these variations in the surface topography of *C. vishniacii* biotypes.

The effects of budding on surface topography are best observed in species with minimal capsular investment. Nulliparous cells of all *C. vishniacii* biotypes displayed similar birth scars. The birth scar (shown in Figs. 1,2) was a smooth, broad, gently convex area capping a distinct neck (a slightly sloping, constricted, elongated region of the cell). Birth scars were never seen in parous cells. Budding was monopolar (Figs. 1,2), almost invariably occurring at the neck end of the cell (rare exceptions were seen in biotypes 4, 11, 12, and 14 of *C. vishniacii* and in *C. bhutanensis*), by protrusion through the birth scar which was thus obliterated. The neck of the newly separated bud in Fig. 2 appears to conform to the internal diameter of the bud scar neck, but, since bud necks were clearly visible in unseparated buds

Figs. 4-7. SEM. 4. *Leucosporidium scottii*. some heavily nodulated cells with bipolar budding. 5. Heavily encapsulated cells of *Cryptococcus himalayensis*. 6. *C. vishniacii* biotype 6, 306Y212, with billowy surface. 7. *C. vishniacii* biotype 9, 303Y206 budding cells with ragged second collar emerging. Arrow indicates the edge of capsular material.

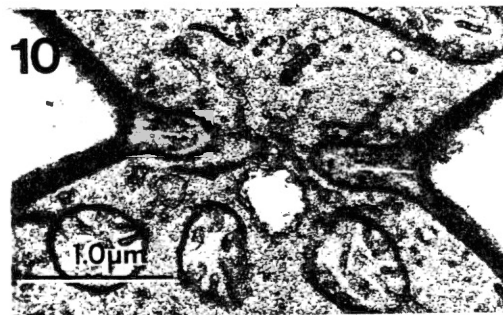
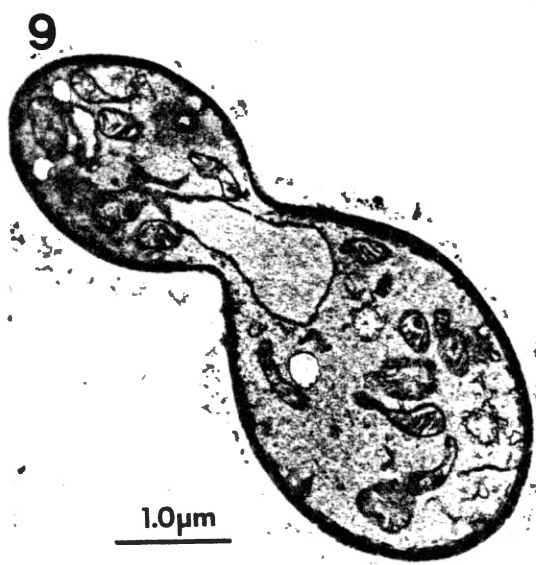
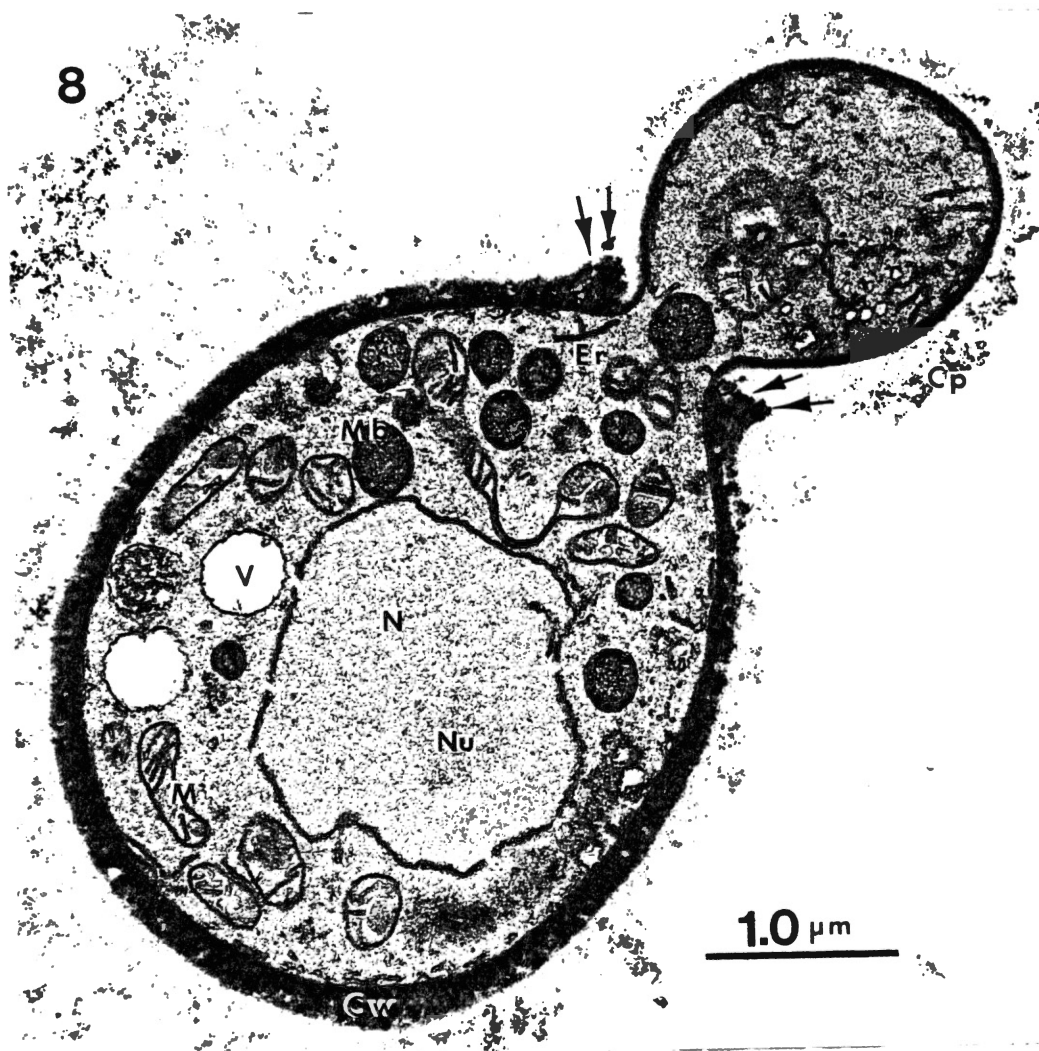


(Figs. 1, 3), this does not indicate the level of septum formation. The shape of budding *C. himalayensis* cells suggests a similar pattern of budding. *Leucosporidium scottii* cells budded bipolarly, with the first bud appearing at the pole opposite to the birth scar; birth scar and bud (or bud scar) could be seen on the same cell. The birth scar resembled that of *L. frigidum* figured by Watson and Arthur (30), as well as those of *C. bhutanensis* and *C. himalayensis*.

The incipient bud broke through a layer of some sort of material on the parent cell (Fig. 1). Budding cells of all strains exhibited a collar, either relatively adherent or protruding, under SEM. The bud scar seen in Fig. 2 consists of the parental neck area, which has typically become somewhat more cylindrical than a slightly sloping birth scar neck, terminated by a roughly level, protruding, collar. The collars of bud scars ranged from merely frayed (Fig. 2) to ragged (Figs. 1, 7) in appearance. Collars so ragged as to appear multiple (Fig. 7) were not uncommon.

The cell in section.- The organelles usual to yeast cells were well preserved by the fixation method used. Surprisingly, cells with numerous microbodies were seen in one preparation of *C. vishniacii* biotype 14 (Fig. 8). These microbodies were seen occasionally in other preparations, but not in such profusion. They resemble, at least superficially, microbodies associated only with growth on methanol, methane, or other alkanes in other yeast species (2,19,31). *Cryptococcus vishniacii* does not utilize decane as sole substrate (27); other microbody inducers have not been tested as substrates for *C. vishniacii*.

Figs. 8-11. TEM micrographs of *Cryptococcus vishniacii*. 8. Budding cell of biotype 14, 302Y259. Cp = capsule, Cw = cell wall, Er = endoplasmic reticulum, M = mitochondria, Mb = microbody, N = nucleus, Nu = nucleolus, V = vacuole. Arrows indicate frayed layers of cell wall making up the bud scar or collar. 9. Nucleus migrating into neck of budding cell: biotype 14, 302Y259 grown at 18 C. Note the continuity of wall between parent and bud. 10. Developing septum, biotype 6, 303Y212. Note the degrees of electron transparency in the layers extending from the common cell wall. 11. Annulate (multi-parous) cell of biotype 14, 302Y259, and bud, with completed septum.



The protoplast (Fig. 8) was bounded by an electron-dense cell wall, definite, but seldom sharp in outline, which was in turn more or less surrounded by the fibrillar remains of the capsule (not well preserved by the fixation method used). The somewhat rayed appearance of the cell wall in Fig. 8 was quite typical. The inner portion of the wall sometimes appeared to be composed of concentric layers, which became frank only in the neck area. There was no evidence of protrusions, pits or grooves in either the inner or outer surface of the cell wall, in contrast to walls figured for *Pityrosporum* species (8).

The erect bud scar collars seen in SEM were seen in TEM (Fig. 8) to be composed of separating, slightly frayed layers of cell wall. The cell wall of the developing bud in Fig. 8 is continuous only with an inner layer of the parental cell wall (enteroblastic). However, in slightly more than half of budding cells, the cell walls of parent cell and bud were continuous (holoblastic), as seen in Figs. 9, 10. This distribution of collars in an exponentially growing population implies that collars are present only after the primary bud has separated. This hypothesis is tenable only if budding time is not significantly dependent upon cell age, i.e. if these yeasts display Flegel's (4) mother-daughter-cell equivalence. Since the proportion of nulliparous cells was roughly 50% in populations observed by all means, one may assume that the time to budding and between budding was substantially the same. Our data were insufficient to establish statistically small differences in cell cycle time (14) or growth rate (25) between parent and bud. The incipient primary bud does not "break through" the outer layers of parent cell wall, but is an extension of the birth scar area. The adherent collar of the primary

Figs. 12-13. *Cryptococcus vishniacii*, biotype 14, 302Y259 TEM.

12. Bud separating from parental cell. 13. Multiannulate budding cell. Note the continuity of bud cell wall with inner layers of parental cell wall and the greater thickness of parental cell wall.



bud in Fig. 1, like the line of demarcation below the frayed collar in Fig. 7 (arrow), must represent a limit of capsular material.

Later in bud development, the nucleus was seen positioned in the connecting neck of the two cells (Fig. 9). The nuclear membrane subsequently disappeared, implying that mitosis is not intranuclear. Still later, the invagination of the cell membrane was accompanied by the formation of a septum. The developing septum (Fig. 10) was a broad ring of material slightly less electron dense than the cell wall, enfolding a much thinner electron transparent circlet, extending from the cell wall. The completed septum (Fig. 11) consisted of a thin electron transparent plate, the rim of which appear to extend slightly under the cell wall common to both cells, and which are bounded by thin electron dense new layers of cell wall in both cells. Since the bud figured is not a primary one, it is obvious that septum formation occurred (typically) at the level of the collar rim. The cells in Fig. 12 appear to be separating with a tearing of the common cell wall creating a second, inner, collar on the parental cell. The septal material appears to be disintegrating. Since birth scars showed no collars, the common wall must be partitioned unequally, possibly because the bud is growing more rapidly. Figure 13 displays a multiparous cell which may have been in the process of producing its fourth collar ring.

The discrete annuli of Fig. 13 represent an extreme. In most budding cells, it was not possible to decide whether the collar represented the completion of one or several budding events. The rupture at a single separation may break cell wall layers at different levels, and, while the bud wall in Fig. 8 is continuous with a thin innermost

layer of parental wall, the developing bud in Fig. 13 appears to have a common multilayered wall with the parental cell. The number of layers, or cluster of layers cannot be assumed equivalent to the parity of the parental cell. It does seem reasonable to assume that this cell (Fig. 13) is more than monoparous, that the cell has expanded slightly between buds (from the height of the successive annuli) and that the cell has laid down additional wall layers since birth (from the perceptibly thicker wall).

Is the cell in Fig. 13 anomalous? The similar annuli seen on other budding cells (Fig. 11) allow the supposition that some correlation between morphology and parity exists. Establishing such a correlation would require the separation of multiparous cells, with their recovery in the proportions expected from the generation number of the population used. Preliminary attempts to do so by density gradient centrifugation were unsuccessful.

Anomalous development was observed. Rudimentary pseudomycelium could be found by searching any culture. Bipolar budding was seen once or twice in biotypes 4, 11, 12, and 14 of *C. vishniacii*. A percurrent cell bore two buds above a single collar in biotype 13. Double budding (at the same pole) was observed in 15 C-grown cells of biotypes 8, 9, 12. The rarity of these events indicated that they did not represent normal development in the *Cryptococcus* species studied.

DISCUSSION

Bud scars consisting of a multilayered cell wall forming a collar

around a site of repeated bud formation have been considered "most conclusive" evidence for basidiomycotinous affinities of anamorphic yeasts (18). The fraying collar of *Rhodospiridium* (10,17) and *Filobasidiella* (15) has never been seen in ascomycotinous yeasts, nor the conspicuous chitinous ring and bud scar of ascomycotinous yeasts in basidiomycotinous ones. Species originally described in *Cryptococcus* have teleomorphs in the Basidiomycetes [*Filobasidium capsuligenum* (23), *Filobasidiella bacillispora* (12), *F. neoformans* (11)] and in the Ascomycetes [*Sporobachydermia cereana*, *S. lactativora* (24)]. We now consider *C. bhutanensis*, *C. himalayensis*, and *C. vishniacii* anamorphs of Basidiomycetes, on the evidence of their bud scars, of our failure to observe intranuclear mitosis [an ascomycetous character (21)], and of their reaction (unpublished data) to diazonium blue B (26).

Cryptococcus bhutanensis and *C. vishniacii*, unlike all yeasts heretofore considered basidiomycotinous, are urease negative (27). While *C. bhutanensis* was originally reported to be "weakly positive" (6), it has not been so in our laboratory, under conditions which allowed *C. himalayensis* to give an unequivocal positive reaction. Since economizing on the production of an enzyme useless in the habitat of *C. vishniacii* is presumably immediately adaptive, one might question whether urease production should be used as a determinative character at any level above species. Other, more complex characters are available.

Descriptions of budding sites have been used (among other characters) to distinguish basidiomycotinous from ascomycotinous yeasts (13), to transfer basidiomycotinous *Candida* species to *Apiotrichum* (sympodial) or to *Rhodotorula* (repetitive) (29), and to characterize *Pityrosporum*

(monopolar)(13). The traits described were 1) the number of budding sites, 2) the site of primary budding with respect to the cell pole indicated by the birth scar and 3) with respect to the site(s) of subsequent buds. Repetitive budding at the same site(s) is typical both of bipolar (i.e. elongated) cells of ascomycotinous and basidiomycotinous yeasts and of monopolar yeasts. The bipolar yeasts typically bud first at the site opposite the birth scar (9). *Leucosporidium scottii* was similar in appearance and budding characters to other species of *Leucosporidium* (30), a basidiomycotinous genus with bipolar cells. The *Cryptococcus* species which we studied typically budded repetitively only through the birth scar. While it is probable that other basidiomycotinous species of *Cryptococcus*, and of other anamorphic genera, do likewise, this behaviour has previously been described adequately only in *Pityrosporum* and keys to this genus. *Pityrosporum*, distinctive in wall pitting and sculpturing, in habitat, and (except for *P. canis*) in lipid requirements, is unlikely to be closely related to *Cryptococcus*.

The polarity of budding appeared in this study to be correlated with cell shape. McCully and Bracker (17) proposed that the vesiculate tips of budding basidiomycotinous yeasts were homologous with hyphal tips. The concentration of vesicles in the distal end of large *L. scottii* buds which they figured suggests that bipolar yeasts derive their shape and their distal primary buds from the same underlying regulatory mechanism. The only concentration of vesicles seen in TEM sections of large buds of the *Cryptococcus* species lay along the developing septum between parent cell and bud. Since width to length ratios in exponentially growing cells of *Pityrosporum* have not been

reported, the correlation of shape and budding polarity requires further study.

A great deal of stress has been placed upon the origin of the wall of the developing bud. This origin is septal, that is, it consists at least in part of previously formed septal material which is distinguishable in TEM from other wall material, in *Pityrosporum* (8) and in the apiculate ascomycotinous yeasts (8,9). In the latter, this septal material remains with the parental cell at the second abscission so that there are external and internal annuli on the percurrent parental cell; in the former, the second abscission occurs at a lower level in the parental cell so that the collar curls inward. The bud wall origin is otherwise described as holoblastic (entirely continuous with the parental cell wall), terms which are ambiguous as regards septal buds. Non-apiculate ascomycotinous yeasts are typically described as holoblastic, basidiomycotinous yeasts as enteroblastic, though enteroblastic budding has been reported in *Saccharomyces cerevisiae*.

"Holoblastic" and "enteroblastic" were perceived in Kananaskis II as referring to a continuum of states which may be present in the same fungus, being manifested according to the physiological age of the wall at the blastic locus (16). A sequence of holoblastic primary buds and enteroblastic secondary buds similar to that which we have described was reported first in haplophase cultures of the smut *Sorosporium consangoineum* (3), but "primary buds" were stated to be "relatively rare". If primary buds are those first produced by the nulliparous cell, such an observation may imply either that cultural parameters have allowed physiological ageing but slowed reproduction or that the

mother-daughter-cell equivalence does not obtain. Enteroblastic budding in *Saccharomyces cerevisiae* seems to be unusual and may be associated with physiological age rather than solely with birth order. Since both physiological age and parity may affect the continuity of bud and parental cell walls, the cultural history of the populations examined is an important variable. In exponentially growing cultures, the layering or thickening of cell walls, as other manifestations of physiological ageing, may be expected to keep step with parity; primary buds can be defined by birth order, regardless of wall origin, and constitute a predictable proportion of the total population.

The *Cryptococcus* species which we have studied are not necessarily closely related to tremellalean fungi (1) or to the smuts (3,22) which bud both holoblastically and enteroblastically. Some species in both groups are capable of indefinite laboratory cultivation as yeasts, but yeasts isolated as such have never been identified with these species. The cultivated yeast phase of both groups is typically derived from haplophase cells. In the dye accumulation test of Yamazaki and Oshima (32), *L. scottii* mating types reacted as haploids, but the *Cryptococcus* species and biotypes as diploids (unpublished data). Furthermore, all yeasts of basidiomycotinous affinity may well have holoblastic primary buds in exponentially growing cultures and exhibit enteroblastic budding otherwise. Many published figures of basidiomycotinous yeasts and their anamorphs have been chosen to display the characteristic collar of multiparous cells. Holoblastic budding can nevertheless be inferred from the similar thickness and continuity of walls in budding cells in published figures of *Aessosporon salmonicolor* (17), *L. scottii* (17), and *F. neoformans* (serotype not given)(15). The correlation of wall

origin with parity in these yeasts has not been attempted.

ACKNOWLEDGMENTS

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CHAPTER II

A FIXATION METHOD FOR VISUALIZATION OF YEAST ULTRASTRUCTURE IN THE ELECTRON MICROSCOPE

A FIXATION METHOD FOR VISUALIZATION OF YEAST ULTRASTRUCTURE
IN THE ELECTRON MICROSCOPE

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ABSTRACT

A primary fixative containing glutaraldehyde (3%), acrolein (1.5%), and paraformaldehyde (1.5%) buffered in 0.05 M sodium cacodylate at pH 7.2 was applied to the cells of *Cryptococcus vishniacii* for 2 hours on ice. The cells were then treated with a 6% aqueous solution of potassium permanganate for 1 hour at room temperature. This method preserves most of the yeast cell fine structural components including cell walls and membrane, nuclear membrane, mitochondria, endoplasmic reticula, microbodies, vacuoles, nucleoli, and ribosomes. However, it leads to disruption of capsular materials and loss of some of the lipid and glycogen granules.

Many investigators have reported that the conventional fixation methods (e.g. application of potassium permanganate, glutaraldehyde, osmium tetroxide, and their combinations) used in yeast electron microscopy cannot be applied universally for all yeast isolates (3,6,8,9,13). While a fixation mixture may be effective on cells of one

species in penetrating and fixing the internal fine structures, it may have very little effect on cells of another (6,8,9). This is probably due to the differences in thickness and composition of cell walls and presence or absence of a slime capsule of different chemical composition and thickness.

We had no success in fixation of *Cryptococcus vishniacii* cells using the previously reported methods {the pre-freezing method of Joshi et al. (8) was not tried}. When cells were treated with a mixture of glutaraldehyde-acrolein-paraformaldehyde as a primary fixation and potassium permanganate as post fixation agents, the ultrastructure of cells was well preserved.

The 16 biotypes of *C. vishniacii* used in this study were originally isolated from the Dry Valleys of South Victoria Land, Antarctica, and have been described by Vishniac and Hempfling (15,16).

Cells from stock cultures grown and maintained on GPYPi (15) slants at 4°C were inoculated into flasks of GPYPi and transferred into fresh medium after growing at 10°C for five to six generations. Exponentially growing cells from the secondary cultures were harvested by centrifugation at 3,000 x g for 5 minutes in a Sorvall RC-2B refrigerated centrifuge and washed twice in glass-distilled water and resuspended either in glass-distilled water (when potassium permanganate was used as the primary fixative) or in 0.2 M sodium cacodylate buffer pH 7.2 (when other primary fixatives were applied) to give an optical density (650 nm) of about 200.

An equal volume of the different fixatives listed in Table I was then added. The conditions under which the fixation was performed are indicated in Table I for individual fixation methods. After the

TABLE I. METHODS OF FIXATION EMPLOYED FOR PRESERVATION OF *CRYPTOCOCCUS VISHNIACII* CELLS

No.	Primary fixative			Secondary fixative ^a
	fixative combination	incubation conditions	incubation time (h)	
I	Potassium Permanganate (2% in water)	room temperature	1 or 2	None
II	Glutaraldehyde (2% in cacodylate) ^b	on ice	2	None
III	Osmium Tetroxide (2% in cacodylate) ^b	on ice	1 or 4	None
IV	Glutaraldehyde (2% in cacodylate) ^b	on ice	2	Potassium Permanganate (1.5% in water)
V	Glutaraldehyde (2% in cacodylate) ^b	on ice	2	Osmium Tetroxide (2% in water)
VI	Glutaraldehyde (3%)- Acrolein (1.5%)- para- formaldehyde (1.5%) in 0.05 M cacodylate	on ice	2	Potassium Permanganate (6% in water)

^a Incubation at room temperature for 1 hour.

^b Sodium cacodylate buffer, 0.2 M, pH 7.2.

primary fixation was completed, cells which were post-fixed (methods IV, V, and VI in Table I), were washed in glass-distilled water and resuspended in the secondary fixative and incubated at room temperature for 1 hour. Fixed cells were then dehydrated in a graded series of ethanol solutions, placed in propylene oxide transition fluid and embedded in Epon 812 (2A:1B). Sections were cut with a DuPont diamond knife on a MT-2 Sorvall ultramicrotome, stained with lead citrate for 15 minutes and examined in an RCA EMU-3G electron microscope operating at an accelerating voltage of 100 KV.

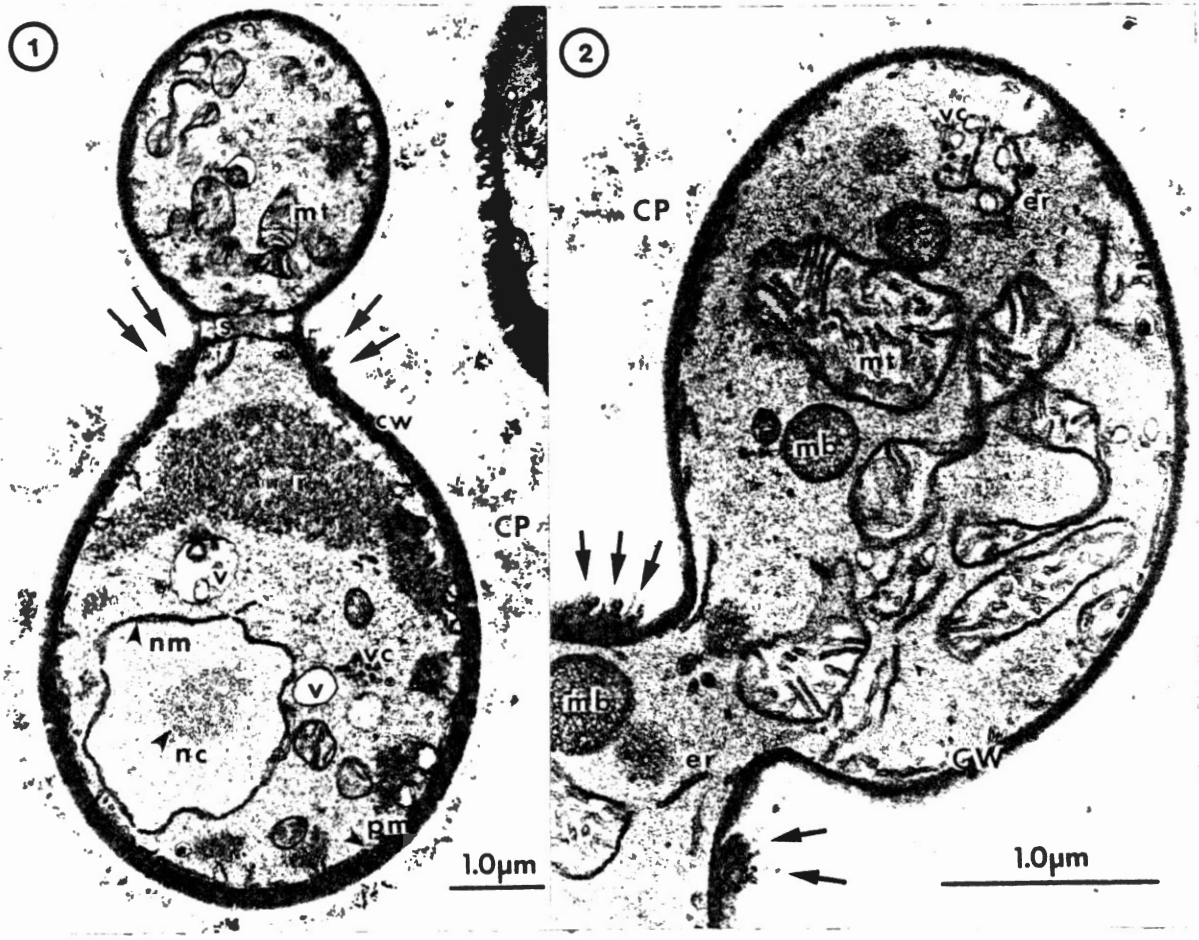
Fixation of cells according to procedures I, II, and III revealed no distinguishable internal structures. Among the combination of these fixatives used, procedures IV and V preserved some of the internal structures the details of which were obscured. The cell wall appeared electron transparent and no layering was observed.

Figures 1 and 2 show cells of *C. vishniacii* fixed according to procedure VI. The cells show most of the fine structural components that one may expect to find in a yeast cell.

Cytoplasm is bounded by an electron dense cell wall comprised of many layers which become frank at the bud formation site (Figs. 1 and 2). Many investigators have reported the presence of a number of cell wall layers (up to seven) with different electron opacity in *Candida albicans* (5,12), and *Cryptococcus neoformans* (4) using a fixative containing Tris-(1-aziridiny)phosphine oxide (TAPO). However, most of the micrographs published in these papers show a poor preservation of the internal structures. Despite the fact that cell walls of *C. vishniacii* (Fig. 1) appeared to be very similar in electron opacity, they clearly demonstrated that the primary buds

Fig. 1. *Cryptococcus vishniacii* biotype 6 (306Y212) fixed with triple aldehyde-potassium permanganate method. The cell shows cell wall (CW) comprised of many layers (arrows), plasma membrane (pm) with occasional invaginations into the cytoplasm and nucleus bounded with a nuclear membrane (nm) and nucleolus (nc). Among the cytoplasmic organelles, mitochondria (mt), vacuoles (v), vesicles (vc) and many ribosomes (r) can also be observed. The septum (s) is complete and the bud is about to separate from the mother cell.

Fig. 2. *Cryptococcus vishniacii* biotype 14 (302Y259). This enteroblastic bud contains many mitochondria (mt), endoplasmic reticula (er) associated with vesicles (vc), and some microbodies (mb). The electron dense cell wall (CW) with many layers (arrows) and fibrillar remains of the slime capsule (CP) are clearly seen.



were holoblastic and the secondary buds enteroblastic (2).

Excellent preservation of internal structures of *C. vishniacii* cells and the overall high contrast of sections are apparently due to the penetration and fixation capabilities of potassium permanganate. However, since this fixative was unsuccessful when used alone, it could be concluded that the triple aldehyde mixture is probably responsible for a rapid, general preservation and increasing the permeability of cells to the post-fixation agents. The more cumbersome method of Joshi *et al.* (8) for increasing permeability by repeated freeze-thawing cycles at -155°C in Freon was therefore not required.

Cell membrane with occasional invaginations into the periplasm, nuclear membrane with pores (Fig. 1), mitochondria with characteristic cristae (Fig. 2), endoplasmic reticulum and vesicles associated with it, vacuoles with some electron dense bodies (possibly polyphosphate) and microbodies are well preserved.

It has been reported that (7,9) fixation of cells with potassium permanganate alone results in a great loss of ribosomes and nucleoli. The presence of nucleoli and many ribosomes in the cells of *C. vishniacii* shows that the triple fixation mixture is suitable for preservation of these structures and the potassium permanganate will have very little effect on them after they are fixed.

The most important disadvantage of the procedure described is the disruption and substantial loss of capsular materials (Figs. 1 and 2). In *C. neoformans*, the conventional fixation procedures, ethanol dehydration, and Epon embedment have been shown to result in loss of capsular integrity and condensation, and its dehydration into filamentous strands projecting from the cell wall (1,6). *C. neoformans*

cell fixed with ruthenium red could however be dehydrated and embedded in Epon by conventional procedures without loss of capsular materials (10). A glutaraldehyde-urea mixture used as an embedding medium, originally described by Pease and Peterson (11), and later employed by Laxalt et al. (10) for preparation of *C. neoformans* cells has also been proven successful in preserving the capsular materials.

Treatment of cells with potassium permanganate results in destruction of some but not all of the lipid and glycogen granules. The fixation procedure introduced by Schwab et al. (14) could preserve these structures but most of the other cytoplasmic organelles such as mitochondria, nuclei, and endoplasmic reticula were obscured.

In summary, the triple aldehyde-potassium permanganate fixative described here reveals most of the internal ultrastructural details of *C. vishniacii* cells including ribosomes and nucleoli which are destroyed by other permanganate-containing fixatives. The procedure does not require pre-freezing to increase the cell wall permeability, it is easy to apply, and relatively safe to prepare. The only major disadvantage that it has is that it causes considerable loss of capsular materials.

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CHAPTER III

THE EVOLUTION OF ANTARCTIC YEASTS:

DNA BASE COMPOSITION AND

DNA-DNA HOMOLOGY

THE EVOLUTION OF ANTARCTIC YEASTS: DNA BASE COMPOSITION
AND DNA-DNA HOMOLOGY

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Baharaeen, S., J. A. Bantle, and H. S. Vishniac. 1982. The evolution of Antarctic yeasts: DNA base composition and DNA-DNA homology. *Can. J. Microbiol.* 28: 406-413.

The 16 biotypes of the *Cryptococcus vishniacii* complex of anamorphic yeasts (Basidioblastomycetes), unique to the Dry Valleys of Antarctica, include 7 species separated by DNA-DNA homologies of less than 52%. Since species belonging to the complex can be as distantly related as *C. bhutanensis* (a Himalayan yeast, G+C 54.18 mol%) is to these species, a common ancestor probably originated and speciated outside of the Dry Valleys. The species *C. vishniacii* (G+C 54.52-55.48 mol%) comprises 7 varieties with greater than 59% DNA-DNA homology and must therefore have been established in the Dry Valleys long enough to have evolved these divergent genomes. In the *C. vishniacii* complex, G+C mol% values differing by more than 1 mol% are accompanied by DNA-DNA homologies of less than 23%.

INTRODUCTION

The *Cryptococcus vishniacii* complex (yeasts of basidiomycetous affinity), isolated from the soil samples of Dr. W. V. Vishniac's 1973 expedition, is, so far as known, peculiar to the Dry Valleys of Antarctica, constituting the only heterotrophic biota demonstrably indigenous to the most severe cold desert on earth (Vishniac and Hempfling, 1979a, 1979b). The examination of genetic relationships can illuminate the nature of microbial adaptation to such extreme environments, as well as the mode of evolution of adapted populations. The characteristics of the Dry Valley yeasts are presumptively adaptive if they are absent in congeners from other habitats or present in yeasts of other evolutionary origins from cold or desert habitats.

Are the 16 biotypes of the *C. vishniacii* complex the result of evolutionary divergence from a single ancestral type which became dominant as the Dry Valleys developed their present climate? While a polyphyletic origin is not entirely incompatible with *in situ* evolution, it would suggest that these yeasts are more likely to be descendants of successive establishments of airspora in niches vacated by catastrophic temperature changes or failure of water and substrate supplies in a multiply stressed, variable, environment.

We have determined the guanine plus cytosine (G+C) mol% of nuclear DNA and the degree of DNA-DNA homology among the biotypes of the *C. vishniacii* complex and between these and *C. bhutanensis*, the yeast most similar to the complex (Vishniac and Hempfling, 1979a; Baharaeen and Vishniac, 1981), in order to establish the extent of relationship and evolutionary divergence which may have occurred.

MATERIALS AND METHODS

Cryptococcus bhutanensis Goto et Sugiyama (ATCC 22461), *C. himalayensis* Goto et Sugiyama (IAM 4963), and isolates representing each biotype of the *C. vishniacii* complex were grown in 6 liters of GPYPi medium (Vishniac and Hempfling, 1979a) supplemented with $10^{-4}\%$ (V/V) of Antifoam A concentrate (Sigma Chemical Co.) in a cold room ($8-11^{\circ}\text{C}$) with vigorous stirring and aeration for about 5-6 generations. Carboys were inoculated to an optical density (650 nm) of about 0.2, from exponentially growing cultures prepared at 10°C in a New Brunswick Scientific Company gyrotory water bath shaker operated at 190 rpm. Cells were harvested by centrifugation at $3,000 \times g$ for 5 minutes in a refrigerated centrifuge at 4°C , washed once in glass-distilled water and resuspended in sucrose buffer (Price et al., 1978) for isolation of DNA.

DNA was isolated and purified by a combination of the procedures of Marmur (1961) and Bernardi et al. (1970) as described by Price et al. (1978). DNA purification was repeated if the preparation deviated more than 0.05 from the absorbance ratios $A_{260}/A_{280} = 1.86$ and $A_{230}/A_{260} = 0.5$ (Mendonca-Hagler and Phaff, 1975). The 80-100 g wet weight of cells obtained yielded about 5-8 mg of highly purified DNA. Analytical ultracentrifugation of the DNA in cesium chloride showed no significant contamination with mitochondrial DNA.

The G+C mol% of nuclear DNA was calculated from three separate determinations of buoyant density in cesium chloride (optical grade, Sigma Chemical Co.) (Schildkraut et al., 1962; Szybalski, 1969) in a Beckman Model L5-50 preparative ultracentrifuge equipped with Beckman

Prep UV Scanner and an An-F analytical rotor modified for the use in the L5-50. *Micrococcus luteus* (obtained from Dr. E. A. Grula, Department of Microbiology, Oklahoma State University) DNA was used as reference. This DNA had a buoyant density of 1.731 g/mL when compared with the DNA of *Escherichia coli* K-12, NX-185, plasmid-free (obtained from A. Rashtchian, Department of Medical Microbiology, University of Nebraska Medical Center) the density of which was calculated to be 1.710 g/mL, using the formula given in Beckman Prep UV Scanner instruction manual LUV-IM-2, 1975.

Purified native DNA was sheared before radioactive labeling by one passage through a French pressure cell (Aminco Model J4-3339) at 36,000 psi, after adjusting to a concentration of 250 µg/mL in Tris-EDTA buffer {tris(hydroxymethyl)-aminomethane hydrochloride, 10 mM; ethylenediamine tetraacetic acid, disodium salt, 1 mM; pH 8.2}. The sheared DNA was passed through a 0.45 µm pore-size Metrical filter. The number average nucleotide size of sheared DNA was determined by sucrose density gradient centrifugation (Bantle and Hahn, 1976), using $\phi\chi$ -174 DNA and λ phage DNA (Miles Laboratories) as references, to be 500 base pairs (3.0×10^5 daltons). Gradients were centrifuged at 38,000 rpm, using a Beckman SW-41 rotor, for 20 hours at room temperature.

The sheared DNA was labeled with methyl, 1',2'- ^3H -thymidine *in vitro* by nick translation techniques described by Rigby *et al.* (1977) and Balmain and Birnie (1979), using the New England Nuclear ^3H -Nick Translation Kit (NEK-005). After incubation for 2.5 hours at 13°C (determined to allow maximum tracer incorporation), 80 µL of carrier DNA (400 µg/mL of sheared calf thymus DNA in 300 mM sodium acetate)

was added to the 20 μ L reaction mixture and the reaction was stopped immediately by the addition of an equal volume of a chloroform-phenol preparation (1:1, phenol, Bethesda Research Laboratories, ultrapure, saturated in Tris-EDTA buffer and treated with 0.01% 8-hydroxyquinoline). The mixture was incubated on ice for 15 minutes with occasional vortexing, then separated into two phases by centrifugation at 12,500 x g (Eppendorf microcentrifuge) for 10 minutes. The upper (aqueous) layer was added to 1 mL of cold 95% ethanol and incubated overnight at -20°C . The DNA precipitate was collected by centrifugation, dried in a vacuum desiccator, and redissolved in 50 μ L of sodium phosphate buffer (140 mM, pH 6.86). The resulting preparations of DNA had a number average nucleotide size of 380 base pairs (1.14×10^5 daltons) and specific activities of 2.2×10^6 to 2.7×10^6 cpm/ μ g.

Single stranded labeled DNA (ssDNA) was prepared and renaturation kinetics and sequence complementarity determined by the following modifications of the method of Price et al. (1978). Sealed siliconized reaction vials containing 0.02 μ g of labeled ssDNA unique sequence probe (separated on hydroxyapatite after incubation to E_{Cot} of 0.7 mol.sec./L) and 20 μ g of sheared homologous or heterologous DNA (in 280 mM sodium phosphate buffer pH 6.86, to a final volume of 50 μ L) were denatured at 105°C for 10 minutes. The temperature was then reduced to 67°C (25°C below the thermal denaturation point, calculated from G+C mol% values) and incubation continued to an E_{Cot} of 280 mol. sec./L). E_{Cot} values were calculated by the method of Britten et al. (1974).

The remaining ssDNA was removed with S_1 nuclease by the method of Maxwell et al. (1978). The vial contents were diluted with 450 μ L

of S_1 nuclease buffer, 200 μ L removed for determination of total radioactivity, and μ L of S_1 nuclease (Bethesda Research Laboratories, 99.995% single stranded specific) added to a total concentration of 8,000 U/mL. After 60 minutes incubation at 37°C, a second 200 μ L sample was removed. Samples were applied onto DEAE cellulose filter discs (Whatman, Inc.) and left undisturbed for 5 minutes at room temperature before washing, drying, and counting.

Filters with "total radioactivity" samples were washed with 5 mL of 140 mM sodium phosphate buffer for 5 minutes, eluting about 2.9% of their radioactivity. Filters with S_1 nuclease treated samples were washed for 15 minutes with three 5 mL changes of 480 mM sodium phosphate buffer and with glass distilled water. All filters were then washed with 95% ethanol. Washing is known to remove very small pieces of DNA (below 15 base pairs) which react non-specifically with heterologous DNA (McConaughy and McCarthy, 1967). The size of the fragments removed in our procedures was determined by horizontal agarose gel electrophoresis modified from the method of McDonnell et al. (1977). Samples (40-50 μ L) and tracking dye mixture (10 μ L; polyethylene glycol, 10%, orange G, 1%, xylene-cyano-fluorophosphate, 0.05%; bromophenol blue, 0.02%) were run into gel (1.2% agarose in Tris-borate buffer: Tris-HCl, 89 mM, boric acid, 89 mM, EDTA, 2.5 mM, pH 8.0) at 50 mA for 1 hour and separated at 30 mA for an additional 5-6 hours. The gel was then sliced (0.5 x 1 cm pieces) and counted. Water washing removed fragments up to and including 7.4×10^4 daltons (245 base pairs) from "total radioactivity" filters; 140 mM sodium phosphate buffer did not remove additional fragments. Washing with 480 mM sodium phosphate buffer removed fragments mostly sized between

1.0 and 2.7×10^4 daltons (50-100 base pairs) from S_1 nuclease treated homologous hybridized samples. Since the size of the 245 base pair peak from washing of nuclease treated and untreated samples was the same, we concluded that the small loss was immaterial, probably representing unattached DNA fragments.

Washed filters were dried under an infra-red lamp, placed in 10 mL of a toluene based scintillation fluid (Beckman, Ready-Solv, HP), vortexed vigorously and counted at the 2% error level in a Beckman LS-7500 liquid scintillation counter equipped with Texas Instruments Silent 700 electronic terminal. Counting efficiency was 30-35%.

Hybridization experiments were conducted in triplicate, unless otherwise indicated. The results were not corrected for duplex formation in probe DNA at zero time or at $E_{Cot} 280$. Duplex formation at zero time in biotype 7 probe DNA was $1.77 \pm 0.05\%$ actual binding, in biotype 10 probe DNA $1.69 \pm 0.00\%$ actual binding. "Self reannealing" in biotype 7 probe DNA was $3.99 \pm 0.01\%$ actual binding.

RESULTS

Base ratios of nuclear DNA of the 16 biotypes of the *C. vishniacii* complex and of *C. bhutanensis* and *C. himalayensis* are shown in Table I.

The renaturation kinetics of homologous DNA (*C. vishniacii* biotype 7, the type of the species) are shown in Figure 1. The Britten-Kohne(1968) plot (Figure 1a) shows that the reaction was essentially complete at $E_{Cot} 210$ with 88.14% renaturation. Genome size, estimated from the $E_{Cot}^{1/2}$ value of 41.8 mol.sec./L by the method of Britten and Kohne(1968), was 13×10^9 daltons. The modified Wetmur-Davidson (1968)

TABLE I. Base composition of nuclear DNA from *Cryptococcus vishniacii* complex and related yeasts

Organisms	Strain designation ^a	Biotype	Mol% G+C	± SD ^b
<i>Cryptococcus vishniacii</i> complex				
	MYSW 302Y216	1	54.52	0.32
	303Y365	2	54.86	0.31
	303Y200	3	55.03	0.06
	309Y215	4	55.48	0.21
	202Y212	5	55.44	0.16
	306Y212	6	55.34	0.06
	304Y268 (type)	7	54.97	0.16
	303Y338	8	54.63	0.06
	303Y206	9	55.68	0.06
	302Y265	10	54.80	0.18
	202Y245	11	55.27	0.31
	202Y252	12	53.27	0.21
	202Y375	13	53.30	0.16
	302Y259	14	54.76	0.21
	303Y336	15	55.82	0.27
	302Y310	16	55.07	0.12
<i>Cryptococcus bhutanensis</i>	ATCC 22461 (type)		54.18	0.21
<i>Cryptococcus himalayensis</i>	IAM 4963 (type)		57.97	0.20

^aMYSW: Dr. H. S. Vishniac, Department of Microbiology, Oklahoma State University, Stillwater, Oklahoma; ATCC: American Type Culture Collection, Rockville, Maryland; IAM: Institute for Applied Microbiology, University of Tokyo, Tokyo, Japan.

^bStandard deviation.

Figure 1. Renaturation kinetics of homologous DNA, *Cryptococcus vishniacii* biotype 7. (a) Britten-Kohne plot. (b) Wetmur-Davidson plot.

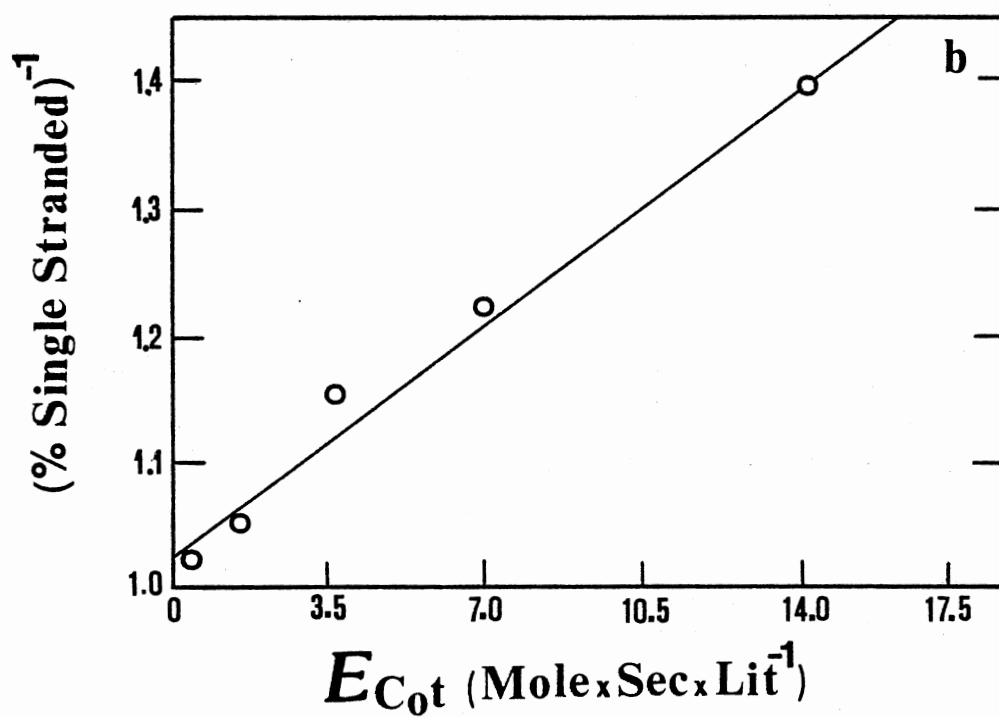
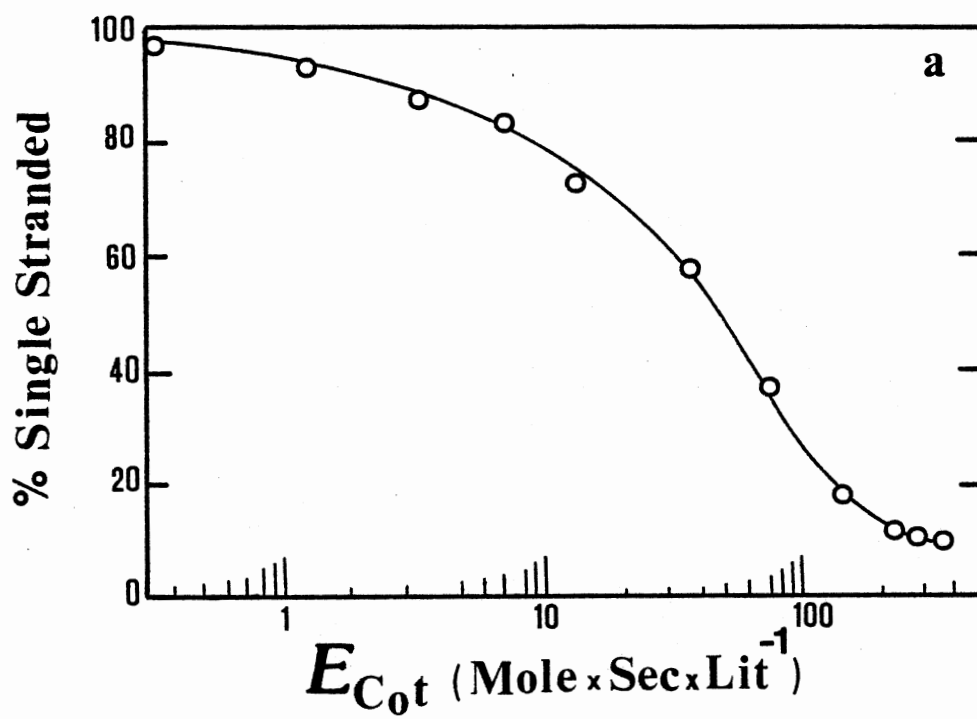


TABLE II. DNA-DNA hybridization: *Cryptococcus vishniacii* complex
 biotype 7 probe DNA

Source of unlabeled DNA		% actual binding \pm SD	% relative binding
strain	biotype		
304Y268	7	87.74 \pm 0.13	100
303Y338	8	85.55 \pm 1.17	97.50
202Y312	5	80.51 \pm 0.44	91.76
303Y200	3	77.16 \pm 0.26	87.94
303Y365	2	74.57 \pm 0.28	84.99
302Y216	1	74.30 \pm 0.25	84.38
309Y215	4	70.82 \pm 0.35	80.72
202Y349	11	66.10 \pm 0.35	75.34
302Y265	10	65.06 \pm 0.39	74.15
302Y259	14	29.13 \pm 0.03	33.20
306Y212	6	24.56 \pm 0.21	27.99
303Y206	9	19.80 \pm 0.13	22.57
302Y310	16	18.75 \pm 0.11	21.37
303Y336	15	17.51 \pm 0.10	19.96
202Y252	12	13.63 \pm 0.13	15.53
202Y375	13	13.63 \pm 0.11	15.53
Calf thymus	--	5.56 \pm 0.05	6.34

"total radioactivity" filters were not washed in this experiment.

TABLE III. DNA-DNA hybridization: *Cryptococcus vishniacii* complex
 biotype 7 probe DNA

Source of unlabeled DNA		% actual binding \pm SD	% relative binding
strain	biotype		
304Y268	7	91.09 \pm 0.34	100
302Y309	4	75.16 \pm 0.69	82.51
303Y202	6	24.63 \pm 0.07	27.04
306Y205	6	23.94 \pm 0.31	26.28
202Y256	12	13.88 \pm 0.12	15.24
<i>C.bhutanensis</i>	-	11.00 \pm 0.04	12.08

TABLE IV. DNA-DNA hybridization: *Cryptococcus vishniacii* complex

biotype 10 probe DNA

Source of unlabeled DNA		% actual binding \pm SD	% relative binding
strain	biotype		
302Y265	10	93.69 \pm 0.12	100
202Y345	11	77.72 \pm 0.48	82.95
303Y338	8	70.28 \pm 0.11	75.01
304Y268	7	70.02 \pm 0.41	74.74
302Y216	1	62.25 \pm 0.43	66.44
303Y200	3	61.13 \pm 0.06	65.25
303Y365	2	57.28 \pm 0.35	61.14
202Y312	5	56.55 \pm 0.72	60.36
309Y215	4	56.27 \pm 0.11	60.06
302Y259	14	44.93 \pm 0.09	47.96
306Y212	6	42.97 \pm 0.52	45.86
303Y336	15	40.38 \pm 0.46	43.10
303Y206	9	32.14 \pm 0.13	34.30
302Y310	16	26.92 \pm 0.06	28.73
202Y252	12	19.36 \pm 0.07	20.66
202Y375	13	19.34 \pm 0.08	20.64
<i>C. bhutanensis</i>	--	12.75 \pm 0.09	13.61

TABLE V. DNA-DNA hybridization: *Cryptococcus vishniacii* complex

biotype 3 probe DNA

Source of unlabeled DNA		% actual binding \pm SD	% relative binding
strain	biotype		
303Y200	3	94.30 \pm 0.98	100
309Y215	4	91.38 \pm 0.28	96.90
303Y216	1	90.70 \pm 1.24	96.18
303Y365	2	90.53 \pm 0.48	96.00
202Y312	5	85.65 \pm 0.43	90.83
304Y268	7	83.57 \pm 1.44	88.62
202Y349	11	64.53 \pm 0.34 ^a	68.43
302Y265	10	58.93 \pm 0.54 ^a	62.49

^a determined from duplicate vials.

TABLE VI. DNA-DNA hybridization: *Cryptococcus vishniacii* complex

biotype 14 probe DNA

Source of unlabeled DNA		% actual binding \pm SD	% relative binding
strain	biotype		
302Y259	14	94.36 \pm 0.36	100
303Y336	15	48.64 \pm 0.47	51.55
302Y265	10	48.05 \pm 0.23	50.92
302Y310	16	35.55 \pm 0.13	37.67
303Y206	9	32.06 \pm 0.31	33.98
306Y212	6	26.69 \pm 0.22	28.29
202Y252	12	21.65 \pm 0.17 ^a	22.94

^adetermined from duplicate vials.TABLE VII. DNA-DNA hybridization: *Cryptococcus vishniacii* complex

biotype 6 probe DNA

Source of unlabeled DNA		% actual binding \pm SD	% relative binding
strain	biotype		
306Y212	6	90.59 \pm 1.52	100
302Y265	10	38.22 \pm 0.92	42.19
302Y259	14	30.26 \pm 0.65	33.40
303Y206	9	28.56 \pm 0.40	31.53
303Y336	15	25.83 \pm 0.14	28.51
303Y310	16	25.76 \pm 0.07	27.09
202Y252	12	18.92 \pm 0.52 ^a	20.89

^adetermined from duplicate vials.

TABLE VIII. DNA-DNA hybridization: *Cryptococcus vishniacii* complex
biotype 16 probe DNA

Source of unlabeled DNA		% actual binding \pm SD	% relative binding
Strain	biotype		
303Y310	16	93.37 \pm 1.08	100
302Y259	14	29.53 \pm 1.72	31.63
306Y212	6	26.56 \pm 0.42	28.45
303Y206	9	26.08 \pm 0.62	27.93
302Y265	10	26.00 \pm 0.52	27.85
303Y336	15	24.60 \pm 1.50	26.35
202Y252	12	19.86 \pm 0.23 ^a	21.27

^a determined from duplicate vials.

TABLE IX. DNA-DNA hybridization: *Cryptococcus bhutanensis* ATCC
22461 probe DNA

Source of unlabeled DNA		% actual binding \pm SD	% relative binding
strain	biotype		
<i>C. bhutanensis</i>	-	92.76 \pm 1.67	100
<i>C. vishniacii</i> complex			
202Y252	12	15.73 \pm 0.31	16.97
202Y375	13	15.61 \pm 0.21	16.83
302Y265	10	15.21 \pm 0.11	16.40
302Y259	14	14.77 \pm 0.33	15.92
302Y310	16	14.68 \pm 0.47	15.83
304Y268	7	14.30 \pm 0.08	15.42

Rapidly reannealing sequences were not removed from the radiolabeled DNA in this experiment and the homology values were determined from duplicate vials.

plot (Figure 1b) of early data points in renaturation indicated that about 1.98% rapidly reannealing sequences remained in the radiolabeled preparation, a value similar to duplex formation at zero time as determined above.

Tables II through IX present the results of hybridization experiments with appropriate radiolabeled DNAs as % actual binding and relative (to the homologous DNA) binding.

DISCUSSION

These data show that more than one species is represented in the *C. vishniacii* complex. Since speciation is an ongoing process in nature, there can be no absolute level of DNA-DNA homology which separates the specific and varietal taxonomic levels. Price et al. (1978) have suggested that 80% (or more) homology should be considered evidence of conspecificity and 20% (or less) homology a conclusive bar to conspecificity, in part because intermediate values were, at the time, rare in yeasts. The correlation of DNA homology and the production of fertile offspring (as an indicator of conspecificity) is further complicated by the probability that very minor changes in total genome may interdict mating while far more extensive changes can leave the mating process intact. Kurtzman et al. (1980) found that base sequence complementarity averaging only 24% between *Issatchenkia scutulata* varieties *scutulata* and *exigua* permitted the production of 3-6% viable, fertile, ascospores which did not appear to be amphidiploids. While the cytology of meiosis at this level of complementarity should be very interesting, the low fertility of

this mating suggests that its products are not likely to be found in nature.

The usefulness of G+C mol% is limited to the exclusion of conspecificity. Among the ascomycetous yeasts, a difference of 1 mol% implies a level of DNA homology which will not permit normal meiosis (Price et al., 1978). The basidiomycetous yeasts are fewer and less extensively studied. The G+C mol% values for *Rhodospiridium* species reported by Nakase and Komagata (1972) are consonant with the 1% exclusion rule, but other reports are not. The interfertility of strains of *Filobasidiella neoformans* differing by more than 1 mol% G+C (Aulakh et al., 1981) could conceivably be an artifact of the method of G+C determination; the apparent interfertility of strains CBS 490, G+C 63.5 mol% (Storck et al., 1969), and CBS 2630, G+C 65.0 mol% (Storck et al., 1969), of *Aessosporon salmonicolor* (Fell and Tallman, 1980) was not followed through meiosis (teliospore germination).

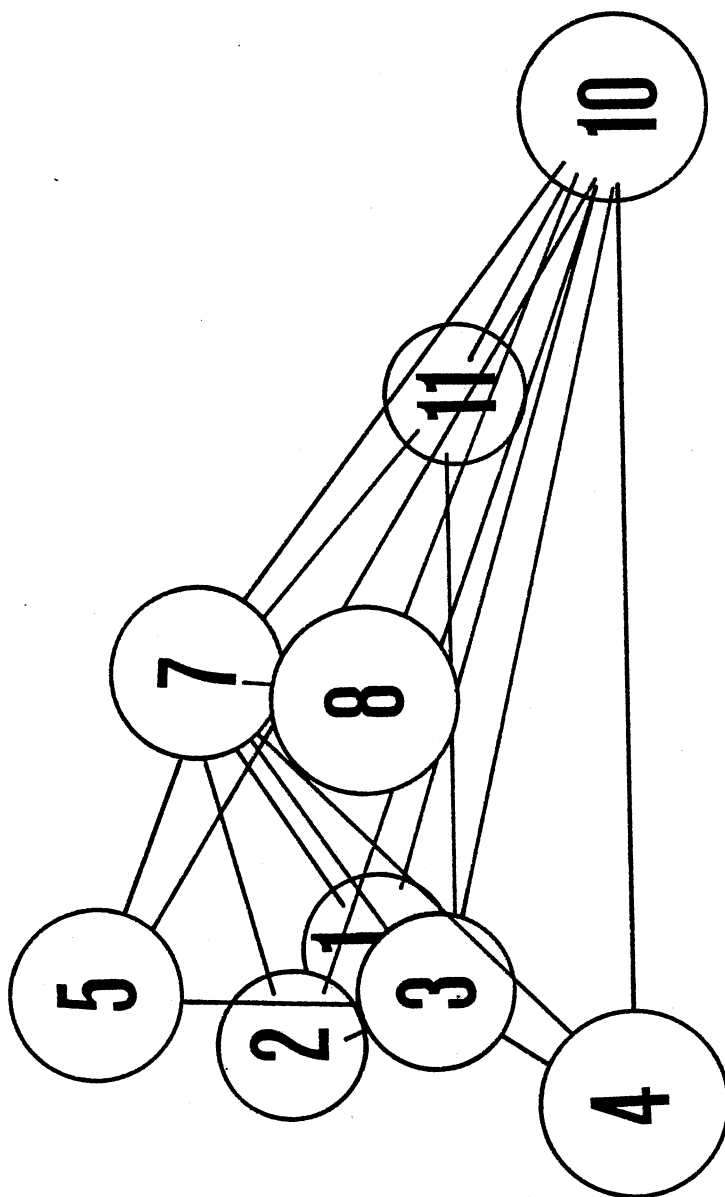
The low DNA-DNA homology between biotypes 12 and 13 of the *C. vishniacii* complex and the remaining biotypes (Tables II-IV, VI-VIII) indicates that the 1 mol% exclusion rule holds in this case. These biotypes, differing by about 1.2 mol% from the rest of the complex (Table I), have been described as *Cryptococcus lupi* (Baharaeen and Vishniac, IN PRESS). *Cryptococcus bhutanensis* is confirmed as a distinct species by the low DNA-DNA homology between the type strain and *C. vishniacii* complex biotypes differing from it by less than 1 G+C mol% (Table IX).

The DNA-DNA homology between biotypes of the *C. vishniacii*

complex frequently have values between 21 and 79%. The fertility of sexual hybrids cannot be determined since sexual reproduction is unknown in these (anamorphic) yeasts. We consider, somewhat arbitrarily, that homology values at 60% and above indicate conspecificity with the type strain of *C. vishniacii*. Above 59% homology, the results of the three probes (biotypes 3, 7, and 10) triangulate neatly into a pattern (Figure 2). Comparison of reciprocal hybridizations indicates the precision with which these phylogenetic distances have been determined. The mean of differences in the 11 reciprocal hybridizations was $2.74 \pm 1.79\%$, indicating a variation considerably greater than that of the individual homology experiments. While the smallest differences (0.59, 0.68%) were found in reciprocal hybridizations between the biotypes with homology above 74%, differences were not correlated with phylogenetic distances. In the group thus circumscribed as the species *C. vishniacii* no single biotype had less than 74% homology with some other biotypes in the group.

Homology between *C. vishniacii* and the remaining biotypes of the complex, and among the remaining biotypes, was lower than 52%. Closely related, but sexually isolated, species of *Filobasidiella* have been reported to show 55-63% relatedness, including 9% relatedness due to base pair mismatch (Aulakh et al., 1981). Base pair mismatch and random fragment match (e.g. the 6.34% homology between calf thymus and biotype 7 probe DNA in Table II) may be assumed to account for larger proportions of the perceived relatedness at greater phylogenetic distances. The unit distance must be sized differently as distance values become larger; biotypes 9 and 15 when

Figure 2. Genetic relationships between biotypes of the species *Cryptococcus vishniacii*. The length of the connecting lines is proportional to the phylogenetic distance (100 minus relative binding percentage of heterologous hybridization) between biotypes.



positioned from the two most homologous probes, are much farther from the other two probes used than the measured phylogenetic distance. Biotypes 9 and 15 cannot be made to come within specific distances of each other in a three-dimensional plot. For these reasons, we consider biotypes 6, 9, 14, 15, and 16, as well as *C. bhutanensis*, *C. lupi*, and *C. vishniacii*, distinct species.

The tools which we have used are inadequate for evaluating relationships at taxonomic levels above species. The informed intuition which has defined the form-genus *Cryptococcus* has not excluded species subsequently found to differ at the class level (reviewed in Baharaeen and Vishniac, 1981). Our data do not reliably demonstrate any greater evolutionary distance between *C. bhutanensis* and the Antarctic yeasts than between *C. lupi* and other species of the *C. vishniacii* complex. *Cryptococcus bhutanensis* is a Himalayan (Laya, Bhutan) yeast (Goto and Sugiyama, 1970). This argues strongly that the Antarctic yeasts are at least diphyletic and that a generic prototype existed and speciated outside of the Dry Valleys.

The biotypes within the species *C. vishniacii* are clearly of monophyletic origin. The biotypes which fail to differ by 6% (the largest reciprocal difference) in responding to one of the alternative probes should not be considered genetically distinct. Biotypes 7 and 8 differ at most by 2.5% (in response to biotype 7 probe DNA); the only phenetic difference was seen in a growth/temperature screen. Biotypes 1 and 2, differing by 5.3% in response to biotype 10 probe DNA, differ phenetically only in ammonium sensitivity (not quantitated) (Vishniac and Hempfling, 1979a). Biotypes 3, 4, 5, 10, and 11 appear

genetically distinct from each other and from 1,2 and 7,8, and should be accorded varietal status. Since the adaptive fit between observed varietal differences and microhabitat is unknown, we cannot differentiate between selection and drift in intraspecific evolution. Since we have not distinguished between functional and non-functional base sequence changes, the time scale of intraspecific evolution can only be guessed at. It is, however, evident that *C. vishniacii* is a species which may well have originated in the Dry Valleys of Antarctica and that it has been established there long enough to have evolved to at least 7 more or less divergent genomes.

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CHAPTER IV

CRYPTOCOCCUS LUPI SP. NOV., AN ANTARCTIC

BASIDIOMYCETE

CRYPTOCOCCUS LUPI SP. NOV., AN ANTARCTIC BASIDIOMYCETE

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Cryptococcus lupi sp. nov. was isolated from soil samples from the Dry Valleys of South Victoria Land, Antarctica. The guanine plus cytosine content (53.3 mol%) of *C. lupi* deoxyribonucleic acid differs by more than 1.5 mol% from the guanine plus cytosine contents of biotypes originally described under the name *Cryptococcus vishniacii*. The type strain of *C. lupi* (MYSW 202Y252 = ATCC 44529) differs phenotypically from the type strain of *C. vishniacii* in that it assimilates cellobiose, methyl- α -D-glucoside, and salicin and fails to assimilate γ -amino butyric acid, gluconate, 2-ketogluconate, 5-ketogluconate, L-rhamnose, and succinate; it also differs by having a higher maximum growth temperature and its cell size and ratio of cell width to length.

The only heterotroph demonstrably indigenous to the barren (lichen-free) sites of the Dry Valleys of Antarctica are yeasts belonging to the *Cryptococcus vishniacii* complex. Determinations of the guanine plus cytosine (G+C) contents of the deoxyribonucleic acids (DNAs) of the organisms in this complex allowed the segregation of

biotypes 12 and 13 as a new species, for which we propose the name *Cryptococcus lupi* (lu'pi. L. noun *lupus* wolf; L. gen. *lupi* of wolf: in honor of Wolf Vladimir Vishniac, 1922-1973, who collected the soil samples from which these yeasts were isolated).

MATERIALS AND METHODS

The methods of collection, isolation, and characterization of the yeasts have been described elsewhere (15,16). For additional physiological studies we used the following methods:

Cycloheximide resistance was determined by visual estimation of growth of GPYPi (0.5% glucose, 0.5% peptone, 0.3% yeast extract, 10 mM potassium phosphate buffer, pH 6.86) agar to which an appropriate amount of cycloheximide stock solution in 95% ethanol was added when the agar was still hot from autoclaving. No growth during the 19-day observation period was referred to as suppressed, and growth delay was referred to as inhibition.

The method of determining the temperature limits of growth has been described previously (15,16). The temperature for optimum growth was determined by transferring to fresh GPYPi medium a volume of exponentially growing (at 10°C) cells sufficient to yield an optical density at 650 nm of 0.2. These secondary cultures were grown at the desired temperatures on a refrigerated gyrotory water bath shaker (New Brunswick Scientific Co.) at 190 rpm. At appropriate intervals, samples (1 ml) were removed without stopping the shaker for determination of the change in optical density (Bausch & Lomb Spectronic 70 spectrophotometer) with time.

The other methods used in the morphological studies have been described previously (1).

Coenzyme Q (CoQ) was isolated by the method of Yamada and Kondo (17). The CoQ was identified by co-chromatography with standard CoQs (Sigma Chemical Co.) in a reverse-phase system, using petrolatum-coated (2.5% in toluene) Whatman No. 1 paper and *N,N*, dimethyl formamide (97:3) in water as the solvent system.

Reactions to diazonium blue B were determined by applying freshly prepared, chilled diazonium blue B reagent to 3-week old colonies on GPYPi agar, as described by van der Walt and Hopsu-Havu (14). A positive reaction was recorded when colonies developed a dark red color within 1 to 2 minutes at room temperature.

DNA extraction and purification was accomplished by a combination of the procedures of Marmur (9) and Bernardi et al. (4) as described by Price et al. (11). DNA purification was repeated if the preparation deviated more than 0.05 units from a ratio of absorbance at 260 nm to absorbance at 280 nm of 1.86 and a ratio of absorbance at 230 nm to absorbance at 260 nm of 0.50 (10). The G+C content of the nuclear DNA was calculated from three separate determinations of buoyant density in cesium chloride (optical grade, Sigma Chemical Co.) (12,13) in a Beckman model L5-50 preparative ultracentrifuge equipped with a Prep UV Scanner (Beckman) and an An-F analytical rotor modified for use in the L5-50 ultracentrifuge (Beckman). *Micrococcus luteus* (kindly provided by E. A. Grula, Department of Microbiology, Oklahoma State University) DNA was used as a reference; this DNA had a buoyant density of 1.731 g/ml compared with DNA of

Escherichia coli K-12 strain NX-185 (plasmid-free; obtained from A. Rashtchian, University of Nebraska Medical Center), the density of which was calculated to be 1.710 g/ml from the formula given in the *Prep UV Scanner Instruction Manual* (3).

RESULTS

Latin diagnosis of *Cryptococcus lupi* sp. nov. Coloniae in agaro colore cremeo, aut politae et mucosae aut asperae et durae; in mediis liquidiis difficiliter crescit, pelliculam haud, annulum exigue, et sedimentum lente formans. Cellulae crescentes forma ova vel lagonae, ca. 5 x 6 μ m, cum capsulis exiguis, e situ cicatricis natalis iterumque iterumque generatae. Parietes typici Basidioblastomycetium. Nec pseudomycelium nec mycelium formatur. Non generant sed per modo blastico asexuali, calore minus 4°C usque ad 25°C.

Fermentatio nulla. Assimilat L-arabinosum, cellobiosum, acidum citricum, fructosum, glucosum, acidum D-glucuronicum, acidum D-glutamicum (variabile), maltosum, mannosum, melezitum, sucrosum, trehalosum, D-xylosumque. Amylosum formatum est. Crescit cum nitrato; crescit sine vitaminis. Gelatinum et urea non finduntur. CoQ₉ continens; diazonium blue B respondens. G+C ca. 53.3 mol%.

Standard description of *Cryptococcus lupi* sp. nov. This species is typified by isolate MYSW 202Y252, which was isolated from coarse, weathered dolerite gravel from the north flank of Mount Baldr (South Victoria Land, Antarctica) at 6,220 feet (1,896 m). The species

comprises isolates originally described as *C. vishniacii* biotypes 12 (the type isolate and from other Dry Valley sites, isolates 302Y253, 303Y267, and 306Y250) and 13 (isolates 202Y375 and 303Y368) (15). The type strain (MYSW 202Y252) has been deposited in the American Type Culture Collection, Rockville, Md., as ATCC 44529.

Colonies on glucose-peptone-yeast extract agar are cream colored, entire, glistening, and somewhat mucoid; on malt extract agar, they are tough and granular. Growth in liquid media is limited unless agitation and aeration are provided; in tubes, a scanty annulus (no pellicle) and slowly produced sediment provide the only evidence of growth. Exponentially growing cultures may produce very small amounts of rudimentary pseudomycelium; pseudomycelium and mycelium are not formed. Sexual reproduction is unknown.

Cells in exponentially growing GPYPi cultures are ovoid to flask shaped, averaging 5.06 (standard deviation ± 0.71) by 6.11 (standard deviation ± 0.71) μm , with a ratio of width to length of 0.83 ± 0.07 . Cells are thinly encapsulated (Fig. 1). Budding is monopolar and repetitive through the birth scar site (1); primary buds are holoblastic (Fig. 2), and secondary buds are enteroblastic, with the septum formed at the level of the characteristic frayed collar of Basidioblastomycetes (Fig. 3).

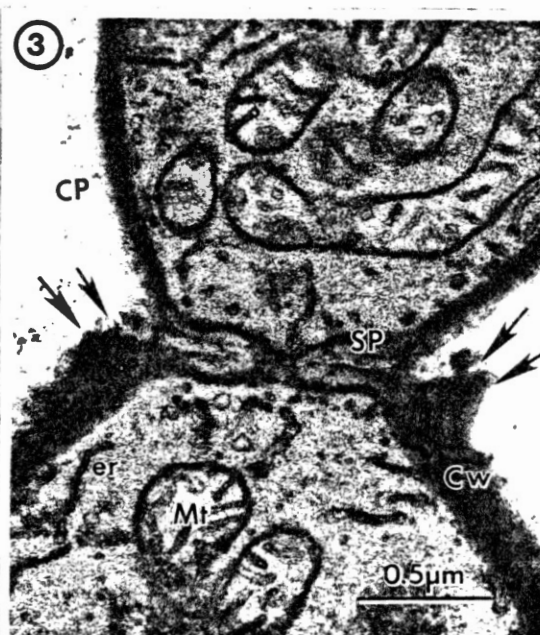
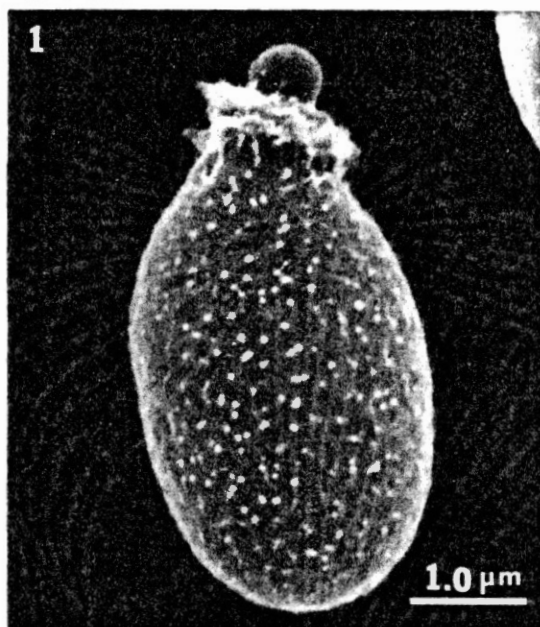
Fermentation does not occur.

Carbon compounds assimilated: L-arabonose, cellobiose, citrate, fructose, glucose, D-glucuronate, L-glutamate (variable response), maltose, mannose, melezitose, methyl- α -D-glucoside, raffinose, salicin, sucrose, tartrate (variable response), trehalose, and D-xylose. Amylose is produced during growth on L-arabinose, glucose, or xylose.

Fig. 1. Scanning electron micrograph of *C. lupi* strain 202Y252 multiparous budding cell showing the characteristic frayed collar of basidioblastomycetes. The capsule, collapsed as a result of critical point drying, appears as nodules and lines on the cell surface.

Fig. 2. Transmission electron micrographs of *C. lupi* strain 202Y252 cell with the primary bud still attached. Note the continuation of the whole mother cell wall with that of the bud (holoblastic). CW, Cell Wall; Mt, mitochondrion, Nu, nucleus; v, vacuole.

Fig. 3. Transmission electron micrograph of *C. lupi* strain 202Y252 showing secondary budding. The bud cell wall is continuous only with the inner mother cell wall layer(s)(enteroblastic). The arrow heads indicate the frayed outer layers making up the bud scar collar. CP, Remains of the capsule; CW, cell wall; er, endoplasmic reticulum, Mt, mitochondrion, SP, septum.



The following compounds were not assimilated as sole source of carbon and energy (arbutin and methanol were not tested): acetate, amino acids other than glutamate (L-alanine, γ -aminobutyrate, L-arginine, L-aspartate, glycine, L-isoleucine, L-lysine, L-methionine, L-ornithine, L-phenylalanine, L-proline, and L-valine), D-arabinose, D-arabitol, L-arabitol, butyrate, decane, erythritol, ethanol, D-fucose, fumarate, galactitol, D-galactose, D- δ -gluconolactone, glycerol, *myo*-inositol, inulin, 2-ketogluconate, 5-ketogluconate, DL-lactate, lactose, L-malate, D-mannitol, melibiose, L-rhamnose, ribitol, D-ribose, L-sorbose, starch, succinate, and xylitol.

Nitrogen sources utilized: NH_4Cl , KNO_3 , L-amino acids (L-alanine, L-arginine, L-aspartate, L-glutamate, glycine, L-histidine, L-isoleucine, L-lysine, L-methionine, L-ornithine, L-phenylalanine, L-proline, and L-valine). Not utilized: D-alanine, creatinine, cytosine, ethylamine, nicotinic acid, thiamine, thymine, and uracil.

Gelatin and urea are not hydrolyzed.

Externally supplied vitamins are not required.

Growth occurs from less than 4 to 25°C; optimal growth is at 17°C.

Growth is inhibited by 0.05 μg of cycloheximide per ml and is suppressed by 0.5 μg of cycloheximide per ml.

Ubiquinone CoQ_9 is produced.

Diazonium blue B: positive.

G+C content of nuclear DNA: 53.27 ± 0.21 mol%.

DISCUSSION

Observation of colony color (not red or pink) and determination

of assimilation of L-arabinose (positive), galactose (negative), 2-ketogluconate (negative), lactose (negative), and mannitol (negative) under conditions (temperature and media) appropriate for *C. lupi* allow presumptive identification of isolates with this species rather than any other yeast species described previously. *C. lupi* differs from the type strain of *C. vishniacii* by the assimilation of cellobiose, methyl- α -D-glucoside, and salicin, by failure to assimilate γ -aminobutyrate (type slow), gluconate (type slow), 2-ketogluconate, 5-ketogluconate (type slow), L-rhamnose (type weak), and succinate, by higher optimal and maximal growth temperatures, and in cell size and the ratios of cell width to length (1). *C. lupi* differs from *C. vishniacii* biotypes 10, 11, and 16 in the following characters: failure to assimilate γ -aminobutyrate (biotypes 10 and 11 slow), L-aspartate (biotypes 10, 11, and 16 slow), 2-ketogluconate (biotypes 10, 11, and 16 slow), L-rhamnose (biotype 16, positive), or succinate (biotypes 10 and 11 positive) and lower susceptibility to cycloheximide (biotype 16, suppressed by 0.05 μ g/ml).

The use of G+C content of the nuclear DNA to exclude conspecificity has become a standard procedure in the taxonomy of yeasts known only as anamorphs. A difference of more than 1 mol% is considered to preclude significant DNA-DNA homology in yeasts (11); that is, such a difference is associated with a failure to mate with the reproduction of fertile recombinant offspring in teleomorphic strains. This information is based on studies with ascomycetous yeasts and asco-blastomycetes and has yet to be confirmed for the less well known basidioblastomycetes. The biotypes assigned here to *C. lupi* show no

significant differences in phenotype (15,16) or in G+C content of nuclear DNA (isolate 202Y252, 53.27 ± 0.21 mol%; isolate 202Y375, 53.30 ± 0.16 mol%) but differ by at least 1.5 mol% from the values determined for other biotypes of *C. vishniacii*. The DNA of the type strain of *C. vishniacii* contains 54.97 ± 0.16 mol% G+C; biotype 10 (the closest biotype) contains 54.80 ± 0.19 mol% G+C. DNA-DNA homology is less than 20% (unpublished data).

Our interest in the taxonomy of these yeasts derives from their importance in analysis of the Antarctic ecosystem. We need genetic measures of relatedness because lack of equivalence of taxonomic levels has been a major problem in the application of theoretical ecology to ecosystems containing both macrobes and microbes. We need (and have attempted) to find easily determined descriptors other than those generally used in yeast identification both because these fail to distinguish recognized species (8) and because we need to identify diversity at subspecific levels. Finally, although there are practical limits to the number of assimilation tests that can be performed, as well as the theoretical objection that habitats may not provide unmixed substrates, extended test series provide a major source of data on resource utilization. Apart from airspora, the closest resources for heterotrophs in the more barren areas of the Dry Valleys of Antarctica are endolithic lichens (*Buellia*) (6) and cyanobacteria (7). The failure of yeasts belonging to the *C. vishniacii* complex to utilize any commercially available polyols makes it unlikely that these organisms can derive immediate benefit from association with the more common chlorophycean lichens (5).

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CHAPTER V

FIVE NEW BASIDIOBLASTOMYCETOUS YEAST SPECIES

SEGREGATED FROM *CRYPTOCOCCUS VISHNIACII*

EMEND. AUCT., AN ANTARCTIC YEAST

SPECIES COMPRISING FOUR

NEW VARIETIES

FIVE NEW BASIDIOMYCETOUS YEAST SPECIES SEGREGATED
FROM *CRYPTOCOCCUS VISHNIACII* EMEND. AUCT., AN ANTARCTIC
YEAST SPECIES COMPRISING FOUR NEW VARIETIES

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ABSTRACT

The *Cryptococcus vishniacii* complex (H. S. Vishniac and W. P. Hempfling, *Int. J. Syst. Bacteriol.* 29: 153-158) consists of seven species possessing less than 52% DNA-DNA homology: *C. lupi*, *C. vishniacii*, and five new species. *Cryptococcus vishniacii* emend. auct. Vishniac et Hempfling (additional characters include assimilation of L-aspartate, L-glutamate, 2-ketogluconate, and succinate) includes *C. vishniacii* var. *vishniacii* var. nov. (MYSW 304Y268 = ATCC 36649) assimilating (besides substrates common to the species) L-arabinose, citrate, L-proline (weakly), sucrose, raffinose, and L-rhamnose (weakly), *C. vishniacii* var. *wolfii* var. nov. (MYSW 303Y216 = ATCC 46404) differing from var. *vishniacii* in assimilating fumarate and L-malate but not L-arabinose, citrate, sucrose, and raffinose, *C. vishniacii* var. *asocialis* var. nov. (MYSW 303Y312 = ATCC 46402) differing from var. *vishniacii* in assimilating fumarate and L-malate but

not L-arabinose or citrate and *C. vishniacii* var. *vladimiri* var. nov. (MYSW 202Y265 = ATCC 46403) differing from var. *vishniacii* in assimilating cellobiose, methyl- α -D-glucoside and salicin but not L-proline or L-rhamnose. *Cryptococcus asgardensis* sp. nov. (MYSW 302Y310 = ATCC 46399) differs from *C. vishniacii* in being suppressed by $0.05 \mu\text{g.ml}^{-1}$ cycloheximide, from all other described yeasts in cream colonies utilizing nitrate-N and assimilating L-arabinose, cellobiose, melezitose, and L-rhamnose but not D-galactose, *myo*-inositol, or D-mannitol. *Cryptococcus baldrensis* sp. nov. (MYSW 302Y259 = ATCC 46400) differs from all other described yeasts in cream colonies utilizing nitrate-N and assimilating L-arabinose, cellobiose, 2-ketogluconate, and melezitose, but not D-mannitol or succinate. *Cryptococcus hempflingii* sp. nov. (MYSW 306Y212 = ATCC 46401) differs from all other described yeasts in utilizing nitrate-N and assimilating melezitose and D-xylose but not D-mannitol, salicin, or succinate. *Cryptococcus tyrolensis* sp. nov. (MYSW 303Y336 = ATCC 46405) differs from all other described yeasts in cream colonies utilizing nitrate-N and assimilating L-arabinose, melezitose, salicin, and D-xylose but not cellobiose or D-mannitol. *Cryptococcus wrightensis* sp. nov. (MYSW 303Y206 = ATCC 46406) differs from all other described yeasts (except *C. vishniacii*) in cream colonies utilizing nitrate-N and assimilating cellobiose, citrate, and melezitose but not L-arabinose, glycerol, or D-mannitol. *C. wrightensis* is phenotypically most similar to *C. vishniacii* var. *vladimiri*, differing in assimilating fumarate (weakly) and L-malate but not L-arabinose. Keys for yeast identification should include varietal listings.

Examination of DNA-DNA homologies among the biotypes originally described as *Cryptococcus vishniacii* (12), an Antarctic Basidiomycete, has shown that only eight of these biotypes can be considered conspecific with the type isolate and its biotypes (2). The description of *C. vishniacii* therefore requires emendation and herewith emended by the original authors. The remaining biotypes include *C. lupi* (4) and five additional species here proposed: *Cryptococcus asgardensis* sp. nov., *Cryptococcus baldrensis* sp. nov., *Cryptococcus hempflingii* sp. nov., *Cryptococcus tyrolensis* sp. nov., *Cryptococcus wrightensis* sp. nov. Since the identification of *C. wrightensis* requires keying at subspecific level, we propose varietal names for those biotypes or biotypical series of *C. vishniacii* which are both genetically distinct and readily identifiable.

MATERIALS AND METHODS

The methods used in characterization have been reported previously (2,3,4,12). Compounds tested as sole source of carbon and energy were: acetate, L-amino acids (L-alanine, L-arginine, L-aspartate, L-glutamate, glycine, L-isoleucine, L-lysine, L-methionine, L-ornithine, L-phenylalanine, L-proline, L-valine), γ -aminobutyrate, D-arabinose, L-arabinose, D-arabitol, L-arabitol, butyrate, cellobiose, citrate, decane, erythritol, ethanol, fructose, D-fucose, fumarate, galactitol, D-galactose, D-glucitol, δ -gluconolactone, D-glucuronate, glucose, glucoseamine, glycerol, *myo*-inositol, inulin, 2-ketogluconate, 5-ketogluconate, DL-lactate, lactose, L-malate, maltose, mannose, melezitose, melibiose, methanol, methyl- α -D-glucos-

side, raffinose, L-rhamnose, ribitol, D-ribose, salicin, L-sorbose, starch, succinate, sucrose, trehalose, xylitol, and xylose. Compounds tested as sole source of nitrogen were: ammonium chloride, potassium nitrate, adenine, D-alanine, L-amino acids (as for carbon sources plus L-histidine and L-tyrosine), creatinine, cytosine, ethylamine, guanine, hypoxanthin, nicotinic acid, thiamine, thymine, and uracil. Results are not reported for adenine, guanine, and hypoxanthin-N since cross-feeding appeared on multiply inoculated plates of all media used. All other substrates should be considered not assimilated in the absence of further mention.

Since yeasts of the *C. vishniacii* complex grow more reliably from small inocula in more dilute media, the utilization of differentiating carbon and nitrogen sources has been repeated using medium Y-2 (mineral base of potassium phosphate, pH 6.0, 1 mM; NaCl, 50 mM; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 mM; chelated trace metal solution (13) $1 \text{ mL} \cdot \text{L}^{-1}$; to which was added vitamins as required, 2 mM NH_4Cl or other nitrogen source, and 0.2% glucose or other substrate) and medium Y-2S (Y-2 supplemented with phosphate buffer to 5 mM, N supplied as 0.2 mM NH_4Cl and 0.2 mM NaHglutamate (pH 6.0), with 0.0125% yeast extract). The supplements in medium Y-2S allow more rapid growth without significantly increasing background growth in the absence of substrates. The results differ from those previously reported with respect to the assimilation of methyl- α -D-glucoside: biotype 3 (weak assimilation reported in 12) does not assimilate this substrate (we assume some growth stimulating factor to have been present in trace amounts), biotype 14 (*C. baldrensis*) does assimilate it, as does

another undescribed biotype giving equivocal results on other media.

When Y-2 or Y-2S media are used, the assimilation of sucrose must be determined in individual containers since cross-feeding may occur on multiply inoculated plates. Amylose is not produced in these media. Amylose formation by these yeasts is not pH dependent, but does require higher concentration of both rapidly available carbon sources and ammonium-N. The critical conditions of this "overflow" phenomenon is ammonium inhibition (unpublished data).

The production of extracellular proteinases clearing skim milk powder (0.25%) was examined using a variant of this medium without inorganic nitrogen (positive controls: *Candida aquatica* ATCC 18805 and *Phaffia rhodozyma* ATCC 24202). Melanin formation from dihydroxy-phenylalanine (DOPA) was examined on solid (2.0% agar) Y-2 medium modified after Nurudeen and Ahearn (9) to contain 0.05% glucose, 0.2 mM NH_4Cl , 0.2 mM NaHglutamate (pH 6.0), 5 mM potassium phosphate buffer (pH 6.0), and 0.2 g.L⁻¹ DOPA (positive control: *Filobasidiella neoformans* NIH-12).

The lists of minimal presumptive characters were compiled with the aid of Dr. R. J. Pankhurst's polyclave (5) as well as subsequent Basidioblastomycete literature.

RESULTS

Cryptococcus vishniacii Vishniac et Hempfling emend. auct. Int. J. Syst. Bacteriol. 29: 153-158.

Descriptioni primae addenda: Acidum asparticum, acidum glutamicum, acidum 2-ketogluconicum, acidum succinumque assimilant omnes

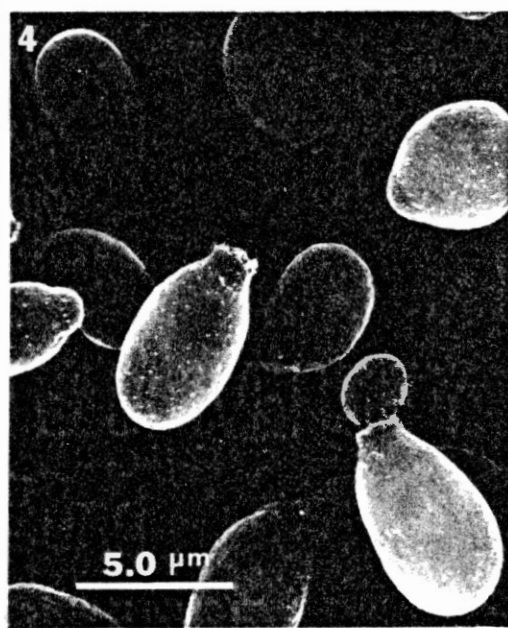
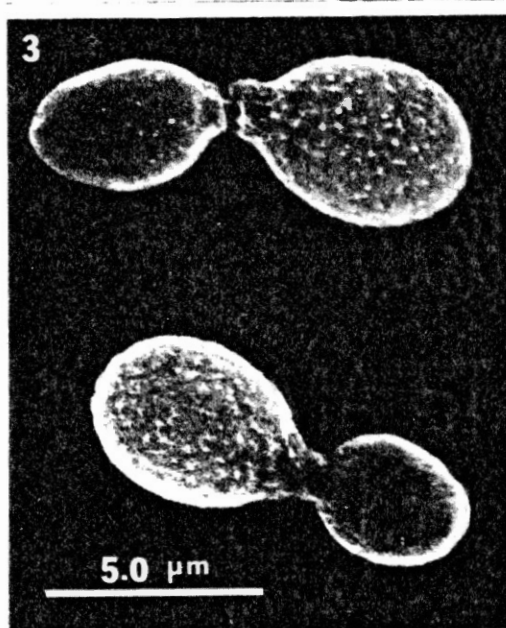
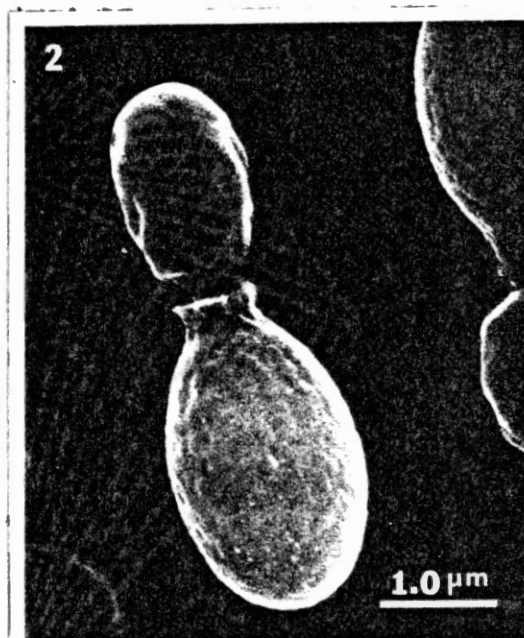
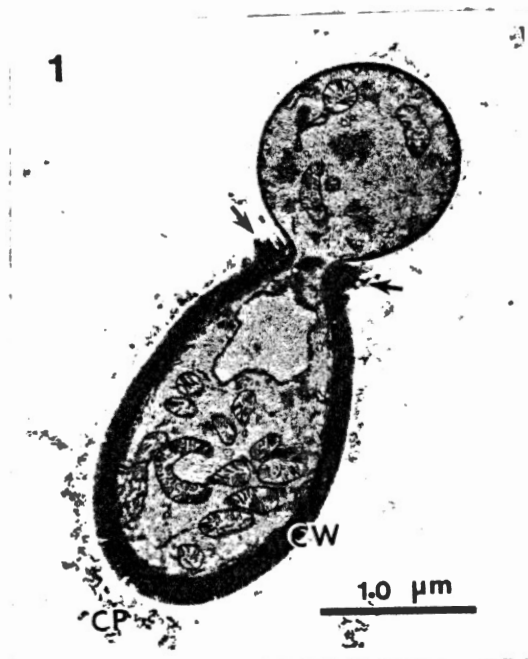
Fig. 1. TEM micrograph of *Cryptococcus vishniacii* var. *vishniacii* (MYSW 304Y268, type of the species and variety). The bud scar consisting of a ragged collar (characteristic of the basidioblastomycetous yeasts) is clearly visible (arrows). The cell wall (CW) is not sharp in outline and is surrounded by the fibrillar remains of the capsule (CP).

Fig. 2. SEM micrograph of *Cryptococcus asgardensis* (MYSW 302Y310, type of the species) showing the typical collar and the smoothly granular capsule.

Fig. 3. SEM micrograph of *Cryptococcus tyrolensis* (MYSW 303Y336, type of the species). Collar and the nodulated capsule are visible.

Fig. 4. SEM micrograph of *Cryptococcus wrightensis* (MYSW 303Y206, type of the species). The curvature observed in some buds is due to the higher growth temperature (15°C rather than 10°C).

The different appearance of capsular materials in Figures 2, 3, and 4 may be artifacts caused by critical point drying.



varietates. Methanolum non assimilant. Media tyrosino atrescentes sed non DOPA. Proteinum lactis non hydrolysans. Cum cycloheximide 0.05 μg per ml haud, 0.5 μg per ml non crescit. CoQ₉ continens; DBB respondens, G+C $54.52 \pm 0.32 - 55.48 \pm 0.21$ mol per centum.

The original description should be modified by the following: all varieties assimilate L-aspartate, L-glutamate, 2-ketogluconate, and succinate but not methanol; darken media containing tyrosine but not DOPA; and do not hydrolyze skim milk protein. All varieties are inhibited by 0.05 $\mu\text{g} \cdot \text{ml}^{-1}$ cycloheximide and suppressed by 0.5 $\mu\text{g} \cdot \text{ml}^{-1}$ cycloheximide, contain CoQ₉ and are diazonium blue B positive. Nuclear DNA contains 54.52 ± 0.32 to 55.48 ± 0.21 mol% G+C. Figure 1 illustrates the type isolate, MYSW 304Y268 (ATCC 36649).

The species comprises the following varieties:

Cryptococcus vishniacii var. *vishniacii* var. nov. Vishniac et Baharaeen. This variety is considered to include all isolates which resemble the type of the species and variety, MYSW 304Y268, in assimilating L-arabinose, citrate, L-proline (weakly), sucrose, raffinose, and L-rhamnose (weakly), but not cellobiose, fumarate, L-malate, methyl- α -D-glucoside, or salicin.

Haec typus speciei et similes quae L-arabinosum, acidum citricum, L-prolinum (lente), sucrosum, raffinolum, L-rhamnosomque (lente) sed non cellobiosum, acidum fumaricum, acidum L-malicum, methyl- α -D-glucosidum, salicinumque assimilant.

Cryptococcus vishniacii var. *wolfii* var. nov. Vishniac et

Baharaeen. (Latinized Wolfius, genitive Wólfi = of wolf, in honor of Wolf Vladimir Vishniac).

The variety includes those isolates (originally described as biotypes 1 and 2) differing from the type of the species in assimilation of fumarate and L-malate but failing to assimilate L-arabinose, citrate, sucrose, and raffinose. It is typified by MYSW 303Y216 (ATCC 46404) from soil, Tyrol Valleys, 4,700 feet (ca. 1,433 m).

Differt a *C. vishniacii* var. *vishniacii* qua acidum fumaricum, L-malicumque assimilatur sed L-arabinosum, acidum citricum, sucrosum, raffinose non assimilatur. Typus MYSW 303Y216 (ATCC 46404) e solo, Vallis Tyrol, 4700 p.

Cryptococcus vishniacii var. *asocialis* var. nov. Vishniac et Baharaeen. (L. a soc i ál is = without companionship, from occurrence in barren soil).

This variety is typified by MYSW 302Y312 (ATCC 46402), from soil, Mt. Baldr, 4,870 feet (ca. 1,485 m), but includes those isolates (biotypes 3, 4, and 5) which are genetically distinct but not distinguishable by substrate assimilation. The variety differs from the type of the species in assimilating fumarate and L-malate but failing to assimilate L-arabinose or citrate.

Differt a var. *vishniacii* qua acidum fumaricum acidum L-malicumque assimilatur sed nec L-arabinosum nec acidum citricum assimilatur. Typus MYSW 302Y312 (ATCC 46402), e solo, Mons Baldr, 4870 p.

Cryptococcus vishniacii var. *vladimiri* var. nov. Vishniac et

Baharaeen. (Latinized Vladimirus, genetive Vlad i' mir i = of Vlad-dimir, in honor of Wolf Vladimir Vishniac).

This variety is typified by MYSW 302Y265 (ATCC 46403), from soil, Mt. Baldr, 4,870 feet (ca. 1,485 m). The variety includes those isolates (biotypes 10 and 11) which though genetically distinct are not distinguishable by substrate assimilation. The variety differs from the type of the species in assimilating cellobiose, methyl- α -D-glucoside, and salicin but not assimilating L-proline or L-rhamnose.

Differt a *C. vishniacii* var. *vishniacii* qua cellobiosum, methyl- α -D-glucosidum, salicinumque assimilatur sed nec L-prolinum nec L-rhamnosumque non assimilatur. Typus MYSW 302Y265 (ATCC 46403), e solo, Mons Baldr, 4870 p.

The new species of the *Cryptococcus vishniacii* complex are:

Cryptococcus asgardensis sp. nov. Vishniac et Baharaeen. (L.-én sis = dwelling in; from occurrence in the Asgard Range, South Victoria Land, Antarctica).

Forma et augmentatione *C. vishniacii* *C. lupique* similes. Cellulae crescentes ca. 5.8 x 7.3 μ m. Cum cycloheximide 0.05 μ g per ml non crescit. CoQ₉ continens; DBB respondens. G+C 55.07 \pm 0.12 mol per centum sed sequentia acidorum deoxyribonucleorum minus quam 38 per centum altribus similes.

Fermentatio nulla. Assimilat L-arabinosum, acidum L-asparticum, cellobiosum, acidum citricum, fructosum, glucosum, acidum glucuronicum, acidum L-glutamicum, acidum 2-ketogluconicum, acidum 5-ketogluconicum (lentissime), maltosum, mannosum, melezitum, methyl- α -D-

glucosidum, raffinum, L-rhamnosum, salicinum, acidum succinicum, sucrosum, trehalosum, D-xylosumque. Media tyrosino atrescentes sed non DOPA. Amylosum formatum est. Crescit cum nitrato, acidibus aminibusque. Crescit sine vitaminis. Nec gelatinum nec lactis proteinum nec urea finduntur.

Typus MYSW 302Y310 (ATCC 46399), e solo, Mons Baldr, 4870 p.

Cryptococcus asgardensis sp. nov. Vishniac et Baharaeen, is typified by isolate MYSW 302Y310 (ATCC 46399), isolated from soil (dolerite terrace) near the middle of the east slope (4,870 feet; ca. 1,485 m) of Mt. Baldr, South Victoria Land, Antarctica. Figure 2 illustrates the type isolate.

Morphology and development are similar to that of *C. vishniacii*, *C. lupi*, and other members of the complex, in which the type was originally described as biotype 16. Exponentially growing cells average approximately $5.8 \times 7.3 \mu\text{m}$. Growth is suppressed by $0.05 \mu\text{g.ml}^{-1}$ cycloheximide. The species contains CoQ_9 and responds positively to DBB. Nuclear DNA contains $55.07 \pm 0.12 \text{ mol\% G+C}$ but exhibits less than 38% homology with other members of the complex.

Fermentation does not occur. L-arabinose, L-aspartate, cellobiose, citrate, fructose, glucose, glucuronate, L-glutamate, 2-ketogluconate, 5-ketogluconate (very slowly), maltose, mannose, melezitose, methyl- α -D-glucoside, raffinose, L-rhamnose, salicin, succinate, sucrose, trehalose, and xylose are assimilated. Amylose may be produced during growth on L-arabinose, glucose, sucrose, or xylose. Media (Y-1 base) containing tyrosine are darkened, but melanin is not produced from L-DOPA. Nitrate and L-amino acids are utilized as

N-sources. Vitamins are not required. Gelatin, skim milk proteins and urea are not hydrolyzed.

Observation of colony color (cream), determination of nitrate utilization (positive) and assimilation of L-arabinose (positive), cellobiose (positive), D-galactose (negative), *myo*-inositol (negative), D-mannitol (negative), melezitose (positive), and L-rhamnose (positive) will allow presumptive identification of isolates with this species rather than any other yeast species heretofore described except *C. vishniacii*, from which this species differs only in greater sensitivity to cycloheximide.

Cryptococcus asgardensis is phenotypically most similar to *C. vishniacii* var. *vladimiri*, from which it differs in L-rhamnose (positive) assimilation and a greater sensitivity to cycloheximide. *C. asgardensis* shows the greatest DNA-DNA homology to *C. baldrensis* sp. nov. (37.7 to 31.6% in reciprocal hybridizations) from which it differs in L-rhamnose (positive) and succinate (positive) assimilation, as well as in cycloheximide sensitivity.

Cryptococcus baldrensis sp. nov. Vishniac et Baharaeen. (L. -én sis = dwelling in; from occurrence in Mount Baldr, South Victoria Land, Antarctica).

Forma et augmentatione *C. vishniacii* *C. lupique* similes. Cellulae crescentes ca. 5.0 x 6.5 μ m. Cum cycloheximide 0.05 μ g per ml haud, 0.5 μ g per ml non crescit. CoQ₉ continens; DBB respondens. G+C 54.76 \pm 0.21 mol per centum sed sequentia acidorum deoxyribonucleorum minus quam 52 per centum altribus similes.

Fermentatio nulla. Assimilat acidum γ -aminobutyricum, L-arabinosum, acidum L-asparticum, cellobiosum, acidum citricum (lente), fructosum, glucosum, acidum glucuronicum, acidum L-glutamicum, acidum 2-ketogluconicum, maltosum, mannosum, melezitiosum, raffiniosum, salicium, sucrosum, trehalosum, D-xylosumque. Media tyrosino atrescentes sed non DOPA. Amylosum formatum est. Crescit cum nitrato, acidibus aminibusque. Crescit sine vitaminis. Nec gelatinum nec lactis proteinum nec urea finduntur.

Typus MYSW 302Y259 (ATCC 46400), e solo, Mons Baldr, 4870 p.

Cryptococcus baldrensis sp. nov. Vishniac et Baharaeen, is typified by isolate MYSW 302Y259 (ATCC 46400), isolated from soil (dolerite terrace) near the middle of the east slope (4,870 feet, ca. 1,485 m) of Mt. Baldr, South Victoria Land, Antarctica. Figures (as biotype 14 of *C. vishniacii*) may be found in (3).

Morphology and development are similar to that of *C. vishniacii*, *C. lupi* and other members of this complex. Exponentially growing cells average about $5.0 \times 6.5 \mu\text{m}$. Growth is inhibited by $0.05 \mu\text{g} \cdot \text{ml}^{-1}$ cycloheximide and suppressed by $0.5 \mu\text{g} \cdot \text{ml}^{-1}$ cycloheximide. The species contains CoQ_9 and responds positively to diazonium blue B. Nuclear DNA contains 54.76 ± 0.21 mol% G+C; homology with other biotypes of the *C. vishniacii* complex is less than 52%.

Fermentation does not occur. γ -aminobutyrate, L-arabinose, L-aspartate, cellobiose, citrate (weakly), fructose, mannose, melezitose, maltose, raffinose, salicin, sucrose, trehalose, and xylose are assimilated. Amylose may be produced during growth on L-arabinose, glucose, sucrose, or xylose. Media (Y-1 base) containing tyrosine

are darkened, but melanin is not produced from L-DOPA. Nitrate and L-amino acids are utilized as N-sources. Vitamins are not required. Gelatin, skim milk protein and urea are not hydrolyzed.

Observation of colony color (cream), determination of nitrate utilization (positive), and assimilation of L-arabinose (positive), cellobiose (positive), 2-ketogluconate (positive), D-mannitol (negative), melezitose (positive) and succinate (negative) will allow presumptive identification of isolates with this species rather than any other yeast species heretofore described.

Cryptococcus baldrensis shows the closest DNA-DNA homology to *C. tyrolensis* sp. nov. (51.5%) from which it differs phenetically in assimilating cellobiose and citrate (weakly) and *C. vishniacii* var. *vladimiri* (50.9-48.9%) from which it differs in failure to assimilate succinate. This species is also phenetically similar to *C. lupi*, from which it differs in assimilating 2-ketogluconate.

Cryptococcus hempflingii sp. nov. Vishniac et Baharaeen. (L. Hempflingius, genitive Hempf ling i i, in honor of W. P. Hempfling who first isolated the *Cryptococcus vishniacii* complex of yeasts).

Forma et augmentatione *C. vishniacii* *C. lupique* similes. Cellulae crescentes ca. 6.5 x 8.0 μ m. Cum cycloheximide 0.05 μ g per ml, 0.5 μ g per ml non crescit. CoQ₉ continens; DBB respondens. G+C 55.34 \pm 0.06 mol per centum sed sequentia acidorum deoxyribonucleorum minus quam 46 per centum altribus similes.

Fermentatio nulla. Assimilat fructosum, acidum fumaricum (lente), glucosum, acidum gluconicum (lentissime, variabile), acidum glucuro-

nicum, acidum L-glutamicum (lente), maltosum, mannosum, melezitum, raffinum, L-rhamnosum (lente), sucrosum, trehalosum, D-xylosumque. Media tyrosino atrescentes sed non DOPA. Amylosum formatum est. Crescit cum nitrato, acidibus aminibusque. Crescit sine vitaminis. Nec geletinum nec lactis proteinum nec urea finduntur.

Typus MYSW 306Y212 (ATCC 46401), e solo, Vallis Tyrol, 4750 p.

Cryptococcus hempflingii sp. nov. Vishniac et Baharaeen, is typified by isolate MYSW 306Y212 (ATCC 46401) from soil (sand, sandstone) from the northwest part of Tyrol Valley (4,750 feet; ca. 1,448 m), South Victoria Land, Antarctica. Figures (as biotype 6 of *C. vishniacii*) may be found in (3).

Morphology and development are similar to that of *C. vishniacii*, *C. lupi*, and other members of this complex. Exponentially growing cells average about $6.5 \times 8.0 \mu\text{m}$. Growth is inhibited by $0.05 \mu\text{g} \cdot \text{ml}^{-1}$ cycloheximide, suppressed by $0.5 \mu\text{g} \cdot \text{ml}^{-1}$ cycloheximide. The species contains CoQ_9 and responds positively to diazonium blue B. Nuclear DNA contains $55.34 \pm 0.06 \text{ mol\% G+C}$; DNA-DNA homology with other biotypes of the *C. vishniacii* complex is less than 46%.

Fermentation does not occur. Fructose, fumarate (weakly), glucose, gluconate (very slowly, variably), glucuronate, L-glutamate (weakly), 2-ketogluconate, L-malate (weakly), maltose, mannose, melezitose, raffinose, L-rhamnose (weakly), sucrose, trehalose, and xylose are assimilated. Amylose may be produced during growth on sucrose, glucose, or xylose. Media (Y-1 base) containing tyrosine are darkened but melanin is not produced from L-DOPA. Vitamins are not required. Gelatin, skim milk protein and urea are not hydrolyzed.

Determination of nitrate utilization (positive) and assimilation of D-mannitol (negative), melezitose (positive), salicin (negative), succinate (negative) and D-xylose (positive) will allow presumptive identification of isolates with this species rather than any other yeast species heretofore described.

Cryptococcus hempflingii is phenotypically most similar to *C. vishniacii* var. *asocialis* from which it differs in failure to assimilate L-aspartate, L-proline (var. *asocialis* weak), and succinate. The species shows most DNA-DNA homology, however, to *C. vishniacii* var. *vladimiri* (45.9 to 42.2% in reciprocal hybridizations with MYSW 302Y265), from which it differs phenotypically by failure to assimilate L-arabinose, L-aspartate, cellobiose, citrate, L-glutamate, methyl- α -D-glucoside, salicin and succinate and by weakly assimilating fumarate, L-malate, and L-rhamnose. *C. hempflingii* and *C. vishniacii* differ in succinate assimilation.

Cryptococcus tyrolensis sp. nov. Vishniac et Baharaeen. (L. -en sis = dwelling in; from occurrence in Tyrol Valleys).

Forma et augmentatione *C. vishniacii* *C. lupique* similes. Cellulae crescentes ca. 4.6 x 5.8 μ m. Cum cycloheximide 0.05 μ g per ml haud, 0.5 μ g per ml non crescit. CoQ₉ continens; DBB respondens. G+ C 55.82 \pm 0.27 mol per centum sed sequentia acidorum deoxyribonucleorum minus quam 52 per centum altribus similes.

Fermentatio nulla. Assimilat L-arabinosum, acidum L-asparticum, fructosum, glucosum, acidum glucuronicum, acidum L-glutamicum, acidum 2-ketogluconicum, acidum 5-ketogluconicum (lentissime) maltosum,

mannosum, melezitum, methyl- α -D-glucosidum, raffinum, salicinum, sucrosum, trehalosum, D-xylosomque. Amylosum formatum est. Crescit cum nitrato, acidibus aminibusque. Media tyrosino atrescentes sed non DOPA. Crescit sine vitaminis. Nec geletinum nec lactis proteinum nec urea finduntur.

Typus MYSW 303Y336 (ATCC 46405), e solo, Vallis Tyrol, 4700 p.

Cryptococcus tyrolensis sp. nov. Vishniac et Baharaeen, is typified by isolate MYSW 303Y336 (ATCC 46405) from soil (dolerite moraine) in the Tyrol Valley (4,700 feet, ca. 1,566 m) South Victoria Land, Antarctica. Figure 3 illustrates the type isolate.

Morphology and development are similar to that of *C. vishniacii*, *C. lupi* and other members of the complex in which this species was originally described as biotype 15. Exponentially growing cells average $4.6 \times 5.8 \mu\text{m}$. Growth is inhibited by $0.05 \mu\text{g.ml}^{-1}$ cycloheximide and is suppressed by $0.5 \mu\text{g.ml}^{-1}$ cycloheximide. The species contains CoQ_9 and responds positively to diazonium blue B. Nuclear DNA contains $55.82 \pm 0.27 \text{ mol\% G+C}$; DNA-DNA homology with other biotypes of the complex is less than 52%.

Fermentation does not occur. L-arabinose, L-aspartate, fructose, glucose, glucuronate, L-glutamate, 2-ketogluconate, 5-ketogluconate (very slowly), maltose, mannose, melezitose, methyl- α -D-glucoside, raffinose, salicin, sucrose, trehalose, and D-xylose are assimilated. Amylose may be produced during growth on L-arabinose, glucose, sucrose, or xylose. Media (Y-1 base) containing tyrosine are darkened, but melanin is not produced from L-DOPA. Nitrate and L-amino acids are utilized as N-sources. Vitamins are not required. Gelatin, skim

milk proteins and urea are not hydrolyzed.

Observation of colony color (cream), determination of nitrate utilization (positive) and assimilation of L-arabinose (positive), cellobiose (negative), D-mannitol (negative), melezitose (positive), salicin (positive) and D-xylose (positive) will allow presumptive identification of isolates with this species rather than any other yeast species heretofore described.

Cryptococcus tyrolensis is phenetically most similar to *C. baldrensis* differing in assimilation of γ -aminobutyrate (negative), cellobiose (negative), citrate (negative), and methyl- α -D-glucoside (negative) and to *C. wrightensis* sp. nov., differing in assimilation of γ -aminobutyrate (negative), L-arabinose (positive), citrate (negative), and succinate (negative). *C. tyrolensis* showed 52.55% DNA-DNA homology with *C. baldrensis*. Homology with *C. wrightensis* was not measured directly but is unlikely to be over 50%. Phylogenetic distances (100-% relative binding of heterologous probe DNA) are not accurate at homologies below roughly 50%, but topological constraints are imposed upon the probable position of loci fixed vectorially in a three-dimensional evolutionary map by the use of a series of probes. The possible position of *C. tyrolensis* is constrained to a phylogenetic distance not less than 50 with respect to *C. wrightensis*.

Cryptococcus wrightensis sp. nov. Vishniac et Baharaeen. (L.
-én sis = dwelling in ; from occurrence in the Wright Valleys).

Forma et augmentatione *C. vishniacii* *C. lupique* similes. Cellulae

crescentes ca. $4.8 \times 6.7 \mu\text{m}$. Cum cycloheximide $0.05 \mu\text{g}$ per ml haud, $0.5 \mu\text{g}$ per ml non crescit. CoQ_9 continens, DBB respondens. G+C 54.63 ± 0.06 mol per centum sed sequentia acidum deoxyribonucleorum minus quam 35 per centum altribus similes.

Fermentatio nulla. Assimilat γ -aminobutyricum, acidum L-asparticum, cellobiosum, acidum citricum, fructosum, acidum fumaricum (lente), glucosum, acidum glucuronicum, acidum L-glutamicum, acidum 2-ketogluconicum, acidum 5-ketogluconicum (lentissime), acidum L-malicum, maltosum, mannosum, melezitum, methyl- α -D-glucosidum, raffinum, salicinum, acidum succinicum, sucrosum, trehalosum, D-xylosumque. Amylosum formatum est. Crescit cum nitrato, acidibus aminibusque. Media tyrosino atrescentes sed non DOPA. Crescit sine vitaminis. Nec gelatinum nec lactis proteinum nec urea finduntur.

Typus MYSW 303Y206 (ATCC 46406), e solo, Vallis Tyrol, 4700 p.

Cryptococcus wrightensis sp. nov. Vishniac et Baharaeen, is typified by isolate MYSW 303Y206 (ATCC 46406), isolated from soil, (dolerite moraine) in the Tyrol Valley (a subsidiary of the Wright Valley), South Victoria Land Antarctica. Figure 4 illustrates the type isolate.

Morphology and development are similar to that of *C. vishniacii*, *C. lupi* and other members of the complex in which this species was originally described as biotype 9. Exponentially growing cells average $4.8 \times 6.7 \mu\text{m}$. Growth is inhibited by $0.05 \mu\text{g} \cdot \text{ml}^{-1}$ cycloheximide and suppressed by $0.5 \mu\text{g} \cdot \text{ml}^{-1}$ cycloheximide. The species contains CoQ_9 and responds positively to diazonium blue B. Nuclear

DNA contains 54.63 ± 0.06 mol% G+C; DNA-DNA homology with other biotypes measured less than 35% and is topologically constrained to less than 50% with respect to those biotypes not used as probes.

Fermentation does not occur. γ -aminobutyrate, L-aspartate, cellobiose, citrate, fructose, fumarate (weakly), glucose, glucuronate, L-glutamate, 2-ketogluconate, 5-ketogluconate (very slowly and weakly), L-malate, maltose, mannose, melezitose, methyl- α -D-glucoside, raffinose, salicin, succinate, sucrose, trehalose, and xylose are assimilated. Amylose may be produced during growth on glucose or xylose. Media (Y-1 base) containing tyrosine are darkened, but melanin is not produced from L-DOPA. Nitrate and L-amino acids are utilized as N-sources. Vitamins are not required. Gelatin, skim milk proteins, and urea are not hydrolyzed.

Observation of colony color (cream), determination of nitrate utilization (positive) and assimilation of L-arabinose (negative), cellobiose (positive), citrate (positive), glycerol (negative), D-mannitol (negative), and melezitose (positive) will allow presumptive identification of isolates with this species rather than any other yeast species heretofore described except *C. vishniacii*.

C. wrightensis is almost equally related by DNA-DNA homology (less than 35%) to the three most homologous probes used (*C. vishniacii* var. *vladimiri*, *C. baldrensis*, and *C. hempflingii*) and may be more homologous (less than 50%) with *C. tyrolensis*, from which it differs phenotypically in assimilating γ -aminobutyrate, cellobiose, citrate, and succinate and failing to assimilate L-arabinose. Phenotypically, this species most closely resembles *C. vishniacii* var.

vladimiri, differing in failure to assimilate L-arabinose and in assimilating fumarate (weakly) and L-malate. Since these characters vary in the species *C. vishniacii*, *C. wrightensis* and *C. vishniacii* can only be distinguished at the varietal level.

DISCUSSION

The erection of anamorphic species on the ground of DNA-DNA homology is mootable; the nature of speciation is such that there can be no absolute homology value which defines conspecificity. We (2) have chosen to regard phylogenetic distances greater than 48 (52% homology) as excluding conspecificity because the depleted information content of lower homology values vitiates the construction of three-dimensional evolutionary maps and because *Filobasidiella neoformans* and *F. bacillispora* (the only model available among basidiomycetous yeasts) are separated by a phylogenetic distance of only 37-45 units (1). The average distance separating normally fertile sexual species of basidiomycetous yeasts may be less than 48 units - the varieties of *C. vishniacii* form a coherent group in which no single biotype is more than 26 units distant from some other biotypes (2). Or it may be more than 48 units - ascomycetous yeasts may mate with markedly diminished fertility at phylogenetic distances as great as 76 units (8). Our method is in any case preferable to the use of phenotypic similarity indices, which do not reflect genetic relationships of many yeasts and are seen (above) to fail in the *C. vishniacii* complex.

The species which we propose are identifiable by appropriate

modifications of standard techniques (11), provided that varietal listings are included in keys. The absence of assimilation profiles at the level of variety or biotype in such widely used compendia as Barnett et al. (6) poses problems for users who are not primarily yeast taxonomists not only in separating *C. vishniacii* from *C. wrightensis* but in discovering the existence of such taxa as *Candida obtusa* var. *arabinosa* {not synonymous with *Clavispora lucitaniae* = *Candida lucitaniae*, *Candida obtusa* (10)}. The inclusion of varietal assimilation profiles might greatly simplify ecological studies of such species as *Candida humicola*, *Cryptococcus albidus*, *Cryptococcus laurentii*, *Rhodotorula glutinis*, *Rhodotorula rubra*, and *Saccharomyces cerevisiae*, species whose assimilation profiles (6) contains roughly as many strain-variable as strain-invariable characters.

It is possible that no non-sexual characters will be found to distinguish *C. vishniacii* and *C. wrightensis*, or to separate the teleomorphs of *Rhodotorula glutinis* {*Rhodosporidium diobovatum*, *R. sphaerocarpum*, *R. toruloids* (7)}. Assimilation profiles can be useful in distinguishing taxa only to the extent to which environmental pressures involving differential resource utilization have been important in their evolution. The success of such techniques at the level of species in the characterization and identification of many relatively featureless microbes appears to confirm the intuitively obvious- for man or microbe, sources of support play a major role in survival and reproduction - but they are not the only factors in speciation. Phenotypic similarity indices do not necessarily reflect genetic relationships in yeasts, or in other microbes.

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CHAPTER VI

PHYLOGENETIC RELATIONSHIPS IN THE BASIDIOMYCETOUS

YEASTS: COMPLEMENTARY DNA-

25S RIBOSOMAL RNA

HOMOLOGY

PHYLOGENETIC RELATIONSHIPS IN THE BASIDIOMYCETOUS YEASTS

COMPLEMENTARY DNA - 25S RIBOSOMAL RNA HOMOLOGY

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INTRODUCTION

The yeasts of the *Cryptococcus vishniacii* complex, indigenous to the Dry Valleys of South Victoria Land, Antarctica, exist at the limits of gean life. The adaptations which enable these organisms to grow in this most extreme cold desert on earth are of interest to those who speculate upon life on other planets as well as to the terrestrial biologists. If *Cryptococcus vishniacii* complex is to be used as a model indigene for ecological investigations, it is desirable to distinguish between characters and molecular mechanisms which are specifically adaptive and those which are relicts of its phylogenetic origin. Presumptively adaptive characters may be identified by their absence in congeners from other habitats or presence in unrelated yeasts of similarly stressed environments. What is the evolutionary origin of *Cryptococcus vishniacii* complex? What are its congeners?

Zymologists have traditionally embraced the methods developed

for bacterial classification; anamorphic yeasts share with prokaryotes the evolutionary and taxonomic problems arising from the absence of zygotic sex. These methods utilize morphological, physiological and biochemical features which reveal only a small fraction of the genome and may not be reliable indicators of evolutionary relationships.

Modern molecular biology has provided methods to work directly with the yeast genome. Determination of DNA base composition constitutes the coarsest genetic probe available and can serve the same exclusionary function in yeast systematics as in bacterial taxonomy. Additional means of comparisons are required to determine whether yeasts which exhibit similar DNA base compositions are phylogenetically related. Studies of DNA-DNA complementarity between single stranded nuclear DNAs from various isolates now constitutes the most reliable means for determination of genetic relationships at the level of species in anamorphic yeasts. Studies of the DNA base composition and DNA-DNA homology in the *Cryptococcus vishniacii* complex allowed the segregation of six distinct species (from *Cr. vishniacii*), some of which were as distantly related to the *Cr. vishniacii* type species as *Cr. bhutanensis* (a Himalayan yeast) (Baharaeen *et al.*, 1982, Baharaeen and Vishniac, 1982; Vishniac and Baharaeen, IN PRESS). A common ancestor probably originated and speciated outside of the Dry Valleys. This common ancestor is related to the *Cr. vishniacii* complex at the generic level, a level which DNA-DNA homology does not penetrate.

The definition of genera and higher taxa in yeasts has been based on informed judgment and convenience. Methods of budding, production of pseudo- and true mycelium, formation of arthrospores, structure of

the hyphal septum, presence of carotenoid pigments, production of extracellular urease and deoxyribonuclease, utilization of nitrate-N, assimilation of inositol, the type of coenzyme Q system, and structural differences in various stages of life cycle have been widely, and in some cases prematurely, used to define genera or higher taxa of yeasts. Although developmental characters (involving a series of biochemical and genetic events rather than a few enzymatic steps) have been traditionally considered most useful for evolutionary studies, the products of the conserved regions of the genome such as the ribosomal RNA sequences could furnish a more reliable and quantitative tool. The homology of ribosomal RNA (or its larger moieties) within total DNA has been investigated in many bacterial genera. Studies by Palleroni et al. (1973) on the species of *Pseudomonas* and by Johnson and Francis (1975) on the species of *Clostridium* for example, have revealed the presence of genetically distinct but phenetically indistinguishable groups each of which could be given a distinct generic (or higher) assignment. From an extensive study among different bacterial genera, Pace and Campbell (1971a, 1971b) concluded that ribosomal RNA homologies can be used as a means of generating a quantitative spectrum of phylogenetic relatedness on the basis of the fact that ribosomal RNA species possessing near relationships to one probe were correspondingly less related to another probe and vice versa. With the exception of the *Pseudomonas* study by Palleroni et al. (1973), all of the homology experiments have examined only linear relationships, using at the most two probes. We have found (Baharaeen et al., 1982) that species are defined in

three-dimensional evolutionary space. Other studies in bacterial systems (Yankofsky and Spiegelman, 1962a, 1962b, 1963; Takahashi et al., 1967; Doi and Igarashi, 1965; Dubnau et al., 1965) have been undertaken to identify ribosomal RNA cistrons by sequence complementarity. The DNA-RNA homology studies have further been applied to protozoa (Gibson, 1966), plants (Matsuda and Siegel, 1967; and yeasts (Bicknell and Douglas, 1970; Schweizer et al., 1969).

The study of 5S ribosomal RNA and cataloging of 16S rRNA sequences have been shown to be useful for molecular evolutionary studies in prokaryotes (Fox et al., 1977, 1980; Ludwig et al., 1981; Tanner et al., 1981; Gojiobori and Nei, 1981; Woese, 1981; Woese and Fox, 1977; Woese et al., 1976). The only published report on this kind of molecular evolution including yeasts is that of Hori (1975) who compared the 5S rRNA sequences of a number of organisms ranging from bacteria to man. From the sequences of the three ascomycetous yeasts (Hindley and Page, 1972, *Saccharomyces calshbergensis*; Miyazaki, 1974, *Saccharomyces cerevisiae*; and Nishikawa and Takemura, 1974, *Torulopsis (Candida) utilis*) he concluded that the fungi diverged from the Chlorella-Animalia stem more than 1.7×10^9 years ago and that these species diverged "very recently" in evolution. Clearly, more 5S rRNA data from other yeasts are required to clarify the relative times of origin of yeasts and other fungi.

Considerably more information can be obtained using larger (18S and 25S) rRNA molecules. The determination of primary structure of these large RNAs is sufficiently difficult at present that full sequence information cannot be used as a basis for evolutionary

studies. It is remarkable that the sequencing of 25S rRNA of the yeast *Saccharomyces cerevisiae* has recently been done (Veldman et al., 1981; Georgiev et al., 1981). A partial sequence characterization in terms of a "comparative cataloging" approach (Fox et al., 1977) is within the range of experimental manageability for a number of 18S rRNAs but too cumbersome to be applied for 25S rRNA molecules. Hybridization techniques are suitable for studying the homologies of 25S ribosomal RNAs.

DNA-RNA homology studies have usually involved the immobilization of the denatured DNA in agar or on membrane filters and its hybridization with *in vivo* radiolabeled RNA (Gillespie, 1968). We have modified this technique by performing the hybridization reactions in solution using radiolabeled complementary DNA synthesized *in vitro* on enzymatically polyadenylated 25S rRNA fragments and subsequently treating the hybrid molecules with S_1 nuclease prior to collection on DEAE cellulose filters for determination of the extent of duplex formation. This technique is employed here to determine the degree of 25S rRNA homology in representatives of basidiomycetous yeast genera and their anamorphs in order to establish the evolutionary relationships of the *Cryptococcus vishniacii* complex, and to develop 25S rRNA-cDNA homology as a new technique in yeast classification.

MATERIALS AND METHODS

Yeast strains and cell cultivation:

The yeast strains used in this study (type of each species when applicable) are listed in Table I. Cells were grown in 6 liters of

TABLE I. YEAST STRAINS AND GROWTH CONDITIONS

Specific names	Strain designation	Medium	Temperature (°C)
<i>Aessosporon salmonicolor</i> van der Walt	ATCC 623 ^{a*}	YM ^f	25
<i>Agaricostilbum palmicolum</i> Wright et al.	UBC 3-14 ^{b*}	YM	30
<i>Candida acutus</i> Goto	ATCC 42713	YM	25
<i>C. aquatica</i> Jones et Slooff	ATCC 18806	YM	20
<i>C. curvata</i> (Diddens et Lodder) Lodder et Kreger van Rij	NRRL-Y-1511 ^c	YM	25
<i>C. fujisanensis</i> Soneda	NRRL-YB-4824	YM	20
<i>C. humicola</i> (Daszewska) Diddens et Lodder	ATCC 14438	YM	25
<i>C. podzolica</i> Babjeva et Reshetova	ATCC 34208	YM	25
<i>C. zeylanoides</i> (Castellani) Langeron et Guerra	NRRL-Y-1774	YM	25
<i>Cryptococcus albidus</i> (Saito) Skinner	ATCC 10666	YM	25
<i>Cr. bhutanensis</i> Goto et Sugiyama	ATCC 22461	GPYPi ^g	20
<i>Cr. himalayensis</i> Goto et Sugiyama	IAM 4963 ^d	GPYPi	10
<i>Cr. laurentii</i> (Kufferath) Skinner	ATCC 18803	YM	25
<i>Cr. lupi</i> Baharaeen et Vishniac	ATCC 44529	GPYPi	10
<i>Cr. vishniacii</i> Vishniac et Hempfling	ATCC 36649	GPYPi	10
<i>Filobasidiella neoformans</i> Kwon-Chung	NIH 12 ^{e*}	YM	30
<i>Filobasidium capsuligenum</i> (Fell et al.) Rodrigues de Miranda	ATCC 14437	YM	25
<i>Fm. uniguttulatum</i> Kwon-Chung	ATCC 24227	YM	25
<i>Hansenula saturnus</i> (Kloecker) Sydow et Sydow	ATCC 18119	YM	25
<i>Leucosporidium antarcticum</i> Fell et al.	ATCC 22177	YM	15
<i>L. frigidum</i> Fell et al.	ATCC 22029	YM	15
<i>L. gelidum</i> Fell et al.	ATCC 22030	YM	15
<i>L. nivalis</i> Fell et al.	ATCC 22031	YM	15
<i>L. scottii</i> Fell et al.	ATCC 22181*	GPYPi	10
<i>L. stokesii</i> Fell et al.	ATCC 22178	YM	15
<i>Phaffia rhodozyma</i> Miller et al.	ATCC 24202*	YM	15
<i>Pityrosporum ovale</i> (Bizzozero) Castellani et Chalmers	ATCC 12078*	Tween ^h	30

TABLE I. (CONTINUED)

Specific names	Strain designation	Medium	Temperature (°C)
<i>Rhodospiridium dacryoidum</i> Fell et al.	ATCC 24502	YM	15
<i>R. infirmo-miniatum</i> Fell et al.	ATCC 16182	YM	20
<i>R. malvinellum</i> Fell et Hunter	ATCC 24058	YM	15
<i>R. toruloides</i> Banno	NRRL-Y-1091	YM	25
<i>Tremella mesenterica</i> Fries	ATCC 24925*	YM	25
<i>Ustilago maydis</i> (DeCandolle) Corda	ATCC 10818	YM	25

- a. ATCC: American Type Culture Collection, Rockville, Maryland, U.S.A.
b. UBC: Dr. R. J. Bandoni. University of British Columbia, Vancouver, British Columbia, Canada.
c. NRRL: Dr. C. P. Kurtzman. Northern Regional Research Laboratories, Peoria, Illinois, U.S.A.
d. IAM: Institute for Applied Microbiology, University of Tokyo, Tokyo, Japan
e. NIH: Dr. K. J. Kwon-Chung. National Institute of Health, Bethesda, Maryland, U.S.A.
f. YM Medium: glucose (1%), peptone (0.5%), yeast extract (0.3%), malt extract (0.3%).
g. GPYPi Medium: glucose (0.5%), peptone (0.5%), yeast extract (0.3%), KPi pH 6.86 (10 mM).
h. Tween Medium: glucose (2%), peptone (1%), Tween 60 (0.25%), Tween 80 (0.25%), olive oil (0.25%).

* indicates type of the genus.

the appropriate medium at the appropriate temperature (Table I) with vigorous stirring and aeration. Exponentially growing cells were harvested by centrifugation at 5,000 x g for 5 minutes (*Filobasidiella neoformans* and *Leucosporidium antarcticum* were harvested at 8,000 x g for 10 minutes) at 4°C in a Sorvall RC-2B refrigerated centrifuge, washed once in glass-distilled water and once in TSM buffer (Tris-HCl, 10 mM; NaCl, 100 mM; MgCl₂, 30 mM, pH 7.4) and resuspended in 2 x volume TSM.

Isolation of ribosomes and ribosomal RNA:

Bentonite (Sigma Chemical Co.) was added to the cell suspension to a final concentration of 0.5%. The suspension was then transferred into the Braun stainless steel vessel (Bronwill Scientific Co.) half-filled with 0.5 mm glass beads and homogenized for 3 minutes at 4,000 rpm in a CO₂-cooled Braun cell homogenizer (Bronwill Scientific Co.). The broken cell suspension was centrifuged at 25,000 x g for 20 minutes to remove cell debris. The supernatant was loaded onto 5 mls of a 15% sucrose solution (Sigma Chemical Co., nuclease-free) in TSM buffer containing 5% ammonium sulfate and centrifuged (Beckman cellulose nitrate tubes) for 12 hours at 26,000 rpm in a SW-28 Beckman rotor in a Beckman L3-50 preparative ultracentrifuge. The transparent ribosomal pellet was resuspended in 40 mls of TSM buffer containing 0.2% SDS using a sterile glass rod, and transferred into a glass-stoppered flask containing 40 mls of a freshly prepared cold phenol solution saturated in Tris-EDTA buffer (Tris-HCl, 10 mM, EDTA, 1 mM, pH 7.0) and shaken at 4°C for 20 minutes. The suspension was

centrifuged at 20,000 x g for 20 minutes, the upper layer was removed and subjected to one additional phenol treatment. The phenol treated supernatant was chilled and the crude ribosomal RNA was alcohol precipitated at -20°C overnight. The pellet was collected by centrifugation at 5,000 x g for 5 minutes, partially air dried and redissolved in 40 mls of TSM buffer. The solution was treated with deoxyribonuclease I (Sigma Chemical Co., $100\text{ }\mu\text{g.ml}^{-1}$) for 1 hour with slow shaking at 100 rpm at 25°C . Pronase (Calbiochem, nuclease free) was then added to a final concentration of $50\text{ }\mu\text{g.ml}^{-1}$; the digest was shaken for an additional 2 hours and then emulsified for 20 minutes with an equal volume of phenol solution. The two layers of emulsion were separated as described above and the purified rRNA was alcohol precipitated from the supernatant at -20°C overnight. The pellet was collected and dried in a vacuum desiccator and redissolved in 140 mM sodium phosphate buffer (PB, an equimolar mixture of monobasic and dibasic sodium phosphate) pH 6.86 to a final concentration of 1 mg.ml^{-1} . The rRNA was considered pure when absorbance ratios A_{260}/A_{280} of about 1.9 and A_{230}/A_{260} of about 0.5 were obtained.

Fractionation of ribosomal RNA:

Approximately 2 mls (2 mg) of rRNA solution was loaded onto 38 mls of a linear 5-20% sucrose gradient in Tris-EDTA buffer in cellulose nitrate tubes and centrifuged at 26,000 rpm for 18 hours (SW-28 rotor, L3-50 ultracentrifuge, 4°C). The gradients were fractionated using an ISCO model 184 gradient fractionator. The peak A_{254} fraction

corresponding to 25S rRNA was pooled. Sucrose was removed from the RNA solution by chromatography on Sephadex G-50 column (1.5 x 50 cm) using a 1:10 dilution of Tris-EDTA buffer (see above). The rRNA fraction was lyophilized, and the lyophilizate was redissolved in sterile glass-distilled water to one-tenth of its original volume and frozen at -20°C .

Partial hydrolysis of 25S rRNA and recovery of fragments:

The 25S rRNA solution was made 50 mM in borate buffer (pH 9.2) and heated at 70°C . After 12 minutes (determined to result in recovery of maximum number of 400-600 nucleotides long fragments by gel electrophoresis), the solution was neutralized by the addition of calculated amounts of 1N hydrochloric acid. The hydrolyzate was run into agarose gel (agarose, 1.75% in Tris-borate buffer: Tris-HCl, 89 mM; boric acid, 89 mM; EDTA, 2.5 mM, pH 8.0) at 50 mA for 1 hour and fractions were separated at 30 mA for 4 hours. A slice of the gel was stained with ethidium bromide ($0.5\text{ }\mu\text{g.ml}^{-1}$) for 1 hour and the zone corresponding to 400-600 nucleotide long fragments {*Saccharomyces cerevisiae* soluble ribosomal RNA (Sigma Chemical Co.) and *Cryptococcus vishniacii* 18S rRNA (isolated in our laboratory) were used as standards} were cut and frozen between two layers of Parafilm at -20°C . The ribosomal RNA fragments were then extracted from the frozen gel by freeze-squeezing method described by Turing et al. (1975). An aliquot which was re-electrophoresed did not show any changes in the fragment size of the extracted material.

Synthesis of poly (A) on 25S rRNA fragments:

A 10 μg portion of the resultant fragments was treated with bacterial alkaline phosphatase (Collaborative Research, 0.5 U. μg^{-1} of RNA) at 65°C for 30 minutes. The reaction was terminated by addition of an equal volume of phenol-chloroform (1:1) mixture. Phenol (Bethesda Research Laboratories, ultrapure) was saturated in Tris-EDTA buffer pH 8.0 and an equal volume of chloroform was added prior to use. The two phases of the emulsion were separated by centrifugation at 12,500 x g for 1 minute at 4°C. The upper (aqueous) layer was extracted with ether twice and ethanol precipitated at -20°C overnight. The pellet was collected by centrifugation at 12,500 x g for 10 minutes, washed once with 95% ethanol, recentrifuged for 1 minute, dried in a vacuum desiccator and redissolved in 85 μl of polynucleotide phosphorylase buffer (Tris-HCl, 50 mM; MgCl_2 , 5 mM; sodium citrate, 10 mM, pH 8.0). A 10 μl aliquot of a 2.36 mg.ml^{-1} adenosine diphosphate (rADP, Sigma Chemical Co.) stock solution and 5 μl of an 8,000 U. ml^{-1} (4U) solution of polynucleotide phosphorylase (Bethesda Research Laboratories) were added. The reaction mixture was incubated at 37°C for 10 minutes. The poly (A) synthesis was terminated by addition of an equal volume of phenol:chloroform. The layers were separated and the rRNA was recovered as previously described. The dried pellet was redissolved in 40 μl of sterile deionized glass-distilled water.

Synthesis of complementary DNA by reverse trascription:

A 25 μl (1 mCi.ml^{-1}) aliquot of (methyl- ^3H)-deoxythymidine-5'-

triphosphate, tetrasodium salt (New England Nuclear) was lyophilized in a vacuum desiccator in a 500 μ l polypropylene microvial. To this vial was added the 40 μ l (about 10 μ g) poly (A)-attached 25S rRNA fragments, 10 μ l of 10 mM dATP, dCTP, and dGTP and 2.5 μ l of 10 mM dTTP (Sigma Chemical Co.), 20 μ l of reverse transcriptase buffer (Tris-HCl, 50 mM; MgCl₂, 10 mM; KCl, 100 mM; pH 8.3), 5 μ l of 1 M dithiothreitol (DTT), 10 μ l of 1 mg.ml⁻¹ poly (dT)₁₀ (Miles Laboratories), and 5 μ l of 12 U. μ l⁻¹ (60 U) of AMV reverse transcriptase (Betheda Research Laboratories). The mixture was incubated at 35°C for 10 minutes to assure A-T base pairing and then at 43°C for 70 minutes. The reaction was terminated by addition of 20 μ l of 100 mM EDTA. To this mixture was added 50 μ l of a carrier DNA solution (400 μ g.ml⁻¹ calf thymus DNA, sheared to 400-500 base-pair long fragments and denatured at 100°C for 10 minutes) and 20 μ l of 3 N NaOH and incubated at 55°C for 40 minutes. After incubation, 20 μ l of 1 M Tris-HCl buffer pH 7.4 and 20 μ l of 3 N HCl were added. The reaction mixture was treated with phenol:chloroform and the cDNA was recovered. The cDNA was further purified from the unincorporated bases on a Sephadex G-50 (0.7 x 20 cm) column equilibrated with 140 mM PB pH 6.86. In order to avoid the loss of cDNA by its binding to Sephadex, the column was prewashed with 100 μ l of the carrier DNA solution. A 2 μ l sample of purified cDNA was precipitated in 1 ml of cold 10% trichloroacetic acid (TCA) and collected on a GF/C filter disc (Whatman Inc.). The precipitate was washed with 1 ml of 10% cold TCA and dried under an infra-red lamp and counted in 10 mls of a toluene based scintillation fluid (Beckman, Ready-Solv HP) in a

Beckman LS-7500 liquid scintillation counter equipped with Texas Instruments Silent 700 electronic terminal at the 2% error level. The specific activity of the cDNA was about 2.2×10^6 cpm. μg^{-1} .

Kinetics of hybridization:

The cDNA-25S rRNA hybridization reactions were carried out in 500 μl polypropylene centrifuge vials which contained 20 μg of 400-600 nucleotide long 25S rRNA fragments and 0.02 μg of homologous labeled cDNA in 50 μl of 280 mM PB. The reaction vials were heated at 100°C for 10 minutes and then quickly transferred to a 65°C constant temperature water bath. At various time intervals, vials were withdrawn and quickly frozen in dry ice-acetone and stored at -20°C . To assay the extent of duplex formation, the vial contents were then thawed at room temperature and diluted to 250 μl by the addition of S_1 nuclease buffer (Maxwell et al., 1978). A 100 μl sample was withdrawn and applied onto a DE-81 (Whatman Inc.) DEAE cellulose filter disc, dried, and prepared for scintillation counting. The remaining 150 μl was treated with 5 μl of S_1 nuclease (20,000 U. ml^{-1} , Bethesda Research Laboratories) at 37°C for 1 hour. Another 100 μl sample was removed and applied onto a DE-81 filter in a small plastic petri plate. After 5 minutes at room temperature, the filter was washed with 5 ml of 480 mM PB three times, with glass-distilled water once, and with 95% ethanol once, then dried and prepared for counting. Comparison of counts on S_1 nuclease treated and untreated (total) filters determined the extent of duplex formation.

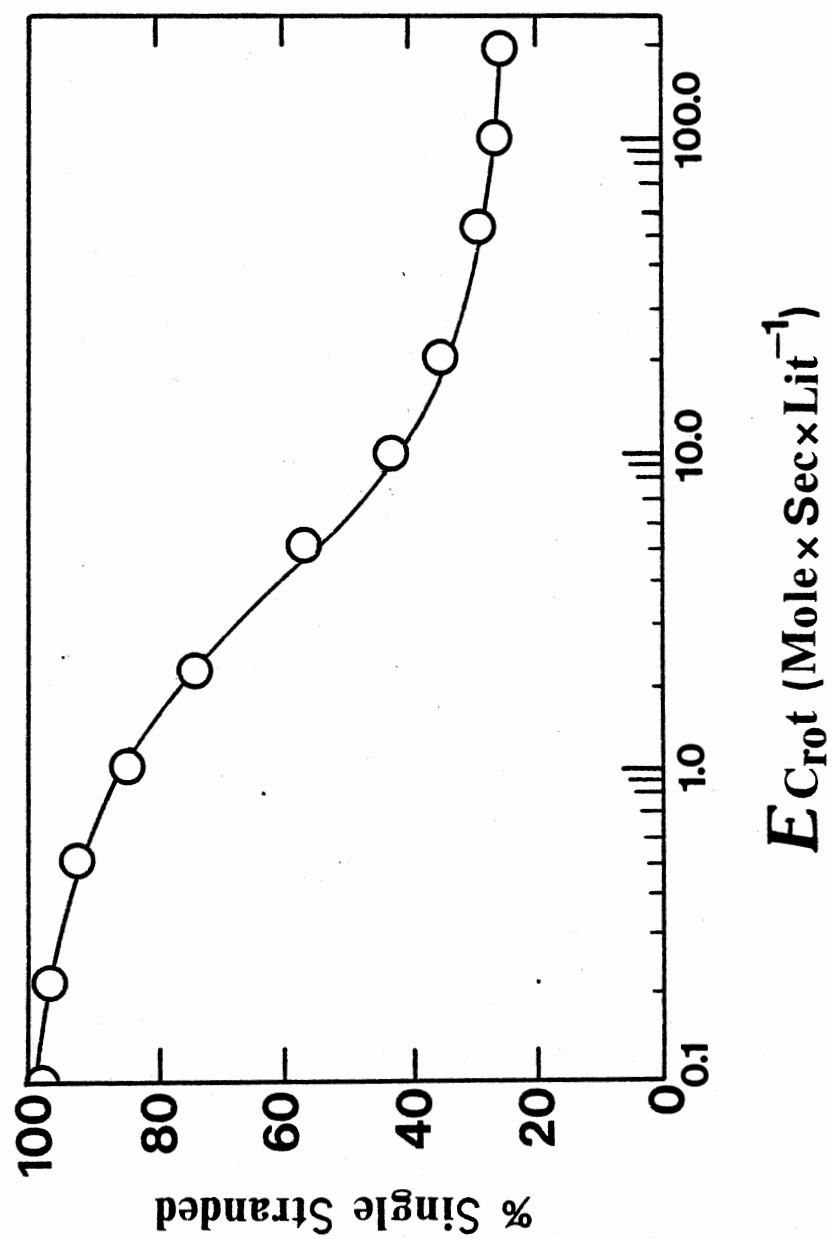
Heterologous cDNA-25S rRNA hybridizations:

Heterologous hybridization vials (prepared in triplicate) contained 0.02 μg of the radiolabeled cDNA and 20 μg of heterologous 25S rRNA fragments. The reactions were continued to E_{Crot} of 220 (calculated by the method of Britten *et al.*, 1974) to ensure maximum duplex formation. The samples were then treated as described for hybridization kinetics experiments. The sequence similarity data were expressed as percentage of relative binding, with the homologous system normalized to 100%.

RESULTS

The hybridization kinetics of homologous cDNA-25S rRNA from *Cryptococcus vishniacii* (Figure 1) show that the reaction was essentially complete at E_{Crot} 100 $\text{mol} \cdot \text{sec} \cdot \text{lit}^{-1}$ with about 72% renaturation. Continuation of reactions to E_{Crot} up to 220 $\text{mol} \cdot \text{sec} \cdot \text{lit}^{-1}$ increased the percent renaturation to slightly above 78% in experiments with *Cr. vishniacii* as the probe and up to about 84% in experiments with *Fa. neoformans* as the source of complementary DNA. The reason for this difference in homologous hybridizations is not clear but it is more likely to be the result of experimental fluctuations rather than the property of the RNA molecules from the different yeasts studied. Comparison of the kinetics of cDNA-25S rRNA hybridization with that of DNA-DNA renaturation (see Baharaeen *et al.*, 1982) indicates that the genes for ribosomal RNA sequences are less complex ($E_{\text{Crot}}^{1/2} = 6.8 \text{ mol} \cdot \text{sec} \cdot \text{lit}^{-1}$ while $E_{\text{Cot}}^{1/2} = 41.8 \text{ mol} \cdot \text{sec} \cdot \text{lit}^{-1}$). While this may seem expectable, the E_{Crot} values may be

Figure 1. Kinetics of hybridization of *Cryptococcus vishniacii*
25S ribosomal RNA with homologous complementary DNA.



distorted by the effects of secondary structure formation in rRNA molecules.

Tables II to V represent the result of cDNA-25S rRNA hybridization experiments with appropriate radiolabeled cDNA as percent actual and relative (to homologous reaction) binding.

DISCUSSION

The definitions of hierarchical categories above species have no other justification than informed judgment and convenience. At this point, however, we may compare the morphological, physiological, and biochemical characters used in defining various levels of taxa with the results of DNA-RNA homology.

A three-dimensional map can be constructed for yeasts which show significant homology to the three probes used (Figure 2). The length of the connecting lines between the two yeasts is proportional to their phylogenetic distance (100 minus % relative binding). The phylogenetic distance of each species from the three probes fixes the position of that species in a unique point in space. This point is uncertain to the extent of the standard deviations of each point. The distance between two points is also uncertain by the standard error of the difference. Standard deviations (to 10.54% relative binding) of determinations and differences (to 5.42% relative binding) between reciprocal determinations tended to be large compared with those we have experienced in DNA-DNA homology experiments (Baharaeen *et al.*, 1982), making each point actually a sphere bounded by the standard error of each difference. The circles shown in Figure 2 are

TABLE II. COMPLEMENTARY DNA-25S RIBOSOMAL RNA HOMOLOGY:

CRYPTOCOCCUS VISHNIACII PROBE COMPLEMENTARY DNA

Source of 25S rRNA	% actual binding ± SD	% relative binding ± SD
<i>Cryptococcus vishniacii</i>	78.01 ± 0.98	100.00 ± 1.26
<i>Cryptococcus lupi</i>	76.92 ± 1.18	98.60 ± 1.53
<i>Cryptococcus bhutanensis</i>	76.64 ± 0.54	98.24 ± 0.70
<i>Cryptococcus himalayensis</i>	74.99 ± 0.53	96.13 ± 0.71
<i>Filobasidiella neoformans</i>	71.71 ± 2.31	91.92 ± 3.22
<i>Phaffia rhodozyma</i>	63.05 ± 1.16	80.82 ± 1.84
<i>Leucosporidium scottii</i>	60.00 ± 0.57	76.91 ± 0.95
<i>Rhodospiridium toruloides</i>	59.88 ± 0.53	76.76 ± 0.89
<i>Candida fujisanensis</i>	55.69 ± 0.69	71.39 ± 1.24
<i>Candida humicola</i>	54.92 ± 0.69	70.40 ± 1.26
<i>Candida acutus</i>	54.74 ± 1.27	70.17 ± 2.32
<i>Aessosporon salmonicolor</i>	20.83 ± 0.44	26.70 ± 2.11
<i>Candida curvata</i>	19.89 ± 0.36	25.50 ± 1.81
<i>Pityrosporum ovale</i>	19.69 ± 0.81	25.24 ± 4.11
<i>Candida aquatica</i>	19.37 ± 0.76	24.83 ± 3.92
<i>Candida podzolica</i>	14.38 ± 0.24	18.43 ± 1.67
<i>Candida zeylanoides</i>	9.49 ± 1.00	12.17 ± 10.54
<i>Hansenula saturnus</i>	8.25 ± 0.52	10.58 ± 6.30

TABLE III. COMPLEMENTARY DNA-25S RIBOSOMAL RNA HOMOLOGY

RHODOSPORIDIUM TORULOIDES PROBE COMPLEMENTARY DNA

Source of 25S rRNA	% actual binding ± SD	% relative binding ± SD
<i>Rhodospiridium toruloides</i>	82.99 ± 2.23	100.00 ± 2.69
<i>Rhodospiridium inf.-miniatum</i>	82.80 ± 0.97	99.77 ± 1.17
<i>Rhodospiridium dacryoidum</i>	82.51 ± 0.84	99.42 ± 1.02
<i>Rhodospiridium malvinellum</i>	80.47 ± 1.52	96.97 ± 1.89
<i>Leucosporidium antarcticum</i>	72.84 ± 0.40	87.77 ± 0.55
<i>Candida fujisanensis</i>	72.56 ± 1.04	87.43 ± 1.43
<i>Leucosporidium nivalis</i>	71.57 ± 1.28	86.24 ± 1.79
<i>Leucosporidium gelidum</i>	71.32 ± 0.63	85.94 ± 0.88
<i>Leucosporidium scottii</i>	71.23 ± 1.04	85.83 ± 1.46
<i>Candida humicola</i>	70.26 ± 1.22	84.66 ± 1.74
<i>Leucosporidium stokesii</i>	70.11 ± 1.05	84.48 ± 1.50
<i>Leucosporidium frigidum</i>	70.06 ± 1.08	84.42 ± 1.54
<i>Candida acutus</i>	68.50 ± 2.88	82.54 ± 4.20
<i>Cryptococcus himalayensis</i>	67.96 ± 0.92	81.89 ± 1.35
<i>Cryptococcus laurentii</i>	67.31 ± 0.57	81.10 ± 0.85
<i>Filobasidiella neoformans</i>	66.84 ± 0.78	80.54 ± 1.17
<i>Cryptococcus albidus</i>	65.96 ± 0.41	79.48 ± 0.62
<i>Phaffia rhodozyma</i>	64.41 ± 1.17	77.61 ± 1.82
<i>Cryptococcus bhutanensis</i>	63.81 ± 0.65	76.89 ± 1.02
<i>Cryptococcus lupi</i>	62.82 ± 2.63	75.69 ± 4.19
<i>Filobasidium uniguttulatum</i>	60.99 ± 0.54	73.48 ± 0.89
<i>Cryptococcus vishniacii</i>	59.36 ± 1.33	71.52 ± 2.24
<i>Filobasidium capsuligenum</i>	58.31 ± 1.12	70.26 ± 1.92
<i>Aessosporon salmonicolor</i>	37.10 ± 0.47	44.70 ± 1.27
<i>Candida curvata</i>	36.80 ± 0.52	44.34 ± 1.41
<i>Candida aquatica</i>	36.38 ± 0.51	43.83 ± 1.40
<i>Candida podzolica</i>	21.64 ± 0.17	26.07 ± 0.79
<i>Candida zeylanoides</i>	18.80 ± 0.21	22.65 ± 1.12
<i>Tremella mesenterica</i>	13.04 ± 0.14	15.71 ± 1.07
<i>Pityrosporum ovale</i>	12.16 ± 0.23	14.65 ± 1.89
<i>Agaricostilbum palmicolum</i>	9.59 ± 0.29	11.56 ± 2.78
<i>Hansenula saturnus</i>	8.32 ± 0.10	10.03 ± 1.20
<i>Ustilago maydis</i>	7.73 ± 0.47	9.31 ± 6.08

TABLE IV. COMPLEMENTARY DNA-25S RIBOSOMAL RNA HOMOLOGU:

FILOBASIDIELLA NEOFORMANS PROBE COMPLEMENTARY DNA

Source of 25S rRNA	% actual binding ± SD	% relative binding ± SD
<i>Filobasidiella neoformans</i>	83.81 ± 1.09	100.00 ± 1.30
<i>Cryptococcus bhutanensis</i>	80.87 ± 1.32	96.50 ± 1.63
<i>Cryptococcus himalayensis</i>	79.80 ± 1.47	95.21 ± 1.84
<i>Cryptococcus lupi</i>	78.48 ± 2.14	93.64 ± 2.73
<i>Cryptococcus vishniacii</i>	78.45 ± 2.01	93.61 ± 2.56
<i>Leucosporidium antarcticum</i>	71.35 ± 0.79	85.14 ± 1.11
<i>Leucosporidium scottii</i>	71.10 ± 0.91	84.84 ± 1.28
<i>Leucosporidium frigidum</i>	70.38 ± 0.78	83.98 ± 1.11
<i>Filobasidium uniguttulatum</i>	70.32 ± 1.00	83.90 ± 1.42
<i>Filobasidium capsuligenum</i>	70.14 ± 0.59	83.68 ± 0.84
<i>Candida fujisanensis</i>	66.61 ± 2.08	79.48 ± 3.12
<i>Candida humicola</i>	65.22 ± 1.70	77.82 ± 2.91
<i>Rhodospiridium toruloides</i>	65.13 ± 1.65	77.71 ± 2.53
<i>Candida acutus</i>	59.93 ± 0.73	71.50 ± 1.78
<i>Phaffia rhodozyma</i>	59.43 ± 1.05	70.91 ± 2.19

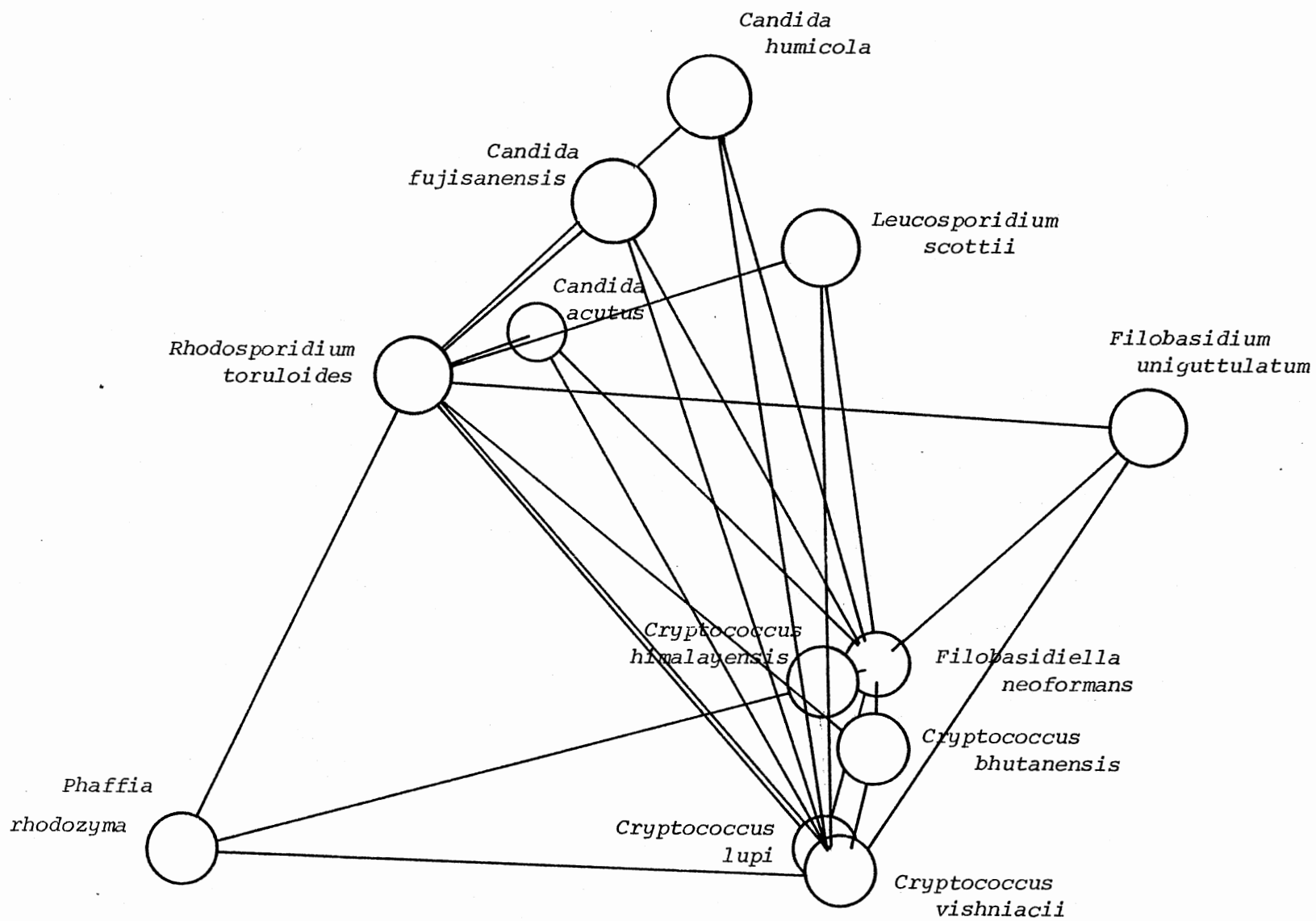
TABLE V. COMPLEMENTARY DNA-25S RIBOSOMAL RNA HOMOLOGU:

CRYPTOCOCCUS VISHNIACII PROBE COMPLEMENTARY DNA

Source of 25S rRNA	% actual binding ± SD	% relative binding ± SD
<i>Cryptococcus vishniacii</i>	78.02 ± 0.71	100.00 ± 0.91
<i>Filobasidium uniguttulatum</i>	61.28 ± 0.68	78.54 ± 0.87
<i>Filobasidium capsuligenum</i>	59.87 ± 0.67	76.73 ± 0.86
<i>Leucosporidium antarcticum</i>	58.41 ± 0.57 ^a	74.87 ± 0.73

^a determined from duplicate vials.

Figure 2. Phylogenetic relationships amongst basidiomycetous yeasts and their anamorphs. The length of the connecting lines is proportional to the "phylogenetic distance" between species or generic clusters.



sized by perspective rather than by the size of these standard errors and were not placed by taking the average of reciprocals but by the empirical fit of all determinations into three dimensions. Two-tailed *t* testing was not employed because of the great differences in variance. The strength of the alternative procedures (e.g. the Fisher Exact Test) was not felt to be sufficient to warrant their calculations from these data. The data mapped in Figure 2 include only homology values above 70%, excluding values with the largest standard deviations.

At what level of hierarchy is this method useful? Divisional and class levels are clearly beyond the scope of this tool. Without wishing to necessarily support one terminology over another, we will refer to the representatives of higher taxa in the terminology used by Alexopoulos and Mims (1979). We did not find any significant difference between the very low homology of the probes used with Hemiascomycetes such as *Hansenula saturnus* of the class Ascomycetes, and basidiomycetous anamorphs such as *Pityrosporum ovale*, Phragmobasidiomycetidae such as *Agaricostilbum palmicolum* and *Tremella mesenterica*, and Teliomycetidae such as *Ustilago maydis* of the class Basidiomycetes. The technique is too new at this point to allow interpretation of intermediate homology values (e.g. below 70% relative binding). The unmapped genus *Aessosporon* is widely separated from *Rhodosporidium* (the closest mapped genus) by about 56 units of phylogenetic distance ($44.7 \pm 1.27\%$ relative binding). This gap may represent an ordinal separation.

Genus and family may be within the reach of more quantitative

definition. The two largest teleomorphic basidiomycetous genera, *Leucosporidium* (6 species) and *Rhodospiridium* (5 species) appear to be point clusters contained within the boundaries of the standard errors of the means of determination (Tables III and IV). The six species of *Leucosporidium* appear $3.28 \pm 4.13\%$ relative binding units (RBU) broad from the *R. toruloides* probe and the four species of *Rhodospiridium* studied (Table III) appear 3.03 ± 4.25 RBU broad from *R. toruloides* probe. The *Leucosporidium* cluster was probed only from a distance at two of the positions which would present the broadest dimension of a linear cluster but the *Rhodospiridium* cluster was probed from within and therefore cannot be broader than roughly twice the indicated dimensions. Thus, it seems unlikely that, in practice, these generic points are larger than they appear. The two other teleomorphic basidiomycetous yeast genera included in these studies, *Filobasidiella* and *Filobasidium*, are smaller, containing two and three described species respectively. Therefore, they are not likely to indicate the largest size which a cluster can attain and still remain generic. The two species of *Filobasidiella* are so closely related as to exhibit DNA-DNA homology (Aulakh et al., 1981). The generic cluster of *Filobasidium* is the same size as that of *Rhodospiridium* and *Leucosporidium* - the two yeast species in this genus, *Fm. capsuligenum* and *Fm. uniguttulatum*, differ at the most by 3.22 ± 2.12 RBU (with *R. toruloides* as probe).

In theory, if any of these clusters are to be recognized as a genus, the gaps which separates them should be significantly larger than the dimensions of the generic clusters themselves. The gap

between *Filobasidiella* and *Filobasidium*, 13.38 ± 3.68 RBU (measured from the nearest member of *Filobasidiella*/*Cryptococcus* anamorph cluster), is of approximately the same magnitude as the gap between *Filobasidium* and *Leucosporidium* (10.94 ± 1.78 RBU between *L. frigidum* and *Fm. uniguttulatum* in respect to *R. toruloides* probe), or that between *Rhodosporidium* and *Leucosporidium* which is 9.0 ± 1.97 RBU (between *R. malvinellum* and *L. antarcticum* using *R. toruloides* as probe), or between *Rhodosporidium* and *Filobasidiella* (16.43 ± 2.22 RBU, between *R. toruloides* and *Fa. neoformans*). Therefore, a gap of more than 9 RBU is certainly consistent with the morphological definitions of teleomorphic genera forming a pin point cluster.

The genus *Filobasidiella* is closely trailed by anamorphic yeasts. As shown in Figure 2, the classically defined anamorphic *Cryptococcus* species (exclusive of those which are anamorphs of *Filobasidium*) form a linear cluster with *Filobasidiella* and show no significant gap. This cluster is 10.37 ± 2.62 RBU broad (from the *R. toruloides* probe) with *Fa. neoformans* to *Cr. himalayensis* 4.79 ± 2.25 RBU broad (including the classically defined *Cryptococcus* species, *Cr. albidus* and *Cr. laurentii*) but *Cr. bhutanensis*, only 3.50 ± 2.08 RBU from *Fa. neoformans*, is in turn only 2.86 ± 3.18 RBU from *Cr. lupi* which in turn is inseparable from *Cr. vishniacii*. The greatest distance between these two latter species by *R. toruloides* probe is 4.17 ± 4.75 RBU. The total length of the *Filobasidiella*/*Cryptococcus* anamorph cluster then becomes 10.37 ± 2.62 (*R. toruloides* probe) RBU which is the same as the dimensions of the generic gap. This implies that isolates at the extremes of the cluster (i.e. *Cr. vishniacii* and *Fa. neoformans*)

would be distinct genera in the absence of connecting taxa. This illustrates a concept long recognized in the definition of species—that real taxa are in the process of evolution and hence cannot be rigidly sized.

Leucosporidium is also closely trailed by anamorphic yeasts. The distance of *Candida humicola* and *C. fujisanensis* from *L. scottii* (Tables II to IV, Figure 2) are barely greater than the standard error of the difference. This suggests that these species are *Leucosporidium* anamorphs and that they are properly placed in *Vanrija* Moore (1980) rather than separated into two genera *Apiotrichum* and *Rhodotorula* as suggested by von Arx and Weijman (1979).

Candida as a genus was so defined as to include unrecognized anamorphic ascomycetous and basidiomycetous yeasts alike; *Vanrija*, defined as including all basidiomycetous "*Candida*" species, is still heterogeneous. "*Candida*" *acutus* appears from our data to belong to a separate genus from *V. humicola* (the type species of *Vanrija*). "*Candida*" *aquatica* and "*C.*" *curvata* do not belong to either genus and are similarly clearly separated from "*C.*" *podzolica* and "*C.*" *zeylanoides* by their homologies with the *R. toruloides* probe. *Vanrija* therefore, contains by RNA homology at least four generic clusters of anamorphic yeasts. The anamorphic genus *Phaffia* Miller et al. (1976) appears by rRNA homology to have been well founded.

The quantitative definition of a yeast genus in Basidiomycetes may not hold true within the ascomycetous yeasts. If Ascomycetes did really arise earlier in geologic time than Basidiomycetes, and if the rate of evolution is the same in ribosomal RNA cistrons, an asco-

mycetous genus would show a larger spread than a basidiomycetous genus. A single ascomycetous genus has been examined; the 25S rRNA homologies observed in the species of *Saccharomyces* by Bicknell and Douglas (1970) ranged linearly over 50%. On the basis of the rRNA homology of the species to the two probes used, they suggested that five of the species (*S. delphensis*, *S. dolbshanskii*, *S. fragilis*, and *S. wickerhamii*) to be segregated and placed in *Kluyveromyces*. The results however, showed no gaps between the two genera, probably illustrating the limitations of two-dimensional mapping. The fact that these species were subsequently placed in five different genera (rather than two) on the basis of phenetic characters (see Barnett et al., 1979), and that the definition of genera in ascomycetes and basidiomycetes is based on different sets of characters, makes it difficult to draw a definite conclusion from these results.

Differences between the methodologies employed by these authors (the competition reactions) and our procedure may also contribute to the varying views of what constitutes a genus.

What are the dimensions of a basidiomycetous family? Well recognized families are represented in our study by Filobasidiaceae Olive (1968) (including the two genera *Filobasidiella* Kwon-Chung and *Filobasidium* Olive), Sporidiaceae Moore (1980) (including the two genera *Leucosporidium* Fell et al. and *Rhodosporidium* Banno), and Sporidiobolaceae Moore (1980) represented by a single genus *Aessosporon* van der Walt. The ribosomal RNA homology values do not appear to separate Filobasidiaceae from teliospore producing family Sporidiobolaceae by more than generic gaps. These results are not consonant

with Moore's (1980) proposal for a wider separation (at the class level) between these two families. One possibility which could be entertained is that the generic gaps observed are actually familial gaps.

The only really large gap observed in our data is between *Aessosporon* and the mapped families (55.30 ± 1.80 RBU to *R. toruloides* probe; 73.30 ± 2.98 RBU to *Cr. vishniacii* probe). One might hypothesize that this gap represents an ordinal (or higher) separation. The position of *Aessosporon* and other ballistospore producing yeasts (family Sporidiobolaceae) has obviously not been thoroughly examined. The use of additional probes may well produce a map in which these yeasts also form a coherent group with the other basidiomycetes, gapped only at the same distance as genera.

The comparison of DNA-RNA homology and phenetic characters used in demarcation of yeasts at taxonomic levels above species indicates that in some cases, inappropriate weight has been given to a delimiting character. Separation of Filobasidiaceae and Spoidiaceae at the class level (Moore, 1980) on the basis of teliospore formation by the latter is belied by DNA-RNA homology values. The production of ballistospores on the other hand, seems to be an important phenetic character in separating Sporidiaceae and Sporidiobolaceae. This conclusion however, requires further confirmation since only one genus was examined within the latter family.

Other aspects of vegetative reproduction also separates *Filobasidiella* (possibly Filobasidiaceae) and Sporidiaceae; *Filobasidiella* and the *Cryptococcus* species examined bud repetitively from a single

site (i.e. they are monopolar) (Baharaeen and Vishniac, 1981), while the Sporidiaceae bud repetitively from both ends of the cell (i.e. they are bipolar) (McCully and Bracker, 1972; Watson and Arthur, 1977). This distinction has been given subfamilial weight in the Saccharomycetaceae (class Ascomycetes). Budding polarity is given additional weight by von Arx and Weijman (1979) who noted a correlation between budding polarity (as examined only in cultures of heterogeneous growth rates, only by light microscopy) and different sugar composition of whole cells of basidiomycetous anamorphic yeasts. The appropriate weight of the budding character in basidiomycetous yeasts is uncertain, but it is evident that not all yeasts with similar budding polarity belong to the same genus; "*Candida*" *acutus* is also monopolar (unpublished data of M. Morgan). The polarity of other *Candida* species examined, with the exception of "*C.*" *zeylanoides* (multipolar in M. Morgan's unpublished data) is not reliably known.

At the generic level, *Leucosporidium* and *Rhodospodium* are segregated by life cycle characters as well as by the presence or absence of carotenoid pigments; the anamorphic isolates are most easily recognized by color. Since all eukaryotic cells contain carotenoids, the amount of carotenoid production has appeared to many mycologists a dubious generic character- notably leading von Arx and Weijman (1979) to place the colorless "*C.*" *fujisanensis* in *Rhodotorula*. The use of astaxanthin production in erecting the genus *Phaffia* does not suffer from the same objection, but colony color as a generic distinction is supported by rRNA homology data in both instances.

The use of nitrogen source utilization and carbon source assimilation as generic characters is not supported by rRNA homology. The utilization of nitrate-N, discarded by Kurtzman (personal communication) in delimiting the ascomycetous genera *Hansenula* and *Pichia*, does not separate the species of *Rhodospiridium* into two genera. The assimilation of inositol has been despite the objection of more broadly experienced microbiologists (van Neil, 1971), the most consistently and insistenty proclaimed character of *Cryptococcus* (see Lodder, 1970). *Cryptococcus bhutanensis*, *Cr. vishniacii*, and *Cr. lupi* do not assimilate inositol.

A very real problem at this point is the weight to be given to rRNA homology data. Does complementary DNA-25S rRNA homology by the techniques used in this study really represent a conserved and phylogenetically reliable character or does it suffer from any significant defects which may affect its value for this sort of study. One of the possible defects of this technique initiates from the presence of double stranded regions in the rRNA molecules (Cantor et al., 1980; Glotz and Brimacombe, 1980; Mankin et al., 1981; Noller and Woese, 1981; Woese et al., 1980). Synthesis of cDNA by reverse transcriptase on enzymatically polyadenylated rRNA fragments will not be completely successful because of the presence of these double stranded regions. It is therefore, more likely that most of cDNA molecules synthesized represent the single stranded regions of the RNA molecules. Although there is no evidence for these regions being more or less conserved than the double stranded portions, strongly conserved regions (specially at the termini) do exist within yeast (*Saccharomyces cere-*

visiae) 25S rRNA molecule- so conserved as to show sequence homology to the mouse 28S rRNA (Michot *et al.*, 1982). It would therefore be desirable to determine the amount of double stranded and single stranded portions in the 25S rRNAs by more sophisticated methodology (comparative sequence analysis, chemical modifications, nuclease susceptibility) in order to find out how much of the RNA sequences are being compared. In spite of this defect, the technique is sufficiently less laborious and time consuming than sequencing or cataloging techniques to have allowed the comparison of many yeasts for phylogenetic studies. Furthermore, it has been useful for a quantitative definition of yeast genera and confirmation of the generic assignment of the *Cryptococcus vishniacii* complex as anamorphs of *Filobasidiella*.

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