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MOLECULAR PROBES FOR THE IDENTIFICATION OF MEDICALLY IMPORTANT *CANDIDA* SPECIES AND DIFFERENTIATION OF *CANDIDA ALBICANS* STRAINS

A Thesis

Presented for the

Master of Science

Degree

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ABSTRACT

Differentiation of the medically important yeasts of the genus Candida is difficult not only for the research laboratory but especially for the clinical laboratory. Likewise, distinguishing C. albicans strains is difficult but necessary for source studies of recurrent vaginitis.

This study explores a new method for identification of Candida species, as well as differentiation of C. albicans strains, using a genetic approach. Whole cell DNA isolated from various species of Candida was digested with restriction endonucleases, electrophoresed, Southern blotted to nitrocellulose and probed with the cloned actin gene of C. albicans SR 1010. Each species gave its own distinct restriction enzyme pattern which can be used to distinguish that species. Mitochondrial DNA was isolated from C. albicans SR 1010 and used as a probe against restriction enzyme digested whole cell DNA of C. albicans strains that was electrophoresed and Southern blotted to nitrocellulose. The EcoRI and Pvu II double digests fragment sizes that were produced were used to place thirty-three C. albicans/ C. stellatoidea strains within fourteen groups. The results of the two types of probes also indicates that C. stellatoidea and C. albicans are synomynous.

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CHAPTER I

INTRODUCTION

Many Candida yeast species are endogenous to their host and under normal circumstances act as commensals. However, if the host is "stressed" or the host immune system is weakened, some of these yeasts can cause diverse infections in the host and are thus opportunistic pathogens.

The genus Candida contains a variety of species, some of which have medical importance. This genus is considered the 'dumping ground' for those yeast species which can not be placed in any other genera of the fungi imperfecti (van Uden and Buckley, 1970). The taxonomy of this genus has varied throughout the twentieth century (van Uden and Buckley, 1970) and is still surrounded by controversy (Yarrow and Meyer, 1978; McGinnis et al., 1984). There are eight major medically important Candida species as described by Odds (1979): C. albicans, C. stellatoidea, C. tropicalis, C. parapsilosis, C. pseudotropicalis, C. krusei, C. guilliermondii, and C. (Torulopsis) glabrata.

The ability to differentiate the various Candida species is based mainly on morphological and physiological characteristics including the distinguishing of C. albicans strains (Odds and Abbott, 1980; Polonelli, 1982). This includes the production of different enzymes and chlamydospores, fermentation of selected carbohydrates, assimilation of carbohydrates and nitrogen sources, susceptibility to the toxic effects of killer yeasts, the requirement of certain vitamins and other

physiological characteristics (Kockova-Kratochvilova and Ondrusova, 1972; Odds, 1979).

Some emphasis has been placed on the use of serological tests using cell wall antigens to help catergorize the medically important Candida. Hasenclever and Mitchell (1961) were able to separate C. albicans into two serological types: group A and group B. Further analysis revealed a close serological relationship between C. albicans group A with C. tropicalis and C. albicans group B with C. stellatoidea (Hasenclever et al., 1961; Fraser, 1977; Gabriel-Bruneau et al., 1982). However, Poulain et al. (1983) found that C. albicans serotype B can express in vitro and in vivo one or several antigens so far considered as specfic for serotype A depending on isolation or growth conditions.

Tsuchiya et al. (1974) and Taguchi et al. (1979) describe a commerically available serological test in Japan used for the identification of various Candida species, including C. (Torulopsis) glabrata. The test uses monospecfic antisera and includes three antisera used to differentiate C. albicans group A, C. albicans group B, C. stellatoidea and C. tropicalis. But as indicated earlier (Poulain et al., 1983) some antigens may only be expressed under certain conditions.

Other antigenic determinants have also been utilized in separating the various Candida species of interest. Cytoplasmic antigens of several Candida species have been used in antibody-antigen reactions. Greenfield and Jones (1982) found some degree of cross-reactivity between antisera of C. albicans and cytoplasmic extracts of C. stellatoidea, C. tropicalis, C. pseudotropicalis, C. krusei, C. parapsilosis, and C. guilliermondii. Gabriel-Bruneau and Gruinet (1984) also used cytoplasmic

extracts in comparing Candida species by a crossed-line immunoelectrophoresis technique. The results of Gabriel-Bruneau and Gruinet showed no differences between group A and group B C. albicans; all group B antigens cross reacted with C. stellatoidea and C. claussenii; a close reactivity between C. tropicalis and C. albicans, C. stellatoidea, or C. claussenii; considerably less reactivity between C. albicans or C. tropicalis with C. krusei, C. pseudotropicalis, C. parapsilosis, and C. guilliermondii.

Another method that has been used is electrophoretic analysis of protein extracts (anionic or cationic protein separation) from yeast cells and subsequent numerical taxonomic analysis (Shechter et al., 1972). The results found C. albicans and C. stellatoidea indistinguishable. They also noted various similarities and differences (some species specific) between the other species tested. In contrast to most other data in the literature they found little similarity between C. tropicalis and the C. albicans/ C. stellatoidea group.

Enzyme profiles of yeasts are also being tested for possible use in clinical identification (Casal and Linares, 1983). However, the results so far remain ambiguous (for the enzymes tested so far) when used to distinguish between species.

Some genetic studies have been carried out to determine the relatedness of certain species. These studies include the elucidation of the DNA base composition (Stenderup and Bak, 1968; Meyer and Phaff, 1972), DNA-RNA homology (Bak and Stenderup, 1969; Segal and Eylan, 1974) and gross DNA homology studies (Meyer, 1979). The results of these homology studies indicate a very close relationship between C. albicans

and C. stellatoidea, but only a distant association between these species and C. tropicalis (in contrast to most antigenic determinant studies), C. pseudotropicalis or C. parapsilosis. The G+C content varies among the medically important Candida from approximately 36% for C. albicans to 45% for C. guilliermondii.

The identification of Candida pathogens is important not only in a clinical sense but also for research and epidemiology purposes. It is also necessary for researchers and epidemiologists to be able to distinguish strains within a species. Researchers need to be able to differentiate strains of a species so that in reporting results, strain variability can be taken into account. Epidemiologists require strain determination to study the epidemiology of initial and recurrent infections of Candida.

The purpose of this study was to explore a genetic approach to the differentiation of medically important Candida species. A preliminary study was also initiated for the differentiation of strains of C. albicans, the most prevalent cause of fungal infections in humans. The approach used in each case was the same except that different probes were used for separating species and in segregating strains.

Whole cell DNA was isolated from either a species of Candida or a strain of C. albicans. The DNA was digested with one or a combination of restriction endonucleases and the resulting fragments were separated electrophoretically on a vertical agarose gel. The DNA was transfered to a solid support (nitrocellulose) after dissociating the DNA. A radioactive DNA probe was hybridized to the DNA on the nitrocellulose, followed by autoradiography using x-ray film. The bands that were

produced on the film represented the fragment(s) of DNA homologous to the radioactive probe. The fragment sizes (in kilobases) were determined and comparisons made either between species (for species differentiation) or between strains (for strain groupings).

The probe used to differentiate Candida species was the C. albicans SR 1010 actin gene cloned into pBR322 (B. A. Lasker, J. Bagash, and W. S. Riggsby, Manuscript in preparation).

Actin is a highly conserved gene product found in all higher eukaryotes. There are various forms of actin produced by higher organisms, but only one has been found in C. albicans. In addition, some higher eukaryotes contain multigene families of actin (Fryberg et al., 1981; Scheller et al., 1981; Engel et al., 1981) including the fungus Dictyostelium (Kindle and Firtel, 1978). C. albicans has been determined to have only one gene for actin (B. A. Lasker, J. Bagash and W. S. Riggsby, Manuscript in preparation).

The conserved nature of the actin protein lies mainly in its amino acid sequence. The nucleotide sequence varies not only between different organisms but also within mutigene families. However, slight homology can still be expressed across kingdom lines. Therefore, the actin gene is a good choice for differentiating a diverse group of 'related' Candida species.

Mitochondrial DNA of C. albicans SR 1010 was used as a probe to distinguish groupings in which the various C. albicans isolates might be placed. Mitochondrial DNA, in contrast to the actin gene, is not highly conserved; mitochondrial DNA evolves approximately ten times faster than nuclear DNA (Brown et al., 1979) where the actin gene is located.

Restriction enzyme analysis of mitochondrial DNA has been used for comparisons of species in primates (Ferris et al., 1981; Brown et al., 1979), Paramecium (Cummings, 1980), rats (Hayashi et al., 1979) Zea (Timothy et al., 1979) and several yeasts (Kozlowski and Stepien, 1982; Clark-Walker and McArthur, 1978). Lansman et al. (1981) describe the potential that mitochondrial DNA restriction enzyme analysis can have, however, Adams and Rothman (1982) warn that a phenetic approach using restriction enzyme data for determining phylogenetic relationships will be biased. Due to the diverse nature of the genus Candida it was more plausible to use mitochondrial DNA for a comparison of restriction enzyme fragment patterns of strain isolates rather than species differentiation.

CHAPTER II

METHODS AND MATERIALS

Growth of Organisms

Cells to be used for whole cell or mitochondrial DNA isolation were grown in the yeast phase in one of the following media:

1) YEPL medium (Zeman and Lusena,1975; Syverson,1981) - this medium contains 1% Bacto-yeast extract (Difco); 2% Bacto-peptone (Difco); and 6% D,L - sodium lactate (Sigma as 60% syrup).

2) YNB medium - this medium contains 0.67% Yeast Nitrogen Base (Difco) and 2% Dextrose (Fisher).

3) YEPG medium - this medium contains the same components as YEPL with the exception that 2% Glycerol (Fisher) was used in place of sodium lactate.

The medium was inoculated from stock cultures maintained on Sabouraud Agar Modified (Difco) at 4°C. The culture was placed at 28-30° C with vigorous shaking for approximately 48 hours. Depending on the organism a yield of 6-12 grams (wet weight) of cells were obtained per liter of medium.

Species/Strains Used

A list of the organisms used in the following experiments is found in Table I. Candida albicans strain H317 (obtained from Dr. Jeffrey M. Becker, University of Tennessee, Knoxville) was used as a standard throughout the experiments and as a source of purified mitochondrial DNA. This strain has the ability to undergo 95% conversion from the

Table I. Strains Used in Study

Strain Designation	Species	Source[a]	Other Designation[b]	Reference[c]
SR 1001	C. albicans	A	ATCC 18804	1
SR 1010	C. albicans	B	H317	2
SR 1017	C. albicans	B	H350	2
SR 1025	C. albicans	B	4W	2
SR 1055	"C. albicans"	S	NUM-2	3
SR 1056	"C. albicans"	S	NUM-43	3
SR 1101	Kloeckra africana	A	ATCC 24232	1
SR 1102	C. (Torulopsis) glabrata	A	ATCC 2001	1
SR 1103	C. tropicalis	A	ATCC 13803	1
SR 1104	C. parapsilosis	A	ATCC 22019	1
SR 1105	C. stellatoidea	A	ATCC 11006	1
SR 1106	C. albicans	A	ATCC 14503	1
SR 1107	C. pseudotropicalis	A	ATCC 2512	1
SR 1110	C. albicans	G	83-57	4
SR 1113	"C. stellatoidea"	C	CLFMC 362	4
SR 1114	C. stellatoidea	C	CLFMC 286	4
SR 1116	C. albicans	C	CLFMC 257	4
SR 1117	C. albicans	C	CLFMC 260	4
SR 1118	"C. albicans"	C	CLFMC 242	4
SR 1119	C. albicans	C	CLFMC 244	4
SR 1120	C. albicans	O	84F151V	4
SR 1121	C. albicans	O	82F371V	4
SR 1122	C. albicans	O	X724V	4

Table I. (cont'd)

Strain Designation	Species	Source[a]	Other Designation[b]	Reference[c]
SR 1123	C. albicans	O	82F2929V	4
SR 1124	C. albicans	O	PERRY	4
SR 1125	C. albicans	O	BOND	4
SR 1131	C. krusei	A	ATCC 6258	1
SR 1132	C. krusei	A	ATCC 14243	1
SR 1133	C. albicans	A	ATCC 24433	1
SR 1134	C. stellatoidea	A	ATCC 36232	1
SR 1135	C. tropicalis	A	ATCC 750	1
SR 1136	C. pseudotropicalis	A	ATCC 46764	1
SR 1137	C. utilis	A	ATCC 9226	1
SR 1201	C. tropicalis	T	PRA	4
SR 1202	C. tropicalis	T	PRZ	4
SR 1203	C. tropicalis	T	PRB	4
SR 1204	C. tropicalis	T	PRY	4
SR 1205	C. guilliermondii	T	PRC	4
SR 1206	C. guilliermondii	T	PRX	4
SR 1207	C. guilliermondii	T	PRD	4
SR 1208	C. guilliermondii	T	PRW	4
SR 1209	Trichosporum beiglii	T	PRE	4
SR 1213	C. parapsilosis	T	PRG	4
SR 1214	C. parapsilosis	T	PRT	4
SR 1215	C. parapsilosis	T	PRH	4
SR 1216	C. parapsilosis	T	PRS	4

Table I. (cont'd)

Strain Designation	Species	Source[a]	Other Designation[b]	Reference[c]
SR 1217	C. parapsilosis	T	PRI	4
SR 1218	C. parapsilosis	T	PRR	4
SR 1219	C. albicans	T	PRJ	4
SR 1220	C. albicans	T	PRQ	4
SR 1221	C. albicans	T	PRK	4
SR 1222	C. albicans	T	PRP	4
SR 1223	C. albicans	T	PRL	4
SR 1224	C. albicans	T	PRO	4
SR 1225	C. albicans	T	PRM	4
SR 1227	C. (Torulopsis) glabrata	T	V29-1	4
SR 1241	C. albicans	T	V31-1	4
SR 1243	C. albicans	T	V33-1	4
SR 1244	C. albicans	T	C11-1	4
SR 1245	C. albicans	T	V86	4

[a] Sources: A, American Type Culture Collection, Rockville, MD; B, J. M. Becker, University of Tennessee, Knoxville, TN; C, M. Casal Roman, University of Cordoba, Spain; G, D. G. Ahearn, Unversity of Georgia, Athens, GA; O, F. C. Odds, Unversity of Leicester, Leicester, England; T, L. J. Torres-Bauza, Unversity of Puerto Rico, San Juan, PR.; S, T. Suzuki, Nagoya University School of Medicine, Nagoya, Japan.

[b] This column lists the strain designation given by the source from whom the strains were obtained.

[c] References: 1 - ATCC catalog; 2 - Riggsby et al., 1982 and Wills et al., 1984; 3 - T. Suzuki et al., 1982; 4 - personal communication.

yeast to the mycelium phase of growth (Torres-Bauza and Riggsby, 1980) and was confirmed by standard tests (Silva-Hutner and Cooper, 1974).

Six other medically important Candida species, as well as Candida utilis and Kloeckra africana, were obtained from the American Type Culture Collection (see Table I). A total of fourteenn strains were obtained from this source.

Additional strains of C. albicans (7 strains), C. tropicalis (4 strains), C. guilliermondii (4 strains), C. parapsilosis (6 strains) and Trichosporum beiglii (1 strain) were obtained from oral clinical isolates from Puerto Rico (Dr. Luis J. Torres-Bauza). Five additional strains of C. albicans and one strain of C. glabrata, isolated from vaginal specimens, were supplied by Dr. Torres-Bauza.

Two putative haploid strains of C. albicans, NUM-2 and NUM-43, were obtained from Dr. Suzuki, University of Kyoto, Japan. Four putative C. albicans and two putative C. stellatoidea were obtained from M. Casal Roman, University of Cardoln, Spain.

Five C. albicans strains were obtained from Dr. F. C. Odds of the United Kingdom . One isolate of C. albicans was obtained from an AIDS patient by Dr. D. G. Ahearn of Atlanta, GA. Two other C. albicans strains were also used which were obtained from Dr. J. M. Becker.

Preparation of Spheroplasts

Spheroplasts of yeast cells were obtained using the procedure of Torres-Bauza and Riggsby (1980). After the yeast cells had been allowed to grow for 48 hours at 30°C the cells were centrifuged for 5 minutes in a GSA rotor, Sorvall RC-2B centrifuge at 4000 rpm at 4°C. The cells were washed twice with distilled water, spinning each time at 4000 rpm for 5

minutes at 4°C. The pellets were resuspended in a Pre-treatment solution at a concentration of 1 gram of cells (wet weight) to 5ml of Pre-treatment solution. The Pre-treatment solution consisted of 0.77 gms of Dithiotheritol (Sigma), 0.186 gms of EDTA (Fisher), 1.21 gms of Trizma Base (Sigma), and 0.1 gms of Pronase (Cal-Biochem) per 100 ml of Osmotic buffer (0.6M KCl, 0.04M K HPO ,pH 6.0). The suspension was allowed to shake at 28-30°C for 1-2 hours.

At this point the cells were centrifuged as before except at 5000 rpm. The cells were washed twice in Osmotic buffer (to prevent osmotic lysis) and centrifuged each time at 5000 rpm for 5 minutes at 4°C.

The pellets were resuspended in Osmotic buffer (at a concentration of 1 gram wet weight of cells per 5 ml of Osmotic buffer) which contained 10,000 units/ ml of beta-glucourinidase (Sigma), 0.2 mg/ ml of chitinase (Cal-Biochem), 0.0005 ml 2-mercaptoethanol per milliliter. The cells were allowed to shake at 28-30°C for 1.5-3 hours.

Spheroplast formation was tested for by the following procedure: 0.02 ml of cell suspension was placed in each of 0.1 ml of Osmotic buffer (as control) and 0.1 ml of distilled water. The test suspensions were shaken and allowed to sit at room temperature for several minutes. The samples were observed under a 100x oil immersion microscope for lysis of the cells in the distilled water. Cells in the Osmotic buffer should not lyse. If lysis was observed in the distilled water, the spheroplasts were centrifuged as before except at 6000 rpm and washed twice in Osmotic buffer, centrifuging each time at 6000 rpm. If no lysis was observed, the cells were allowed to continue shaking until a postive lysis was observed.

Spheroplasts were either stored in SS medium (0.05M 2-(N-Morpholino)ethanesulfonic acid (Cal-Biochem), 0.05M Dextrose,0.2M KCl, 0.6M Sorbitol (Fisher), pH 6.0 with KOH) at 4°C or used immediately in a DNA isolation procedure.

Whole Cell DNA Isolation

Whole cell DNA was extracted from spheroplasts by the method of Cryer et al. (1975), as modified by Riggsby et al. (1982). Spheroplasts were centrifuged at 6000 rpm, GSA rotor, 5 minutes at 4°C. The pellet was resuspended in approximately five volumes of TEN buffer (0.1M Trizma base, 0.1M EDTA, 0.2M NaCl, pH 8.0 with HCl) which contained 0.1 mg/ ml Proteinase K (Boehinger-Mannheim) and 1% SDS (Sigma) from a 25% stock solution. The spheroplasts were allowed to lyse for 1-1.5 hours at 37°C. The lysate was extracted with an equal volume of phenol (TEN buffer saturated)-chloroform-isoamyl alcohol (25:24:1). The sample was shaken for 5 minutes and centrifuged in Corex glass tubes, Sorvall centrifuge, SS-34 rotor at 9000 rpm. The top layer was extracted twice with chloroform-isoamyl alcohol (24:1) and centrifuged each time for 5 minutes at 9000 rpm. The top layer was precipitated with 2.5 volumes of ethanol. If a precipitate formed immediately it was centrifuged at 4000 rpm, the ethanol suctioned off and the nucleic acid pellet resuspended in 0.1x SSC (0.015M NaCl, 0.015M NaCitrate(Fisher), pH 7.0 with HCl) and stored at 4°C. If a precipitate did not form immediately the ethanol suspension was placed at -20°C overnight and centrifuged for 30 minutes at 10,000 rpm. The ethanol was suctioned off and the pellet resuspened in 0.1x SSC and stored at 4°C.

After the DNA was dissolved in 0.1x SSC the solution was made 1x SSC by the addition of 0.11 volumes of a 10x SSC stock. The following enzymes were added to the DNA solution at the indicated concentrations and allowed to incubate at 37° C for 1-2 hours: Ribonuclease T1 (Boehinger-Mannheim) 100 units/ ml, Ribonuclease A (Sigma) 0.025 mg/ ml, alpha-amylase (Boehinger-Mannheim) 0.1 mg/ ml, beta-amylase (Boehinger-Mannheim) 0.1 mg/ ml.

The enzyme treated DNA solution was extracted once with phenol-chloroform-isoamyl alcohol as described above as well as two chloroform-isoamyl alcohol extractions, ethanol precipitated and resuspended in 0.1x SSC. After the pellet had dissolved, the Ribonuclease-amylase enzyme treatment was repeated along with the organic extractions and ethanol precipitations. Instead of resuspending the DNA pellet in 0.1x SSC at this point, the pellets were resuspended in 1x TE buffer (10mM Trimza base, 1mM EDTA, pH 7.4 with HCl).

Once resuspended, the DNA samples were made 0.3M NaAcetate, .0001M EDTA (pH 7.0) by the addition of a 10x concentrate. The DNA samples were precipitated with 0.54 volumes of Isopropanol, centrifuged for 5 minutes at 4000 rpm, resuspended in 1x TE buffer and stored at -20°C till used.

Mitochondria Isolation

Mitochondria were isolated by a modified procedure of Zeman and Lusena (1975) as described by Wills et al. (1984). Spheroplasts were prepared as described ealier. The spheroplasts were either a) used immediately after spherplast formation or b) centrifuged out of the SS medium they were stored in at 6000 rpm, 5 minutes, GSA rotor, at 4°C.

From this point on the sample was kept on ice until mitochondrial DNA was obtained.

The spheroplasts were resuspended in Lysis buffer (0.25M Sucrose, 10mM Trizma base, 1mM EDTA, pH 7.5 with HCl) at a concentration of approximately 1 gram of speroplasts/ 7.5 ml. The spheroplast suspension was homogenized in a Dounce homogenizer and stirred for 30 minutes surrounded with ice. The lysate was returned to a pH of 7.5 with the addition of 10N NaOH, centrifuged for 10 minutes , 5000 rpm, SS-34 rotor, at 4°C and the supernatant placed in clean tubes. The pellets were resuspended in Lysis buffer at approximately 60% of the original volume used and homogenized as before. Both the supernatant and the pellet suspension were centrifuged as above. All supernatants were pooled, placed in clean Corex tubes and centrifuged for 40 minutes, 10,000 rpm, SS-34 rotor, at 4°C to obtain a crude mitochondrial pellet.

The crude mitochondrial pellet was resuspended in DNAse buffer (0.3M Sucrose, 20mM Trizma base, 0.15% BSA (Pentex), 5mM MgCl (Sigma 4.9M stock), pH 7.5 with HCl) and homogenized gently. DNAse I (Boehinger-Mannheim) was added to a final concentration of 0.01 mg/ ml and placed at 4°C for 30 minutes. An equal volume of DNAse Wash buffer (0.3M Sucrose, 20mM Trizma base, 0.15% BSA, 1mM EDTA, pH 7.5 with HCl) was added to the crude prep which was centrifuged for 30 minutes, 10,000 rpm, SS-34 rotor, at 4°C. The supernatant was discarded and the pellet resuspended in more DNAse Wash buffer and gently homogenized. The washed prep was centrifuged as before. The supernatant was discarded and the pellet resuspended in 25% Sucrose buffer (25% Sucrose, 10mM Hepes (Sigma), 1mM EDTA, pH 7.0 with NaOH) with gentle homogenizing. The

mitochondrial prep was layered upon continuous 25-50% Sucrose gradients prepared from 25% and 50% Sucrose buffers. The gradients were centrifuged for 1.5 hours, 21,000 rpm, SW-28 rotor, L5-75 ultracentrifuge, at 4°C.

After centrifugation the gradient material was removed to the mitochondrial band. The mitochondrial band was removed and placed in heavy thick-walled polycarbonate tubes. 25% Sucrose buffer was used to fill the remaining portion of the tubes. The mitochondria were centrifuged for 30 minutes, 35,000 rpm, Type 42.1 rotor, L5-75 ultracentrifuge, at 4°C into a pellet.

Mitochondrial DNA Isolation

The mitochondrial pellet that was obtained was immediately resuspended in TEN buffer with Proteinase K at 0.1 mg/ ml and 1% SDS from a 25% stock. At this point the mitochondrial lysate was either phenol extracted or placed on a CsCl gradient.

If the lysate was phenol extracted it was done so with an equal volume of phenol (TEN buffer saturated)-chloroform-isoamyl alcohol (25:24:1) and gently shaken for several minutes. The extracted lysate was centrifuged for 5 minutes, 9000 rpm, at 4° C. and chloroform extracted twice as outlined above for whole cell DNA isolation. The top layer was precipitated with 2 volumes of ethanol and placed at -20°C overnight. The precipitin was collected by centrifugation for 30 minutes, 10,000 rpm, at 4°C, resuspended in 1x TE buffer and stored at -20°C.

For CsCl gradient isolation the mitochondrial lysate was added to approximately 11 ml of 1x TE buffer with CsCl dissolved at a

concentration of 0.86 gms/ ml. 1 ml of EtBr (10 mg/ ml) was also added. The density was adjusted by the addition of either more CsCl or 1x TE buffer to 1.3865 gms/ cm^3. The density gradients were centrifuged for 48 hours, 40,000 rpm, Ti50 rotor, L5-75 ultracentrifuge, at 4°C.

The gradients were viewed with an ultraviolet light source to detect the mitochondrial DNA band. The band was taken off with a 5 ml syringe with an 18 gauge needle punctured through the side of the sealable polyallomer tube. The band was placed in a siliconized, baked glass tube and the EtBr extracted from the DNA by a series of extractions using sec-Butanol saturated with 5M NaCl, 10mM Trizma base, 1mM EDTA, pH 7.9. The DNA was placed in dialysis tubing and dialyzed against 1x TE buffer for 24 hours at 4° C using several changes of buffer. The mitochondrial DNA was taken from the dialysis tubing and stored at -20°C.

Actin DNA Isolation

The C. albicans actin gene was isolated by Brent A. Lasker (Manuscript in preparation) and cloned into pBR322. The resulting plasmid, pCaAct, carries the C. albicans actin gene within the Sal I site of pBR322. pCaAct was isolated by a maxi-prep procedure of Davis et al (1980) and Norgard (1981).

Restriction Endonuclease Digestion of DNA

Restriction endonuclease digestions were carried out as follows: 3 units of enzyme were used per 0.0005 mg of DNA and placed at 37°C for 3 hours. The reaction buffer in all cases except for the enzyme Sal I was 1x TA buffer (33mM Trizma base, 66mM Potassium Acetate, 10mM Magnesium

Acetate, 5mM Dithiothreitol, pH 8.6 with glacial acetic acid) prepared from a 10x stock (O'Farrel et al,1980). Sal I digestion was carried out in 6mM Trizma base, 150mM NaCl, 6mM MgCl , 6mM 2-mercaptoethanol, pH 7.9. All reactions contained BSA at a concentration of 0.1 mg/ ml. All restriction endonucleases were obtained from either Bethesda Research Laboratories or New England Biolabs.

Labeling of DNA with 32-P

MtDNA and pCaAct were labeled with 32-P dCTP by the *in vitro* method of nick translation as described by Davis et al (1980) or by the manufactures' instructions (BRL). Essentially, the reaction mixture contained the DNA to be labeled in a 1x nick translation buffer (50mM Trizma base, 50mM MgCl , 50mM Dithiothreitol, 50% Glycerol, pH 7.8 with HCL) along with unlabeled deoxynucleotides (0.25 mM ATP, GTP, TTP) and 0.025 mCi of 5'-[alpha-32-P] deoxycytidine triphosphate. A DNAse I - DNA polymerase enzyme mixture was added and the sample allowed to incubate for 2 hours at 15°C.

The nick translation reaction was placed on a Sephadex-G50 column to separate the nick translated DNA from unincorporated nucleotides. A 4 ml column of Sephadex-G50 was formed in a 5 ml syringe and column buffer (0.05M Trizma base, 0.25M NaCl, 0.002M EDTA, 0.5% SDS, pH 7.9 with HCl) was passed through several column volumes before adding the sample. The nick translation reaction was placed on the column and 1 ml of column buffer was put on top. Approximately 20 fractions of 0.2 ml were collected by addition of more column buffer to the column. A geiger counter was used to determine the relative amount of radioactivity in

the fractions and those fractions which contained the first peak to come off the column were pooled and stored at -20°C.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out using vertical gel apparatuses (150 x 140 x 4 mm). The buffer used in all cases and to make the agarose solutions was 1x 'E' buffer (40mM Trizma base, 5mM Na Acetate, 1mM EDTA, pH 7.8 with glacial acetic acid). In all cases 1% agarose gels were used with 2% agarose solutions used for sealing purposes. All samples to be run contained 20% by volume of a loading and tracking solution (25% Ficoll, 0.1% Bromophenol blue, 0.1% Xylene cyanole FF, 10mM trizma base, 1mM EDTA, pH 7.9). Digested and undigested DNA samples were placed on the gel and run under constant voltage till the bottom dye (Bromophenol blue) reached just above the 2% plug. The gel apparatus was disassembled and the gel stained with EtBr in 500 ml of distilled water at a concentration of 0.0005 mg/ ml for 30 minutes while shaking gently. The gel was photographed using Polariod Type 57 film and a Kodak number 23A Wratten gelatin filter upon illumination by a Mineralight 302 nm, long wave ultraviolet light transilluminator (Fisher).

Southern Blot Transfer of DNA

Restriction fragments separated on an agarose gel were transfered to Nitrocellulose by the method of Southern (1979). After the gel was photographed it was placed under short wavelength ultraviolet for 15-20 seconds to produce nicks in the DNA and was submersed in 500 ml of Denaturation solution (0.4M NaOH, 0.8M NaCl) for 30 minutes with

shaking. The gel was transfered to 500 ml of Neutralization buffer (0.5M Trizma base, 1.5M NaCl, pH 7.6 with HCl) and allowed to shake for 30 minutes. During this time the nitrocellulose filter (Schleicher and Schuell, Type BA85) was allowed to soak in distilled water and the rest of the Southern Blot apparatus was set up. The gel was transfered to the wick and the remaining apparatus was constructed. DNA transfer was accomplished overnight at room temperature. The nitrocellulose filter was washed in 4x SSC, blotted dry on 3MM Whatman filter paper, placed between two sheets of the 3MM Whatman filter paper, wrapped in aluminum foil and baked at 70°C in a vacuum oven for 1-2 hours. The blots could now be stored at room temperature until ready for use.

Hybridization of Nick-Translated DNA to DNA on Nitrocellulose

Hybridization of nick-translated DNA's to Southern blots followed the procedure of Southern (1979). Southern blots of single-stranded DNA attached to nitrocellulose (as described above) were prepared for hybridization to nick-translated DNA as follows (pre-hybridization): a single nitrocellulose sheet was placed inside a plastic re-sealable pouch with 15 ml of '1.5x' buffer (1.334M NaCl, 66mM Trizma base, 1.25mM EDTA, 0.1334% N-Lauryl Sarcosine, 2.99mM Sodium pyrophosphate, pH 7.9 with HCl), 4 ml of 50x Denhardt's solution (1% Ficoll (M.W. 400,000), 1% BSA, 1% Polyvinyl pyrollidone (M.W. 360,000), filter sterilized), 0.2 ml of single-stranded salmon sperm DNA (10 mg/ ml). The pouch was heat sealed and placed at 65°C in a rotary oven overnight.

After at least 12 hours of pre-hybridization of the Soutern blot, the nick-translated DNA probe to be used was prepared in the following manner: approximately 2.5-5 millon counts per minute were added to 0.4

ml of distilled water and placed in boiling water for 2-3 minutes. The radioactive probe was added to the pouch with a 1 ml syringe with a 26 gauge needle and the pouch re-sealed. The pouch containing the blot and the probe was placed inside another plastic bag and this bag was sealed to prevent possible leakage of the radioactive material. The pouch was placed at 65°C for approximately 48 hours in a rotary oven.

Autoradiography of Hybridized Southern Blots

After hybridization the pouch containing the nitrocellulose filter was emptied of the radioactive solution and two successive 10 ml washes were performed with 1x Low buffer (50mM NaCl, 50mM Trizma base,0.01% N-Lauryl Sarcosine, pH 7.9 with HCl) while the filter remained in the pouch. The filter was taken out of the pouch and washed twice with 100 ml of 1x Low buffer at room temperature for 10 minutes. The filter was washed twice with 150 ml of 1x Low buffer for 15 minutes at 65°C and then washed twice for 30 minutes with 200 ml of 1x Low buffer at 65°C. The filter was blotted dry on Whatman 3MM paper , placed in plastic wrap, and either immediately autoradiographed or stored in a drawer until ready for use .

Autoradiography was carried out at -70°C in Kodak film holders using Kodak XAR-5 x-ray film placed between two DuPont Cronex Lighting-Plus intensifing screens.

CHAPTER III

RESULTS

A. Mitochondrial DNA as Probe

Digested whole cell DNA isolated from each of the strains listed in Table I (page 8) was probed with labeled mitochondrial DNA obtained from C. albicans SR 1010. Whole cell DNA was either digested with: a) EcoRI b) Pvu II or c) EcoRI and Pvu II. In most cases all three digestion options were used. The digested DNA was electrophoresed on vertical 1% agarose gels, transfered to nitrocellulose, and probed with 4-5 x 10^6 cpm for 48-72 hours at 65°C. The probed genomic blots were subsequently washed and autoradiographed.

All Strains

Upon autoradiography of the probed whole cell DNA of the various strains it was noted that most non-C. albicans strains would not hybridize well with the C. albicans mitochondrial DNA probe (as seen by lack of bands or the requirement for prolonged exposure time). In contrast to the other Candida species , C. stellatoidea did hybridize well with the C. albicans probe. Experiments were subsequently performed at less stringent hybridization and wash conditions to enhance the hybridization of the other non-C. albicans strains. EcoRI and Pvu II double digests were utilized. These experiments involved placing the probed blot at 55°C during hybridization and washing the blot with 5x 'Low' buffer at 55°C (as opposed to 65 C and washing in 1x 'Low' buffer at 65°C). Representative results can be seen in Figures 1 and 2. Figure

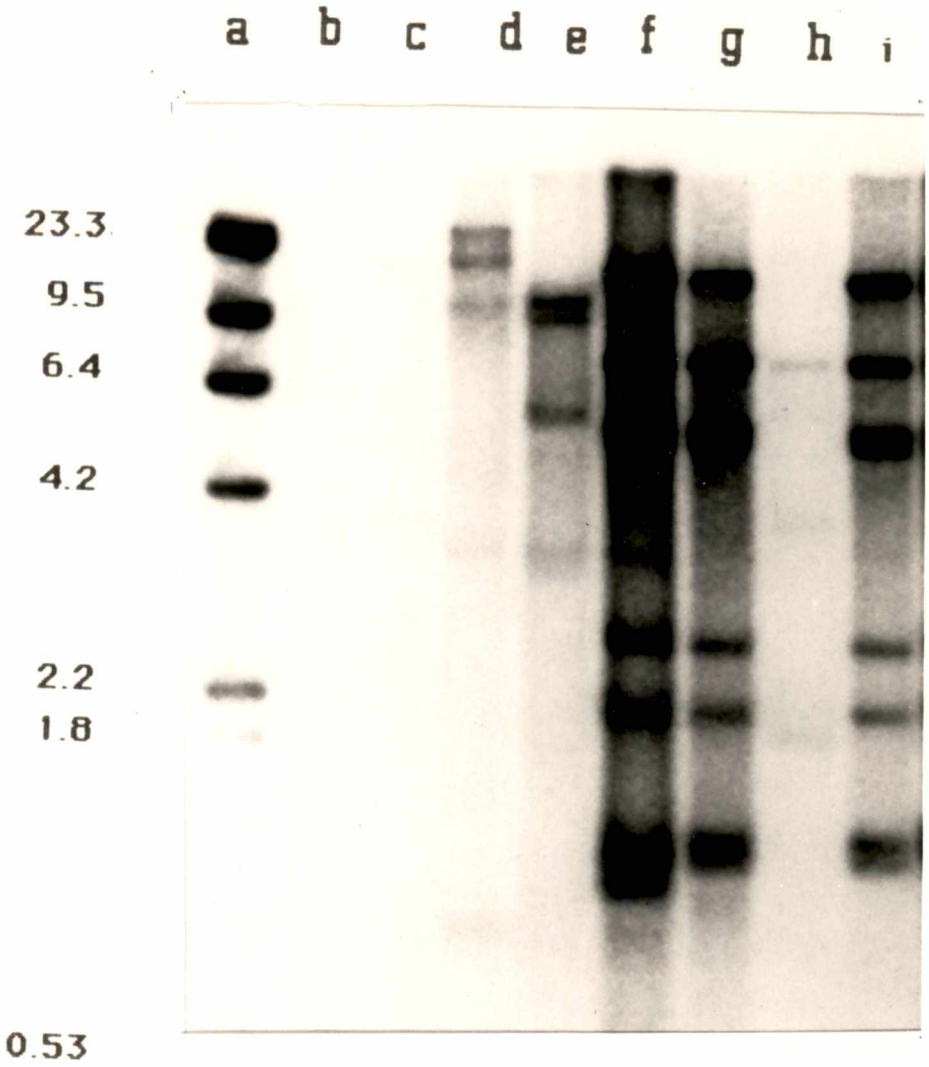


Figure 1. One hour exposure of various Candida species wcDNA digested with EcoRI and Pvu II and probed with C. albicans mtDNA. Lane a, λ-Hind III marker; b, Kloeckra africana SR 1101; c, C. (Torulopsis) glabrata SR 1102; d, C. tropicalis SR 1103; e, C. parapsilosis SR 1104; f, C. stellatoidea SR 1105; g, C. albicans SR 1106; h, C. pseudotropicalis SR 1107; i, C. albicans SR 1010.

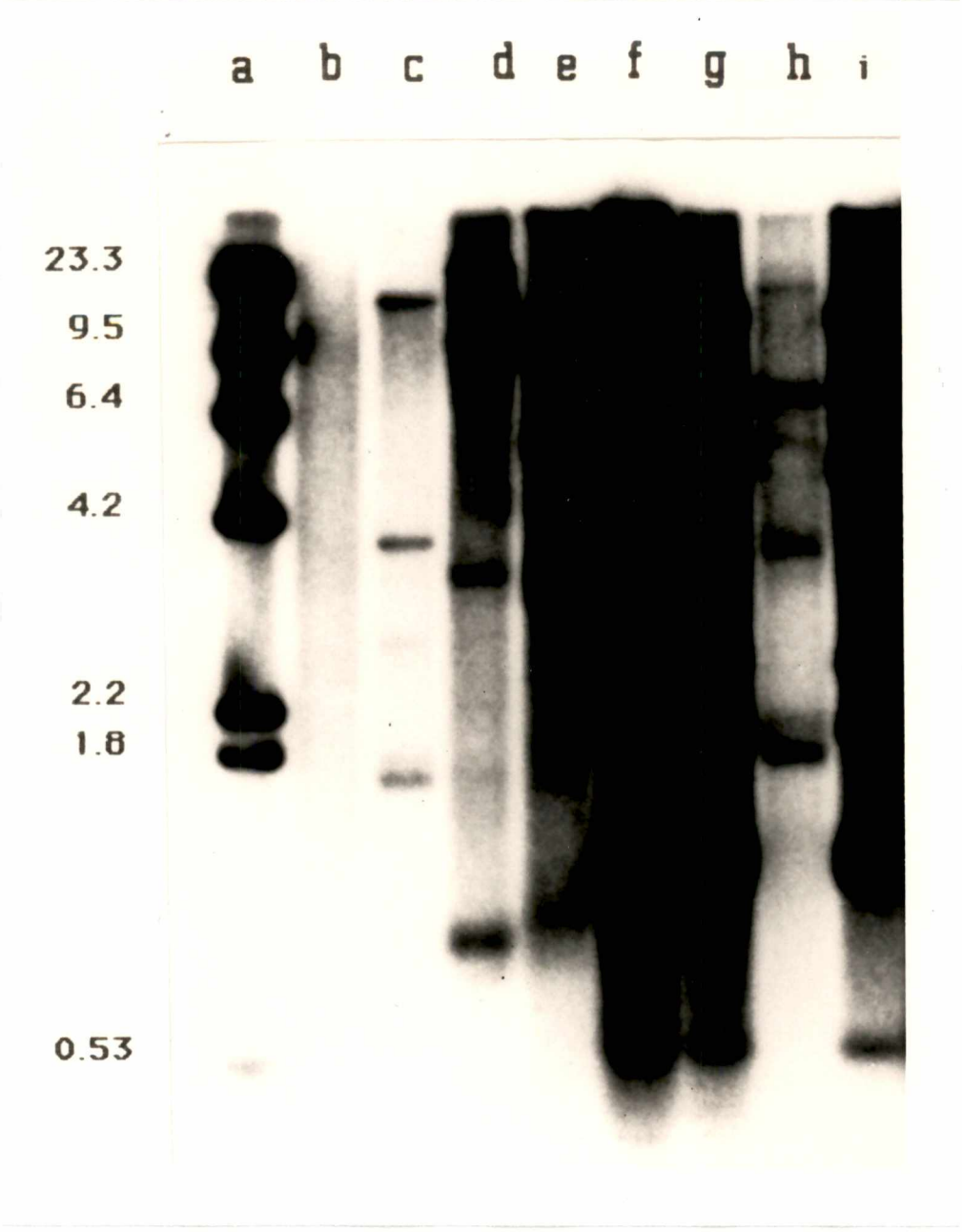


Figure 2. Ten hour exposure of the probed blot shown in Figure 1. All lanes are the same as Figure 1.

1 shows a one hour exposure and Figure 2 a ten hour exposure. In each case C. (Torulopsis) glabrata is in lane c; C. tropicalis in lane d; C. parapsilosis in lane e; C. stellatoidea in lane f; C. albicans in lanes g and i; and C. pseudotropicalis in lane h. Only with less stringent conditions and prolonged exposure time could these "good" results be obtained. Representative autoradiographs of C. albicans SR 1010 mitochondrial DNA hybridized to C. guilliermondii, C. krusei, and C. parapsilosis are not shown. Three putative C. albicans strains (SR 1055, SR 1056 and SR 1118) and one putative C. stellatoidea strain (SR 1113) did not hybridize well, indicating the possiblity that they were not of the C. albicans/ C. stellatoidea group; by using the actin DNA probe this hypothesis was verified (see 'Actin DNA as Probe' in this Chapter).

C. albicans/ C. stellatoidea

Digestions with EcoRI. Fragment size measurements were made for all C. albicans/ C. stellatoidea strains banding patterns from autoradiographs (data not shown). The banding patterns varied slightly. Out of twenty-nine C. albicans/ C. stellatoidea strains (excluding the four strains that did not hybridize, as described above), only three classes of varying fragment sizes could be resolved. Most strains produced fragment sizes identical to those of C. albicans SR 1010 in Table II. Strains SR 1117 and SR 1245 varied enough to be placed in their own size classes.

Digestions with Pvu II. Again, all C. albicans/ C. stellatoidea strains banding patterns were measured (data not shown). Twelve of the strains have identical fragment sizes as those shown in Table II. The other strains produced fragment sizes that resulted in the formation of ten additional size classes or groups. In most cases the variation seen

Table II. Fragment Sizes of *C. albicans* SR 1010 Mitochondrial DNA.[a]

EcoRI		EcoRI and Pvu II		Pvu II	
Fragment	kbp	Fragment	kbp	Fragment	kbp
E 1	12.2	EP 1	12.2	P 1	14.8
E 2	10.0	EP 2	7.4	P 2	13.0
E 3	8.1	EP 3	5.4	P 3	5.2
E 4	5.7	EP 4	5.1	P 4	3.4
E 5	4.7	EP 5	2.6	P 5	2.7
E 6	0.34	EP 6	2.1	P 6	1.28
		EP 7	1.36		
		EP 8	1.28		
		EP 9	0.60		

[a] Wills, J. W., B. Troutman and W. S. Riggsby, Submitted to Journal of Bacteriology.

from the standard strain SR 1010 occured in the two smallest Pvu II fragments: P 5 and P 6.

Digestion with EcoRI and Pvu II. Whole cell DNA from the various C. albicans/ C. stellatoidea strains was digested with both EcoRI and Pvu II, electrophoresised, transfered to nitrocellulose and probed. The resulting banding patterns were measured and fourteen different fragment size groups could be obtained from the twenty-nine strains as shown in Table III. Representative banding patterns for each group can be seen in Figures 3, 4, 5 and 6. Figure 3 contains C. stellatoidea SR 1105 in lane e (Group N) and C. albicans in all other lanes. Also in Figure 3 is Group A in lanes a and h; Group B in lanes b, c, f, g, i and j; Group C in lane d; and Group L in lane k. Figure 4 shows Group B in lanes a, d, e and h; Group L in lane c; Group K in lane g. Figure 5 shows Group B in lanes a and i; Group D in lane d; Group N in lane c; Group E in lane e; Group F in lane g; Group G in lane h; and Group H in lanes j and k. Figure 6 shows Group B in lanes a and f; Group I in lane b; Group J in lane c and Group M in lane g.

Table III also gives the number of strains that can be placed into each group. The strains that fall into each group are listed in Table IV. As in the measurements obtained from the Pvu II digestions, twelve strains fall into the group that contains the standard strain SR 1010. Three additional groups are formed from the double digestion giving fourteen fragment size groups, as opposed to eleven groups from the Pvu II digests. Variations occured mostly in fragments EP 5 and EP 8 with some divergence in fragments EP 3 and EP 7. Strains 1117 and 1245 are unique in that they have variations in one or both of the two largest fragments, EP 1 and EP 2 respectively.

Table III. Mitochondrial DNA Fragment Sizes (Pvu II and EcoRI) and Their Subsequent Groupings.

Fragment	Group													
	A	B	C	D	E	F	G	H	I	J	K	L	M	N
EP 1	12.2	12.2	12.2	12.2	7.6	12.2	12.2	12.2	12.2	12.2	12.2	12.2	12.2	12.2
EP 2	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4	6.9	7.4	7.4
EP 3	5.3	5.4	5.4	5.4	6.9	5.4	5.4	5.4	5.4	5.4	5.4	5.3	5.3	5.3
EP 4	5.1	5.1	5.1	5.1	5.4	5.1	5.1	5.1	5.1	5.1	5.1	5.1	5.1	5.1
EP 5	2.6	2.6	2.75	2.65	5.1	2.6	2.65	2.6	2.7	2.6	2.7	2.7	2.6	2.65
EP 6	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1
EP 7	1.36	1.36	1.36	1.36	1.36	1.28	1.36	1.28	1.36	1.36	1.34	1.34	1.34	1.36
EP 8	1.21	1.28	1.28	1.15	1.15	1.15	1.28	1.10	1.21	1.21	1.21	1.24	1.15	1.15
No. of strains in group.	3	12	1	1	1	1	1	2	1	2	1	1	2	1

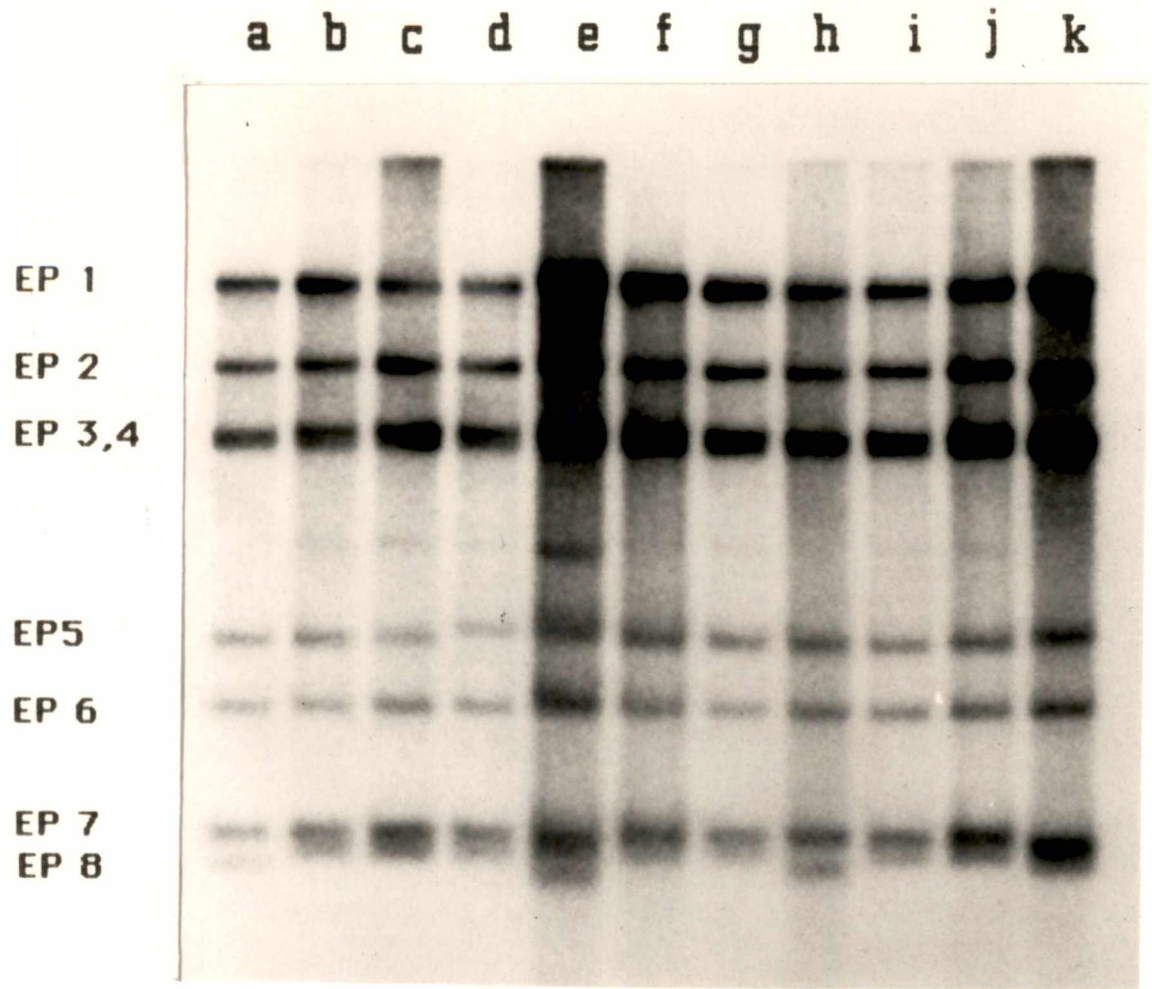


Figure 3. Various C. albicans strains and one C. stellatoidea wcDNA probed with mtDNA of C. albicans SR 1010. The wcDNA was digested with EcoRI and Pvu II. Lane a, SR1001; b, SR 1010; c, SR 1017; d, SR 1025; e, C. stellatoidea SR 1105; f, SR 1106; g, SR 1110; h, SR 1219; i, SR 1223; j, SR 1222; k, SR 1245.

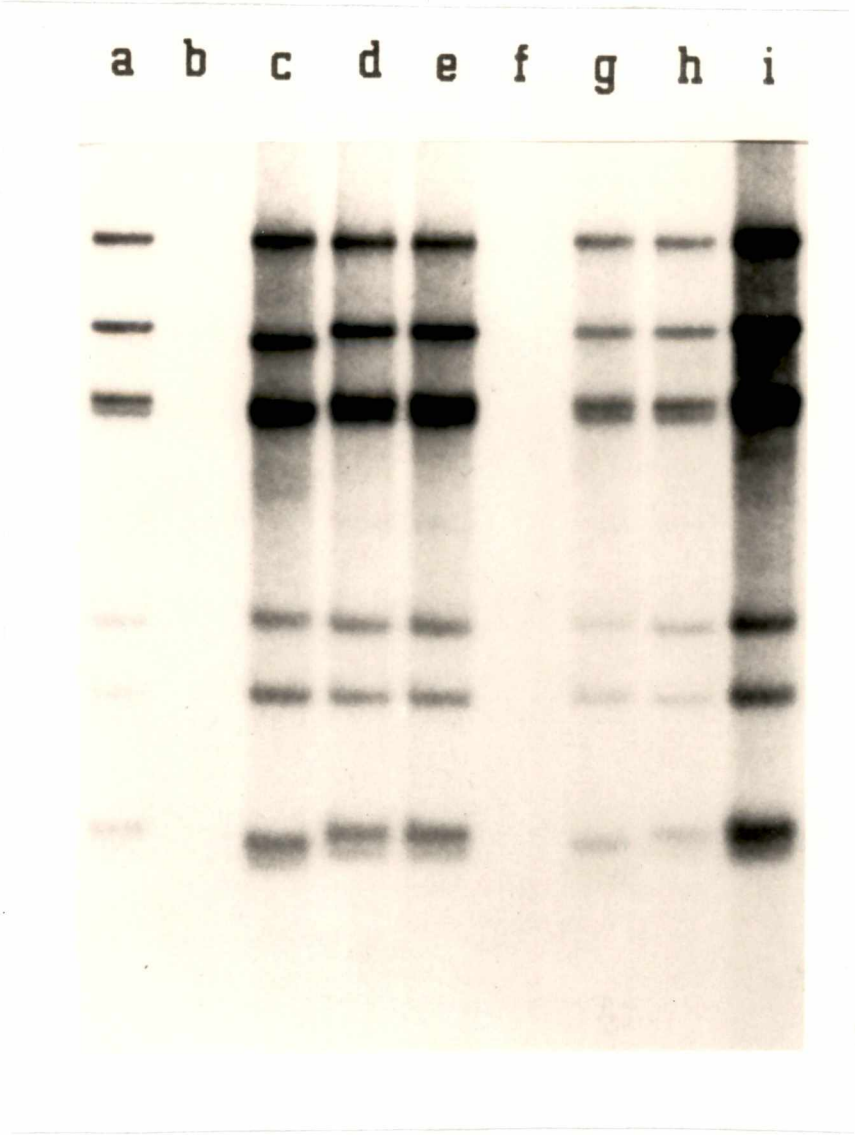


Figure 4. Various vaginal C. albicans strains wcDNA probed with mtDNA of C. albicans SR 1010. The wcDNA was digested with EcoRI and Pvu II. Lane a, SR 1110; b, C. guillermondii SR 1207; c, SR 1245; d, SR 1241; e, SR 1243; f, C. (Torulopsis) glabrata SR 1227; g, SR 1244; h, SR1010; i, SR 1010 mtDNA.

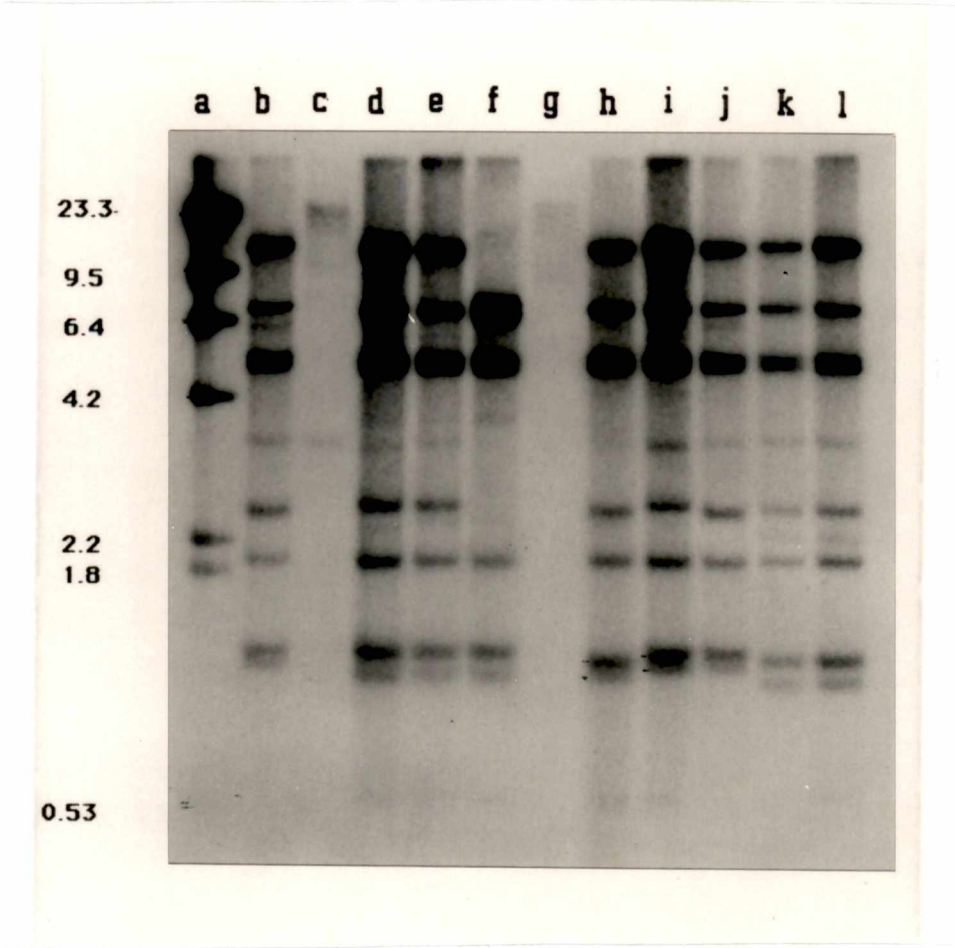


Figure 5. Various C. albicans/ C. stellatoidea strains and putative C. albicans/ C. stellatoidea strains wcDNA probed with C. albicans SR 1010 mtDNA. The wcDNA was digested with EcoRI and Pvu II. Lane a, λ-Hind III marker; b, SR 1010; c, "C. stellatoidea" SR 1113; d, C. stellatoidea SR 1114; e, SR 1116; f, SR 1117; g, "C. albicans" SR 1118; h, SR 1119; i, SR 1120; j, SR 1121; k, SR 1122; l, SR 1123.

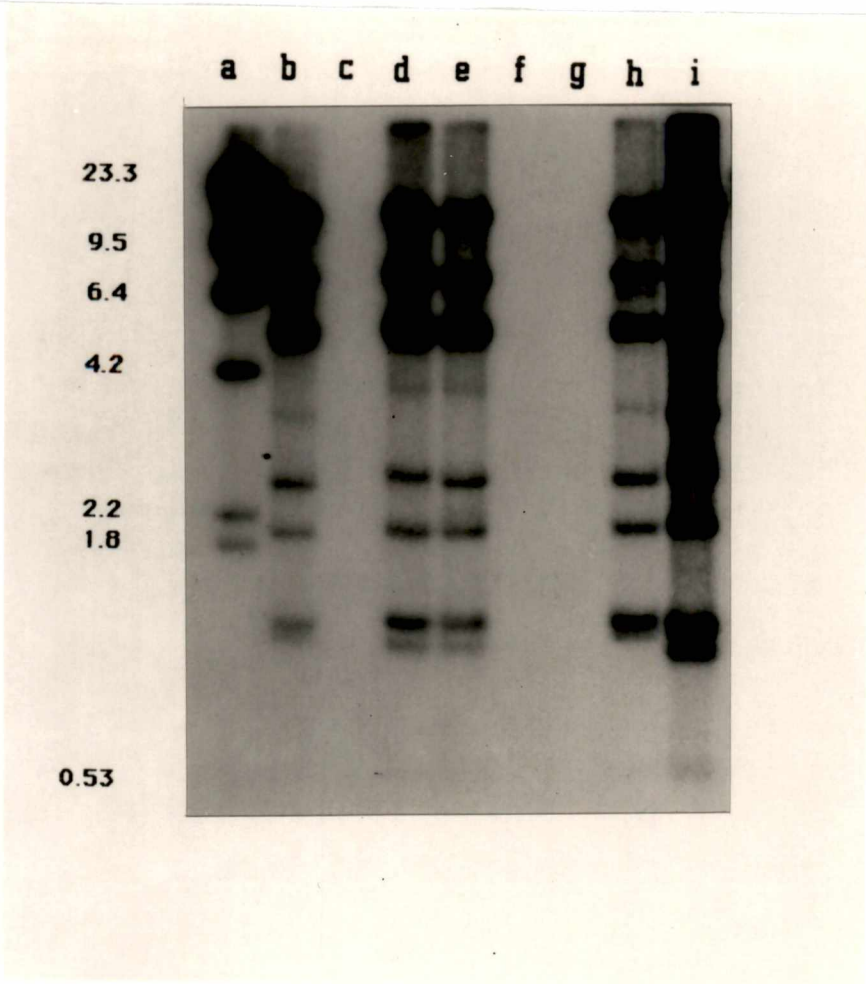


Figure 6. C. albicans, C. stellatoidea and C. krusei strains wcDNA probed with C. albicans SR 1010 mtDNA. The wcDNA was digested with EcoRI and Pvu II. Lane a, λ-Hind III marker; b, SR 1010; c, empty; d, SR 1124; e, SR 1125; f, C. krusei SR 1131; g, C. krusei SR 1132; h, SR 1133; i, C. stellatoidea SR 1134.

Table IV. Strains Included in the Various Fragment Size Groups

Group	Strains			
A	SR 1001	SR 1219	SR 1220	
B	SR 1010	SR 1017	SR 1106	SR 1110
	SR 1121	SR 1133	SR 1222	SR 1223
	SR 1224	SR 1225	SR 1241	SR 1243
C	SR 1025			
D	SR 1116			
E	SR 1117			
F	SR 1119			
G	SR 1120			
H	SR 1122	SR 1123		
I	SR 1124			
J	SR 1125	SR 1221		

Table IV. (cont'd)

Group	Strains	
K	SR 1244	
L	SR 1245	
M	SR 1105	SR 1134
N	SR 1114	

B. Actin DNA as Probe

Genomic blots of 49 strains of Candida that are listed in Table I were probed with nick-translated actin DNA. At least two representatives of each species of Candida studied, excluding C. (Torulopsis) glabrata and C. utilis, were probed. Whole cell DNA was obtained from each of these strains, digested with one of five different restriction endonucleases, electrophoresed on 1% agarose vertical gels, and transfered to nitrocellulose sheets. The genomic blots were probed with approximately 2.5-4 millon cpm for 48 hours, washed and autoradiographed for various times at -70 C.

Restriction Endonuclease: EcoRI

Autoradiography of the probed genomic blots of DNA digested with EcoRI revealed one band for each of the Candida species tested (C. utilis was not tested with EcoRI). The various patterns obtained for each species are shown in the right half of each lane in Fig. 7 (C. albicans, lanes a and c;C. pseudotropicalis, lane b;C. stellatoidea, lane d;C. parapsilosis, lane e;C. tropicalis, lane f;C. (Torulopsis) glabrata, lane g) and Fig. 8 (C. guilliermondii, lane a;C. krusei, lanes b and c). The different fragment sizes for each species are recorded in Table V.

Restriction Endonuclease: Hind III

Whole cell DNA was digested with Hind III and probed with the actin gene as described. The resulting patterns are shown in the left half of

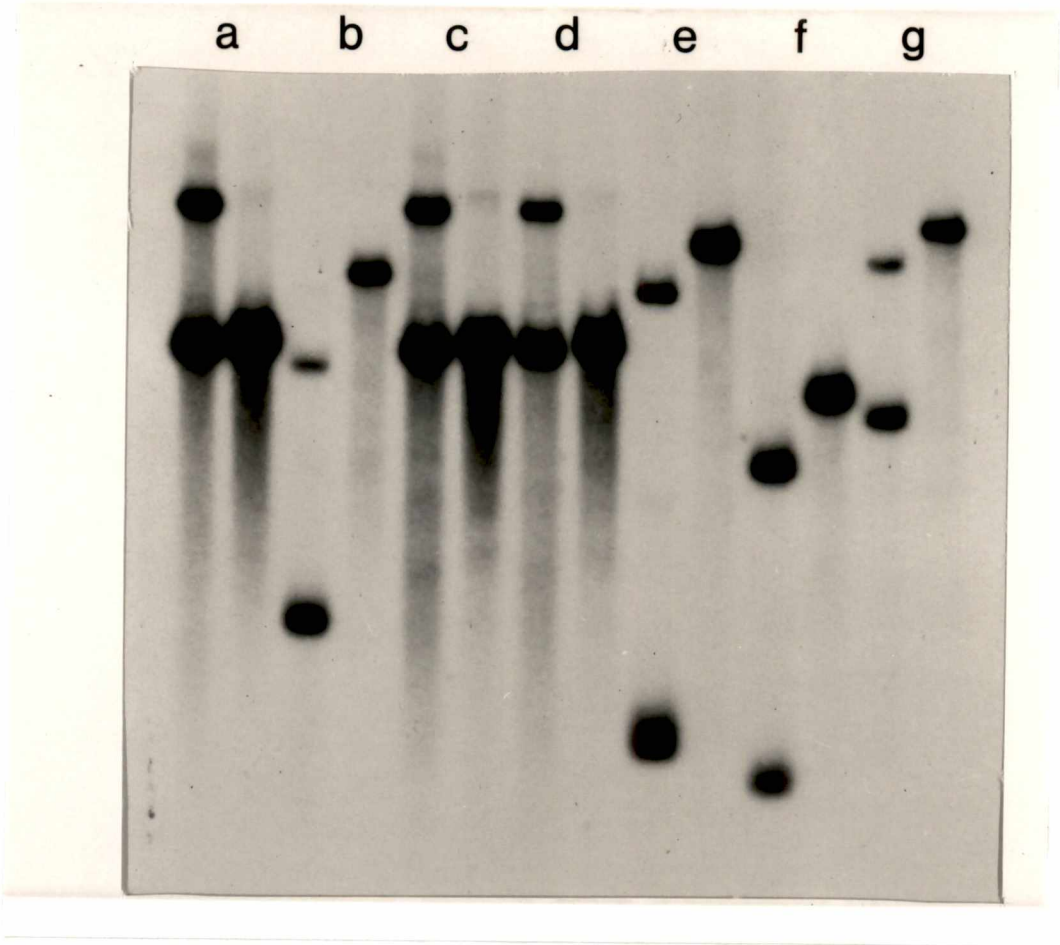


Figure 7. Several Candida species wcDNA probed with C. albicans SR 1010 cloned actin DNA. The left hand portion of each lane was digested with EcoRI and the right hand portion was digested with Hind III. Lane a, SR 1010; b, C. pseudotropicalis SR 1107; c, C. albicans SR 1106; d, C. stellatoidea SR 1105; e, C. parapsilosis SR 1104; f, C. tropicalis SR 1103; g, C. (Torulopsis) glabrata SR 1102.

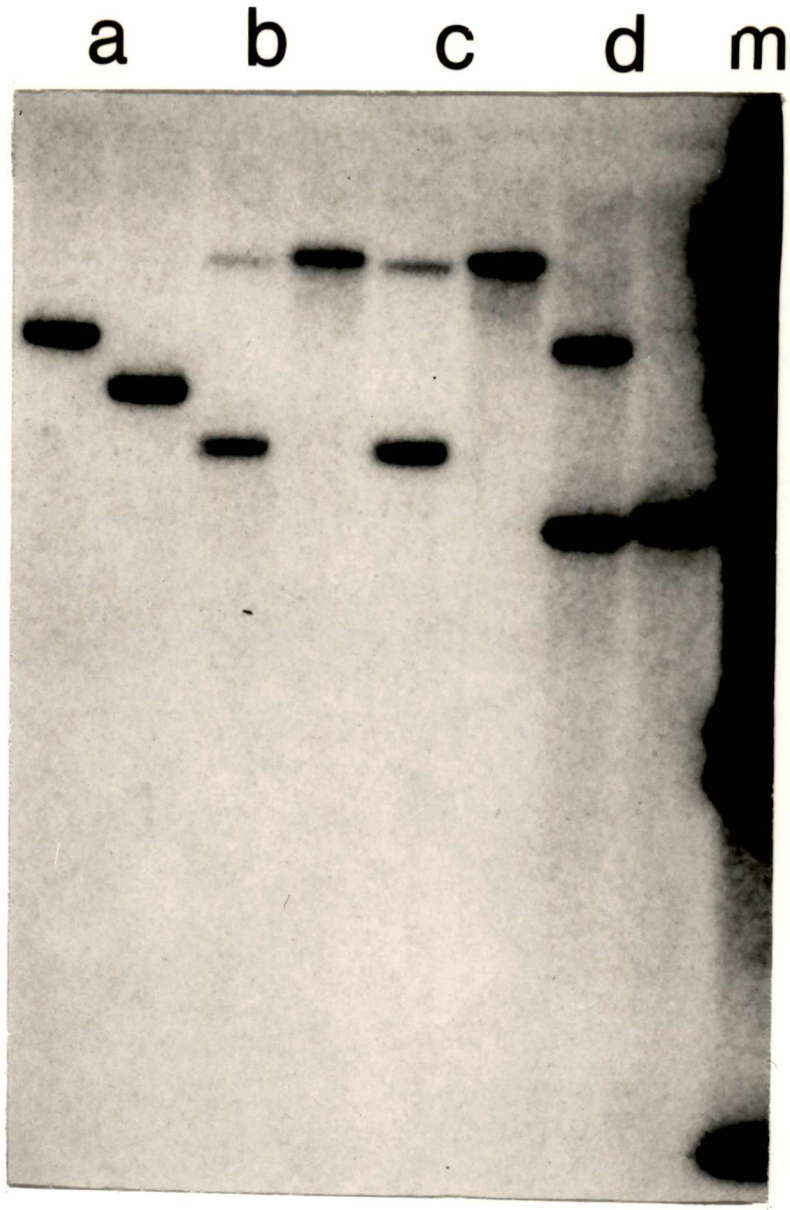


Figure 8. C. guillermondii and C. krusei strains wcDNA probed with C. albicans SR 1010 cloned actin DNA. The wcDNA was digested with either Hind III in the left hand portion of the lane or EcoRI in the right hand portion. Lane a, C. guillermondii SR 1207; b, C. krusei SR 1132; c, C. krusei SR 1131; d, C. albicans SR 1010; m, λ-Hind III marker.

Table V. Actin-containing DNA Fragments of Certain *Candida* Species.

Species	Fragment Size (kbp)				
	EcoRI	Hind III	Cla I	Pvu II	Sal I
C. albicans	4.0	8.2 3.9	1.5 1.3	4.0 1.1	1.9
C. stellatoidea	4.0	8.2 3.9	1.5 1.3	4.0 1.1	1.9
C. tropicalis	2.8	2.6 0.7	1.3	17	6.6
C. pseudotropicalis	6.0	3.6 1.3	1.9 0.8	16.5	23
C. guilliermondii	6.0	7.9	5.1	4.1 2.0	5.7
C. parapsilosis	7.0	5.6 0.8	1.0 0.5	6.1	23
C. krusei	17	16.5 5.0	10.5	21.5	7.2
C. (*Torulopsis*) *glabrata*	7.8	6.5 2.8	ND[a]	ND[a]	ND[a]
C. utilis	ND[a]	3.7 2.5	9.2 1.2	11.5	21

[a] ND: Not determined.

each lane in Fig. 7 (C. albicans, lanes a and c;C. pseudotropicalis, lane b;C. stellatoidea, lane d;C. parapsilosis, lane e;C. tropicalis, lane f;C. (Torulopsis) glabrata, lane g) and Fig. 8 (C. guilliermondii, lane a;C. krusei, lanes b and c). Table V gives the sizes of the fragments generated by Hind III.

Restriction Endonuclease: Sal I

Figures 9 and 13 show the results of the actin probe on Sal I genomic blots (Fig. 9,C. albicans, lanes a,b,c, and g;C. krusei, lanes d and e;C. stellatoidea, lane f;C. tropicalis, lane h;C. pseudotropicalis, lane i;C. utilis, lane j; and Fig. 13 shows C. guilliermondii in lanes a and b). Representative C. parapsilosis banding patterns for Sal I are not shown. Actin Sal I fragment sizes are shown in Table V.

Restriction Endonucleases: Cla I and Pvu II

Representative Cla I and Pvu II actin fragments are shown in Figures 10 and 11 respectively. In each case C. albicans is in lanes a,b,c, and g;C. krusei in lanes d and e;C. stellatoidea in lane f;C. tropicalis in lane h;C. pseudotropicalis in lane i; and C. utilis in lane j. C. parapsilosis patterns can be seen in Figure 12, lanes d, e and f with Cla I in the left half and Pvu II in the right. The Pvu II fragments for C. guilliermondii can be seen in Figure 13, lanes f and g.C. guilliermondii banding patterns for Cla I are not shown. All fragment sizes are shown in Table V.

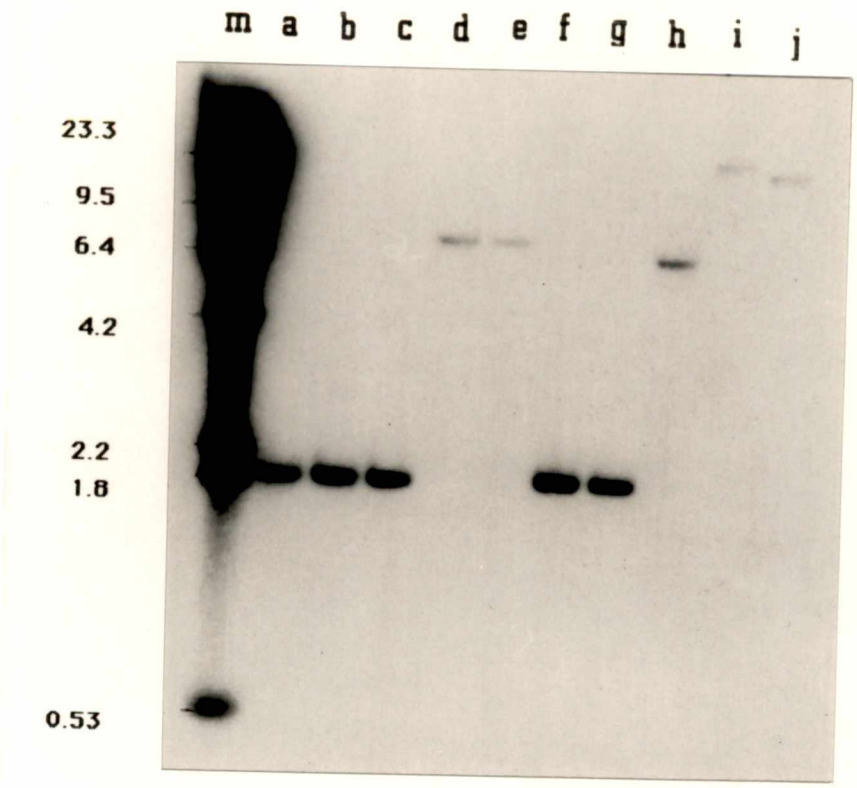


Figure 9. wcDNA of various Candida species digested with Sal I and probed with C. albicans SR 1010 cloned actin DNA. Lane a, SR 1010; b, C. albicans SR 1124; c, C. albicans SR 1125; d, C. krusei SR 1131; e, C. krusei SR 1132; f, C. albicans SR 1133; g, C. stellatoidea SR 1134; h, C. tropicalis SR 1135; i, C. pseudotropicalis SR 1136; j, C. utilis SR 1137. Lane m is λ-Hind III marker.

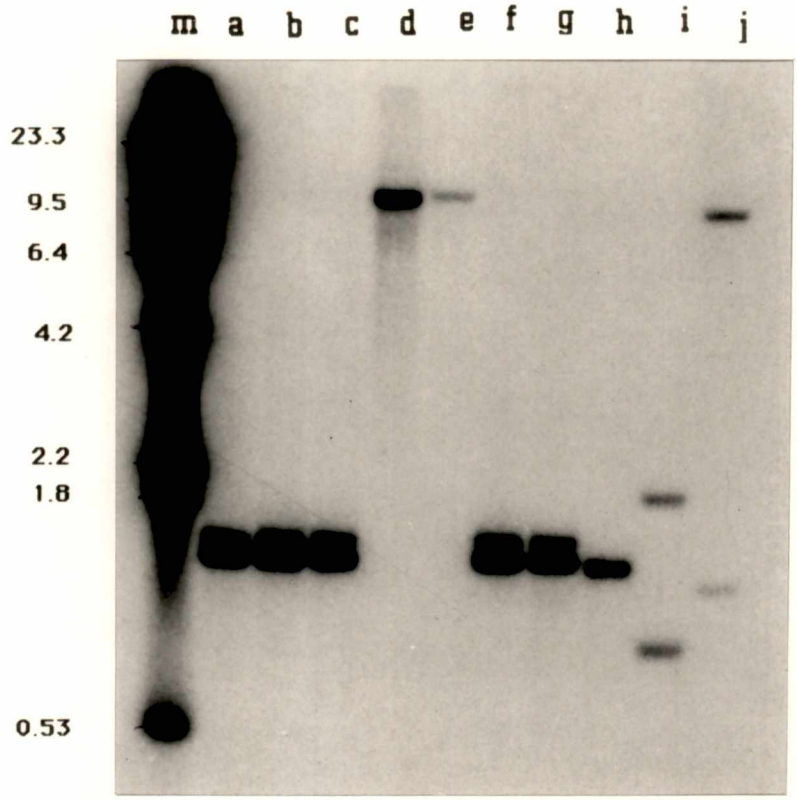


Figure 10. wcDNA of various Candida species digested with Cla I and probed with C. albicans cloned actin DNA. All lanes are the same as Figure 9.

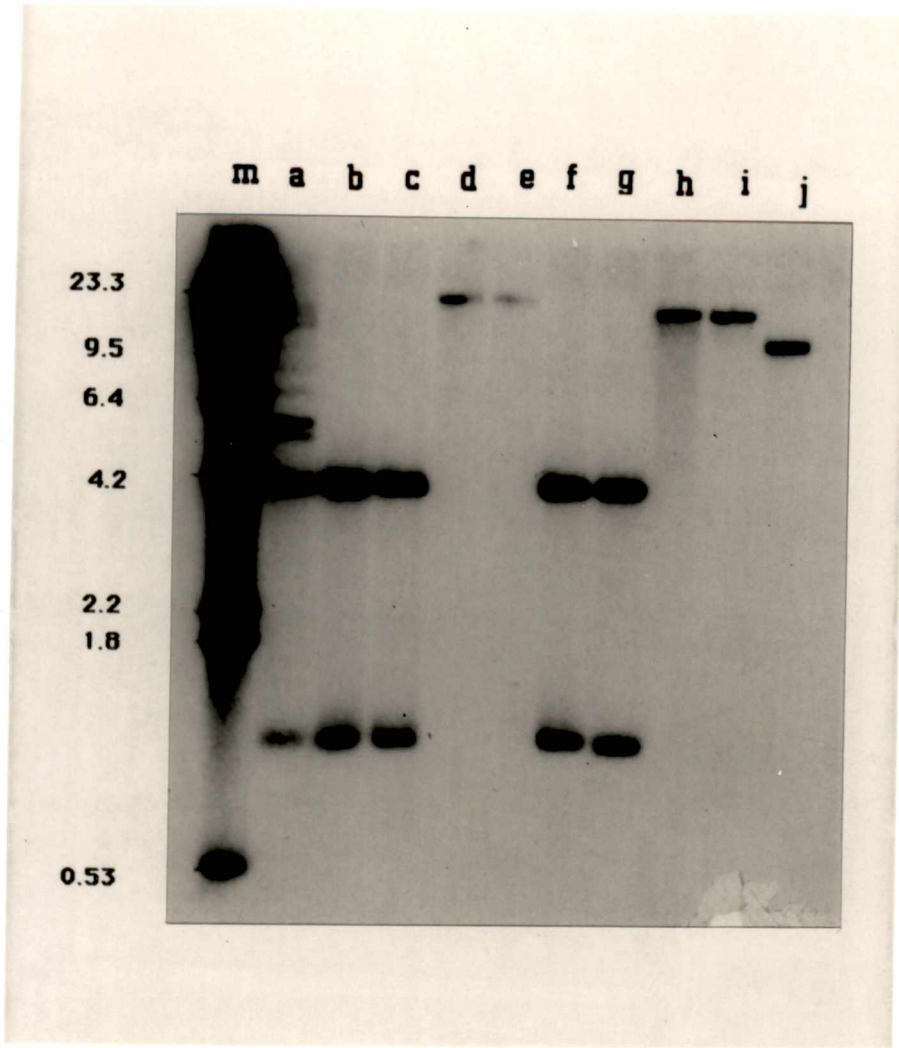


Figure 11. wcDNA of various Candida species digested with Pvu II and probed with C. albicans cloned actin DNA. All lanes are the same as Figure 9.

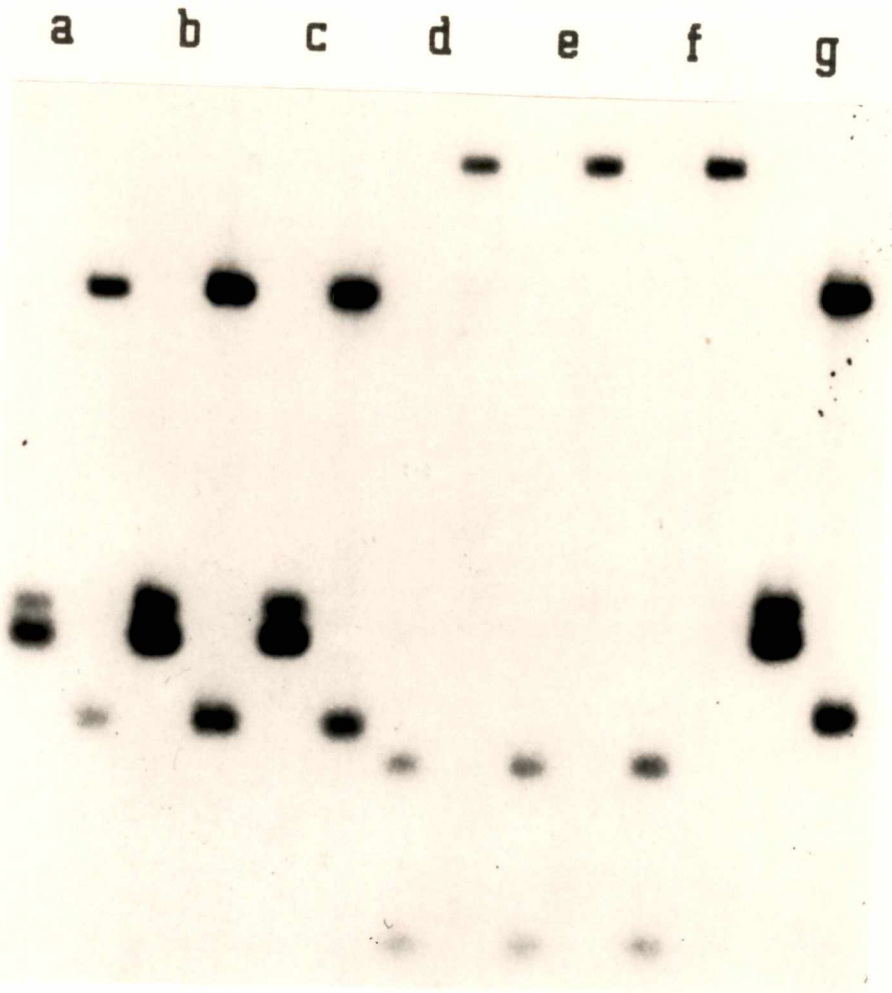


Figure 12. Several C. albicans and C. parapsilosis strains wcDNA digested with Cla I or Pvu II and probed with C. albicans SR 1010 cloned actin DNA. wcDNA digested with Cla I is in the left hand portion of each lane and digestion with Pvu II is in the right hand portion. Lanes a,b andc are C. albicans SR 1224, SR 1222 and SR 1220. Lanes d,e and f are C. parapsilosis SR 1218, SR 1216 and SR 1214. Lane g is C. albicans SR 1010.

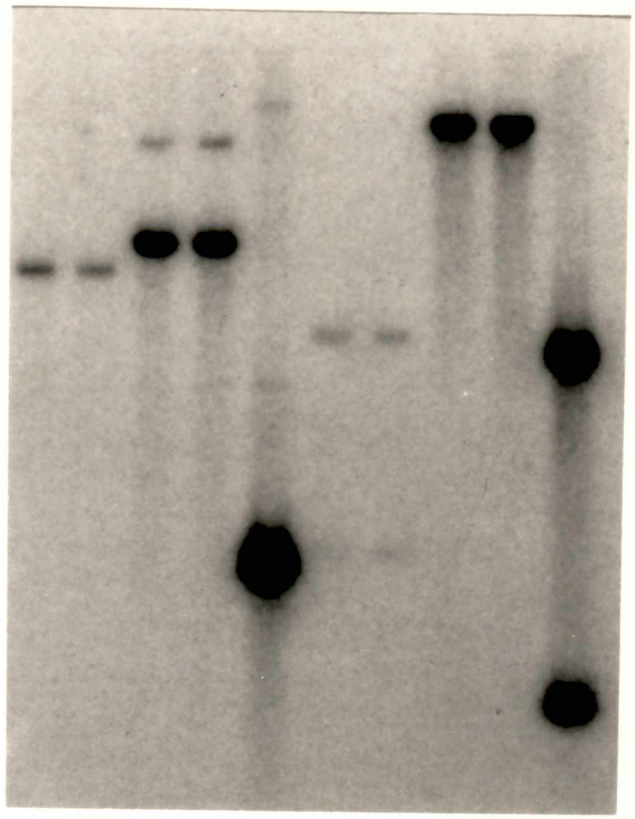


Figure 13. wcDNA of C. tropicalis and C. guillermondii digested with Cla I or Pvu II and probed with C. albicans SR 1010cloned actin DNA. Cla I digested DNA is in lanes a-e and Pvu II digested DNA in lanes f-j. Lanes a and f are C. guillermondii SR 1208; lanes b and g are C. guillermondii SR 1206; lanes c and h are C. tropicalis SR 1204; lanes d and i are C. tropicalis SR 1202. Lanes e and j are C. albicans SR 1010.

CHAPTER IV

DISCUSSION

Mitochondrial DNA

The results indicate that mitochondrial DNA is a possible probe for strain differentiation, but probably not for species identification. This preliminary study shows that the faster evolving mitochondrial DNA will hybridize well to the species that it is obtained from , but poorly to other species, and with the generation of completely different restriction enzyme patterns (except C. stellatoidea). The total genome size obtained by adding the fragment sizes of C. parapsilosis (Figures 1 and 2, lane e; 33kb) and C. (Torulopsis) glabrata (Figures 1 and 2, lane c; 19.2kb) agree well with those obtained by O' Connor et al. (1975) and O' Connor et al. (1976) respectively.

The data shown here along with those obtained with the Actin DNA probe indicate that C. stellatoidea should be considered a variant of C. albicans. The restriction endonuclease patterns obtained from C. albicans and C. stellatoidea differed only slightly with respect to each other. This variation can be attributed to strain differences as shown in Table III (page 29). No difficulties were encountered with C. stellatoidea strains in detecting hybridization bands with autoradiography as with other species (special conditions or prolonged autoradiography were unnecessary). These results are in agreement with the gross DNA homology studies preformed by Meyer (1979).

The results of the EcoRI digestion series using C. albicans/ C. stellatoidea strains revealed very little variation in the restriction

enzyme patterns between the different strains. This is presumably due to the detection limits of the procedure. Small insertions or deletions (100 base pairs or less) would be difficult to determine from the measurements of the large restriction enzyme fragments obtained from an EcoRI digestion.

Digestion with the restriction enzyme Pvu II produces two fragments whose size allows the determination of small variations. These two fragments are P 5 at 2.7 Kbp and P 6 at 1.28 Kbp. Using this enzyme it was possible to differentiate eleven fragment size groups within the twenty-nine strains used.

Double digestions using EcoRI and Pvu II made it possible to distinguish fourteen different fragment size groups. Variations were seen to occur in six of the eight EcoRI and Pvu II double digestion fragments. These variations were seen even in large fragments (> 5kb).

This preliminary study only exploitated two restriction endonucleases and was able to generate fourteen fragment size groups from thirty C. albicans/ C. stellatoidea isolates used. The use of more restriction enzymes, including those with four nucleotide recognition sites, will help facilitate the differentiation of strain types.

The system described here for the differentiation of C. albicans strains has an advantage over other systems. This system does not rely on the phenotypic expression of the organism which may vary or cause the selection of mutant organisms within a population depending on the growth or isolation conditions.

Actin DNA

After examining the various restriction fragment sizes obtained from probing whole cell DNA of nine different Candida with C. albicans actin DNA, it becomes apparent that each species has its own distinctive pattern of restriction endonuclease sites, except C. albicans and C. stellatoidea. C. albicans and C. stellatoidea could not be resolved with the mitochondrial DNA probe as indicated earlier and therefore were not expected to differ in any extent with the actin probe.

A total of twenty-six different strains of C. albicans were probed with actin DNA and twenty-three gave identical results. The three strains that varied were SR 1055, SR 1056 and SR 1118. SR 1055 and SR 1056 are putative haploid strains. According to the data presented, the three strains that did not resemble the other strains used were probably not C. albicans (also see data in Chapter III: Mitochondrial DNA as Probe). This conclusion has also been reached by others who have examined a different genetic element in this strain (A. Gillum, personal communication). SR 1118 was identified as C. tropicalis on the basis of its restriction endonuclease fragment size patterns obtained from the actin probe (data not shown), again in agreement with the results with the mitochondrial DNA probe. Moreover, it has subsequently been identified as C. tropicalis by standard clinical techniques (L. J. Torres-Bauza, personal communication).

Four strains of C. stellatoidea were tested with all giving identical results except for SR 1113. SR 1113 as noted earlier did not hybridize well with the mitochondrial DNA probe as all other C. albicans

and C. stellatoidea did, indicating it does not belong in this group. Using the actin DNA probe , SR 1113 resembles C. tropicalis as SR 1118 did mentioned above. SR 1113 and SR 1118 both produced germ tubes (a low percentage of germ tubes to budding yeasts) after 3 hours at 37 C, but with obviously different morphological characteristics from those of C. albicans/C. stellatoidea when observed microscopically.

Nineteen other strains were tested involving seven species. Each species gave its own distinguishable fragment sizes with no strain variation. Only one similiarity can be noted (between C. pseudotropicalis and C. guilliermondii) concerning fragment sizes, and this is probably coincidental since the other restiction enzyme fragment sizes of these organisms have nothing in common.

Although the fragment sizes obtained from each specie differed, there are similiarities in their banding patterns. A common characteristic seen for each species is only one EcoRI band and one Sal I band. All species except C. guilliermondii contain an internal Hind III site. No consistency is seen with respect to the banding patterns generated by Pvu II or Cla I (species specific for either one or two bands).

The origin of C. albicans and C. stellatoidea strains varied widely in regards to their geographical and clinical source. Stains were obtained from the U. S. A., Puerto Rico, Spain, Japan, and the United Kingdom (Table I, page 9). Some strains were isolated from pateints with oral thrush (SR 1225), dermatosis (SR 1133), systemic infection (SR 1106) and Aquired Immune Deficiency Syndrome (A.I.D.S.; SR 1110).

The significance of the actin DNA probe can be seen not only in identification of Candida in the research laboratory (where accurate identification is required for all experiments), but in clinical diagnosis as well. However, modifications of the procedure will be necessary in order to carry out this technique in a clinical situation. Such a modification would require an easier method of DNA extraction other than the one described here(which was used for quantitative reasons and is more rigorous than that needed in a clinic). A possible alternative for quicker and easier DNA extraction could be either a zymolase spheroplast procedure (Kitamura and Yamamoto, 1972) or the use of liquid nitrogen for cell lysis (Garber and Yoder, 1983). Radioactive phosphorous which was used to label the DNA probe, is not a reagent that can be effectively or safely used in a clinical situation. A viable substitution would be the biotinylated, nick-translation probe described by Leary (1983) and Langer (1981) which gives the reagent a long storage life and excludes the use of potentially hazardous radioactive material.

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